USER GUIDE

Ion TargetSeq™ Exome Enrichment for the Ion Proton™ System

For capturing exons identified in the GenCode, RefSeq, Ensembl, UCSC, VEGA, and CCDS databases, functional RNA genes from the miRBase and UCSC databases, predicted micro-RNA binding sites and COSMIC-annotated tumor variants from human genomic DNA for sequencing on the Ion Proton™ System

Catalog Number 4477742, 4477743, and 4477744

Publication Number MAN0006730

Revision A.0

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Contents

About this guide

IMPORTANT! Before using this kit, read and understand the information in Appendix D, "Safety" of this document.

Revision history

Revision	Date	Description of change
A.0	24 April 2014	• Changed the recommendation for final library dilution to 100 pM to unify the guide with other Ion library and template user guides. • BED file download and installation procedure updated. • Version numbering reset to A.0 in conformance with internal document control.
5.0	7 June 2013	• Support for multiple barcoded libraries added • Changed to 260–280 bp (non-barcoded libraries) or 275–295 bp (barcoded libraries) target length for 200-base-read sequencing
4.0	5 March 2013	• New accelerated 16–18-hour hybridization protocol, with supporting data in new Appendix C

Revision	Date	Description of change
3.0	18 December 2012	• Changed to 220-bp target length for 150-base-read sequencing • Changed references to template kit • Added E-Gel® Gel size selection
2.0	14 November 2012	• Corrected amount of Ion TargetSeq™ Amplification Primer Mix provided
1.0	19 October 2012	• New user guide

Purpose of the guide

The *Ion TargetSeq™ Exome Enrichment for the Ion Proton™ System User Guide* (Pub. no. MAN0006730) provides a procedure and reference information for using Ion TargetSeq™ Exome Kits (Cat. nos. 4477742, 4477743, and 4477744) to enrich gDNA fragment libraries that have been prepared using the Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269) or Ion Plus Fragment Library Kit (Cat. no. 4471252).

1 Product information

Product description

Ion TargetSeq™ Exome Kits provide qualified reagents for capturing most highly annotated exons (protein coding sequences), functional RNA genes, predicted microRNA-binding sites, and COSMIC-annotated tumor variants from human genomic DNA for sequencing on the Ion Proton™ System. The following table provides a summary of the targeted features:

Feature type	Number of targeted features
Protein coding genes	>21,500
RNA genes (miRNA, snoRNA, tRNA, and other ncRNA)	>8,000
Predicted miRNA binding sites	>44,000
COSMIC positions	>33,500

To use the Ion TargetSeq™ system, you first construct a fragment library from genomic DNA using the Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269) or Ion Plus Fragment Library Kit (Cat. no. 4471252), and then capture the library fragments in solution using ~2 million Ion TargetSeq™ capture probes, biotinylated oligos that range in size from ~50 bases to ~120 bases. Hybridization specificity is ensured by the use of blocker DNA sequences (Human Cot-1 DNA® Fluor QC and Ion TargetSeq™ Blockers).

The bound DNA is isolated using streptavidin-coated Dynabeads® paramagnetic beads, and then amplified and purified. The purified, exome-enriched library is then used to prepare templated Ion PI™ Ion Sphere™ Particles (ISPs) for sequencing on an Ion PI™ Chip to obtain the necessary data coverage.

Protocol timeline

Day	Step	Description	Time (hours)
Day 1	1	Prepare library	6
	2	Amplify and purify	1.5
	3	Quantify	1.5
	4	Set up and start hybridization	1
Days 2–4	5	Hybridize at 47°C	64–72
Day 4	6	Wash and recover beads with enriched DNA	2
	7	Amplify and purify	1.5
	8	Quantify the enriched library	1.5

Accelerated protocol timeline

Refer to Appendix C, "Expected performance with 16–18-hour hybridization" for more detail.

Day	Step	Description	Time (hours)
Day 1	1	Prepare library	6
	2	Amplify and purify	1.5
	3	Quantify	1.5
	4	Set up and start hybridization	1
Night 1	5	Hybridize at 47°C overnight	16-18
Day 2	6	Wash and recover beads with enriched DNA	2
	7	Amplify and purify	1.5
	8	Quantify the enriched library	1.5

Kit contents and storage

Ion TargetSeq™ Exome Kits are available in three sizes, depending on the number of enrichment reactions you require. Catalog numbers and volumes are listed below:

	Component volume			Storage
Kit Cat. no.	**4477742**	**4477743**	**4477744**	
No. of enrichments/kit	**4**	**12**	**48**	
Ion TargetSeq™ Exome Reagents				
Ion TargetSeq™ Exome Probe Pool	1 × 18 µL	3 × 18 µL	1 × 216 µL	–30°C to –10°C
Human Cot-1 DNA® Fluor QC	1 × 1 mL	3 × 1 mL	10 × 1 mL	
Ion TargetSeq™ Amplification Primer Mix	2 × 100 µL	3 × 1 mL	2 × 1 mL	
Low TE	2 × 1.8 mL	6 × 1.8 mL	13 × 1.8 mL	
Ion TargetSeq™ Multi Blocker Kit (also sold separately; see below)				
Ion TargetSeq™ Blocker P1	132 µL	132 µL	4 × 132 µL	–30°C to –10°C
Ion TargetSeq™ Blocker A	132 µL	132 µL	4 × 132 µL	
Ion TargetSeq™ Blocker BC1-16	132 µL	132 µL	4 × 132 µL	
Dynabeads® M-270 Streptavidin (also sold separately; see below)				
Dynabeads® M-270 Streptavidin	1 × 2 mL	1 × 2 mL	1 × 10 mL	2°C to 8°C
TargetSeq™ Hybridization & Wash Kit (also sold separately; see below)				
TargetSeq™ Wash Solution A (10X)	750 µL	750 µL	2 × 750 µL	–30°C to –10°C
TargetSeq™ Wash Solution B (10X)	500 µL	500 µL	2 × 500 µL	
TargetSeq™ Wash Solution C (10X)	500 µL	500 µL	2 × 500 µL	
TargetSeq™ Stringent Wash Solution (10X)	1000 µL	1000 µL	2 × 1000 µL	
TargetSeq™ Hybridization Solution A (2X)	190 µL	190 µL	2 × 190 µL	
TargetSeq™ Hybridization Enhancer B	75 µL	75 µL	2 × 75 µL	

	Component volume			Storage
Kit Cat. no.	**4477742**	**4477743**	**4477744**	
No. of enrichments/kit	**4**	**12**	**48**	
TargetSeq™ Magnetic Bead Wash (2.5X)	5 mL	5 mL	2 × 5 mL	–30°C to –10°C

Additional kits sold separately

The following component kits are also sold separately:

- Ion TargetSeq™ Multi Blocker Kit (Cat. no. 4477113)
- Dynabeads® M-270 Streptavidin (Cat. no. 65305 or 65306)
- TargetSeq™ Hybridization & Wash Kit (Cat. no. A138230)

Aliquot and store the Ion TargetSeq™ Exome Probe Pool

Store the Ion TargetSeq™ Exome Probe Pool in 4.5-µL aliquots as described below:

1. If frozen, thaw the Ion TargetSeq™ Exome Probe Pool vial on ice.
2. Vortex for 3 seconds.
3. Centrifuge the tube at 10,000 × *g* for 30 seconds to ensure that the liquid is at the bottom of the tube before opening the tube.
4. Aliquot the probe pool into single-use aliquots (4.5 µL/aliquot) in 0.2-mL MicroAmp® Reaction Tubes and store at –30°C to –10°C until use. Some residual volume after dispensing all single-use aliquots is normal.

Library kit compatibility

Use either of the following kits to prepare fragment libraries from genomic DNA (gDNA) for target enrichment using the Ion TargetSeq™ Exome Kits:

Ion Plus Fragment Library Kit (Cat. no. 4471252)

This kit includes reagents for end-repair of physically fragmented gDNA and reagents for library preparation from the end-repaired DNA.

Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269)

This kit includes Ion Shear™ Plus Reagents for enzymatic fragmentation of gDNA plus the contents of the Ion Plus Fragment Library Kit (see above) for library preparation from the enzymatically fragmented DNA.

Template kit compatibility

Ion TargetSeq™ Exome Kits are compatible with the Ion PI™ Template OT2 200 Kit v2 (Cat. no. 4485146) and the Ion PI™ Template OT2 200 Kit v3 (Cat. no. 4488318).

Expected coverage of the Ion TargetSeq™ Exome Kits

The capture probes provided with Ion TargetSeq™ Exome Kits have been optimized to provide high specificity of capture and high uniformity of coverage.

Using the standard 64–72-hour probe hybridization time, 75-85% of mapped reads can be expected to overlap targeted regions by at least one base. With 4.7 Gb of aligned sequence data per barcode, at least 80% of the target bases will be covered at a depth of 20X or more. This value rises to >85% with more than 6 Gb of aligned sequence and to ~90% with >7 Gb of aligned sequencing output.

Reference BED files

Three BED files that describe the content enriched by Ion TargetSeq™ Exome Enrichment Kits are available for download from the Ion Community. Each file contains chromosomal coordinates that match the GRCh37/hg19 assembly of the human genome. The files are described below.

Ion-TargetSeq-Exome-50Mb-hg19_revA.bed

Note: This file is available as a zipped file on the Ion Community, and can be downloaded and installed on the Torrent Server as described on page 11. This BED file is in a non-standard, 6-column format that is compatible with Torrent Suite™ software.

This is the default file to use when assessing coverage over the biologically-relevant bases that comprise the Ion TargetSeq™ Exome design. It lists all of the regions (exons, miRNA genes, etc) targeted for enrichment. All protein-coding exons have been extended 3 bases upstream and downstream to ensure that bases at splice-sites are also enriched. The minimum region size is 100 bases, and all targeted loci that are less than 100 bases have been centered in 100-base regions. In total, this file spans 46.2 Mb of the human genome. The file contains a header line that is compatible with display as a "Custom Track" in the UCSC Genome Browser.

The BED file is provided in a 6-column format can be uploaded directly into Torrent Suite™ software. The columns are:

Column	Contains
1	Standard chromosome of targeted region
2	Start position of targeted region
3	End position of targeted region
4	Regional identifier name, in the format exome_target_N
5	Text identifying non-exonic targeted feature or the gene name if the feature is an exon
6	Simplified gene ID that corresponds to the overlapping or nearest annotated gene

Ion-TargetSeq-Exome-50Mb-probe-regions-hg19.bed

Note: This file is available as a zipped file on the Ion Community, and can be downloaded and installed on the Torrent Server as described on page 11. This BED file is in a non-standard, 6-column format that is compatible with Torrent Suite™ software.

This file lists the exact bases that are complementary to the biotinylated hybridization capture probes in the kit. Because the proprietary hybridization probe sequences are designed algorithmically to maximize specificity within the genome, the positions of the probes are not always coincident with the biologically desired bases. However, in most cases, the probes overlap with each other and the target regions (i.e., tile the targets) or are closely proximal to the desired target regions. Close proximity maximizes the likelihood that the targeted bases will be co-captured on fragment library molecules that hybridize to the probe molecules. This file is provided for those customers who want to assess the specificity and efficiency of the Ion TargetSeq™ technology relative to exact bases probed. In total, this file spans 52.7 Mb of the human genome. This file contains a header line that is compatible with display as a "Custom Track" in the UCSC Genome Browser. The BED file is provided in a 6-column format that is compatible with upload and use in Torrent Suite™ software. The columns are:

Column	Contains
1	Standard chromosome of clustered probe region
2	Start position of clustered probe region
3	End position of clustered probe region
4	Regional identifier name, in the format exome_probe_region_N
5	Place holder
6	Same information as in column 4

Ion-TargetSeq-Exome-50Mb-hg19-complete-annotation.bed

Note: This file is for reference only. It is available as a zipped file on the Ion Community, but should **not** be loaded on the Torrent Server.

This file is a tab-delimited text file that can be opened with spreadsheet software that comprehensively lists the database identifiers of the biological regions that were targeted. Protein coding exons were extracted from the following gene-model databases: GENCODE, RefSeq, CCDS, Ensembl, UCSC, and VEGA. MicroRNA genes were gathered from miRBase. Other non-coding RNA genes including snoRNAs, tRNAs, rRNAs, SRP RNAs, and lncRNA genes TERC and XIST were retrieved from the UCSC genome table browser as were human microRNA binding sites predicted by TargetScan (**http://www.targetscan.org**). Finally, documented positions of somatic tumor variants of 100 bases or smaller were retrieved from the Catalogue of Somatic Mutations in Cancer (COSMIC).

Download and unzip the BED files

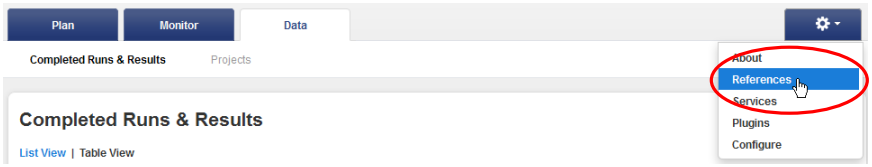
To download the files:

1. Go to **http:// ioncommunity.lifetechnologies.com**.
2. Navigate through the **Protocols ▸ Ion Proton™ Sequencer ▸ Construct Library** menu, and click on the BED file links in the TargetSeq™ Library Preparation section to download the files.
3. The BED files will have a **.zip** extension. Download and unzip the files using a file compression utility.

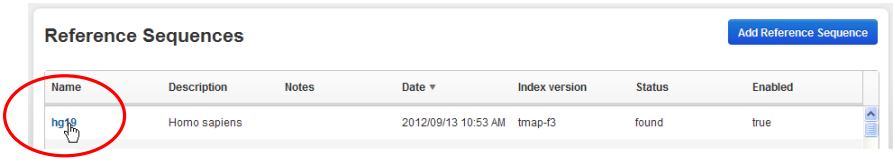
Install the BED files on the Torrent Server

Brief instructions for installing the **Ion-TargetSeq-Exome-50Mb-hg19.bed** and **Ion-TargetSeq-Exome-50Mb-probe-regions-hg19.bed** files on your Torrent Server are provided below. Detailed instructions are provided in the *Torrent Browser User Interface Guide*.

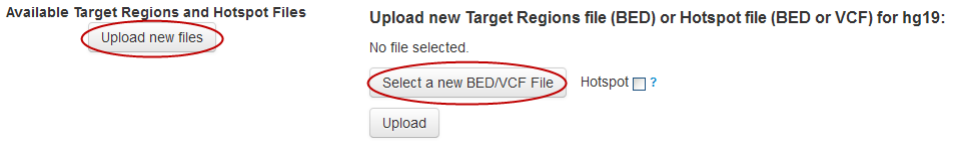
1. After downloading and unzipping the BED files, open the Torrent Browser, click the **Settings** button ( ) on the right side of the screen, and select **References**.



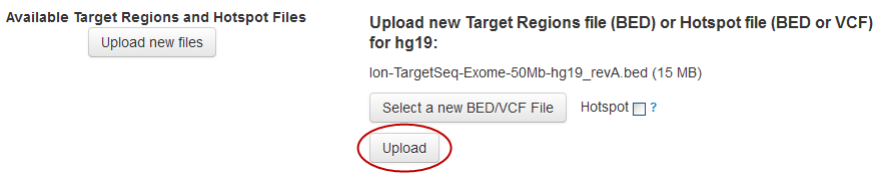
2. Click on the **hg19** reference sequence.



3. Scroll down and click on **Upload new files**, then click on **Select a new BED/VCF File**.



4. Navigate to the location containing the unzipped BED file and select it, then click on the **Upload** button.



Required materials and equipment (not provided)

✓	Description	Supplier	Cat. no.	Quantity
	Ion Xpress™ Plus Fragment Library Kit Ion Plus Fragment Library Kit	Life Technologies	4471269 4471252	1 kit 1 kit
	Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1 kit
	Platinum® PCR SuperMix High Fidelity	Life Technologies	12532016	4.5 mL [1]
	Qubit® 2.0 Fluorometer	Life Technologies	Q32866	1
	Qubit® dsDNA HS Assay	Life Technologies	Q32851 or Q32854	100 assays 500 assays
	Ion Library Quantitation Kit	Life Technologies	4468802	1
	Agilent® 2100 Bioanalyzer® instrument	Agilent	G2939AA	1
	Agilent® High Sensitivity DNA Kit	Agilent	5067-4626	1 kit
	DynaMag™-2 magnet (magnetic rack)	Life Technologies	12321D	1
	DynaMag™-PCR magnet (for 0.2-mL tubes)	Life Technologies	49-2025	1
	1.5-mL Eppendorf LoBind® Tubes	Eppendorf	022431021	1 box
	0.2-mL MicroAmp® Reaction Tubes	Life Technologies	N8010540	1 package
	Microcentrifuge	Major laboratory supplier (MLS)	N/A	1
	Thermal cycler (GeneAmp® PCR System 9700 and Veriti® Systems from Life Technologies are recommended)	MLS	N/A	1
	Nuclease-free Water	Life Technologies	AM9932	1000 mL
	Low TE (10 mM Tris, 0.1 mM EDTA), pH 8.0	MLS	N/A	Varies
	Ethanol, absolute	MLS	N/A	Varies
	Vacuum concentrator (for 1.5-mL tubes)	MLS	N/A	1
	Water bath or heat block	MLS	N/A	1
	Calibrated thermometer	MLS	N/A	1
	Rotator	MLS	N/A	1

✓	Description	Supplier	Cat. no.	Quantity
	Vortex mixer	MLS	N/A	1
	Pipettors 1-1000 µL	MLS	N/A	1 each
Optional (for preparing barcoded libraries)				
	Ion Xpress™ Barcode Adapters 1-16 Kit	Life Technologies	4471250	1 kit
Optional (for preparing control libraries)				
	Ion Proton™ Controls Kit	Life Technologies	4478328	1 kit
Optional (for FFPE samples)				
	MagMAX™ FFPE Total Nucleic Acid Isolation Kit	Life Technologies	4463365	96 preps
	RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	Life Technologies	AM1975	40 preps
	E-Gel® EX Starter Kit, 2%	Life Technologies	G6512ST	1 kit
	E-Gel® EX Gel, 2%, 20-pak	Life Technologies	G402002	20 gels

[1] Amount needed per Ion TargetSeq™ Exome enrichment reaction is 200 µL.

Required for physical fragmentation of gDNA

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	Cat. no.	Quantity
	Bioruptor® UCD-200 Standard Sonication System with accessories for 12 x 0.5 mL tubes (Microtube Attachment & Gearplate)	Life Technologies *or* Diagenode	4465622 [1] UCD-200 TS [1]	1 1
	Bioruptor® NGS Sonication System with accessories for 12 x 0.5 mL tubes (Microtube Attachment & Gearplate) [2]	Diagenode	UCD-600 TS	1
	Bioruptor® NGS 0.65-mL MicroTubes for DNA Shearing	Diagenode	WA-005-0500	500 tubes

[1] These catalog numbers include a soundproof box.

[2] The Bioruptor® NGS Sonication System is discontinued and has been replaced by the Bioruptor® Pico Sonication System (Life Technologies Cat. no. 4486162 or Diagenode Cat. no. B0106001).

Required for size selection

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

✓	Description	Supplier	Cat. no.	Quantity
	Pippin Prep™ instrument	Life Technologies	4471271	1
	2% Agarose Gel Cassettes for the Pippin Prep™ instrument	Life Technologies	4472170	10 cassettes
	OR			
	E-Gel® iBase™ unit and E-Gel®Safe Imager™ transilluminator combo kit	Life Technologies	G6465	1
	E-Gel® SizeSelect™ 2% Agarose Gel	Life Technologies	G6610-02	10/pack
	50 bp DNA ladder (1 µg/µL) for E-Gel® Agarose Gel size selection	Life Technologies	10416-014	1

2 Procedure guidelines and DNA input requirements

Procedure guidelines

- Each fragment library should be constructed from **1 µg of high-quality, RNA-free genomic DNA,** as quantified with a Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay. See "Input DNA requirements" on page 19 below for more information.
- First-time users may prepare and enrich a control library to familiarize themselves with the procedure prior to using their own samples. Use the Human CEPH Genomic DNA Control supplied in the Ion Proton™ Controls Kit (Cat. no. 4478328) or the Ion PI™ Controls 200 Kit (Cat. no. 4488985) to prepare control libraries for sequencing on the Ion Proton™ 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 Cat. no. 022431021).
- Thaw reagents on ice before use, and keep enzymes at –30°C to –10°C until ready to use.
- Mix reagents thoroughly before use, especially if frozen and thawed.

Input DNA requirements

Genomic DNA

Each fragment library should be constructed from **1 µg of high-quality, RNA-free genomic DNA**, as quantified with a Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay. Quantification with the Qubit® system is required to achieve expected performance.

A number of commercially available kits are available for isolation of high molecular weight, RNA-free genomic DNA. See **Appendix C** in the *Ion Xpress™ Plus Fragment Library Preparation User Guide* (Pub. no. MAN0009847) for more information about assessing the integrity and size of your input DNA material and performing an optional RNase I treatment procedure.

Libraries are constructed from the input DNA using materials from the Ion Xpress™ Plus Fragment Library Kit (Catalog no. 4471269) or Ion Plus Fragment Library Kit (Catalog no. 4471252), as described in this user guide.

DNA extracted from FFPE samples

During the fixation processes used in preparing formalin-fixed, paraffin-embedded (FFPE) tissue samples, formaldehyde reacts primarily with the nitrogen atoms of lysine, arginine, and histidine, resulting in extensive cross-linking of the protein matrix within each cell. Some of these crosslinks extend to the nucleic acid species present as well. The subsequent embedding process further accelerates this chemistry by heating the samples. The resulting extensive web of protein-protein and protein-nucleic acid crosslinks tends to physically entrap and chemically fragment the genomic DNA to a greater or lesser extent, depending on the methods and reagents used and the duration and conditions of sample storage.

For these reasons, the quantity and quality of genomic DNA recovered from FFPE specimens can vary widely and it is important to assess both the quantity and quality of the DNA before moving forward with library construction for exome enrichment and sequencing. We recommend two kits for the preparation of genomic DNA from FFPE samples for fragment library preparation:

- MagMAX™ FFPE Total Nucleic Acid Isolation Kit (Catalog no. 4463365)
- RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Catalog no. AM1975)

Quantification of DNA extracted from FFPE samples

It is important to measure the quantity of DNA to be used in fragment library construction with an assay that is highly sensitive to and specific for double-stranded DNA. For this reason, we recommend using the Qubit® 2.0 Fluorometer (Cat. no. Q32866) with the Qubit® dsDNA HS Assay (Cat. no. Q32851). Ensure that you have at least 1 µg of dsDNA, as measured by the Qubit® assay, before starting fragment library construction. Consult the user guide for your genomic DNA extraction kit for expected yields. In general, 1 µg of genomic DNA can be extracted from eight 10-µm thick slices of FFPE-preserved tissue with a surface area of 20-50 mm^2. However, yield can vary widely depending upon the preservation and storage of the sample and the specific tissue type used.

Assessment of quality of DNA from FFPE samples

For fragment library construction prior to exome enrichment, it is important to start with genomic DNA that is of relatively high apparent molecular weight. This can be assessed either by agarose gel electrophoresis or using an Agilent® 2100 Bioanalyzer® instrument.

- **Agarose gel electrophoresis**. DNA from FFPE samples will always be a population of sheared fragments. You can assess the average size of this population using standard electrophoresis methods. We recommend using 2% E-Gel® EX Gels. This will require ~20 ng of DNA; however, it is a rapid method to view the apparent molecular weight of genomic DNA prior to library construction. Only DNA that runs at a relatively high molecular weight — comparable to that of DNA extracted from fresh or frozen samples—should be used for library construction. See Figure 1 below for a comparison of the electrophoretic mobilities of (A) low-quality and (B) high-quality FFPE-extracted DNA.

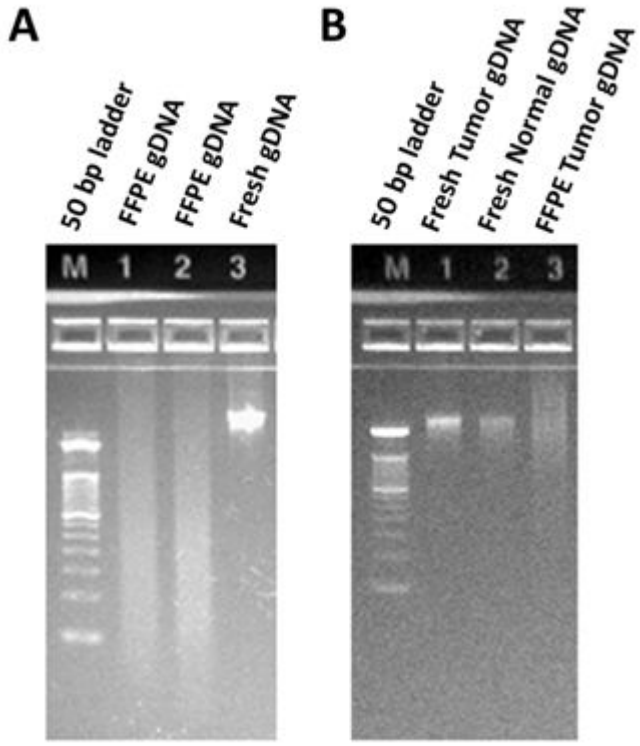
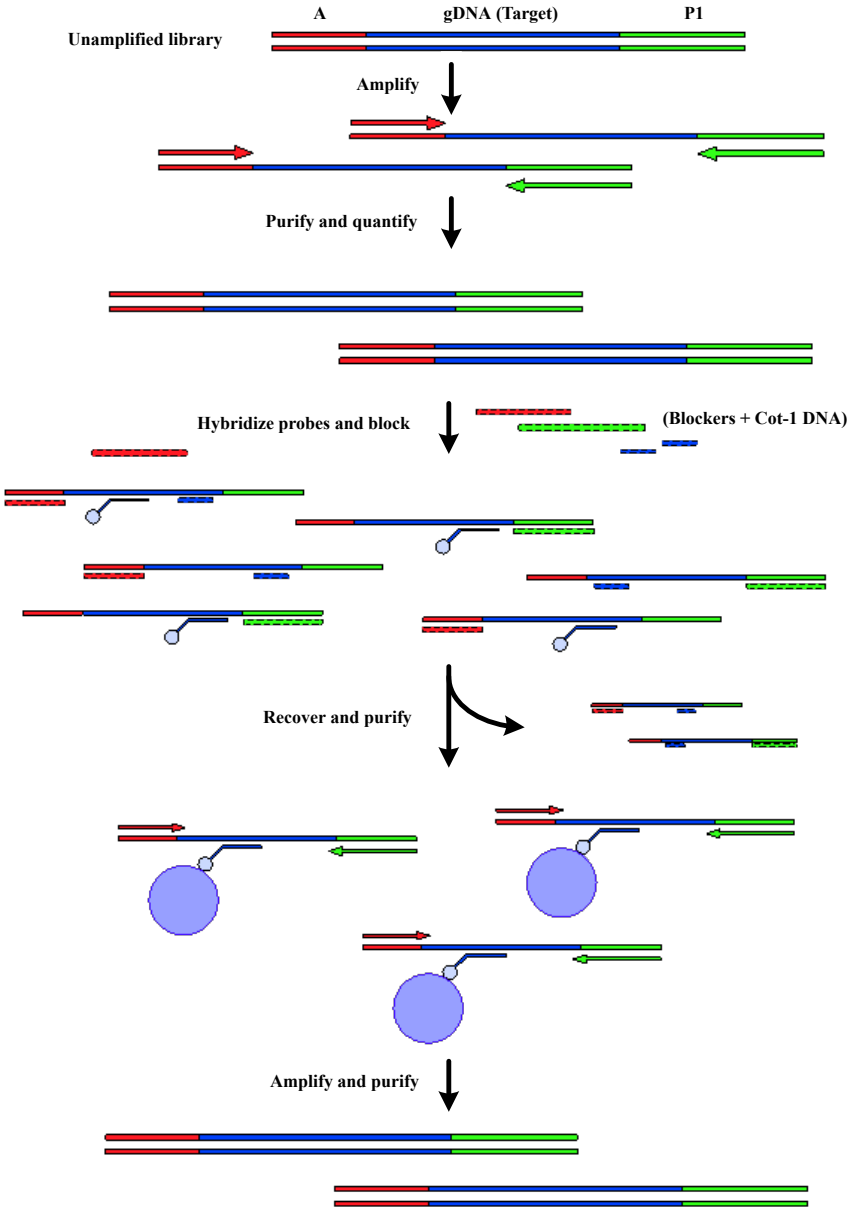


Figure 1 E-Gel® EX, 2% analysis of FFPE-extracted genomic DNA. A) Example of FFPE-extracted gDNA (lanes 1 and 2) that is degraded to low molecular-weight fragments. B) High quality FFPE-extracted gDNA (lane 3) runs as a high molecular weight smear of similar mobility to that of DNA extracted from fresh tissue samples (lanes 1 and 2).

- **Agilent® Bioanalyzer® instrument**. The Agilent® Bioanalyzer® instrument requires a much smaller amount of recovered DNA than does agarose gel electrophoresis. The recovered DNA typically appears as a broad "smear," covering a size range up to thousands of base pairs, with the modal size varying from less than 100 bp to ~3000 bp, depending on the age and condition of the original sample.

Workflow diagram



3 Prepare adapter-compatible DNA

This section describes two methods for preparing adapter-compatible DNA from 1 µg of input DNA:

Method	Kit used	Starting on...
Enzymatic fragmentation with Ion Shear™ Plus Reagents	Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269)	Below
Physical fragmentation with the Bioruptor® Sonication System	Ion Plus Fragment Library Kit (Cat. no. 4471252)	"Method 2: Fragment gDNA with the Bioruptor Sonication System, end-repair, and purify" on page 28

Note: Physical fragmentation with a Covaris® System may also be used. Consult with the manufacturer or look for guidance on the Ion Community at **http://ioncommunity.lifetechnologies.com**.

Method 1: Fragment gDNA with Ion Shear™ Plus Reagents

This section describes the enzymatic fragmentation of gDNA into blunt-ended fragments using Ion Shear™ Plus Reagents. Purification with AMPure® XP Reagent is required before and after fragmentation. No end-repair is required.

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.

Purify the gDNA with AMPure® XP Reagent

Note: The Ion Shear™ Plus reaction has very good tolerance of the G+C content of a sample, but is very sensitive to EDTA concentration, the integrity of the sample, and operator handling. The following purification procedure is designed to reduce the EDTA concentration of the sample and normalize sample-to-sample fragmentation. We recommend that you confirm the best reaction time for your laboratory conditions.

IMPORTANT! Use **freshly prepared 70% ethanol** (1 mL plus overage per sample) for the following procedure. A higher percentage of ethanol causes inefficient removal of smaller-sized molecules. A lower percentage of ethanol may lead to sample loss.

1. Dilute 1 µg of concentrated gDNA to a final volume of 50 mL (20 ng/µL) with nuclease-free water or 10 mM Tris, pH 7.5-8.5.

   (*Optional*) In a separate tube, prepare a control sample of 1 µg of Human CEPH Genomic DNA Control (supplied in the Ion Proton™ Controls Kit or Ion PI™

Controls 200 Kit) in 50 µL Nuclease-free Water or 10 mM Tris, pH 7.5-8.5. Process the control in parallel for all steps.

2. Add 90 µL of Agencourt® AMPure® XP Reagent (1.8X sample volume) to the 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.

3. 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.

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

5. Repeat step 4 for a second wash.

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

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

8. Remove the tube from the magnet and add 35 µL of **Nuclease-free Water** 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.

9. Pulse-spin the tube, then proceed directly to "Fragment the DNA and purify" on page 24.

IMPORTANT! Do not remove the supernatant containing the eluted DNA from the beads before fragmentation, as some DNA may be lost on the beads due to poor elution of long DNA from the beads. The protocol has been optimized for fragmenting DNA with beads present in the reaction.

Fragment the DNA and purify

Fragmentation conditions have been optimized for the following library size. Once fragmented the DNA is ready for adapter ligation.

Library insert size	Modal fragment size
~200 bp	180–220 bp

Fragment the DNA

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

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

2. Add the following reagents in the indicated order to a 1.5-mL Eppendorf LoBind® Tube, and mix vigorously by vortexing for 5 seconds. Pulse-spin to bring the contents to the bottom of the tube. Do not scale up the reaction volumes or prepare a master mix.

Component	Volume
Purified gDNA with beads	35 µL
Ion Shear™ Plus 10X Reaction Buffer	5 µL
Total	**40 µL**

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

4. 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.**

5. 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.

Purified DNA sample origin	Modal fragment size	Default Reaction time	Optimization range
Cultured cells	200-base-read library	45 minutes	30–45 minutes
Fresh or fresh-frozen tissue	200-base-read library	30 minutes	30–45 minutes
FFPE tissue	200-base-read library	15 minutes	15–30 minutes

6. Add 5 µL of Ion Shear™ Stop Buffer immediately after incubation, for a total volume of 55 µL and mix thoroughly by vortexing for at least 5 seconds. Store the reaction tube on ice.

Purify the fragmented DNA

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

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

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

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

4. Repeat step 3 for a second wash.

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

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

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

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

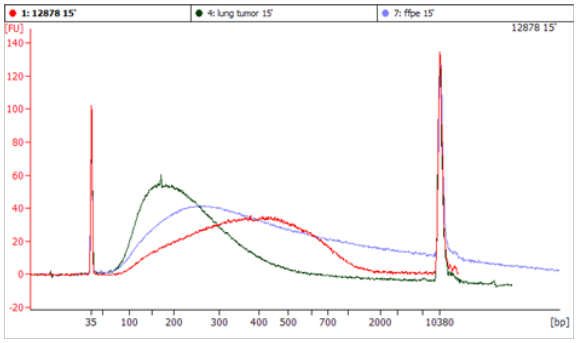
   IMPORTANT! The supernatant contains your sample. **Do not discard!**

9. *(Optional)* Check the fragment size using 1 µL of 1:10 dilution of eluted DNA in nuclease-free water and the Agilent® Bioanalyzer® and Agilent® High Sensitivity DNA Kit. Confirm the desired DNA fragment size range as follows:

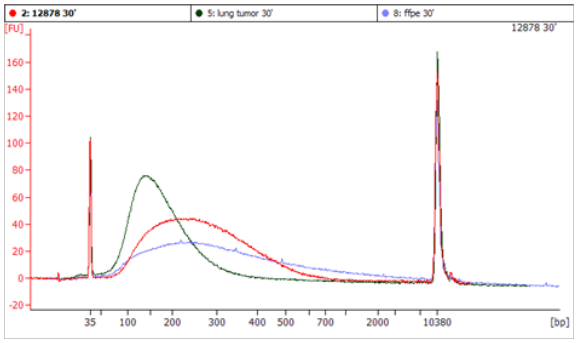
Modal fragment size	Fragment size range
200-base-read library	70–400 bp

   See Figure 2 for example traces:

(A)



(B)



(C)

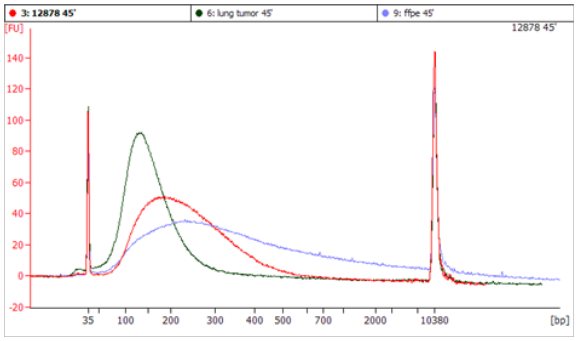


Figure 2 Typical fragmentation profiles of genomic DNA, prepared from cultured cells (red traces), fresh-frozen tumor tissue (green traces), or FFPE-derived tumor tissue (blue traces) using Ion Shear™ Plus Reagents at 37°C for (A) 15 minutes, (B) 30 minutes, and (C) 45 minutes. Highest yields of fragments in the size-range of 180–220 bp are generally obtained from 15–30 minute reactions for FFPE-derived DNA and 30–45 minute reactions for DNA derived from non-embedded tissue or cells. The 35-bp and 10380-bp peaks represent molecular weight markers.

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

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

Method 2: Fragment gDNA with the Bioruptor® Sonication System, end-repair, and purify

This section describes conditions for using 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 suitable for preparing 100 base-read libraries 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 with a cooling recirculating bath. 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: **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, to achieve volume compatibility with the end-repair reaction.

Prepare the samples

1. In a 0.65-mL microcentrifuge tube for the Bioruptor® Sonication System, prepare 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 for physical fragmentation of gDNA" on page 17.

2. (*Optional*) In a separate tube, prepare a control sample of 1 µg of Human CEPH Genomic DNA Control (supplied in the Ion Proton™ Controls Kit) in 50 µL Low TE. Keep the control on ice, and process in parallel for all steps.

3. You can 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 or water to fill all empty slots.

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

5. Proceed to "Option 1: Sonicate the DNA with the Bioruptor UCD-200 TS Sonication System" on page 29 in the following section or to "Option 2: Sonicate the DNA with the Bioruptor UCD-600 NGS Sonication System" on page 30.

Option 1: 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:

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

4. 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.

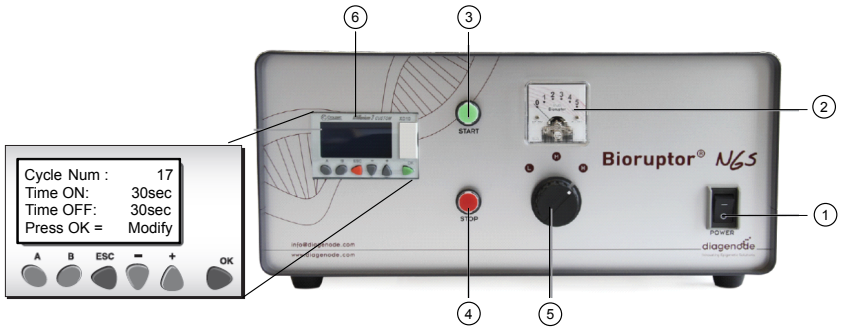
5. Remove the tubes from the rotor and store on ice.

6. Proceed to "Assess the fragmentation profile" on page 30.

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

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

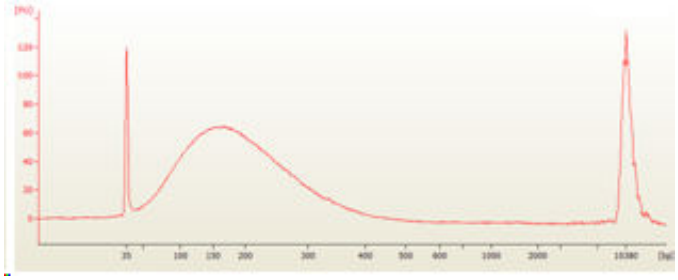
   Note: Ensure the temperature of the cooler stays below 10°C during the run.
5. Remove the tubes from the rotor and store on ice.
6. Proceed to "Assess the fragmentation profile" on page 30.

Assess the fragmentation profile

1. Analyze the following volume of fragmented DNA to confirm a fragment size range between 50–500 bp, with a peak around 200 bp (see Figure 3).

Bioanalyzer® instrument with Agilent® High Sensitivity DNA Kit	**Agarose gel**
1 µL 1:10	5 µL

(A)



(B)

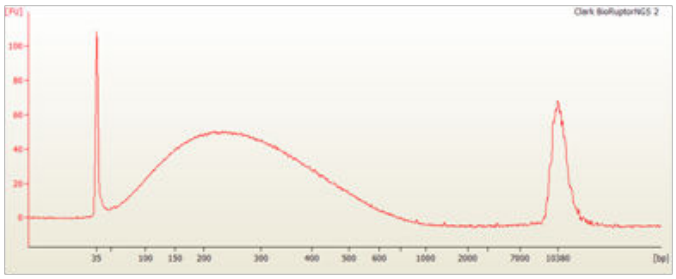


Figure 3 Typical fragmentation profile of genomic DNA (prepared from frozen cells) produced by the (A) Bioruptor® UCD 200 TS Sonication System or (B) Bioruptor® UCD 600 NGS Sonication System. Each profile indicates fragmentation suitable for size-selection prior to adaptor ligation.

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

End-repair the DNA

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 158 µL.

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

Component	Volume
Fragmented gDNA (step 1)	158 µL
5X End Repair Buffer	40 µL
End Repair Enzyme	2 µL
Total	**200 µL**

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

Purify with the Agencourt® AMPure® XP Kit

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

1. Add 360 µL of Agencourt® AMPure® XP Reagent beads (1.8X sample volume) to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes.
2. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the bead pellet.
3. Without removing the tube from the magnet, dispense 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
4. Repeat step 3 for a second wash.
5. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
6. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
7. Remove the tube from the magnet, and add 25 µL of Low TE to the sample. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
8. Pulse-spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, **transfer the supernatant** containing the eluted DNA to a new 1.5-mL Eppendorf LoBind® Tube without disturbing the pellet.

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

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

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

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

Ligate and nick repair

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

Non-barcoded libraries		Barcoded libraries	
Component	**Volume**	**Component**	**Volume**
DNA	~25 µL	DNA	~25 µL
10X Ligase Buffer	10 µL	10X Ligase Buffer	10 µL
Adapters	10 µL	Ion P1 Adapter	10 µL
		Ion Xpress™ Barcode X[1]	10 µL
dNTP Mix	2 µL	dNTP Mix	2 µL
Nuclease-free water	41 µL	Nuclease-free water	31 µL
DNA Ligase	4 µL	DNA Ligase	4 µL
Nick Repair Polymerase	8 µL	Nick Repair Polymerase	8 µL
Total	**100 µL**	**Total**	**100 µL**

[1] X = barcode chosen.

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

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

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

Stage	Temperature	Time
Hold	25°C	15 min
Hold	75°C	20 min
Hold	4°C	Hold[1]

[1] 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-translated DNA

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

1. Add 180 µL (1.8X sample 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.

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

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

4. Repeat step 3 for a second wash.

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

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

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

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

9. Proceed to Chapter 5, “Size-select the unamplified library”.

5 Size-select the unamplified library

This section describes two options for size-selection:

- Pippin Prep™ instrument, starting below.
- E-Gel® SizeSelect™ Agarose Gel, starting on page 35.

The target peak size of the non-barcoded size-selected library should be ~260–280 bp (for a 200-base-read library).

The target peak size of a barcoded size-selected library should be ~275–295 bp (for a 200-base-read library).

Option 1: Size-select the library with the Pippin Prep™ instrument

Start with unamplified library, prepared and purified as described in "Purify the adapter-ligated and nick-translated DNA" on page 34.

IMPORTANT! The protocol below closely follows the Pippin Prep™ instrument manual. Novice users may want to review training materials at **http://www.sagescience.com/products/pippin-prep/** 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.

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

1. 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.
2. Remove the two adhesive strips covering the loading wells and elution wells.
3. Fill the loading wells with Electrophoresis Buffer to the top so that a concave meniscus forms.
4. Remove all liquid from the elution wells, and then add 40 µL of Electrophoresis Buffer.
5. Seal the elution wells with the adhesive tape strips supplied with the cassette packaging.
6. Following the Pippin Prep™ instrument manual, apply current across the cassette and confirm that the current across both the separation ports and the elution ports is within specifications.

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

1. From the cassette type drop-down menu, choose **2% Marker B No Overflow Detection**.
2. Press **Ref Lane** (reference lane) to define lane 5 as the ladder lane.
3. Define lanes 1–4 as sample lanes. Select the "Tight" collection mode for each lane and then define the **BP Target** setting as 270 bp (non-barcoded library) or 285 bp (barcoded library) for each of sample lanes 1–4 used.
4. For every sample lane, press the **Sig Mon** (signal monitoring) button to inactivate lane monitoring. Each button for an inactivated lane turns dark.
5. Set the run time for 1.5 hours.

Load the sample

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

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

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

Run the instrument

1. When the ladder and all samples are loaded, close the lid of the Pippin Prep™ instrument.
2. On the Main screen, press **Start** to initiate the run.
3. When the separation is complete, transfer the DNA from the elution wells (typically 40–60 µL) with a pipette to new 1.5-mL Eppendorf LoBind® Tubes.
4. Add Nuclease-free Water to the DNA to bring the volume to 60 µL.
5. Proceed to “Purify the size-selected DNA” on page 40.

Option 2: Size-select the library with the E-Gel® SizeSelect™ Agarose Gel

Start with unamplified library, prepared and purified as described in "Purify the adapter-ligated and nick-translated DNA" on page 34.

Note: When using E-Gel® SizeSelect™ 2% Agarose Gels, the goal is to obtain library DNA that is ~270 bp (non-barcoded library) or ~285 bp (barcoded library) in the collection wells. This is best achieved when the collection well in the center marker lane is flanked above by the 300-bp band and below by the 200-bp band of the 50-bp DNA ladder as described in the following protocol.

Prepare the E-Gel® SizeSelect™ Agarose Gel and iBase™ unit

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

1. Place the iBase™ unit on top of the Safe Imager™ transilluminator, and plug the short cord from the Safe Imager™ transilluminator into the power inlet of the iBase™ unit.
2. Plug the connector of the power cord with the transformer into the Safe Imager™ transilluminator and connect the other end of the power cord to an electrical outlet.
3. Verify that the iBase™ unit has the "SizeSelect™ 2%" program. If not, refer to "Downloading upgrade" from the *E-Gel® Technical Guide.*
4. Remove the gel from the package and gently remove the combs from the SizeSelect™ cassette.
5. Insert the gel cassette into the E-Gel® iBase™ unit right edge first.
6. Press firmly at the left edge of the cassette to seat the gel in the base. A steady light illuminates on the iBase™ unit when the cassette is properly inserted.

Load the gel

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 wells #4 and #5 right next to the ladder well in the center (to avoid potential cross-contamination with the DNA ladder).

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

1. Add 20 µL of Low TE buffer to the purified, ligated DNA from "Purify the adapter-ligated and nick-translated DNA" on page 34 to dilute the volume to 40 µL.

2. Load *either* adjacent loading wells #2 and #3 *or* adjacent loading wells #6 and #7 with 20 µL each of diluted, ligated DNA.

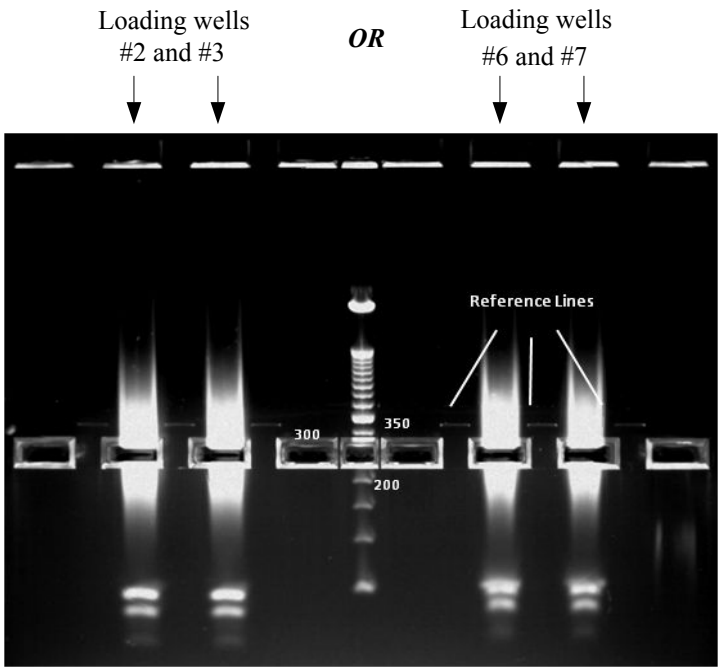


Figure 4 Use either of two pairs of adjacent loading wells.

3. 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 DNA Ladder.

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

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

Run the gel

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

2. Select the **Run SizeSelect 2%** program.

3. The run time to the reference line for a 200-bp DNA ladder band is in the range of 11–14.5 minutes, based on the Run Time Estimation Table in the *E-Gel® SizeSelect™ Agarose Gels Quick Reference*. If you are a new user, select the shorter run time.

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

5. Run the gel until the 200-bp ladder band reaches the reference line, then press **Go** again to stop the run.

   Note: Monitor the gel periodically during the run. If needed, extend the run time by entering very short run time settings.

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

7. Enter a run time to the collection well of 1–2 minutes, based on the Run Time Estimation Table in the *E-Gel® SizeSelect™ Agarose Gels Quick Reference*, and press **Go**.

8. Monitor the middle marker well (M) frequently, and stop the run when the 250-bp band is centered in the middle marker well. The 200-bp ladder band should be just below the middle marker well (M) and the 300-bp ladder band should be just above it, as shown in Figure 5.

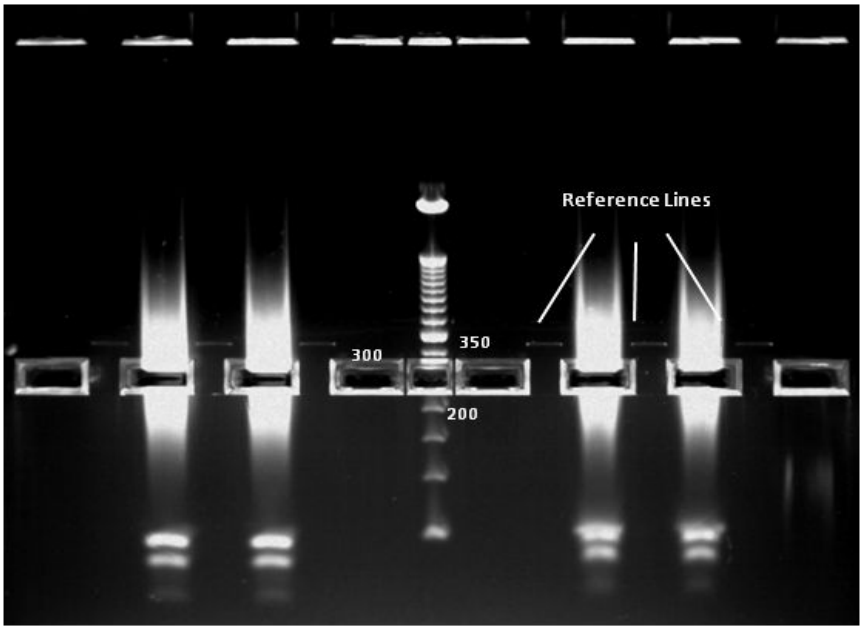


Figure 5 200--base-read library gel.

Collect the sample

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

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

3. Combine the recovered DNA from the two wells, for a total volume of ~60 µL. If the volume is below 60 µL, add Nuclease-free Water to bring it to a final volume of 60 µL. If the volume is more than 60 µL, aliquot only 60 µL for use in exome library preparation.

4. Dispose of the used gels as hazardous waste.

5. Proceed to "Purify the size-selected DNA" on page 40.

Purify the size-selected DNA

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

1. Add 108 µL of Agencourt® AMPure® beads (1.8X sample volume) to the 60-µL 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.

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

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

4. Repeat step 3 for a second wash.

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

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

7. Remove the tube from the magnetic rack, and add 30 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.

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

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

9. Proceed to Chapter 6, "Amplify and purify the size-selected fragment library".

6 Amplify and purify the size-selected fragment library

1. Add the following to an appropriately sized tube:

Component	Volume
Platinum® PCR SuperMix High Fidelity[1]	200 µL
Library Amplification Primer Mix[2]	20 µL
Purified, size-selected library DNA	30 µL
Total	**250 µL**

[1] Included in the Ion Plus Fragment Library Kit, or may also be purchased separately.
[2] From the Ion Plus Fragment Library Kit or from the Ion TargetSeq™ Exome Kit.

2. Mix by pipetting. Split the reaction mix into two 0.2-mL MicroAmp® Reaction Tubes, each containing 125 µL.

3. Place the tubes into a thermal cycler and run the following PCR cycling program:

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
8–10 cycles [1] (14–16 cycles for FFPE samples)	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Holding	Extend	70°C	5 min
Holding	—	4°C	Hold[2]

[1] Average yield for libraries fragmented with Ion Shear™ Plus Reagents amplified with 8 cycles of PCR has been ~1.0 µg.
[2] Not a stopping point; continue directly to the next steps.

4. Combine the previously split PCRs into a new 1.5-mL Eppendorf LoBind® Tube (250 µL total volume).

IMPORTANT! Use freshly prepared 70% ethanol for the next steps.

5. Add 375 µL of Agencourt® AMPure® XP Reagent (1.5X the 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.

6. 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.

7. 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.

8. Repeat step 7 for a second wash.

9. 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.

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

11. Remove the tube from the magnetic rack, and add 50 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.

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

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

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

STOPPING POINT Proceed immediately to quantification or store the library at –20°C. If storing, thaw on ice before use. To reduce the number of freeze-thaw cycles, store the library in several aliquots.

7 Quantify and qualify the amplified library

1. Quantify each purified, pre-capture library using the Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay.

2. Analyze the library on the Agilent® Bioanalyzer® instrument with an Agilent® High Sensitivity DNA Kit. Use 1 µL of a 1-ng/µL dilution (based on the Qubit® measurement; see Figure 6 below).

IMPORTANT! Ensure that there are minimal primer-dimers immediately adjacent to the marker or that there is minimal evidence of high molecular weight products due to over-amplification (concatemers). A small amount of high molecular weight species (as shown in Figure 6) will not adversely affect the enrichment.

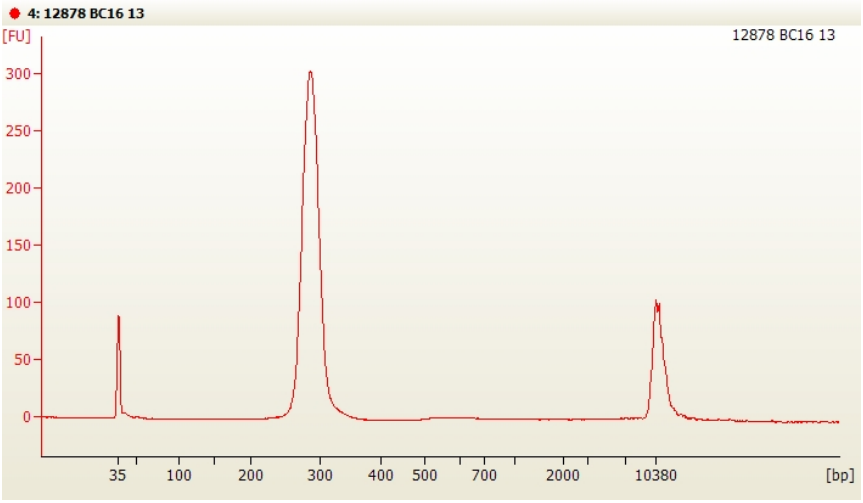


Figure 6 Example of a successfully amplified pre-capture library analyzed using an Agilent® 2100 Bioanalyzer® instrument and an Agilent® High Sensitivity DNA Kit. The 35-bp and 10380-bp peaks represent molecular weight markers.

Library amount needed

Use a total of **500 ng of amplified, size-selected library DNA** in each Ion TargetSeq™ capture reaction. You can process a single library or multiple barcoded libraries in a single capture.

The library amount(s) to use in each capture reaction are listed in the table below:

Library Type	Library amount needed
Single (non-barcoded) library	500 ng
Two barcoded libraries	250 ng each, pooled
Three barcoded libraries	167 ng each, pooled
Four barcoded libraries	125 ng each, pooled

Note: The number of barcoded libraries that you can pool in a single capture reaction depends on the depth of coverage over the targeted bases that you require for a given experiment. With 4.7 Gb of aligned sequence data per barcode for a single Ion PI™ chip loaded with two barcoded Ion TargetSeq™ Exome libraries, at least 80% target bases will be covered at a depth of 20X or more. Hybridizing more than two barcoded samples in a capture reaction is acceptable, but will require sequencing data to be merged from multiple Ion PI™ chip runs to obtain per-barcode coverage equivalent to a single two-barcode Ion PI™ chip run.

8 Exome enrichment

This section includes a probe hybridization reaction, followed by recovery of the probe-hybridized DNA and amplification and purification of the exome-enriched library.

The standard hybridization reaction is 64–72 hours. An accelerated hybridization reaction of 16–18-hours may also be used. See Appendix C, "Expected performance with 16–18-hour hybridization" for a discussion of the expected performance with the accelerated protocol.

Preventing sample loss

IMPORTANT! During probe hybridization, if the reaction tube is not completely sealed, or if the weight of the thermal cycler lid compresses the reaction tube and creates an air gap between the tube and cap, the reaction will dry out and your sample will be lost. **Carefully follow the guidelines described below to avoid sample loss.**

Before proceeding:

- Always make sure your tube is completely sealed before placing it in the thermal cycler.
- Test your tubes and thermal cycler by incubating 15 µL of water at 47°C overnight and measuring any loss of liquid. (Recovery of ~12.5 µL is acceptable.)
- To prevent reaction tubes from being compressed by the lid, place a rack supplied with your thermal cycler inside the instrument to support the weight of the lid.
- Alternatively, place empty tubes in the thermal cycler to evenly distribute the pressure of the lid.

Note: The Veriti® Thermal Cycler (Cat. no. 4375786, 4375305, or 438844) has a heated lid that can be set to 57°C, resulting in less risk of sample loss than thermal cyclers with lids that heat to 100°C or higher.

Hybridize the probes

Before starting

1. Remove a 4.5-µL aliquot of the Ion TargetSeq™ Exome Probe Pool (aliquoted as described on page "Aliquot and store the Ion TargetSeq Exome Probe Pool" on page 12 from –20°C storage and thaw on ice.
2. Thaw Ion TargetSeq™ Blockers P1 and A on ice or at room temperature.

3. Thaw Human Cot-1 DNA® Fluor QC on ice or at room temperature.
4. Thaw the Ion TargetSeq™ Hybridization Solution A and Hybridization Enhancer B at room temperature. If necessary, heat to 47°C to dissolve any precipitate.
5. Equilibrate a heat block to 95ºC.

Hybridization procedure

1. Add the following to a 1.5-mL Eppendorf LoBind® Tube:

Reagent	Volume
1 mg/mL Human Cot-1 DNA®	5 µL
Amplified non-barcoded library or pool of amplified barcoded libraries (500 ng total)	X µL[1]
Ion TargetSeq™ Blocker P1	5 µL
Ion TargetSeq™ Blocker A **or** Ion TargetSeq™ Blocker BC1-16 for barcoded samples	5 µL

[1] The volume that contains 500 ng of amplified, size-selected library, as determined in Chapter 7, "Quantify and qualify the amplified library".

2. Close the tube cap and make a hole in the cap with a clean 18–20 gauge or smaller needle.

   Note: The closed tube cap with a hole permits the sample to be dried down in a vacuum concentrator while minimizing the risk of cross-contamination.

3. Dry the sample in a vacuum concentrator set at 60°C (high heat) until completely dry (typically ~30 minutes).
4. Following dry-down, remove the tube from the concentrator and add the following:

Reagent	Volume
TargetSeq™ Hybridization Solution A (2X)	7.5 µL
TargetSeq™ Hybridization Enhancer B	3 µL

5. Cover the hole in the tube cap with a small piece of laboratory tape.
6. Vortex the tube for 10 seconds and centrifuge at maximum speed for 10 seconds.
7. Place the tube in a 95°C heat block for 10 minutes to denature the DNA.
8. Centrifuge the tube at maximum speed for 10 seconds at room temperature.
9. Transfer the sample to the 0.2-mL MicroAmp® Reaction Tube containing the 4.5-µL aliquot of the Ion TargetSeq™ Custom Probe Pool.
10. Close the cap on the tube tightly.

11. Vortex for 5 seconds and centrifuge at maximum speed for 10 seconds.

IMPORTANT! Before proceeding, make sure the tube cap is sealed tight to minimize the risk of sample evaporation. See also "Preventing sample loss" on page 45.

12. Transfer the reaction mixture to a thermal cycler with a heated lid and program for standard or accelerated hybridization as follows:

    - **Standard hybridization:** Incubate at 47°C for **64–72 hours** with the heated lid turned on and preferably set to maintain 57°C (+10°C above the hybridization temperature).
    - **Accelerated hybridization:** See Appendix C Appendix C, "Expected performance with 16–18-hour hybridization" for expected performance with accelerated hybridization time. Incubate at 47°C for **16–18 hours** with the heated lid turned on and preferably set to maintain 57°C (+10°C above the hybridization temperature).

    Note: The Veriti® 96-Well Fast Thermal Cycler is recommended because it includes a heated lid that may be set at 10°C higher than the hybridization temperature, which reduces the risk of evaporation. We have also verified that the GeneAmp® PCR System 9700 thermal cycler may be used in "9600 mode" with its default lid heating.

Wash and recover the probe-hybridized DNA

IMPORTANT! In the following procedure, it is critical that the water bath/heat block temperature be **closely monitored and maintained at 47°C**. Because the displayed temperature is often imprecise, we recommend that you use an external, calibrated thermometer.

Prepare the beads and solutions

1. Equilibrate the Dynabeads® M-270 Streptavidin to room temperature for 30 minutes before use.
2. Thaw the 10X Wash Solutions (A, B, C, and Stringent) and Magnetic Bead Wash provided in the TargetSeq™ Hybridization and Wash Kit.

   Note: Some of the wash solutions may appear cloudy after thawing. Warm at 47°C until they clarify.
3. Dilute the 10X Wash Solutions and Magnetic Bead Wash in Nuclease-free Water to create 1X working solutions, as shown in the table below. The volumes shown are for a single capture reaction. Scale up accordingly if you are processing additional libraries.

   Note: 1X solutions may be stored at room temperature for up to 2 weeks.

Stock solution	Volume of stock solution	Amount of Nuclease-free Water	Total volume of 1X Buffer
Wash Solution A (10X)	30 µL	270 µL	300 µL
Wash Solution B (10X)	20 µL	180 µL	200 µL
Wash Solution C (10X)	20 µL	180 µL	200 µL
Stringent Wash Solution (10X)	40 µL	360 µL	400 µL
Magnetic Bead Wash (2.5X)	200 µL	300 µL	500 µL

4. Preheat the following volumes of 1X wash buffers to 47°C for ~30 minutes in a water bath or heat block before use:
   - 400 µL of 1X Stringent Wash Solution
   - 100 µL of 1X Wash Solution A (the remaining 200 µL of 1X Wash Solution A should be kept at room temperature)

Prepare the Dynabeads® M-270 Streptavidin

1. Resuspend the Dynabeads® M-270 Streptavidin (warmed to room temperature as above) thoroughly by vortexing.
2. Transfer 100 µL of beads per library into a new 1.5-mL Eppendorf LoBind® Tube. (Up to 600 µL of beads may be prepared at once in a single tube, if you will be processing multiple tubes of libraries.)
3. Place the tube in a DynaMag™-2 magnet for ~3 minutes until the liquid becomes clear. Remove and discard the supernatant without disturbing the pellet. (Residual bead solution will be removed in the following wash steps.)
4. With the tube still on the magnet, add 1X Magnetic Bead Wash at twice the initial volume of beads (i.e., for 100 µL of beads, use 200 µL of wash).
5. Cap the tube, remove it from the magnet, and vortex for 10 seconds.
6. Place the tube back in the magnet ~1 minute until the solution clears, then remove and discard the supernatant.
7. Repeat steps 4–6 one more time.
8. Add 1X Magnetic Bead Wash at the same volume as the initial volume of beads (i.e., for 100 µL of beads, use 100 µL buffer).
9. Resuspend the beads by vortexing.
10. Aliquot 100 µL of resuspended beads into a new 0.2-mL MicroAmp® Reaction Tube. Repeat with separate tubes if you are processing >100 µL of beads.
11. Place the tube(s) in a DynaMag™-PCR magnetic rack until the solution clears, then remove and discard the supernatant. Leave the tube on the magnet. A small amount of residual wash solution may remain and will not interfere with binding.

12. The Dynabeads® M-270 Streptavidin is now ready to bind the captured DNA.

IMPORTANT! Proceed immediately to "Bind the DNA to the beads." Do not allow Dynabeads® M-270 SA Streptavidin to dry out.

Bind the DNA to the beads

1. Following incubation of the hybridization sample on the thermal cycler, transfer the complete sample to the tube of Dynabeads® M-270 Streptavidin prepared above.
2. Mix thoroughly by pipetting up and down 10 times.
3. Transfer the tube to a thermal cycler set to 47°C for 45 minutes (heated lid set to 57°C or higher). At 15 minute intervals, remove the tube and mix by vortexing for 3 seconds followed by a pulse spin to ensure that the beads remain in suspension. Immediately return the tube to the thermal cycler after each mixing.

   Note: We recommend moving the vortex mixer close to the thermal cycler for this step.

Heated bead wash

IMPORTANT! Throughout the following heated wash procedure, work quickly to ensure that the temperature of the wash solutions and beads do not drop much below 47°C.

---

1. After the 45-minute incubation, add 100 µL of the heated **1X Wash Solution A** (at 47°C) to the beads/DNA complex.
2. Mix by vortexing for 10 seconds.
3. Transfer the entire contents of each 0.2-mL tube to a 1.5-mL Eppendorf LoBind® Tube.
4. Place the tube in the DynaMag™-2 magnet until the solution clears, then remove and discard the supernatant.
5. Remove the tube from the magnet and add 200 µL of the heated **1X Stringent Wash Solution** (at 47°C).
6. Pipet up and down 10 times to mix.
7. Immediately incubate in a water bath or heat block at 47°C for 5 minutes.
8. Place the tube back in the magnet until the solution clears, then remove and discard the supernatant.
9. Repeat wash steps 5–8 with the heated **1X Stringent Wash Solution** one more time.

Room-temperature bead wash

1. Add 200 µL of room-temperature **1X Wash Solution A** and mix by vortexing for 2 minutes, followed by a pulse spin.
2. Place the tube in the DynaMag™-2 magnet until the solution clears, then remove and discard the supernatant.

3. Add 200 µL of room-temperature **1X Wash Solution B** and mix by vortexing for 1 minute, followed by a pulse spin.
4. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
5. Add 200 µL of room-temperature **1X Wash Solution C** and mix by vortexing for 30 seconds.
6. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
7. Add 30 µL of Nuclease-free Water to each tube.
8. Store the beads at –15°C to –25°C or proceed to amplification.

   Note: There is no need to elute DNA off the beads. The captured DNA on the beads will be used as template in the amplification reaction.

Amplify and purify the exome library

1. Thaw the Ion TargetSeq™ Amplification Primer Mix and Platinum® PCR SuperMix High Fidelity on ice or at room temperature.
2. To the 1.5-mL Eppendorf LoBind® Tube that contains the captured bead/DNA mixture, add the following (prepare a master mix for multiple reactions):

Component	Volume
TargetSeq™ capture beads with DNA	30 µL
Platinum® PCR SuperMix High Fidelity	200 µL
Ion TargetSeq™ Amplification Primer Mix	20 µL
Total	**250 µL**

3. Vortex the reaction for 5 seconds, then pulse-spin.
4. Split the volume into two PCR tubes (125 µL each).
5. Transfer the tubes to a thermal cycler and run the following program:

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
6–8 cycles	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Holding	Extend	70°C	5 min
Holding	—	4°C	Hold[1]

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

6. Pool the PCR reaction replicates into a single ~250-µL volume in a new 1.5-mL Eppendorf LoBind® Tube.

IMPORTANT! In the following step, the supernatant contains your sample. **Do not discard!**

---

7. Place the tube in a DynaMag™-2 magnet for at least 1 minute until the solution clears, then **remove and save the supernatant** in a new 1.5-mL Eppendorf LoBind® Tube. Discard the pellet.

IMPORTANT! Use freshly prepared 70% ethanol for the next steps.

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8. Add 375 µL of Agencourt® AMPure® XP Reagent (1.5X the 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.

9. 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.

10. 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.

11. Repeat step 10 for a second wash.

12. 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.

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

14. Remove the tube from the magnetic rack, and add 25 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.

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

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

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

17. Perform a second library cleanup to remove any residual primer dimers. Add 37.5 µL of Agencourt® AMPure® XP Reagent (1.5X the sample volume) to each 25 µL of elution of the exome library. 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.

18. Repeat steps 9-16 one more time for the rest of the cleanup and elution procedure.

STOPPING POINT Proceed immediately to Chapter 9, "Prepare the exome-enriched library for template preparation" or store the library at –20°C. If storing, thaw on ice before use.

9 Prepare the exome-enriched library for template preparation

This section describes how to assess the size distribution of the exome-enriched library, and quantify and dilute the library prior to template preparation using the Ion PI™ Template OT2 200 Kit v2 (Cat. no. 4485146) or the Ion PI™ Template OT2 200 Kit v3 (Cat. no. 4488318).

Note: Prior to template preparation, the optional qPCR-based assay described in Appendix B, "qPCR analysis of control loci" can be used to determine whether exome enrichment was successful.

Assess the size distribution of the exome-enriched library

Analyze 1 µL of undiluted exome-enriched library on the Agilent® Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit (Cat. no. 5067-4626), as shown in the figure below.

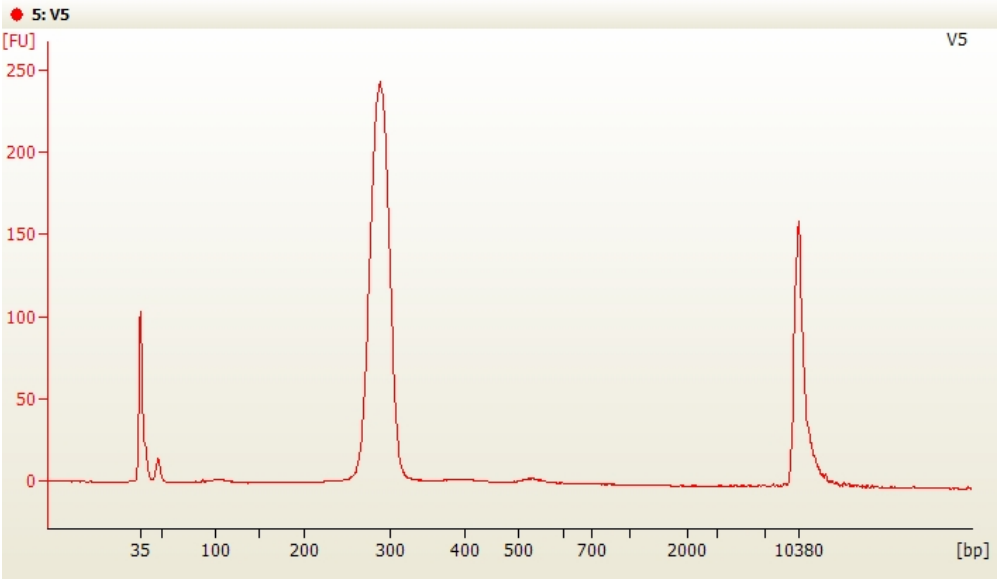

Figure 7 Example of a successfully amplified post-capture exome-enriched library analyzed using an Agilent® 2100 Bioanalyzer® instrument and an Agilent® High Sensitivity DNA Kit. The low molecular-weight species that are less than 100 bp are excess primers, and do not interfere significantly with use of the library in template preparation. The 35-bp and 10380-bp peaks represent molecular weight markers.

Quantify the exome-enriched library and determine the dilution required for template preparation

Quantify the exome-enriched library and determine the library dilution that results in a concentration within the optimal range for template preparation. You can quantify the library by qPCR using the Ion Library Quantitation Kit or by Agilent® Bioanalyzer® analysis.

Quantitation method	Features
Ion Library Quantitation Kit (qPCR)	• Higher sensitivity: Recommended for low-yield libraries. • Higher precision: A single library dilution is usually sufficient for optimized template preparation. • Kit includes standards for direct determination of the library dilution. • Accurately quantifies libraries with minimal impact from background material.
Bioanalyzer® analysis	• Determines the molar concentration of the library, from which the library dilution is calculated. • The same Bioanalyzer® analysis can be used to assess the library size distribution and determine the library concentration. • Lower sensitivity: Requires more amplified library to obtain sufficient material for quantitation. • Lower precision: Libraries quantified by Bioanalyzer® analysis must be titrated over a 4--fold concentration range for optimized template preparation.

Ion Library Quantitation Kit method

1. Use the Ion Library Quantitation Kit (Cat. no. 4468802) to directly determine the correct library dilution using real-time quantitative PCR (qPCR). This kit uses serial dilutions of an *E. coli* DH10B Control Library to generate a standard curve, from which the exome-enriched library concentration is calculated.

2. From the results, determine the dilution factor that results in a concentration of ~100 pmol/L. This concentration is suitable for template preparation using the Ion PI™ Template OT2 200 Kit v2 or v3 following the recommendation for Ion TargetSeq™ library input in the *Ion PI™ Template OT2 200 Kit v2 User Guide* (Pub. no. MAN0007624) or *Ion PI™ Template OT2 200 Kit v3 User Guide* (Pub. no. MAN0009133).
   To determine the dilution factor, use the following formula:
   Dilution factor = (Library concentration in pM)/100 pM

Example

The library concentration is 15,000 pM.

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

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

Agilent® 2100 Bioanalyzer® quantitation method

1. Use the Agilent® 2100 Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit to determine the molar library concentration in pmol/L. 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. From the results, determine the dilution factor that results in a concentration of ~100 pmol/L. This concentration is suitable for template preparation using the Ion PI™ Template OT2 200 Kit v2 or v3 following the recommendation for Ion TargetSeq™ library input in the *Ion PI™ Template OT2 200 Kit v2 User Guide* (Pub. no. MAN0007624) or *Ion PI™ Template OT2 200 Kit v3 User Guide* (Pub. no. MAN0009133).

   To determine the dilution factor, use the following formula:

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

Example

The library concentration is 15,000 pM.

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

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

Note: Because Bioanalyzer® instrument 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 ½X library dilution (~50 pM), 1X library dilution (~100 pM), and 2X library dilution (~200 pM) to ensure that one or more dilutions are in the optimal concentration range.

Proceed to template preparation

Prior to template preparation, dilute an appropriate aliquot of the library to the correct concentration as described in the previous sections. Refer to the *Ion PI™ Template OT2 200 Kit v2 User Guide* (Pub. no. MAN0007624) or the *Ion PI™ Template OT2 200 Kit v3 User Guide* (Pub. no. MAN0009133) for the volume of diluted library needed for template preparation.

Note: Diluted libraries should be stored at 4°C to 8°C and used within 48 hours. Store undiluted libraries at –30°C to –10°C.

A

Troubleshooting

Observation	Possible cause	Recommended action
Hybridization reaction dries out	Tube is not sealed well	Prepare a new exome-enriched library. When setting up the hybridization reaction, carefully follow the recommendations under "Preventing sample loss" on page 45.
Amount of pre-enriched fragment library is low following amplification	Inefficient thermal cycler	Amplify for two more cycles, though sample complexity may decrease.
		Use a different thermal cycler.
High off-target rate and poor coverage depth	Blockers not included in hybridization reaction	Repeat enrichment procedure, including blockers.
	1X wash buffers not properly warmed to 47°C	Preheat the 1X wash buffers to 47°C for at least 2 hours in a water bath or heat block as described on page "Prepare the beads and solutions" on page 47. To avoid buffer cooling, wash the DNA bound to the beads rapidly and thoroughly and do not attempt to process too many samples at once (4-6 per operator maximum).
Too few unique sequencing reads (low complexity)	Started with less than 1 µg high-quality DNA	Start with at least 1 µg of DNA. To ensure the quality of the DNA, treat the DNA with RNase I and analyze integrity and size by gel electrophoresis as described in Appendix C of the *Ion Xpress™ Plus Fragment Library Preparation User Guide* (Pub. no. MAN0009847).

B qPCR analysis of control loci

Measure enrichment by qPCR

Use the qPCR assay described in this section to assess sample enrichment with three positive control primer pairs. The genomic loci targeted by the primer pairs are included as capture targets in every exome library.

Following qPCR, you can subtract the C_T value of the exome-enriched DNA library from the ΔC_T value of the unenriched DNA library to determine the DC_T for each control.

qPCR control primers

The forward and reverse control primer sequences are listed below. Use all four sets when comparing the enriched library to the unenriched library.

Gene	Primer type	Primer sequence
RUNX2	Forward	5′-CGC ATT CCT CAT CCC AGT ATG-3′
	Reverse	5′-AAA GGA CTT GGT GCA GAG TTC AG-3′
PRKG1	Forward	5′-CCC ACC GCC TTC GAC AT-3′
	Reverse	5′-CCT GCT TAC TGT GGG CTC TTG-3′
SMG1	Forward	5′-CTC GCT TAA CCA GAC TCA TCT ACT GT-3′
	Reverse	5′-ACT TGG CTC AGC TGT ATG AAG GT-3′

Note: The following protocol has been optimized for the StepOnePlus™ Real Time PCR System and SYBR® GreenER™ qPCR SuperMix. Using a different thermal cycler or reagents could require altering these conditions to achieve optimal results.

qPCR reaction setup

Prepare 20-µL qPCR reactions for each exome-enriched and unenriched library (3 positive control primer-pairs and 1 NTC). We recommend running each templated reaction in triplicate along with no-template controls (NTC) in duplicate. So if you are comparing an exome-enriched to an unenriched library along with no-template

controls (NTC), you will be running a minimum of 24 qPCR reactions (2 libraries × 3 primer sets × 3 triplicate reactions + 1 NTC × 3 primer sets × 2 duplicate reactions).

1. Prepare the following qPCR master mix for each primer set and transfer to the appropriate wells of a PCR plate.

Component	Single reaction	5 reactions
SYBR® GreenER™ qPCR SuperMix	10 µL	50 µL
Forward Primer (10 µM)	0.4 µL	2 µL
Reverse Primer (10 µM)	0.4 µL	2 µL
Nuclease-free Water	4.2 µL	21 µL
Total	**15 µL**	**75 µL**

2. Dilute each unenriched library and exome-enriched library to 0.1 ng/mL. Prepare enough of each diluted library to run at least 9 reactions (three primer sets in triplicate). Add 5 mL of diluted library to each appropriate qPCR reaction.

3. Transfer the plate to a thermal cycler and run the following program (may vary by thermal cycler):

Stage	Step	Temperature	Time
Holding	Denature	95°C	10 min.
40 cycles	Denature	95°C	10 sec.
	Extend	60°C	1 min.
Melting Curve	Denature	95°C	15 sec.
		60°C	1 min.
		95°C	Continuous
Holding	Hold	4°C	Hold [1]

[1] Not a stopping point; continue directly to data analysis.

Data analysis

Calculate the ΔC_T for each of the four test loci by subtracting the C_T value of the exome-enriched DNA library from the C_T value of the unenriched DNA library. For each of the three positive qPCR tests, values should fall within the parameters shown in Figure 8 and the table on the next page.

ΔC_T values that are two standard deviations below the average (see the table on the next page) for positive control loci or greater than zero for the negative locus indicate that the enrichment was inefficient or of low specificity and may yield poor sequencing and coverage results.

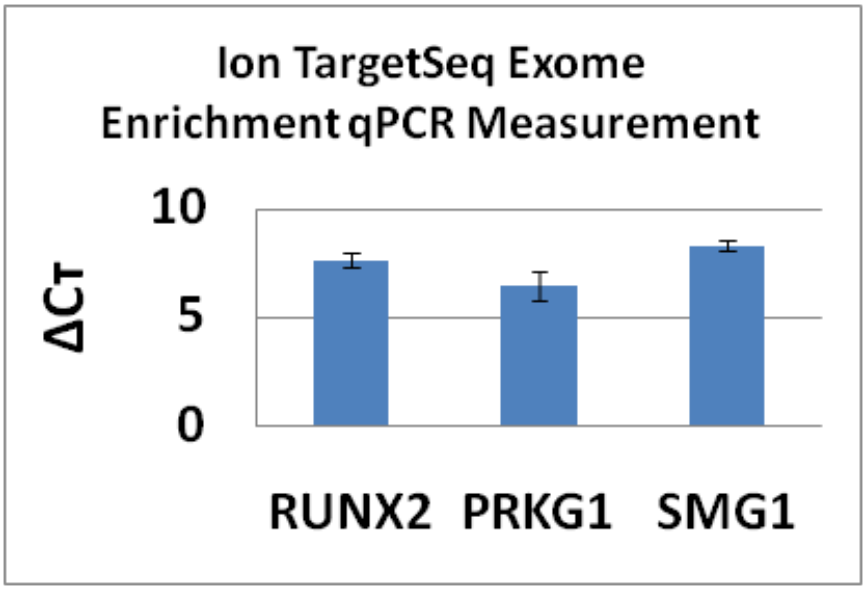

Figure 8 Example qPCR results; ΔC_T for each control locus.

Gene/Amplicon	Expected ΔC_T (measured from 41 libraries)
RUNX2	7.6 ± 0.3
PRKG1	6.5 ± 0.7
SMG1	8.3 ± 0.2

These results were generated from exome-enriched libraries prepared from NA12878 and NA12891 genomic DNA samples.

Note: If the ΔC_T value for any one of the test loci is significantly lower, it does not necessarily indicate a failure to enrich the exome but may be due to genomic polymorphism specific to a given sample DNA.

C

Expected performance with 16–18-hour hybridization

When speed of data acquisition is favored, you can make a trade-off between depth of exome coverage and time to results. The following section provides empirical data on the magnitude of this trade-off.

The capture probes provided with Ion TargetSeq™ Exome Kits have been optimized to provide high specificity of capture and high uniformity of coverage using the standard 64–72-hour hybridization time. Reducing the hybridization time to 16–18 hours will result in a shorter protocol but reduced specificity. With the accelerated hybridization time, 65–75% of mapped reads can be expected to overlap targeted regions by at least one base, compared to a typical "on-target" rate of 75–85% for the standard hybridization time. The net effect is that the accelerated protocol will produce reduced average coverage of target bases from a given amount of aligned sequence data.

To illustrate, the coverage profiles of two exome datasets obtained on separate Ion PI™ Chips and each corresponding to 6.8 Gb of aligned sequence data are shown in the figure below.

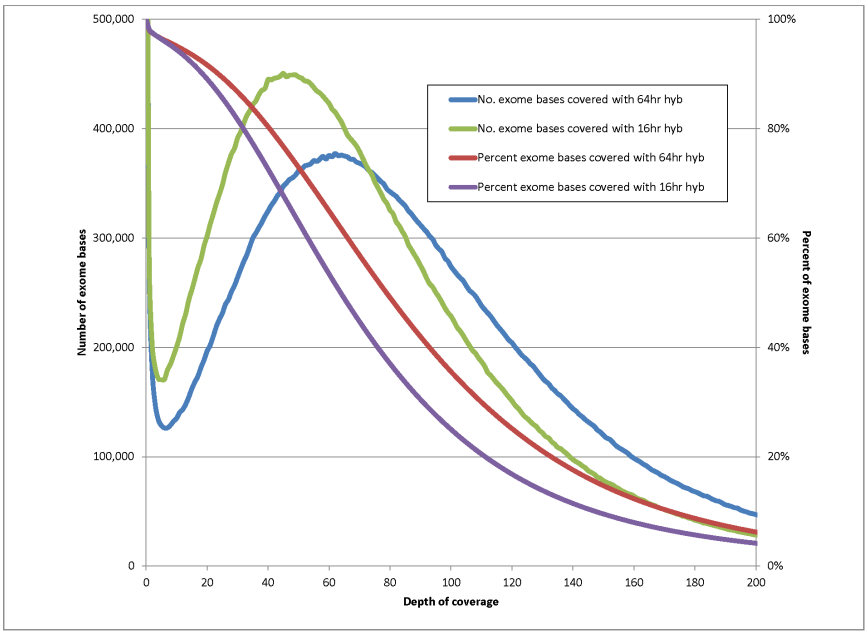

Figure 9 A comparison of 6.8-Gb datasets obtained on an Ion Proton™ System. Ion TargetSeq™ exome libraries were prepared using either the default 64-hour hybridization protocol or an "accelerated" 16-hour hybridization protocol.

In this case, the accelerated 16-hour hybridization protocol yielded an average coverage depth of 77X. In contrast, the standard 64-hour hybridization yielded an average coverage depth of 92X. The green and purple curves correspond to data obtained from an Ion TargetSeq™ Exome library prepared with the 16-hour hybridization, while the blue and red curves correspond to data from the 64-hour hybridization.

At moderate depths—20X for example—the 16-hour protocol yields data that covers ~4–5% less of the exome than data from the 64-hour protocol. At greater depths (e.g., 50X–100X), this disparity grows slightly to ~10%, as can be seen by comparison of the red and purple curves; however, this reduction in coverage is typically considered minimal.

D

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

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- 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).
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- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

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

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

Documentation and support

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

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