

TargetSeq™ Custom Enrichment Kits

For capturing user-defined target regions, ranging from 20 kb to 80 Mb in total, from human genomic DNA for sequencing on the SOLiD™ 4 System or 5500 Series Genetic Analysis Systems

Catalog Numbers A14224, A14225, A14226, and A14227

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Product Information

Overview

TargetSeq™ Custom Enrichment Kits provide optimized reagents for capturing user-defined target regions, ranging from 20 kb to 80 Mb in total, from genomic DNA (gDNA) that has been fragmented and constructed into libraries for sequencing on the SOLiD® 4 System or 5500 Series Genetic Analysis Systems.

To use the TargetSeq™ system, you first construct a barcoded fragment library following the standard workflow. The library fragments are then captured in solution using ~2 million TargetSeq™ capture probes targeting a user-defined region, biotinylated oligos that range in size from ~50 to 120 bases. Hybridization specificity is ensured by the use of blocker DNA sequences (Human Cot-1 DNA® and library-adaptor-specific oligos). The bound DNA is isolated using streptavidin-coated Dynabeads® paramagnetic beads, and then amplified and purified. The purified, enriched sample is then returned to the SOLiD® System or 5500 Series workflow for emulsion PCR, enrichment, and sequencing.

Workflow timelines

Target regions > 2 Mb (single 3-day hybridization)				Target regions < 2 Mb (two overnight hybridizations)			
Day	Step	Description	Time (hours)	Day	Step	Description	Time (hours)
Day 1	1	Prepare library	3	Day 1	1	Prepare library	3
	2	Amplify and purify	1.5		2	Amplify and purify	1.5
	3	Quantitate	1.5		3	Quantitate	1.5
	4	Set up and start hybridization	1		4	Set up and start first hybridization at 47°C (overnight, >12 hours)	1
Days 2-4	5	Hybridize at 47°C	64-72	Day 2	5	Wash and recover beads with enriched DNA	2
Day 4	6	Wash and recover beads with enriched DNA	2		6	Amplify and purify	1.5
	7	Amplify and purify	1.5		7	Set-up and start second hybridization at 47°C (overnight, >12 hours)	1
	8	Quantify enriched library	1.5	Day 3	8	Wash and recover beads with enriched DNA	2
	9	Set-up and start emulsion PCR (may leave overnight)	3-4		9	Amplify and purify	1.5
Day 5	10	Break emulsion and enrich templated beads	1.5	Day 4	10	Quantify enriched library	1.5
		Proceed to sequencing and analysis			11	Set-up and start emulsion PCR (may leave overnight)	3-4
					12	Break emulsion and enrich templated beads	1.5
					13	Proceed to sequencing and analysis	

Kit Contents and Storage

TargetSeq™ Custom Enrichment Kit

Each TargetSeq™ Custom Enrichment Kit provides sufficient reagents for 96 capture reactions. There are four kit configurations, depending on the size of the target region you are enriching for:

- TargetSeq™ Custom Enrichment Kit, 20–500 kb, catalog no. A14227
- TargetSeq™ Custom Enrichment Kit, 500 kb–2 Mb, catalog no. A14226
- TargetSeq™ Custom Enrichment Kit, 2–10 Mb, catalog no. A14225
- TargetSeq™ Custom Enrichment Kit, >10 Mb, catalog no. A14224

The components of each catalog number are as follows:

Components	A14226 and A14227	A14224 and A14225	Storage
TargetSeq™ Custom Probe Pool (custom-designed per your order)	2 × 216 µL	2 × 216 µL	–30°C to –10°C
TargetSeq™ Blocker P1B (sold separately as TargetSeq™ Oligo Blocker P1)	8 × 1 kit	4 × 1 kit	
TargetSeq™ Blocker BC 1–16 (also sold as TargetSeq™ Oligo Blocker BC 1–16)	8 × 1 kit	4 × 1 kit	
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	2–8°C
Dynabeads® M-270 Streptavidin	2 × 10 mL	10 mL	

Aliquot and store the TargetSeq™ Custom Probe Pool

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

1. If frozen, thaw the 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.

Required Materials

This section identifies the kits, reagents, and equipment needed for this protocol. Vendor and catalog information are provided in the following tables.

SOLiD® Fragment Library Construction Kits

The TargetSeq™ system is compatible with library construction kits for the SOLiD® 4 System or 5500 Series Genetic Analysis System. For a list of kits, visit www.appliedbiosystems.com.

SOLiD® Fragment Library Barcode Adaptors

This kit is compatible with the following adaptor kits.

Note: Libraries must be constructed using barcode adaptors, even if you are enriching only a single fragment library.

SOLiD® Fragment Library Barcoding Kit Module 1–16 (for the SOLiD® 4 System) (catalog no. 4444836)

5500 Series Fragment Library Standard Adaptors (catalog no. 4464411)

5500 Series Fragment Library Barcode Adaptors 1–16 (catalog no. 4464405)

Library PCR Primers 1 and 2

Library PCR Primers 1 and 2 are required for this procedure, and are available in the following kits:

SOLiD® Fragment Library Oligos Kit (catalog no. 4401151)

5500 Series Fragment Library Standard Adaptors (catalog no. 4464411)

5500 Series Fragment Library Barcode Adaptors 1–16 (catalog no. 4464405)

SOLiD® Library TaqMan® Quantitation Kit

All components are needed from the SOLiD® Library TaqMan® Quantitation Kit (catalog no. 4449639).

Additional TargetSeq™ Blockers

TargetSeq™ Blocker P1B and BC 1–16 are included with all custom orders. Extra blockers can be ordered separately from Life Technologies, under the names and catalog numbers shown below.

Each tube provides enough blockers for 24 capture reactions:

TargetSeq™ Oligo Blocker P1 (0.2 mM) (Catalog no. A14178) (Note that this is equivalent to Blocker P1B provided in the kit.)

TargetSeq™ Oligo Blocker 1–16 (0.2 mM) (Catalog no. A14177)

IMPORTANT!

A blocker sequence for the SOLiD® 4 System P2 adaptor is **not provided** and has not been verified experimentally. This kit is only recommended for use with libraries constructed using barcode adaptors.

Platinum® PCR SuperMix High Fidelity

Platinum® PCR SuperMix High Fidelity (100-rxn kit, catalog no. 12532-016) is required to amplify the enriched library. The number of kits required to perform 96 capture reactions depends on the TargetSeq™ kit used, and is shown in the table below:

TargetSeq™ Custom Enrichment Kit catalog numbers	Number of Platinum® PCR SuperMix High Fidelity kits (Catalog no. 12532-016) needed
A14226 and A14227	9
A14224 and A14225	4

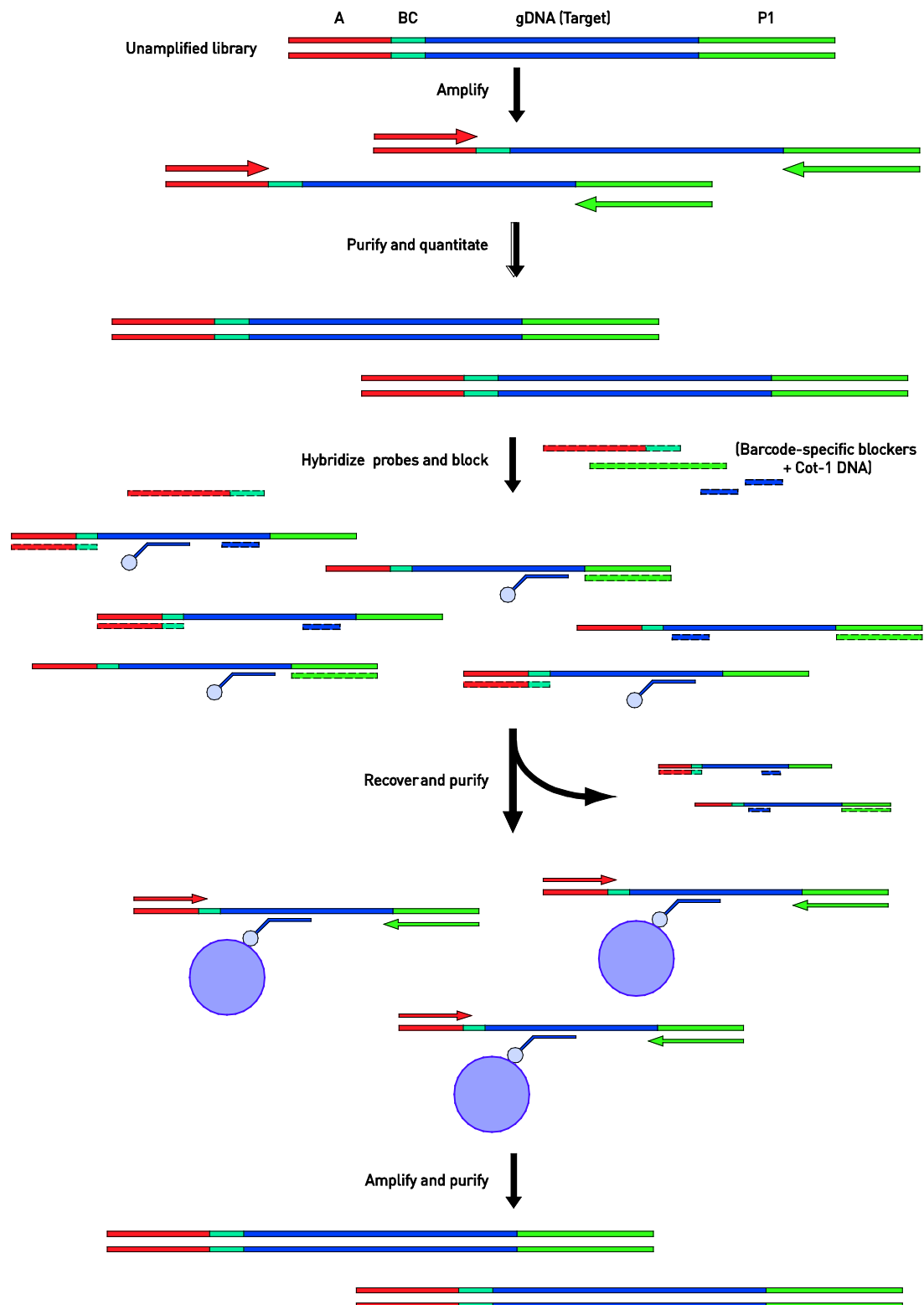
Other equipment and materials

Description	Supplier	Part number	Quantity
Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1
Thermal cycler‡ (e.g., Veriti® 96-Well Fast Thermal Cycler or Applied Biosystems GeneAmp® PCR System 9700)	Many	Varies	1
Agilent 2100 Bioanalyzer™ Instrument	Agilent	G2939AA	1
Agilent High Sensitivity DNA Kit	Agilent	5067-4626	1 kit
1.5-mL LoBind Tubes	Eppendorf	022431021	1 box
0.2-mL MicroAmp® Reaction Tubes	Life Technologies	N8010540	1 package
PCR plates (as recommended by qPCR instrument vendor)	Many	Varies	Varies
Ethanol, absolute	Many	Varies	Varies
Nuclease-free water	Applied Biosystems	AM9932	1000 mL
Vortex mixer	Many	Varies	1
Vacuum concentrator (for 1.5-mL tubes)	Many	Varies	1
DynaMag™-2 magnet (for 1.5-mL tubes)	Life Technologies	12321D	1
DynaMag™-PCR magnet (for 0.2-mL tubes)	Life Technologies	49-2025	1
Water bath or heat block	Many	Varies	1
Microcentrifuge	Many	Varies	1
Calibrated thermometer	Many	Varies	1

‡ 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 with the default lid heating.

Workflow diagram

5500 System/ SOLiD® 4 barcoded libraries are shown in the diagram. The workflow is the same for non-barcoded libraries.



Prepare and Quantitate the Library

Library preparation: SOLiD® 4 System

SOLiD® 4 System library preparation guidelines

Follow the library preparation instructions in the *SOLiD® 4 System Library Preparation Guide* (Publication part no. 4445673) or *Fragment Library Preparation Using the AB Library Builder™ System—SOLiD® 4 System* (Publication part no. 4462553), except as noted below.

Important guidelines:

- **Starting material:** Prepare each fragment library from 3 µg of DNA.
- **Barcoding:** Libraries must be constructed using barcode adaptors, even if you are enriching only a single fragment library. Construct each library using an adaptor from the SOLiD® Fragment Library Barcoding Kit Module 1–16, catalog no. 4444836.
- **Color-balancing:** When preparing four fragment libraries for multiplex sequencing, be sure to follow the instructions for color-balancing the barcodes in the SOLiD® 4 System user guide (i.e., use one of the following color-balanced sets of barcodes: 1–4, 5–8, 9–12, or 13–16 for each set of four libraries, up to 16 libraries).
- **Amplification:** During the nick translation/amplification step, amplify for 6 cycles using the Multiplex Library PCR-1 and Multiplex Library PCR-2 primers included in the SOLiD® Fragment Library Barcoding Kit Module 1–16.
- **Purification:** Following amplification, we recommend purifying the library using Agencourt® AMPure® XP beads instead of the gel purification recommended in the *SOLiD® 4 System Library Preparation Guide*. See the following protocol.

AMPure® XP purification for SOLiD® 4 libraries

Following nick translation/amplification of the SOLiD® 4 library, use the AMPure® XP bead purification protocol below **instead of** the gel purification recommended in the *SOLiD® 4 System Library Preparation Guide*.

1. Resuspend the Agencourt AMPure® XP Reagent beads and allow the mixture to come to room temperature (20–25°C).
2. Prepare 2 mL of fresh 70% ethanol per sample:

Component	Amount
Nuclease-free water	600 µL
Ethanol, absolute	1400 µL
Total	2000 µL

3. Add 375 µL of Agencourt® AMPure® XP Reagent (1.5X the sample volume) to the post-capture amplified DNA from the previous page.
4. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin.
5. Incubate the mixture at room temperature for 5 minutes.
6. Place the tube in a DynaMag™-2 magnetic rack for 3 minutes or until the solution clears.

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

7. Without disturbing the pellet, remove and discard the supernatant. Save the pellet, which contains the DNA.
8. Wash the pellet 2 times. For each wash:
 - a. Add 500 µL of freshly prepared 70% ethanol to the tube, cap the tube and mix by inverting a few times, then pulse-spin.
 - b. Place the tube in a DynaMag™-2 magnetic rack for at least 1 minute until the solution clears, then remove and discard the supernatant.
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. Open the tube, and then dry the beads at room temperature (20–25°C) for 5–10 minutes.
11. Remove the tube from the DynaMag™-2 magnetic rack, then add 50 µL Low TE Buffer directly to the pellet. Pipette up and down to mix.
12. Vortex the beads for 10 seconds, then pulse-spin. Place the tube in a magnetic rack for at least 1 minute until the solution clears.

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

13. Transfer the supernatant containing the size-selected DNA to a new 1.5-mL LoBind Tube.

Library preparation: 5500 Series Genetic Analysis Systems

5500 Series System library preparation guidelines

Follow the library preparation instructions in the *Fragment Library Preparation User Guide—5500 Series Genetic Analysis Systems* (Publication part no. 4460960) or *Fragment Library Preparation Using the AB Library Builder™ System—5500 Series Genetic Analysis Systems* (Publication part no. 4460965), except as noted below.

Important guidelines:

- **Starting material:** Prepare each fragment library from 3 µg of DNA.
- **Barcoding:** Libraries must be constructed using barcode adaptors, even if you are enriching only a single fragment library. Construct each library using an adaptor from the 5500 Series Fragment Library Barcode Adaptors 1–16 (Catalog no. 4464405).
- **Color-balancing:** When preparing four fragment libraries for multiplex sequencing, be sure to follow the instructions for color-balancing the barcodes in the 5500 Series Genetic Analysis System user guide (i.e., use one of the following color-balanced sets of barcodes: 1–4, 5–8, 9–12, or 13–16 for each set of four libraries, up to 16 libraries).
- **Amplification:** During the nick translation/amplification step, amplify for 6 cycles using the Library PCR Primer 1 and Library PCR Primer 2 included in the 5500 Series Fragment Library Barcode Adaptors 1–16. (Amplification is listed as optional in the 5500 Series Genetic Analysis Systems user guide, but is **required** for this workflow.)

Quantitate the amplified DNA library

Quantitation methods

Use an Agilent Technologies 2100 Bioanalyzer™ instrument to verify the integrity of the DNA and determine the average molecule length. Use a 1-µL sample diluted to a concentration of 1–10 ng/µL.

Quantitate the number of amplifiable library molecules using the SOLiD® Library TaqMan® Quantitation Kit (Catalog no. 4449639).

IMPORTANT!

-
- If you are pooling multiple libraries, it is **critical** that you quantitate them in the same qPCR experiment.
 - We do not recommend quantitation by spectrophotometry (e.g., with a NanoDrop® ND-1000 Spectrophotometer), because it is not specific for double-stranded DNA.
-

Determine the mass of the library

Typically, the average library molecule is ~260 bp long. Use the peak value from the Bioanalyzer™ instrument measurement and the results of qPCR quantitation to calculate the mass of amplifiable molecules, as described below.

A = Library concentration (nmol/L), based on SOLiD® Library TaqMan® Quantitation

B = Average library molecule length (typically ~260 bp), estimated from the Bioanalyzer™ instrument peak

C = Library concentration by mass (ng/μL)

$$C \text{ (ng/}\mu\text{L)} = A \text{ nmol/L} \times 660 \text{ ng/(nmol}\cdot\text{bp)} \times (1 \text{ L}/10^6 \mu\text{L}) \times B \text{ bp}$$

$$\text{Mass of library (ng)} = \text{volume of library } (\mu\text{L}) \times C \text{ (ng/}\mu\text{L)}$$

Example: Assuming A= 75 nmol/L, B is 260 bp, and library volume is 50 μL

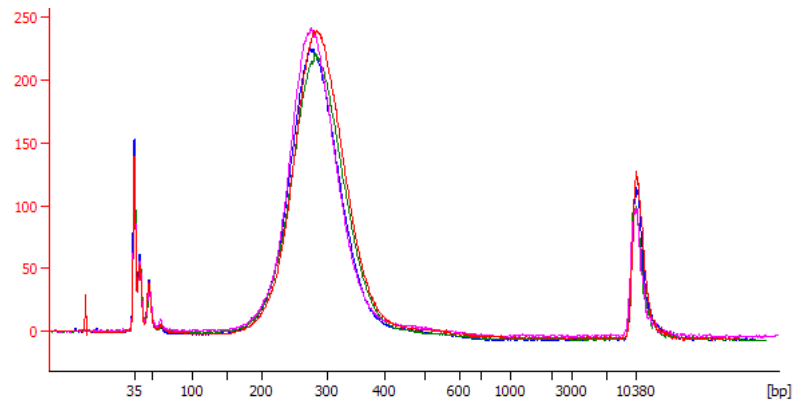
$$C \text{ (ng/}\mu\text{L)} = 75 \text{ nmol/L} \times 660 \text{ ng/(nmol}\cdot\text{bp)} \times (1 \text{ L}/10^6 \mu\text{L}) \times 260 \text{ bp}$$

$$C \text{ (ng/}\mu\text{L)} = \mathbf{12.9 \text{ ng/}\mu\text{L}}$$

$$\text{Mass of library (ng)} = 50 \mu\text{L} \times 12.9 \text{ ng/}\mu\text{L} = \mathbf{645 \text{ ng}}$$

**Example data—
2100
Bioanalyzer™
instrument**

The graph below shows data from a Bioanalyzer™ instrument and Agilent High Sensitivity DNA Chip with replicate human barcoded SOLiD® 4 DNA libraries after adaptor ligation, double Agencourt® AMPure® XP bead-based purification, nick-translation, 6 cycles of PCR amplification, and Agencourt® AMPure® XP bead-based purification. Samples were diluted to ~2 ng/μL prior to loading.



**Example data—
SOLiD® Library
TaqMan®
Quantitation Kit**

The table below shows yields from replicate human DNA libraries as measured by the SOLiD® Library TaqMan® Quantitation Kit (with back-calculation of mass). All preparations started from 3 μg of pure genomic DNA. Following adaptor ligation and nick translation, libraries were amplified for 6 cycles of PCR amplification followed by Agencourt® AMPure® XP bead-based purification.

DNA [†] yields after PCR amplification and AMPure® XP purification	
Replicate library	Yield in ng
Library A (BC1)	821
Library B (BC2)	837
Library C (BC3)	944
Library D (BC4)	975

† Starting material = 3 μg human genomic DNA.

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.

First hybridization

Preventing sample loss

IMPORTANT! Each 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 ≥12.5 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.

Library amounts needed

TargetSeq™ Custom Enrichment Kits require a total of **500 ng of amplified library DNA** in each capture reaction. (To determine the library yield in nanograms, see [Quantitate the amplified DNA library](#) on page 11.) You can process a single library or pooled color-balanced libraries in a single capture reaction. (See [Library preparation: SOLiD® 4 System](#) on page 9 or [Library preparation: 5500 Series Genetic Analysis Systems](#) on page 11.)

Note: For pooled libraries, it is important that color balance is maintained by using at least four barcodes from within a color-balanced set. For example, if you want to pool five libraries, use BC 1–4 and BC 5 or BC 5–8 and BC 9.

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

Library Type	Amount Needed
Single library	500 ng
Four barcoded libraries	125 ng per library
Eight barcoded libraries	62.5 ng per library
Twelve barcoded libraries	41.7 ng per library
Sixteen barcoded libraries	31.3 ng per library

**TargetSeq™
Blockers**

TargetSeq™ blockers are included in the kit. These pre-designed and diluted oligos can be used to block the P1 and barcode adaptors for **either** the SOLiD® 4 or 5500 Series Genetic Analysis System. Each tube provides enough blockers for 24 capture reactions.

- **TargetSeq™ Blocker P1B** (also sold as TargetSeq™ Oligo Blocker P1) is used to block the P1 Adaptor.
- **TargetSeq™ Blocker 1–16** (also sold as TargetSeq™ Oligo Blocker 1–16) is a pool of oligos designed to block the corresponding barcode adaptors (e.g., Barcodes 001–016 on the SOLiD® 4 System **or** Barcodes T-001–T-016 on the 5500 Series).

IMPORTANT!

A blocker sequence for the SOLiD® 4 System P2 adaptor is **not provided** and has not been verified experimentally.

Before starting

1. Remove a 4.5-µL aliquot of the TargetSeq™ Custom Probe Pool (aliquoted as described on page 5) from –20°C storage and thaw on ice.
2. For multiplex sequencing, pool equal masses of color-balanced libraries as described in **Library amounts needed** on page 13.
3. Thaw TargetSeq™ Blocker P1B and TargetSeq™ Blocker BC 1–16 on ice or at room temperature.
4. Thaw Human Cot-1 DNA® on ice or at room temperature.
5. Thaw TargetSeq™ Hybridization Solution A and Hybridization Enhancer B at room temperature. If necessary, heat to 47°C to dissolve any precipitate.
6. 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
Single library <i>or</i> pool of color-balanced libraries	_____ µL (500 ng total)
TargetSeq™ Blocker P1B	5 µL
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 samples 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:

Component	Amount
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 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(s) 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 13.

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

IMPORTANT!

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

Prepare Sequence Capture and Bead Wash Buffers

1. Thaw the 10X Wash Solutions (A, B, C, and Stringent) and Magnetic Bead Wash provided in the TargetSeq™ Hybridization and Wash Kit.
Note: Some solutions may appear cloudy after thawing. Warm at 47°C until the solution clarifies.
2. Dilute the 10X Wash Solutions and Magnetic Bead Wash in nuclease-free water to create 1X working solutions, as shown in the following table. The volumes shown are for a single capture reaction. Scale up accordingly if you are processing additional reactions.

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. Repeats 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 first capture reaction

Library PCR Primers 1 and 2

For the following protocol, use the Library PCR Primers 1 and 2 from one of the following kits:

SOLiD® Fragment Library Oligos Kit (catalog no. 4401151)

5500 Fragment Library Standard Adaptors (catalog no. 4464411)

5500 Fragment Library Barcode Adaptors 1–16 (catalog no. 4464405)

Amplify the first capture

- 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	Amount
TargetSeq™ capture beads with DNA	40 µL
Platinum® PCR SuperMix High Fidelity	200 µL
Library PCR Primer 1, 50 µM	5 µL
Library PCR Primer 2, 50 µM	5 µL
Total	250 µL

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

Stage	Step	Temp	Time
Holding	Denature	95°C	5 min.
	Denature	95°C	15 sec
5 cycles	Anneal	62°C	15 sec
	Extend	70°C	1 min
Holding	Extend	70°C	5 min
Holding	Hold	4°C	∞

- 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!**

- 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!

IMPORTANT! The Agencourt AMPure® XP Reagent beads must be at room temperature for the following steps.

Purify the first capture

1. Resuspend the Agencourt AMPure® XP Reagent beads and allow the mixture to come to room temperature (20–25°C).
2. Prepare 2 mL of fresh 70% ethanol per sample:

Component	Amount
Nuclease-free water	600 µL
Ethanol, absolute	1400 µL
Total	2000 µL

3. Add 375 µL of Agencourt® AMPure® XP Reagent (1.5X the sample volume) to the post-capture amplified DNA from the previous page.
4. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin.
5. Incubate the mixture at room temperature for 5 minutes.
6. Place the tube in a DynaMag™-2 magnetic rack for 3 minutes or until the solution clears.

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

7. Without disturbing the pellet, remove and discard the supernatant. Save the pellet, which contains the DNA.
8. Wash the pellet 2 times. For each wash:
 - a. Add 500 µL of freshly prepared 70% ethanol to the tube, cap the tube and mix by inverting a few times, then pulse-spin.
 - b. Place the tube in a DynaMag™-2 magnetic rack for at least 1 minute until the solution clears, then remove and discard the supernatant.
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. Open the tube, and then dry the beads at room temperature (20–25°C) for 5–10 minutes.
11. Remove the tube from the DynaMag™-2 magnetic rack, then add 50 µL Low TE Buffer directly to the pellet. Pipette up and down to mix.
12. Vortex the beads for 10 seconds, then pulse-spin. Place the tube in a magnetic rack for at least 1 minute until the solution clears.

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

13. Transfer the supernatant containing the size-selected DNA to a new 1.5-mL LoBind Tube.

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

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.

Second hybridization

Before starting

1. Equilibrate a heat block to 95°C.
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 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
TargetSeq [™] Blocker P1B	5 µL
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 samples 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:

Component	Amount
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 remaining half (2.25 µL) of the 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(s) tightly.

- 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 13.

- 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

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 Sequence Capture and Bead Wash Buffers

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

Note: Some solutions may appear cloudy after thawing. Warm at 47°C until solutions clarify.

- 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 reactions.

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

- 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
- 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. Repeats 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 μ L 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 μ L 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 μ L 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 μ L 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 μ L 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 μ L 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

Library PCR Primers 1 and 2

For the following protocol, use the Library PCR Primers 1 and 2 from one of the following kits:

SOLiD® Fragment Library Oligos Kit (catalog no. 4401151)

5500 Fragment Library Standard Adaptors (catalog no. 4464411)

5500 Fragment Library Barcode Adaptors 1–16 (catalog no. 4464405)

Amplify the second capture

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	Amount
TargetSeq™ capture beads with DNA	40 µL
Platinum® PCR SuperMix High Fidelity	200 µL
Library PCR Primer 1, 50 µM	5 µL
Library PCR Primer 2, 50 µM	5 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 program below:

Stage	Step	Temp	Time
Holding	Denature	95°C	5 min.
	Denature	95°C	15 sec
12-14 cycles	Anneal	62°C	15 sec
	Extend	70°C	1 min
Holding	Extend	70°C	5 min
Holding	Hold	4°C	∞

* Average yield for captured libraries amplified with 12 cycles of PCR on an GeneAmp® PCR System 9700 has been ~25 ng for a target capture size of 100 kb .

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!

The Agencourt AMPure® XP Reagent beads must be at room temperature for the following steps.

Purify the second capture

1. Resuspend the Agencourt AMPure® XP Reagent beads and allow the mixture to come to room temperature (20–25°C).
2. Prepare 2 mL of fresh 70% ethanol per sample:

Component	Amount
Nuclease-free water	600 µL
Ethanol, absolute	1400 µL
Total	2000 µL

3. Add 375 µL of Agencourt® AMPure® XP Reagent (1.5X the sample volume) to the post-capture amplified DNA from the previous page.
4. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin.
5. Incubate the mixture at room temperature for 5 minutes.
6. Place the tube in a DynaMag™-2 magnetic rack for 3 minutes or until the solution clears.

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

7. Without disturbing the pellet, remove and discard the supernatant. Save the pellet, which contains the DNA.
8. Wash the pellet 2 times. For each wash:
 - a. Add 500 µL of freshly prepared 70% ethanol to the tube, cap the tube and mix by inverting a few times, then pulse-spin.
 - b. Place the tube in a DynaMag™-2 magnetic rack for at least 1 minute until the solution clears, then remove and discard the supernatant.
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. Open the tube, and then dry the beads at room temperature (20–25°C) for 5–10 minutes.
11. Remove the tube from the DynaMag™-2 magnetic rack, then add 50 µL Low TE Buffer directly to the pellet. Pipette up and down to mix.
12. Vortex the beads for 10 seconds, then pulse-spin. Place the tube in a magnetic rack for at least 1 minute until the solution clears.

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

13. Transfer the supernatant containing the size-selected DNA to a new 1.5-mL LoBind Tube.

STOPPING POINT Store the purified DNA in Low TE Buffer at 4°C. Proceed to [Quantitate the library molecules](#) on page 34.

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.

Hybridization reaction

Preventing sample loss

IMPORTANT! Each 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 ≥12.5 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.

Library amounts needed

TargetSeq™ Custom Enrichment Kits require a total of **500 ng of amplified library DNA** in each capture reaction. (To determine the library yield in nanograms, see [Quantitate the amplified DNA library](#) on page 11.) You can process a single library or pooled color-balanced libraries in a single capture reaction. (See [Library preparation: SOLiD® 4 System](#) on page 9 or [Library preparation: 5500 Series Genetic Analysis Systems](#) on page 11.)

Note: For pooled libraries, it important that color balance be maintained by using at least four barcodes from within a color-balanced set. For example, if you want to pool five libraries, use BC 1–4 and BC 5 or BC 5–8 and BC 9.

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

Library Type	Amount Needed
Single library	500 ng
Four barcoded libraries	125 ng per library
Eight barcoded libraries	62.5 ng per library
Twelve barcoded libraries	41.7 ng per library
Sixteen barcoded libraries	31.3 ng per library

TargetSeq™ Blockers

TargetSeq™ blockers are included in the kit. These pre-designed and diluted oligos can be used to block the P1 and barcode adaptors for **either** the SOLiD® 4 or 5500 Series Genetic Analysis System. Each tube provides enough blockers for 24 capture reactions.

- **TargetSeq™ Blocker P1B** (also sold as TargetSeq™ Oligo Blocker P1) is used to block the P1 Adaptor.
- **TargetSeq™ Blocker 1–16** (also sold as TargetSeq™ Oligo Blocker 1–16) is a pool of oligos designed to block the corresponding barcode adaptors (e.g., Barcodes 001–016 on the SOLiD® 4 System **or** Barcodes T-001–T-016 on the 5500 Series).

IMPORTANT!

A blocker sequence for the SOLiD® 4 System P2 adaptor is **not provided** and has not been verified experimentally.

Before starting

1. Remove a 4.5-µL aliquot of the TargetSeq™ Custom Probe Pool (aliquoted as described on page 5) from –20°C storage and thaw on ice.
2. For multiplex sequencing, pool equal masses of four color-balanced libraries as described in **Library amounts needed** on page 26.
3. Thaw TargetSeq™ Blocker P1B and TargetSeq™ Blocker BC 1–16 on ice or at room temperature.
4. Thaw Human Cot-1 DNA® on ice or at room temperature.
5. Thaw TargetSeq™ Hybridization Solution A and Hybridization Enhancer B at room temperature. If necessary, heat to 47°C to dissolve any precipitate.
6. Equilibrate a heat block to 95°C.

Hybridization procedure

1. Add the following to a 1.5-mL tube.

Reagent	Volume
1 mg/mL Human Cot-1 DNA®	5 µL
Single library or pool of color-balanced libraries	_____ µL (500 ng total)
TargetSeq™ Blocker P1B	5 µL
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 samples 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:

Component	Amount
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. Add the 4.5-µL single-use aliquot of the TargetSeq™ Custom Probe Pool to the probe pool.
10. Transfer the sample to the 0.2-mL PCR tube containing the 4.5-µL aliquot of the TargetSeq™ Custom Probe Pool.
11. Close the cap on the tube(s) tightly.
12. 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 26.

13. 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.

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

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 Sequence Capture and Bead Wash Buffers

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

Note: 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 reactions.

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. Repeats 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 μ L of beads, use 100 μ L buffer).
9. Resuspend the beads by vortexing.
10. Aliquot 100 μ L of resuspended beads into a new 0.2-mL PCR tube. Repeat with separate tubes if you are processing > 100 μ L 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 μ L 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 μ L 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 reaction

Library PCR Primers 1 and 2

For the following protocol, use the Library PCR Primers 1 and 2 from one of the following kits:

SOLiD® Fragment Library Oligos Kit (catalog no. 4401151)

5500 Fragment Library Standard Adaptors (catalog no. 4464411)

5500 Fragment Library Barcode Adaptors 1–16 (catalog no. 4464405)

Amplify the first capture

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	Amount
TargetSeq™ capture beads with DNA	40 µL
Platinum® PCR SuperMix High Fidelity	200 µL
Library PCR Primer 1, 50 µM	5 µL
Library PCR Primer 2, 50 µM	5 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 program below:

Stage	Step	Temp	Time
Holding	Denature	95°C	5 min.
	Denature	95°C	15 sec
10 cycles	Anneal	62°C	15 sec
	Extend	70°C	1 min
Holding	Extend	70°C	5 min
Holding	Hold	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!

IMPORTANT! The Agencourt AMPure® XP Reagent beads must be at room temperature for the following steps.

Purify the first capture

1. Resuspend the Agencourt AMPure® XP Reagent beads and allow the mixture to come to room temperature (20–25°C).
2. Prepare 2 mL of fresh 70% ethanol per sample:

Component	Amount
Nuclease-free water	600 µL
Ethanol, absolute	1400 µL
Total	2000 µL

3. Add 375 µL of Agencourt® AMPure® XP Reagent (1.5X the sample volume) to the post-capture amplified DNA from the previous page.
4. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin.
5. Incubate the mixture at room temperature for 5 minutes.
6. Place the tube in a DynaMag™-2 magnetic rack for 3 minutes or until the solution clears.

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

7. Without disturbing the pellet, remove and discard the supernatant. Save the pellet, which contains the DNA.
8. Wash the pellet 2 times. For each wash:
 - a. Add 500 µL of freshly prepared 70% ethanol to the tube, cap the tube and mix by inverting a few times, then pulse-spin.
 - b. Place the tube in a DynaMag™-2 magnetic rack for at least 1 minute until the solution clears, then remove and discard the supernatant.
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. Open the tube, and then dry the beads at room temperature (20–25°C) for 5–10 minutes.
11. Remove the tube from the DynaMag™-2 magnetic rack, then add 50 µL Low TE Buffer directly to the pellet. Pipette up and down to mix.
12. Vortex the beads for 10 seconds, then pulse-spin. Place the tube in a magnetic rack for at least 1 minute until the solution clears.

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

13. Transfer the supernatant containing the size-selected DNA to a new 1.5-mL LoBind Tube.

STOPPING POINT Store the purified DNA in Low TE Buffer at 4°C. Proceed to “**Quantitate the library molecules**” on page 34.

Quantitate the Library Molecules

Determine molecule length and quantitate

Use an Agilent Technologies 2100 Bioanalyzer™ instrument to verify the integrity of the DNA and determine the average molecule length. Use a 1-μL sample diluted to a concentration of 1–10 ng/μL.

Quantitate the number of amplifiable library molecules using the SOLiD® Library TaqMan® Quantitation Kit (Catalog no. 4449639).

IMPORTANT! We do not recommend quantitation by spectrophotometry (e.g., with a NanoDrop® ND-1000 Spectrophotometer), because it is not specific for double-stranded DNA.

Determine the mass of the library

Typically, the average library molecule is ~260 bp long. Use the peak value from the Bioanalyzer™ instrument measurement and the results of qPCR quantitation to calculate the mass of amplifiable molecules, as described below.

A = Library concentration (nmol/L), based on SOLiD® Library TaqMan® Quantitation

B = Average library molecule length (typically ~260 bp), estimated from the Bioanalyzer™ instrument peak

C = Library concentration by mass (ng/μL)

$$C \text{ (ng/}\mu\text{L)} = A \text{ nmol/L} \times 660 \text{ ng}/(\text{nmol}\cdot\text{bp}) \times (1 \text{ L}/10^6 \mu\text{L}) \times B \text{ bp}$$

$$\text{Mass of library (ng)} = \text{volume of library } (\mu\text{L}) \times C \text{ (ng/}\mu\text{L)}$$

Example: Assuming A= 25 nmol/L, B is 260 bp, and library volume is 50 μL

$$C \text{ (ng/}\mu\text{L)} = 25 \text{ nmol/L} \times 660 \text{ ng}/(\text{nmol}\cdot\text{bp}) \times (1 \text{ L}/10^6 \mu\text{L}) \times 260 \text{ bp}$$

$$C \text{ (ng/}\mu\text{L)} = \mathbf{4.3 \text{ ng/}\mu\text{L}}$$

$$\text{Mass of library (ng)} = 50 \mu\text{L} \times 4.3 \text{ ng/}\mu\text{L} = \mathbf{215 \text{ ng}}$$

Proceed to sequencing

Following enrichment evaluation by qPCR, proceed to emulsion PCR (ePCR), bead enrichment, and SOLiD® System or 5500 Series sequencing.

Documentation and Support

Obtaining SDSs

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

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

Obtaining support

For the latest services and support information for all locations, go to: www.lifetechnologies.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

Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at www.lifetechnologies.com/termsandconditions. If you have any questions, please contact Life Technologies at www.lifetechnologies.com/support.

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