USER GUIDE

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by life technologies™

Ion TargetSeq™ Custom Enrichment Kits

Catalog Numbers A14228, A14229, and A14230

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life technologies™

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Contact information

Phone:

In North America: 1-87-SEQUENCE (1-877-378-3623)

Outside of North America: +1-203-458-8552

Address:

246 Goose Lane
Suite 100
Guilford, CT 06437
USA

7000 Shoreline Court
Suite 201
South San Francisco, CA 94080
USA

Web site: www.iontorrent.com

Ion community: ioncommunity.iontorrent.com

Support email: ionsupport@lifetech.com

Table of Contents

Product information

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

Product description

Ion TargetSeq™ Custom Enrichment Kits provide optimized reagents for capturing defined target regions from genomic DNA (gDNA) that has been fragmented and constructed into libraries using the Ion Xpress™ Plus Fragment Library Kit (part no. 4471269) or the Ion Plus Fragment Library Kit (part no. 4471252).

When you order an Ion TargetSeq™ Custom Enrichment Kit, you specify the target region that you want to enrich as described in the *TargetSeq™ Custom Enrichment Kit Guide for TargetSeq™ Orders* (available for download at **ioncommunity.iontorrent.com**). The kit will depend on the size of the region you are targeting (20–500 kb, 500 kb – 2 Mb, or >2 Mb). Ion TargetSeq™ Custom Probes—biotinylated oligos that range in size from ~50 to 120 bases—are part of the kit and are specifically designed for the region that you specify.

You then construct an unamplified fragment library from gDNA following the procedure provided in the *Ion Xpress™ Plus gDNA and Amplicon Library Preparation User Guide* (part no. 4471989).

Following library size selection, you amplify and quantitate the library as described in this manual, then hybridize the Ion TargetSeq™ Custom Probes to the library fragments using either a double hybridization or single hybridization procedure, depending on the size of the target region. Hybridization specificity is ensured by the use of blocker DNA sequences (Human Cot-1 DNA® and adapter-specific oligos).

The bound DNA is isolated using streptavidin-coated Dynabeads® paramagnetic beads, and then amplified and purified. The purified, target-enriched sample is then returned to the Ion Torrent™ system workflow for emulsion PCR, enrichment, and sequencing.

Components and storage conditions

The Ion TargetSeq™ Custom Enrichment Kits are available as three separate catalog numbers, depending on the size of the target region that your custom probe pool is designed for:

- Ion TargetSeq™ Custom Enrichment Kit 20–500 kb (Catalog no. A14230)
- Ion TargetSeq™ Custom Enrichment Kit 500 kb – 2 Mb (Catalog no. A14229)
- Ion TargetSeq™ Custom Enrichment Kit >2 Mb (Catalog no. A14228)

The components of each catalog number are as follows:

Components	A14230 and A14229	A14228	Storage
Ion TargetSeq™ Custom Probe Pool (custom-designed per your order)	2 × 216 µL	2 × 216 µL	–30°C to –10°C
Ion TargetSeq™ Amplification Primer Mix	2 × 1 mL	1 mL	
Ion TargetSeq™ Blocker P1	1 mL	1 mL	
Ion TargetSeq™ Blocker A	1 mL	1 mL	
Ion TargetSeq™ Blocker BC 1–16	1 mL	1 mL	
Human Cot-1 DNA®	1 mL	1 mL	
Low TE	25 mL	25 mL	
TargetSeq™ Wash Solution A (10X)	8 × 750 µL	4 × 750 µL	
TargetSeq™ Wash Solution B (10X)	8 × 500 µL	4 × 500 µL	
TargetSeq™ Wash Solution C (10X)	8 × 500 µL	4 × 500 µL	
TargetSeq™ Stringent Wash Solution (10X)	8 × 1 mL	4 × 1 mL	
TargetSeq™ Hybridization Solution A (2X)	8 × 190 µL	4 × 190 µL	
TargetSeq™ Hybridization Enhancer B	8 × 75 µL	4 × 75 µL	
TargetSeq™ Magnetic Bead Wash (2.5x)	8 × 5 mL	4 × 5 mL	
Dynabeads® M-270 Streptavidin	2 × 10 mL	10 mL	2–8°C

Aliquot and store the Ion TargetSeq™ Custom Probe Pool

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

1. If frozen, thaw the Ion TargetSeq™ Custom 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 PCR tubes and store at –30°C to –10°C until use. The presence of some residual volume after dispensing all single-use aliquots is normal.

Materials and equipment required but not included

✓	Description	Supplier	Part number	Quantity
	Platinum® PCR SuperMix High Fidelity	Life Technologies	12532016	4.5 mL[†]
	Ion Xpress™ Plus Fragment Library Kit (for libraries prepared by enzymatic fragmentation)	Life Technologies (Ion Torrent)	4471269	1
	Ion Plus Fragment Library Kit (for libraries prepared by physical fragmentation)	Life Technologies (Ion Torrent)	4471252	1
	Ion Xpress™ Barcode Adapters 1–16 Kit (for barcoded libraries)	Life Technologies (Ion Torrent)	4471250	1
	Qubit® 2.0 Fluorometer	Life Technologies	Q32866	1
	Qubit® dsDNA HS Assay	Life Technologies	Q32851 or Q32854	100 assays or 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
	Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1
	DynaMag™-2 magnet (magnetic rack)	Life Technologies (Invitrogen)	123-21D	1
	DynaMag™-PCR magnet (for 0.2-mL tubes)	Life Technologies (Invitrogen)	49-2025	1
	1.5-mL LoBind Tubes	Eppendorf	022431021	1 box
	0.2-mL PCR tubes **or** 8-tube strips and 8-cap strips	MLS[‡]	N/A	—
	Microcentrifuge	MLS	N/A	1
	Thermal cycler	MLS	N/A	1
	Nuclease-free water	Life Technologies (Applied Biosystems)	AM9932	1000 mL
	Ethanol, absolute	MLS	Varies	Varies
	Vacuum concentrator (for 1.5-mL tubes)	MLS	Varies	1
	Water bath or heat block	MLS	Varies	1
	Microcentrifuge	MLS	Varies	1
	Calibrated thermometer	MLS	Varies	1
	Rotator	MLS	N/A	1
	Vortex mixer	Rotator	MLS	N/A
	Pipettors 1–1000 µL	MLS	N/A	1 each

[†]Amount needed per TargetSeq™ workflow varies: 400 µL for target regions < 2 MB, 200 µL for target regions > 2 Mb

[‡]Major laboratory supplier

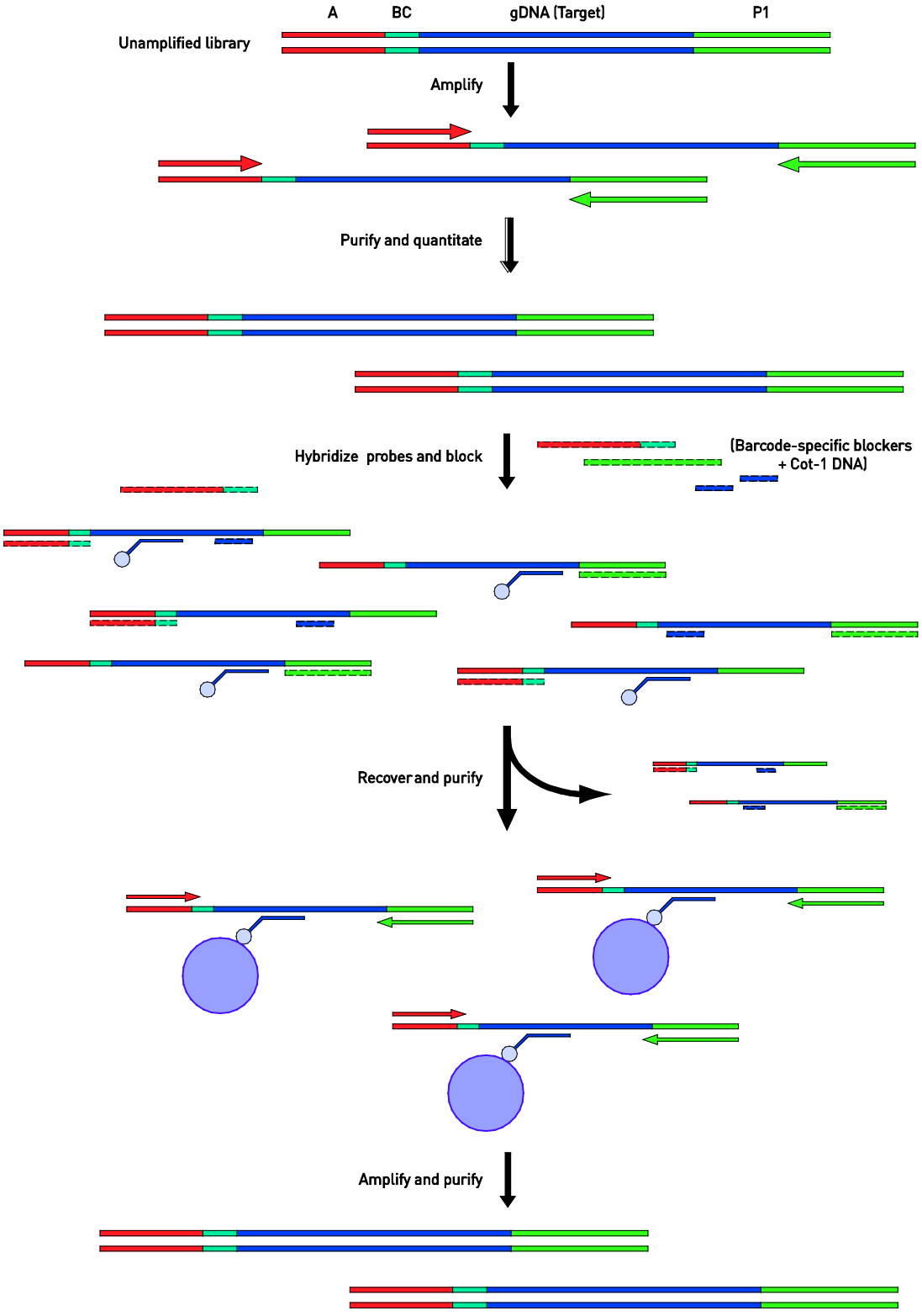
Workflow timelines

Target regions > 2 Mb (single 3-day hybridization)			
Day	**Step**	**Description**	**Time (hours)**
Day 1	1	Prepare library	3
	2	Amplify and purify	1.5
	3	Quantitate	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 enriched library	1.5
	9	Set-up and start emulsion PCR (usually leave overnight)	3-4
Day 5	10	Break emulsion and enrich ISPs	1.5
	11	Sequence on the PGM™ System	2
	12	Analysis	1-2

Target regions < 2 Mb (two overnight hybridizations)			
Day	**Step**	**Description**	**Time (hours)**
Day 1	1	Prepare library	3
	2	Amplify and purify	1.5
	3	Quantitate	1.5
	4	Set up and start first hybridization (overnight, >12 hours)	1
Day 2	5	Wash and recover beads with enriched DNA	2
	6	Amplify and purify	1.5
	7	Set-up and start second hybridization (overnight, >12 hours)	1
Day 3	8	Wash and recover beads with enriched DNA	2
	9	Amplify and purify	1.5
	10	Quantify enriched library	1.5
	11	Set-up and start emulsion PCR (usually leave overnight)	3-4
Day 4	12	Break emulsion and enrich ISPs	1.5
	13	Sequence on the PGM™ System	2
	14	Analysis	1-2

Workflow diagram

Barcoded libraries are shown in the diagram. The workflow is the same for non-barcoded libraries.



Prepare the fragment library

Library preparation requirements

- **Starting material:** Each fragment library should be constructed from **1 µg of genomic DNA** using the Ion Xpress™ Plus Fragment Library Kit (part no. 4471269) or Ion Plus Fragment Library Kit (part no. 4471252).
- **Shearing method:** Libraries may be created from enzymatically or mechanically fragmented DNA, as described in the *Ion Xpress™ Plus gDNA and Amplicon Library Preparation User Guide* (part no. 4471989).
- **Barcoding:** Barcoding is optional. Libraries may be prepared using *either* the standard (non-barcoded) adapters provided with the Ion Fragment Library Kits *or* with the barcode adapters provided in the Ion Xpress™ Barcode Adapters 1–16 Kit (part no. 4471250).
- **Size selection:** Proceed with library preparation through size selection. Libraries size selected using E-Gel® SizeSelect™ Agarose Gels require an additional purification procedure as described below.
- **Amplification: Do not use the library amplification procedure described in the *Ion Xpress™ Plus gDNA and Amplicon Library Preparation User Guide*.** Instead, follow the amplification instructions starting on page 10 of this user guide.

For libraries prepared using E-Gel® SizeSelect™ Gels:

If you size-selected the library using the E-Gel® SizeSelect™ Gel, you must purify the size-selected DNA with AMPure® XP Reagent before proceeding to amplification. Follow the steps below:

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

1. Bring the volume of the combined recovered DNA from the E-Gel® SizeSelect™ Gel to exactly 60 µL by adding Nuclease-free Water or by taking only 60 µL from the total volume recovered.
2. Add 108 µL of Agencourt® AMPure® XP Reagent (1.8× sample volume) to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse-spin the tube, and incubate the mixture for 5 minutes at room temperature.
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. Carefully remove and discard the supernatant without disturbing the pellet.
4. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads. After the solution clears, remove and discard the supernatant without disturbing the pellet.
5. Repeat step 4 for a second wash.
6. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
7. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
8. Remove the tube from the magnetic rack, and add 40 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.
9. Pulse spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. **Transfer the supernatant** containing the eluted DNA to a new 1.5-mL LoBind tube without disturbing the pellet.

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

Amplify and purify the library

After library size selection (as described in the *Ion Xpress™ Plus gDNA and Amplicon Library Preparation User Guide*), amplify each library using the instructions provided in this section.

Materials provided in the kit

- Ion TargetSeq™ Amplification Primer Mix
- Low TE

Other materials and equipment

- Platinum® PCR SuperMix High Fidelity
- Thermal cycler
- 0.2-mL PCR tubes
- 1.5-mL LoBind tubes
- Agencourt® AMPure® XP Kit
- Freshly prepared 70% ethanol
- DynaMag™-2 magnet
- 1.5-mL LoBind tubes

IMPORTANT! For libraries size-selected using the E-Gel® SizeSelect™ Gel, first purify the size-selected DNA as described on page 9.

1. Add the following to an appropriately sized tube:

Component	Volume (for 1 µg input DNA)
Platinum® PCR SuperMix High Fidelity†	200 µL
Library Amplification Primer Mix‡	10 µL
Purified, size-selected library DNA	40 µL
Total	**250 µL**

†Included in the Ion Plus Fragment Library Kit, or may also be purchased separately (see page 6).
‡From the Ion Plus Fragment Library Kit.

2. Mix by pipetting. Split the reaction mix into two 0.2-mL PCR tubes, each containing 125 µL.

   Note: For barcoded libraries, the amount of each library needed depends on the number of libraries being pooled, as shown in the table under "Library amounts needed" on page 13. Consequently, the amount of amplification (number of cycles) that is needed per library will vary accordingly. This is reflected in the range given for the recommended number of PCR cycles in the table below.

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
12–14 cycles	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	72°C	1 min
Holding	—	4°C	∞

* Average yield for libraries amplified with 12 cycles of PCR has been ~ 500 ng to 1 µg.

4. Combine the previously split PCRs into a new 1.5-mL 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 8 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 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 LoBind tube without disturbing the pellet.

STOPPING POINT Proceed immediately to quantitation 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.

Quantitate and qualify each amplified library

Required materials and equipment

- Qubit® 2.0 Fluorometer
- Qubit® dsDNA HS Assay
- Ion Library Quantitation Kit
- Agilent Bioanalyzer™ instrument
- Agilent High Sensitivity DNA Kit
- Low TE

Quantitate each library using the Qubit® 2.0 Fluorometer and the Agilent Bioanalyzer™ instrument

1. Quantitate 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 1:5 dilution in Low TE.

IMPORTANT! Ensure that there are no excessive primer-dimers immediately adjacent to the marker or that there are no excessive over-amplification products (concatemers).

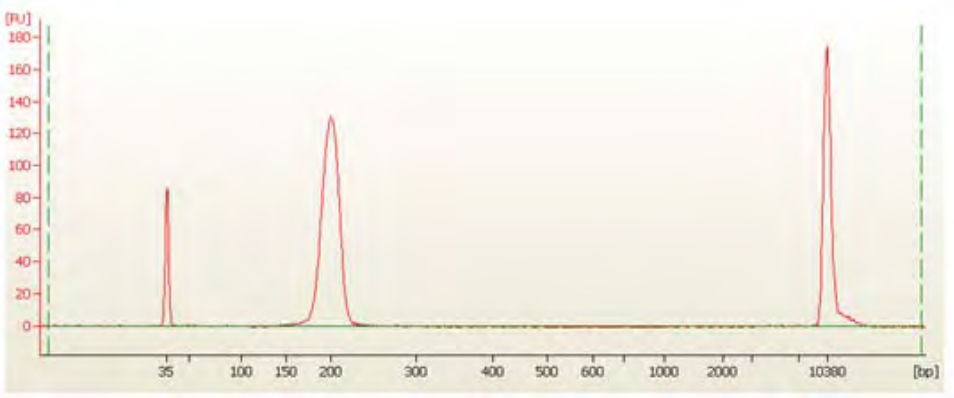


Figure 1: Example of a successfully amplified pre-capture library analyzed using an Agilent High Sensitivity DNA Kit.

Pool barcoded libraries

When pooling barcoded libraries, use each library quantity as determined with the Qubit® 2.0 Fluorometer. Mix equal masses of each library to obtain a total pooled library mass of 500 ng for each TargetSeq™ enrichment reaction, as described below.

Library amounts 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 up to 16 pooled barcoded libraries in a single capture reaction.

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
...	...
Sixteen barcoded libraries	31.3 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. For example, with ~130 Mb of AQ17 data, 4 barcoded samples have each been sequenced to an average depth of ≥182X with > 86% of target bases covered at ≥ 20X depth, while 12 barcoded samples have each been sequenced to an average depth of ≥ 64X with > 70% of target bases covered at ≥ 20X depth for a target that is 200 kb in size.

Capture procedure for target regions < 2 Mb

Use the following double-hybridization procedure for target regions < 2 Mb. This procedure takes ~3 days, with two overnight hybridization reactions.

Ion TargetSeq™ Blockers

The Ion TargetSeq™ Blockers provided in each kit are pre-designed and diluted oligos used to block the non-barcoded and barcoded adapters used in library construction.

- Use **Ion TargetSeq™ Blocker P1** and **Ion TargetSeq™ Blocker A** if you are blocking the standard Adapters provided in the Ion Xpress™ Plus Fragment Library Kit.
- Use **Ion TargetSeq™ Blocker P1** and **Ion TargetSeq™ Blocker BC 1–16** if you are blocking the barcoded adapters provided in the Ion Xpress™ Barcode Adapters 1–16 Kit.

First hybridization

Materials provided

- Ion TargetSeq™ Custom Probe Pool
- Ion TargetSeq™ Blocker P1
- Ion TargetSeq™ Blocker A ***or***
  Ion TargetSeq™ Blocker BC 1–16
- Human Cot-1 DNA® (1 mg/mL)

Other materials and equipment

- 18–20 gauge or smaller needle
- Vacuum concentrator
- Heat block
- Calibrated thermometer
- Thermal cycler
- Microcentrifuge
- 0.2-mL PCR tubes/strip tubes/plate wells

Preventing sample loss

IMPORTANT! The probe hybridization reaction runs overnight at 47°C. **If the tube is not completely sealed, the reaction will dry out and your sample will be lost.**

In particular, be careful to avoid the following:

- The tube cap is not completely sealed before the reaction is placed in the thermal cycler
- The weight of the thermal cycler lid compresses the tube and creates an air gap between the tube and cap

Before proceeding, we recommend that you test your tubes and thermal cycler by incubating 15 µL of water at 47°C overnight and measuring any loss of sample. (Recovery of ~14 µL is acceptable.) We also recommend that you use any rack supplied with your thermal cycler that is designed to prevent tube compression when the lid is closed. Alternatively, you can add empty tubes to the thermal cycler to evenly distribute the pressure of the lid.

Before starting

1. Remove a 4.5-µL aliquot of the Ion TargetSeq™ Custom Probe Pool (aliquoted as described on page 5) from –20°C storage and thaw on ice.
2. If necessary, pool equal masses of barcoded libraries as described in "Library amounts needed" on page 13. Total library mass = 500 ng.
3. Thaw Ion TargetSeq™ Blocker P1 on ice or at room temperature.

4. Thaw Ion TargetSeq™ Blocker A (for a single library) ***or*** Ion TargetSeq™ Blocker BC 1–16 (if using pooled barcoded libraries) on ice or at room temperature.
5. Thaw Human Cot-1 DNA® on ice or at room temperature.
6. Thaw the TargetSeq™ Hybridization Solution A and Hybridization Enhancer B at room temperature. If necessary, heat to 47°C to dissolve any precipitate.
7. Equilibrate a heat block to 95°C.

First hybridization procedure

1. Add the following to a 1.5-mL 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)	_____ µL
Ion TargetSeq™ Blocker P1	5 µL
Ion TargetSeq™ Blocker A ***or*** Ion TargetSeq™ Blocker BC 1–16 (if using library pool)	5 µL

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 half (2.25 µL) of the 4.5-µL single-use aliquot of the Ion TargetSeq™ Custom Probe Pool to a new 0.2-mL PCR tube. Store the remaining aliquot at –20°C for use the next day in the second hybridization reaction.
10. Add 2.25 µL of nuclease-free water to the probe pool.
11. Transfer the library or library pool to the 0.2-mL PCR tube containing the diluted probe pool.
12. Close the cap on the tube tightly.
13. 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 14.

14. Transfer the reaction mixture to a thermal cycler with heated lid. Incubate at 47°C overnight (≥ 12 hours) with the heated lid turned on.

    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 tested the GeneAmp® PCR System 9700 thermal cycler from Applied Biosystems® with the default lid heating.

Wash and recover the probe-hybridized DNA

Materials provided

- TargetSeq™ Hybridization and Wash Kit
- Dynabeads® M-270 Streptavidin (10 mg/mL)

Other materials and equipment

- Nuclease-free water
- 1.5-mL LoBind tubes
- DynaMag™-2 magnet
- DynaMag™-PCR magnetic rack
- 0.2-mL PCR tubes/strip tubes/plate wells
- Vortex mixer
- Thermal cycler

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

Prepare the wash solutions

1. Thaw the 10X Wash Solutions (A, B, C, and Stringent) and Magnetic Bead Wash provided in the TargetSeq™ Hybridization and Wash Kit.

   Note: The 10X Wash Solution A may appear cloudy after thawing. Warm at 47°C until it clarifies.

2. 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

3. Preheat the following 1X wash buffers to 47°C for at least 2 hours in a water bath or heat block:
   - 400 µL 1X Stringent Wash Solution
   - 100 µL 1X Wash Solution A
4. Equilibrate the Dynabeads® M-270 Streptavidin to room temperature for 30 minutes prior to use.

Prepare the Dynabeads® M-270 Streptavidin

1. Resuspend the Dynabeads® M-270 Streptavidin thoroughly by vortexing.
2. Transfer 100 µL of beads per sample into a new 1.5-mL LoBind tube. (Up to 600 µL of beads may be prepared at once in a single tube, if you will be processing multiple samples.)
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. (Any 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 PCR 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 are 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.

Wash the bound DNA

1. After the 45-minute incubation, add 100 µL of the 1X Wash Solution A heated to 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 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 **1X Stringent Wash Solution** heated to 47°C.
6. Pipet up and down 10 times to mix. Work quickly so that the temperature does not drop much below 47°C.
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 the **1X Stringent Wash Solution** wash (steps 5–8) one more time.
10. Add 200 µL of room-temperature **1X Wash Solution A** and mix by vortexing for 2 minutes, followed by a pulse spin.

11. Place the tube in the DynaMag™-2 magnet until the solution clears, then remove and discard the supernatant.
12. Add 200 µL of room-temperature **1X Wash Solution B** and mix by vortexing for 1 minute, followed by a pulse spin.
13. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
14. Add 200 µL of room-temperature **1X Wash Solution C** and mix by vortexing for 30 seconds.
15. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
16. Add 40 µL of nuclease-free water to each tube.
17. 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 first capture reaction

Materials provided

- Ion TargetSeq™ Amplification Primer Mix
- Low TE

Other materials and equipment

- Platinum® PCR SuperMix High Fidelity
- Thermal cycler
- 0.2-mL PCR tubes
- Agencourt® AMPure® XP Kit
- Freshly prepared 70% ethanol
- DynaMag™-2 magnet
- 1.5-mL LoBind tubes

1. To the 1.5-mL 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	40 µL
Platinum® PCR SuperMix High Fidelity	200 µL
Ion TargetSeq™ Amplification Primer Mix	10 µL
Total	**250 µL**

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

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
5 cycles	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	72°C	1 min
Holding	—	4°C	∞

5. Pool the PCR reaction replicates into a single ~250-µL volume in a new 1.5-mL LoBind tube.

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

6. 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 LoBind tube. Discard the pellet.

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

7. 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.
8. 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.
9. 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. After the solution clears, remove and discard the supernatant without disturbing the pellet.
10. Repeat step 9 for a second wash.
11. 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.
12. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
13. 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.
14. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the **supernatant containing the eluted DNA** to a new 1.5-mL LoBind tube without disturbing the pellet.

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

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

    Note: Since samples were only amplified with 5 cycles, they are too dilute to register a measureable reading on a NanoDrop™ spectrophotometer. If desired, a subset of samples and a no template control can be measured to verify this and that there is no contamination. Additionally, samples will only show a small peak if run on an Agilent Bioanalyzer™ instrument with a High Sensitivity DNA Chip.

STOPPING POINT Proceed immediately to **Second Hybridization** or store the sample at –20°C. If storing, thaw on ice before use.

Second hybridization

Materials provided

- Ion TargetSeq™ Custom Probe Pool
- Ion TargetSeq™ Blocker P1
- Ion TargetSeq™ Blocker A ***or*** Ion TargetSeq™ Blocker BC 1–16
- Human Cot-1 DNA® (1 mg/mL)

Other materials and equipment

- 18–20 gauge or smaller needle
- Vacuum concentrator
- Heat block
- Calibrated thermometer
- Thermal cycler
- Microcentrifuge
- 0.2-mL PCR tubes/strip tubes/plate wells

Before starting

1. Equilibrate a heat block to 95ºC.
2. If necessary, thaw Human Cot-1 DNA® and TargetSeq™ Hybridization Solution A and TargetSeq™ Hybridization Enhancer B at room temperature.
3. Thaw the remaining half of the 4.5-µL aliquot of the Ion TargetSeq™ Custom Probe Pool that was used in the first hybridization reaction.

Second hybridization procedure

1. Add the following to the 1.5-mL LoBind tube containing your sample:

Reagent	Volume
1 mg/mL Human Cot-1 DNA®	5 µL
Ion TargetSeq™ Blocker P1	5 µL
Ion TargetSeq™ Blocker A ***or*** Ion TargetSeq™ Blocker BC 1–16	5 µL

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	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 remaining half (2.25 µL) of the Ion TargetSeq™ Custom Probe Pool from the first hybridization reaction to a new 0.2-mL PCR tube.
10. Add 2.25 µL of nuclease-free water to the probe pool.
11. Transfer the library or library pool to the 0.2-mL PCR tube containing the diluted probe pool.

12. Close the cap on the tube tightly.
13. 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 "Preventing sample loss" on page 14.

14. Transfer the reaction mixture to a thermal cycler with heated lid. Incubate at 47°C overnight (≥ 12 hours) with the heated lid turned on.

    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 tested the GeneAmp® PCR System 9700 thermal cycler from Applied Biosystems® with the default lid heating.

Wash and recover the probe-hybridized DNA

Materials provided

- TargetSeq™ Hybridization and Wash Kit
- Dynabeads® M-270 Streptavidin (10 mg/mL)

Other materials and equipment

- Nuclease-free water
- 1.5-mL LoBind tubes
- DynaMag™-2 magnet
- DynaMag™-PCR magnetic rack
- 0.2-mL PCR tubes/strip tubes/plate wells
- Vortex mixer
- Thermal cycler

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

Prepare the wash solutions

1. Thaw the 10X Wash Solutions (A, B, C, and Stringent) and Magnetic Bead Wash provided in the TargetSeq™ Hybridization and Wash Kit.

   Note: The 10X Wash Solution A may appear cloudy after thawing. Warm at 47°C until it clarifies.

2. 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

3. Preheat the following 1X wash buffers to 47°C for at least 2 hours in a water bath or heat block:

- 400 µL 1X Stringent Wash Solution
- 100 µL 1X Wash Solution A

4. Equilibrate the Dynabeads® M-270 Streptavidin to room temperature for 30 minutes prior to use.

Prepare the Dynabeads® M-270 Streptavidin

1. Resuspend the Dynabeads® M-270 Streptavidin thoroughly by vortexing.
2. Transfer 100 µL of beads per sample into a new 1.5-mL 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. (Any 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 PCR 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 are 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 the second hybridization incubation, 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 thermo cycler after each mixing.

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

Wash the bound DNA

1. After the 45-minute incubation, add 100 µL of the 1X Wash Solution A heated to 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 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 **1X Stringent Wash Solution** heated to 47°C.
6. Pipet up and down 10 times to mix. Work quickly so that the temperature does not drop much below 47°C.
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 the **1X Stringent Wash Solution** wash (steps 5–8) one more time.
10. Add 200 µL of room-temperature **1X Wash Solution A** and mix by vortexing for 2 minutes, followed by a pulse spin.
11. Place the tube in the DynaMag™-2 magnet until the solution clears, then remove and discard the supernatant.
12. Add 200 µL of room-temperature **1X Wash Solution B** and mix by vortexing for 1 minute, followed by a pulse spin.
13. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
14. Add 200 µL of room-temperature **1X Wash Solution C** and mix by vortexing for 30 seconds.
15. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
16. Add 40 µL of nuclease-free water to each tube.
17. 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 second capture reaction

Materials provided

- Ion TargetSeq™ Amplification Primer Mix
- Low TE

Other materials and equipment

- Platinum® PCR SuperMix High Fidelity
- Thermal cycler
- 0.2-mL PCR tubes
- Agencourt® AMPure® XP Kit
- Freshly prepared 70% ethanol
- DynaMag™-2 magnet
- 1.5-mL LoBind tubes

1. To the 1.5-mL 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	40 µL
Platinum® PCR SuperMix High Fidelity	200 µL
Ion TargetSeq™ Amplification Primer Mix	10 µL
Total	**250 µL**

2. Vortex the reaction for 5 seconds, then pulse-spin.

3. Split the volume into two PCR tubes (125 µL each).
4. Transfer the tubes to a thermal cycler and run the following program:

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
12–14 cycles*	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	72°C	1 min
Holding	—	4°C	∞

* Average yield for captured libraries amplified with 12 cycles of PCR has been ~25 ng.

5. Pool the PCR reaction replicates into a single ~250-µL volume in a new 1.5-mL LoBind tube.

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

6. 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 LoBind tube. Discard the pellet.

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

7. 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.
8. 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.
9. 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.
10. Repeat step 9 for a second wash.
11. 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.
12. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
13. 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.
14. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the **supernatant containing the eluted DNA** to a new 1.5-mL LoBind tube without disturbing the pellet.

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

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

STOPPING POINT Proceed immediately to "Qualify the capture library" or store at –20°C. If storing, thaw on ice before use.

Capture procedure for target regions > 2 Mb

Use the following single-hybridization procedure for target regions > 2 Mb. This procedure includes a 64–72-hour hybridization reaction.

Ion TargetSeq™ Blockers

The Ion TargetSeq™ Blockers provided in each kit are pre-designed and diluted oligos used to block the non-barcoded and barcoded adapters used in library construction.

- Use **Ion TargetSeq™ Blocker P1** and **Ion TargetSeq™ Blocker A** if you are blocking the standard Adapters provided in the Ion Xpress™ Plus Fragment Library Kit.
- Use **Ion TargetSeq™ Blocker P1** and **Ion TargetSeq™ Blocker BC 1–16** if you are blocking the barcoded adapters provided in the Ion Xpress™ Barcode Adapters 1–16 Kit.

Hybridization

Materials provided

- Ion TargetSeq™ Custom Probe Pool
- Ion TargetSeq™ Blocker P1
- Ion TargetSeq™ Blocker A ***or***
  Ion TargetSeq™ Blocker BC 1–16
- Human Cot-1 DNA® (1 mg/mL)

Other materials and equipment

- 18–20 gauge or smaller needle
- Vacuum concentrator
- Heat block
- Calibrated thermometer
- Thermal cycler
- Microcentrifuge
- 0.2-mL PCR tubes/strip tubes/plate wells

Preventing sample loss

IMPORTANT! The probe hybridization reaction runs 64–72 hours at 47°C. **If the tube is not completely sealed, the reaction will dry out and your sample will be lost.**

In particular, be careful to avoid the following:

- The tube cap is not completely sealed before the reaction is placed in the thermal cycler
- The weight of the thermal cycler lid compresses the tube and creates an air gap between the tube and cap

Before proceeding, we recommend that you test your tubes and thermal cycler by incubating 15 µL of water at 47°C overnight and measuring any loss of sample. (Recovery of ~14 µL is acceptable.) We also recommend that you use any rack supplied with your thermal cycler that is designed to prevent tube compression when the lid is closed. Alternatively, yous can add empty tubes to the thermal cycler to evenly distribute the pressure of the lid.

Before starting

1. Remove a 4.5-µL aliquot of the Ion TargetSeq™ Custom Probe Pool (aliquoted as described on page 5) from –20°C storage and thaw on ice.
2. If necessary, pool equal masses of barcoded libraries as described in "Library amounts needed" on page 13. Total library mass = 500 ng.
3. Thaw Ion TargetSeq™ Blocker P1 on ice or at room temperature.

4. Thaw Ion TargetSeq™ Blocker A (for a single library) ***or*** Ion TargetSeq™ Blocker BC 1–16 (if using pooled barcoded libraries) on ice or at room temperature.
5. Thaw Human Cot-1 DNA® on ice or at room temperature.
6. Thaw the TargetSeq™ Hybridization Solution A and Hybridization Enhancer B at room temperature. If necessary, heat to 47°C to dissolve any precipitate.
7. Equilibrate a heat block to 95ºC.

Hybridization procedure

1. Add the following to a 1.5-mL 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)	_____ µL
Ion TargetSeq™ Blocker P1	5 µL
Ion TargetSeq™ Blocker A ***or*** Ion TargetSeq™ Blocker BC 1–16 (if using library pool)	5 µL

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 PCR 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 14.

12. Transfer the reaction mixture to a thermal cycler with heated lid. 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).

    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 tested the GeneAmp® PCR System 9700 thermal cycler from Applied Biosystems® with the default lid heating.

Wash and recover the probe-hybridized DNA

Materials provided

- TargetSeq™ Hybridization and Wash Kit
- Dynabeads® M-270 Streptavidin (10 mg/mL)

Other materials and equipment

- Nuclease-free water
- 1.5-mL LoBind tubes
- DynaMag™-2 magnet
- DynaMag™-PCR magnetic rack
- 0.2-mL PCR tubes/strip tubes/plate wells
- Vortex mixer
- Thermal cycler

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

Prepare the wash solutions

1. Thaw the 10X Wash Solutions (A, B, C, and Stringent) and Magnetic Bead Wash provided in the TargetSeq™ Hybridization and Wash Kit.

   Note: The 10X Wash Solution A may appear cloudy after thawing. Warm at 47°C until it clarifies.

2. 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

3. Preheat the following 1X wash buffers to 47°C for at least 2 hours in a water bath or heat block:
   - 400 µL 1X Stringent Wash Solution
   - 100 µL 1X Wash Solution A
4. Equilibrate the Dynabeads® M-270 Streptavidin to room temperature for 30 minutes prior to use.

Prepare the Dynabeads® M-270 Streptavidin

1. Resuspend the Dynabeads® M-270 Streptavidin thoroughly by vortexing.
2. Transfer 100 µL of beads per library into a new 1.5-mL 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. (Any 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 PCR 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 are 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 thermo cycler after each mixing.

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

Wash the bound DNA

1. After the 45-minute incubation, add 100 µL of the 1X Wash Solution A heated to 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 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 **1X Stringent Wash Solution** heated to 47°C.
6. Pipet up and down 10 times to mix. Work quickly so that the temperature does not drop much below 47°C.
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 the **1X Stringent Wash Solution** wash (steps 5–8) one more time.
10. Add 200 µL of room-temperature **1X Wash Solution A** and mix by vortexing for 2 minutes, followed by a pulse spin.

11. Place the tube in the DynaMag™-2 magnet until the solution clears, then remove and discard the supernatant.
12. Add 200 µL of room-temperature **1X Wash Solution B** and mix by vortexing for 1 minute, followed by a pulse spin.
13. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
14. Add 200 µL of room-temperature **1X Wash Solution C** and mix by vortexing for 30 seconds.
15. Place the tube in the magnet until the solution clears, then remove and discard the supernatant.
16. Add 40 µL of nuclease-free water to each tube.
17. 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 capture library

Materials provided

- Ion TargetSeq™ Amplification Primer Mix
- Low TE

Other materials and equipment

- Platinum® PCR SuperMix High Fidelity
- Thermal cycler
- 0.2-mL PCR tubes
- Agencourt® AMPure® XP Kit
- Freshly prepared 70% ethanol
- DynaMag™-2 magnet
- 1.5-mL LoBind tubes

1. To the 1.5-mL 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	40 µL
Platinum® PCR SuperMix High Fidelity	200 µL
Ion TargetSeq™ Amplification Primer Mix	10 µL
Total	**250 µL**

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

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
10 cycles	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	72°C	1 min
Holding	—	4°C	∞

5. Pool the PCR reaction replicates into a single ~250-µL volume in a new 1.5-mL LoBind tube.

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

6. 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 LoBind tube. Discard the pellet.

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

7. 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.
8. 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.
9. 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.
10. Repeat step 9 for a second wash.
11. 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.
12. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
13. 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.
14. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the **supernatant containing the eluted DNA** to a new 1.5-mL LoBind tube without disturbing the pellet.

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

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

STOPPING POINT Proceed immediately to "Qualify the capture library" or store the library at –20°C. If storing, thaw on ice before use.

Qualify the capture library

Required materials and equipment

- Ion Library Quantitation Kit
- Agilent Bioanalyzer™ instrument
- Agilent High Sensitivity DNA Kit
- Low TE

Determine the library dilution required for Template Preparation

Quantitate the library or pooled libraries to determine the dilution (Template Dilution Factor) that results in a concentration within the optimized target range for template preparation (PCR-mediated addition of library molecules onto Ion Sphere™ Particles).

The final library or library pool can be quantitated for downstream template preparation in two ways:

Quantitation method	Features
Ion Library Quantitation Kit (qPCR)	• Quantitate the library using real-time quantitative PCR (qPCR) to determine the Template Dilution Factor. • Higher precision for quantitation. A single dilution of the library, based on the Template Dilution Factor determined with the Ion Library Quantitation Kit, is usually sufficient for an optimized Template Preparation procedure. • Higher sensitivity for detection. The Ion Library Quantitation Kit is recommended for low-yield libraries, such as are generated using the Ion TargetSeq™ Custom Enrichment Kits. Libraries with insufficient material for detection by Bioanalyzer™ analysis may have material that is detectable by qPCR and sufficient for sequencing.
Qubit® and Bioanalyzer™ analysis	• Determine the average molecule length using the Bioanalyzer™ instrument and library concentration using the Qubit® 2.0 fluorometer, then calculate the Template Dilution Factor.

Determine the Template Dilution Factor with the Ion Library Quantitation Kit

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

Determine the Template Dilution Factor from Qubit® and Bioanalyzer™ analysis

The optimal number of library molecules to achieve 10–25% positive Ion Sphere™ Particles may vary between libraries. Generally, approximately 280×10^6 molecules per reaction is recommended. To estimate the library concentration in terms of molecules per µL, do the following:

1. Quantitate the DNA library using a Qubit® 2.0 fluorometer. The concentration will be in units of ng/mL.
2. Analyze an aliquot of the library on the Bioanalyzer™ instrument with an Agilent High Sensitivity DNA Kit. Dilute the library to between 1 and 10 ng/µL with Low TE prior to analysis. Determine the molar library concentration using the Bioanalyzer™ software, following the manufacturer's instructions. Use the position of the peak as an estimate of the average library molecule's length.
3. Using the example equations below, determine the estimated concentration of the library in terms of molecules per µL.

Determine the number of molecules per µL within the library

Use the peak value from the Bioanalyzer™ measurement and the results of Qubit® 2.0 quantitation to calculate the number of library molecules per µL as described below.

A = Library concentration (ng/µL) from Qubit® 2.0 measurement.

B = Average library molecule length (bp), estimated from the Bioanalyzer™ peak

C = Library concentration (molecules/µL).

D = Number of microliters of library that contains 280×10^6 library molecules

C (molecules/µL) = A ng/mL ÷ [660 ng/(nmol•bp)] ÷ (B bp) × [6×10^{14} (molecules/nmol)] ÷ (1000 µL/ mL)

D (µL) = 280×10^6 molecules ÷ C (molecules/µL)

Example: Assuming A= 15 ng/µL and B is 300 bp

C (molecules/µL) = 15 ng/µL ÷ [660 ng/(nmol•bp)] ÷ (300 bp) × [6×10^{14} (molecules/nmol)] ÷ (1000 µL/ mL)

C = 45×10^6 (molecules/µL)

D = 280×10^6 molecules ÷ 45×10^6 (molecules/µL)

D = 6.2 µL

Use D µL of library for template preparation.

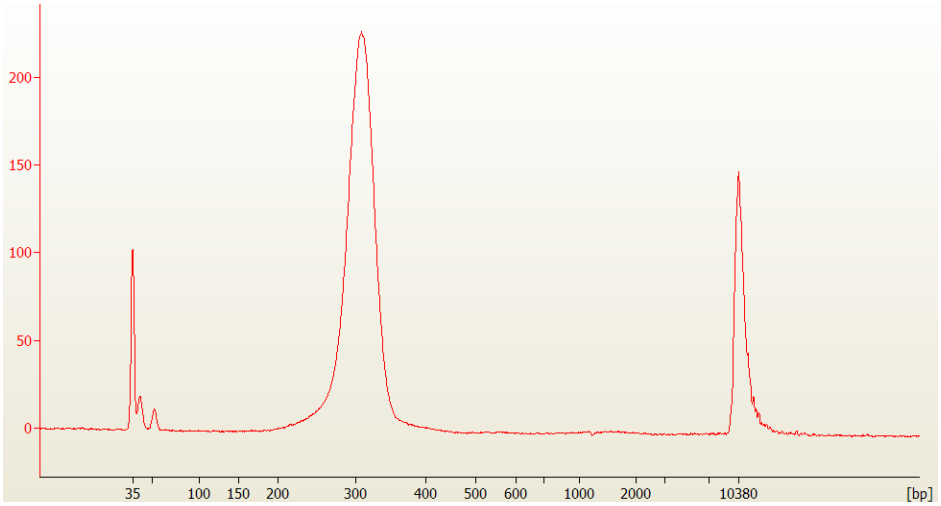


Figure 2. Example of a successfully amplified Ion TargetSeq™ enriched library analyzed on a Bioanalyzer™ instrument using an Agilent High Sensitivity DNA Kit

Proceed to template preparation

The library or pooled libraries are ready for template preparation as described in the user documentation for the Ion Xpress™ Template 200 Kit.

Appendix A. Safety

Chemical safety

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

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

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Biohazard. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the information below:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at:
  www.cdc.gov/biosafety

- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

  Additional information about biohazard guidelines is available at: **www.cdc.gov**

  In the EU:

  Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Appendix B. Documentation and support

Related documentation

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

Obtaining SDSs

Safety Data Sheets (SDSs) for Life Technologies products are available online at **www.appliedbiosystems.com/sds** and **www.invitrogen.com/msds**.

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

Obtaining support

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

At the website, you can:

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

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit www.iontorrent.com/support

www.lifetechnologies.com

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