USER GUIDE

TargetSeq™ Exome Enrichment System

For capturing the exons identified in the VEGA, CCDS, and RefSeq databases and >700 microRNA genes from human genomic DNA for sequencing on the SOLiD™ 4 System or 5500 SOLiD™ Series Systems

Catalog Numbers A14060, A14061, A14062, and A14063

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life technologies™

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Kit Contents and Storage

TargetSeq™ Exome Enrichment System

The TargetSeq™ Exome Enrichment System contains the following components and is available in four sizes, as shown in the table below:

	Catalog number (Kit size)			
Component	**A14060 (4 reactions)**	**A14061 (12 reactions)**	**A14062 (48 reactions)**	**A14063 (96 reactions)**
TargetSeq™ Exome Probe Pool	18 µL	3 × 18 µl	216 µL	2 × 216 µL
TargetSeq™ Hybridization and Wash Kit (see below for details)	1 kit	1 kit	2 kits	4 kits
Human Cot-1 DNA® (1 mg/mL)	1 mg	1 mg	1 mg	1 mg
Dynabeads® M-270 Streptavidin (10 mg/mL)	2 mL	2 mL	10 mL	10 mL

TargetSeq™ Hybridization and Wash Kit

The TargetSeq™ Hybridization and Wash Kit is part of the kits above and is also sold separately (catalog no. A138230). Each kit contains the following components, as shown in the table below:

Component	Volume per Kit
TargetSeq™ Wash Solution A (10X)	750 µL
TargetSeq™ Wash Solution B (10X)	500 µL
TargetSeq™ Wash Solution C (10X)	500 µL
TargetSeq™ Stringent Wash Solution (10X)	1000 µL
TargetSeq™ Hybridization Solution A (2X)	190 µL
TargetSeq™ Hybridization Enhancer B	75 µL
TargetSeq™ Magnetic Bead Wash (2.5x)	5 mL

Aliquoting and storage

Upon receipt, store the TargetSeq™ Exome Probe Pool in 4.5-µL aliquots as described on page 13.

IMPORTANT! Avoid multiple freeze/thaw cycles of the TargetSeq™ Exome Probe Pool.

Intended use

For research use only. Not intended for any human or animal diagnostic or therapeutic uses.

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:

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).

TargetSeq™ Blockers

The TargetSeq™ blockers used in the exome capture reaction can be ordered separately from Life Technologies. These pre-designed and diluted oligos can be used to block adaptors for **either** the SOLiD® 4 or 5500 Series Genetic Analysis System. Each tube provides enough blockers for 24 exome capture reactions.

- **TargetSeq™ Blocker P1** is used to block the P1 Adaptor.
- **TargetSeq™ Blockers 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). Use this blocker pool whenever you are using any combination of these barcodes.

TargetSeq™ Blocker P1 (0.2 mM) (Catalog no. A14178)
TargetSeq™ Blockers 1–16 (0.2 mM) (Catalog no. A14177)

Blocker sequences

You can design and order individual blocker oligos yourself using the sequences below. The name of the blocker sequence matches the corresponding SOLiD® 4 adaptor or 5500 Series adaptor.

For example: Use the Barcode-001 Blocker to block Barcode-001 (SOLiD® 4) **or** Barcode-T-001 (5500 Series). The P1 Blocker is used to block the P1 Adaptor in either SOLiD® System.

IMPORTANT! HPLC purification following synthesis is required. The oligos should have no modifications.

Blocker name	Sequence
P1 Blocker	5'-ATCACCGACTGCCCATAGAGAGGAAAGCGGAGGCGTAGTGG-3'
Barcode-001 Blocker	5'-CGCCTTGGCCGTACAGCAGCCTCTTACACAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-002 Blocker	5'-CGCCTTGGCCGTACAGCAGACCACTCCCTAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-003 Blocker	5'-CGCCTTGGCCGTACAGCAGTATAACCTATAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-004 Blocker	5'-CGCCTTGGCCGTACAGCAGGACCGCATCCAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-005 Blocker	5'-CGCCTTGGCCGTACAGCAGCTTACACCACAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-006 Blocker	5'-CGCCTTGGCCGTACAGCAGTGTCCCTCGCAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-007 Blocker	5'-CGCCTTGGCCGTACAGCAGGGCATAACCCAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-008 Blocker	5'-CGCCTTGGCCGTACAGCAGATCCTCGCTCAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-009 Blocker	5'-CGCCTTGGCCGTACAGCAGGTCGCAACCTAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-010 Blocker	5'-CGCCTTGGCCGTACAGCAGAGCTTACCGCAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-011 Blocker	5'-CGCCTTGGCCGTACAGCAGCGTGTCGCACAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-012 Blocker	5'-CGCCTTGGCCGTACAGCAGTTTTCCTCTTAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-013 Blocker	5'-CGCCTTGGCCGTACAGCAGGCCTTACCGCAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-014 Blocker	5'-CGCCTTGGCCGTACAGCAGTCTGCCGCACAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-015 Blocker	5'-CGCCTTGGCCGTACAGCAGCATTCAACTCAGAGAATGAGGAACCCGGGGCAG-3'
Barcode-016 Blocker	5'-CGCCTTGGCCGTACAGCAGAACGTCTCCCAGAGAATGAGGAACCCGGGGCAG-3'

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 SOLiD® 4 libraries constructed using Barcodes 001–016.

Other equipment and materials

Description	Supplier	Part number	Quantity
SOLiD® Library TaqMan® Quantitation Kit	Life Technologies	4449639	250 reactions
SYBR® GreenER™ qPCR Supermix Universal	Life Technologies	11762100	250 reactions
Thermal cycler (e.g., Veriti® 96-Well Fast Thermal Cycler or Applied Biosystems GeneAmp® PCR System 9700*)	Many	Varies	1
qPCR instrument (e.g., the StepOnePlus™ Real-Time PCR System)	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 PCR tubes **or** 8-tube strips and 8-cap strips	As recommended by thermal cycler vendor	Varies	1 box
PCR plates (as recommended by qPCR instrument vendor)	Many	Varies	Varies
Agencout® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1
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 from Applied Biosystems™ with the default lid heating.

Product Qualification

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to **www.invitrogen.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Product Description

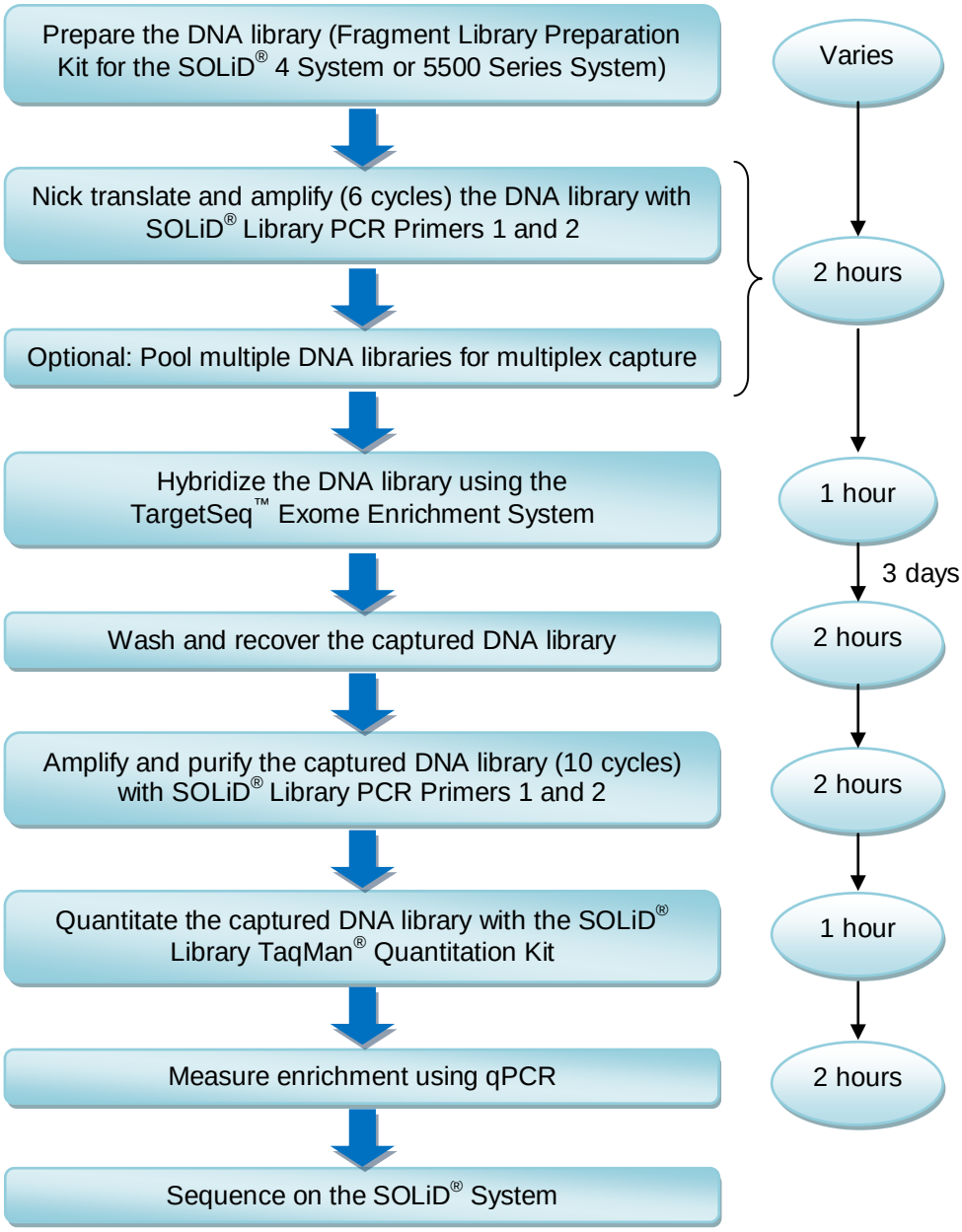
Overview

The TargetSeq™ Exome Enrichment System provides optimized reagents for capturing the exons identified in the VEGA, CCDS, and RefSeq databases (downloaded on October 1, 2009) and >700 microRNA genes from human genomic DNA for sequencing on the SOLiD® 4 System or 5500 Series Genetic Analysis Systems.

To use the TargetSeq™ system, you first construct a SOLiD® barcoded fragment library following the standard SOLiD® workflow. The library fragments are then captured in solution using ~2 million TargetSeq™ capture probes, 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, exome-enriched sample is then returned to the SOLiD® System workflow for emulsion PCR, enrichment, and sequencing.

Reference BED files are available for download from **www.invitrogen.com** for the primary targeted sequences of the human genome assembly. TargetSeq™ BED files are provided for hg18 and hg19 assemblies.

Workflow Summary



Methods

Prepare the sample library

Starting material

Use **3 µg of DNA** as starting material for preparing a SOLiD® fragment library for exome enrichment.

SOLiD® library construction

The TargetSeq™ Exome Enrichment System is compatible with barcoded fragment libraries constructed for the SOLiD® 4 System or 5500 Series Genetic Analysis System. You can enrich either a single library or four color-balanced, barcoded libraries in a single capture reaction.

Note: The TargetSeq™ Blockers listed on pages 4–5 are compatible with libraries constructed using SOLiD® Barcodes 001–016 for the SOLiD® 4 System or Barcodes T-001–T-016 for the 5500 Series Genetic Analysis System.

Prepare barcoded fragment libraries for your SOLiD® System using the instructions provided in the appropriate library preparation guide.

Manual library preparation guides:

- SOLiD® 4 System Library Preparation Guide (Publication part no. 4445673)
- Fragment Library Preparation User Guide—5500 Series Genetic Analysis Systems (Publication part no. 4460960)

Automated library preparation guides:

- Fragment Library Preparation Using the AB Library Builder™ System—SOLiD® 4 System (Publication part no. 4462553)
- Fragment Library Preparation Using the AB Library Builder™ System—5500 Series Genetic Analysis Systems (Publication part no. 4460965)

IMPORTANT!

- Fragment libraries **must be amplified** to obtain sufficient mass before exome enrichment. (Amplification is listed as optional in the *Fragment Library Preparation User Guide—5500 Series Genetic Analysis Systems*, but is **required** for this workflow.)
- We recommend amplifying for **6 cycles**.
- Before exome enrichment, amplify barcoded SOLiD® 4 System libraries using Multiplex Library PCR-1 and Multiplex Library PCR-2 primers to generate full-length library molecules. (These primers are included in the SOLiD® Fragment Library Barcoding Kit Module 1–16, catalog no. 4444836.) After enrichment, amplify the barcoded SOLiD® 4 libraries with Library PCR Primer 1 and Library PCR Primer 2 from the SOLiD® Fragment Library Oligos Kit (catalog no. 4401151). For 5500 Series libraries, use Library PCR Primer 1 and Library PCR Primer 2 for all amplification steps.

IMPORTANT!

- Following amplification, we recommend purifying SOLiD® 4 fragment libraries using Agencourt® AMPure® XP beads **instead of** the gel purification recommended in the *SOLiD® 4 System Library Preparation Guide*. AMPure® bead purification results in greater recovery of library material. Follow the AMPure® bead purification protocol provided on page 20 of this user guide for purifying your SOLiD® 4 fragment library.
- When preparing four barcoded fragment libraries for multiplexing, be sure to follow the instructions for color-balancing the barcodes in the appropriate SOLiD® System user guide (i.e., use the following full sets of four barcodes: 1–4, 5–8, 9–12, or 13–16)

Library amount needed

You can process either a single barcoded library or four pooled barcoded libraries in a single capture reaction. This kit requires a total of 500 ng of library DNA in each capture reaction, i.e. 500 ng of a single library or 125 ng each of four libraries.

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™ 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 Bioanalyzer™ peak

C = Library concentration by mass (ng/µL)

C (ng/µL) = A nmol/L × 660 ng/(nmol•bp) × (1 L/10^6 µL) × B bp

Mass of library (ng) = volume of library (µL) × C (ng/µL)

Example: Assuming A= 75 nmol/L, B is 260 bp, and library volume is 45 µL

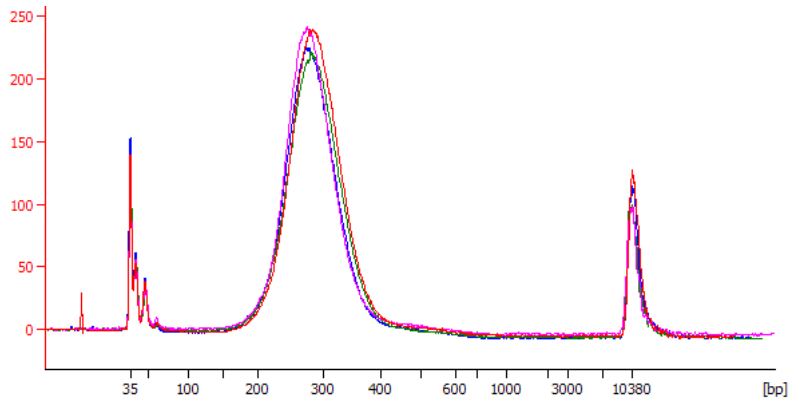
C (ng/µL) = 75 nmol/L × 660 ng/(nmol•bp) × (1 L/10^6 µL) × 260 bp

C (ng/µL) = **12.9 ng/µL**

Mass of library (ng) = 50 µL × 12.9 ng/µL = **645 ng**

Example data—2100 Bioanalyzer™ instrument

The graph below shows data from a 2100™ 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.

Exome probe hybridization

In this procedure, add Human Cot-1 DNA® and the appropriate blocker(s) to the fragment library or pooled libraries.

IMPORTANT!

The probe hybridization reaction runs for 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, you can add empty tubes to the thermal cycler to evenly distribute the pressure of the lid.

Aliquoting and storage of probe pools

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

1. If frozen, thaw the TargetSeq™ Exome Probe Pool vial on ice.
2. Vortex for 3 seconds.
3. Centrifuge the tube at 10,000 × g for 30 seconds to ensure that the liquid is at the bottom of the tube before opening the tube.
4. Aliquot the Probe Pool into single-use aliquots (4.5-µL/aliquot) in 0.2 ml PCR tubes or 96-well plates and store at –15°C to –25°C until use. The presence of some residual volume after dispensing all single-use aliquots is normal.

Library amount to be used in exome capture

The TargetSeq™ Exome Enrichment System can be used for exome enrichment of either a single library or four color-balanced, barcoded libraries in a single capture reaction. A total of 500 ng of library DNA is required in each capture reaction.

- When enriching a single library, use 500 ng of library.
- When enriching four libraries simultaneously, pool 125 ng of each library (in color-balanced barcode groups, as described in the **Important** note on page 10).

Note: For multiplex experiments of four barcoded libraries, be careful to use the following full sets of four barcodes: Barcodes 1-4, 5-8, 9-12, or 13-16.

Before starting

1. Dilute the stock Human Cot-1 DNA® provided in the kit (1 mg/mL) 10-fold in nuclease-free water, to 0.1 mg/mL.
2. Remove a 4.5-µL aliquot of the TargetSeq™ Exome Probe Pool from –20°C storage and thaw on ice.
3. Equilibrate a heat block to 95ºC.
4. Thaw the TargetSeq™ Hybridization Solution A and Hybridization Enhancer B at room temperature. If necessary, heat to 47°C to dissolve any precipitate.

Diluting blocker oligos (if necessary)

If you designed your own blocker oligos from the sequences listed on page 5, dilute each blocker to a concentration of 0.2 mM in Low TE Buffer. Then substitute these oligos as appropriate for the TargetSeq™ Blockers used in the following protocol.

For multiplex reactions:

After diluting the blockers to 0.2 mM, pool the four blockers specific for the barcodes in the libraries. Pool the equivalent of 1.25 µL of each blocker per reaction, for a total volume of 5 µL per reaction.

Add Human Cot-1 DNA® and TargetSeq™ Blockers

For a pool of four libraries:

1. Determine the volume of each library that contains 125 ng of amplifiable library molecules using the mass calculations from page 11.
2. In a new 1.5-mL LoBind tube, pool the appropriate volumes of the four libraries, for a total of 500 ng in the pool.
3. Add 5 µL of 0.1 mg/mL Human Cot-1 DNA®.
4. To the library pool, add 5 µL of TargetSeq™ Blocker P1 and 5 µL of TargetSeq™ Blockers 1–16 (or 5 µL each of your own 0.2-mM P1 blocker/blocker pool, designed from the sequences on page 5).

For a single library:

1. Determine the library volume that contains 500 ng of amplifiable library molecules using the mass calculations from page 11. Transfer that volume to a new 1.5-mL LoBind tube.
2. Add 5 µL of 0.1 mg/mL Human Cot-1 DNA®.
3. To the library pool, add 5 µL of TargetSeq™ Blocker P1 and 5 µL of TargetSeq™ Blockers 1–16 (or 5 µL each of your own 0.2-mM P1 blocker and the appropriate barcode blocker oligo, designed from the sequences on page 5).

Probe hybridization

1. 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.

2. Dry the sample in a vacuum concentrator set at 60°C (high heat) until completely dry (typically ~30 minutes).
3. 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

4. Cover the hole in the tube cap with a small piece of laboratory tape.
5. Vortex the tube for 10 seconds and centrifuge at maximum speed for 10 seconds.
6. Place the tube in a 95°C heat block for 10 minutes to denature the DNA.
7. Centrifuge the tube at maximum speed for 10 seconds at room temperature.
8. Transfer the pooled sample library to a 0.2-mL PCR tube or strip tube containing a 4.5-µL aliquot of the TargetSeq™ Exome Probe Pool, as prepared on page 3 (the entire volume can also be transferred to a well of a 96-well PCR plate).
9. Close the cap on the tube(s) tightly.
10. 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 the IMPORTANT note on page 13.

11. 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

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.

   Note: Store working solutions at room temperature for up to 2 weeks. The volumes in this table are calculated for a single exome capture reaction of a library or library pool. Scale up accordingly if you are performing multiple capture reactions.

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 or library pool 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/pools.)
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).

Continued on the next page

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.

Continued on the next page

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 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 15 µL of nuclease-free water to each tube.
17. Store the beads at –15°C to –25°C or proceed to "Amplify the libraries."

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 the libraries

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)

Amplification Procedure

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
Post-TargetSeq™ capture beads with DNA	15 µL
Platinum® PCR Amplification Mix	200 µL
Library PCR Primer 1, 50 µM	5 µL
Library PCR Primer 2, 50 µM	5 µL
Nuclease-free water	25 µL
Total	250 µL

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

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

4. 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!**

5. 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.

Post-amplification purification

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

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 library):

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

3. Add 375 µL (~1.5 volumes) of Agencourt® AMPure® XP Reagent to the post-capture amplified DNA from the previous page.
4. Vortex the beads for 10 seconds, then pulse-spin.
5. Incubate the mixture at room temperature (20–25°C) for 5 minutes.
6. Place the tube in a DynaMag™-2 magnetic rack for at least 1 minute 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 3 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. Remove the tube from the DynaMag™-2 magnetic rack, pulse-spin the tube, return the tube to the magnetic rack, then remove and discard the supernatant with a 20-µL pipettor.
10. Open the tube, and then dry the beads at room temperature (20–25°C) for 5–10 minutes.
11. Elute the DNA:
    a. Remove the tube from the DynaMag™-2 magnetic rack, then add 50 µL Low TE Buffer directly to the pellet to disperse the beads.
    b. Pipette the suspension up and down to mix.
    c. Vortex the beads for 10 seconds, then pulse-spin.
    d. Place the tube in a magnetic rack for at least 1 minute until the solution clears.
    e. 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 4ºC.

Quantitate the amplified DNA library

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™ 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 Bioanalyzer™ peak

C = Library concentration by mass (ng/µL)

C (ng/µL) = A nmol/L × 660 ng/(nmol•bp) × (1 L/10^6 µL) × B bp

Mass of library (ng) = volume of library (µL) × C (ng/µL)

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

C (ng/µL) = 21.6 nmol/L × 660 ng/(nmol•bp) × (1 L/10^6 µL) × 260 bp

C (ng/µL) = **3.7 ng/µL**

Mass of library (ng) = 50 µL × 4.1 ng/µL = **185 ng**

Measure enrichment by qPCR

Use the qPCR assay described in this section to assess sample enrichment using four positive control and two negative control primer pairs. The genomic loci targeted by the positive primers are included as capture targets in every exome library.

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

qPCR control primers

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

Gene	Primer type	Primer sequence	Control type
RUNX2	Forward	5′– CGC ATT CCT CAT CCC AGT ATG –3′	Positive
	Reverse	5′– AAA GGA CTT GGT GCA GAG TTC AG –3′	
PRKG1	Forward	5′– CCC ACC GCC TTC GAC AT –3′	
	Reverse	5′– CCT GCT TAC TGT GGG CTC TTG –3′	
SMG1	Forward	5′– CTC GCT TAA CCA GAC TCA TCT ACT GT –3′	
	Reverse	5′– ACT TGG CTC AGC TGT ATG AAG GT –3′	
PLAU	Forward	5′– GTG GCC AAA AGA CTC TGA GG –3′	
	Reverse	5′– CCT CCA CAC ACG TAG GTG AC –3′	
PLAU-3'UTR	Forward	5′– CAA ATC TCC CTG GTG CTT GT –3′	Negative
	Reverse	5′– CCT GCC CTA CAG CTC TCC TA –3′	
PLAU-Promoter	Forward	5′– AGC TGG GCG AGG TAG AGA GT –3′	
	Reverse	5′– CAG CGT CTG GAC TGA GGA AT –3′	

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

qPCR Reaction Setup

Prepare six 20-µL qPCR reactions for each enriched and unenriched library (4 positive controls and 2 negative controls). We recommend running each reaction in at least duplicate. So if you are comparing an unenriched to an enriched library, you will be running at a minimum 24 qPCR reactions (2 libraries × 6 primer sets × duplicate reactions = 24 reactions).

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

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

2. Dilute each unenriched library and enriched library to 0.1 ng/µL. Prepare enough of each diluted library to run at least 12 reactions (six primer sets in duplicate). Add 5 µL of diluted library to each appropriate qPCR reaction.

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

Stage	Step	Temp	Time
Holding	Denature	95 ºC	10 min.
40 cycles	Denature	95 ºC	10 sec
	Extend	60 ºC	1 min
Melting Curve	Denature	95 ºC	15 sec
		60 ºC	1 min
		95 ºC	Continuous
Holding	Hold	4 ºC	∞

Data analysis

Calculate the ΔC_T for each of the six test loci by subtracting the C_T value of the exome-enriched DNA library from the C_T value of the unenriched DNA library. For each of the four positive qPCR tests, values should fall within the parameters shown in Figure 1 and Table 1 on the next page.

ΔC_T values that are **two standard deviations below** the average (see Table 1 on the next page) for positive control loci or **greater than zero** for the negative control loci indicate that the capture reaction was inefficient or of low specificity and may yield poor sequencing results.

Figure 1: Example qPCR results—ΔCT for each control

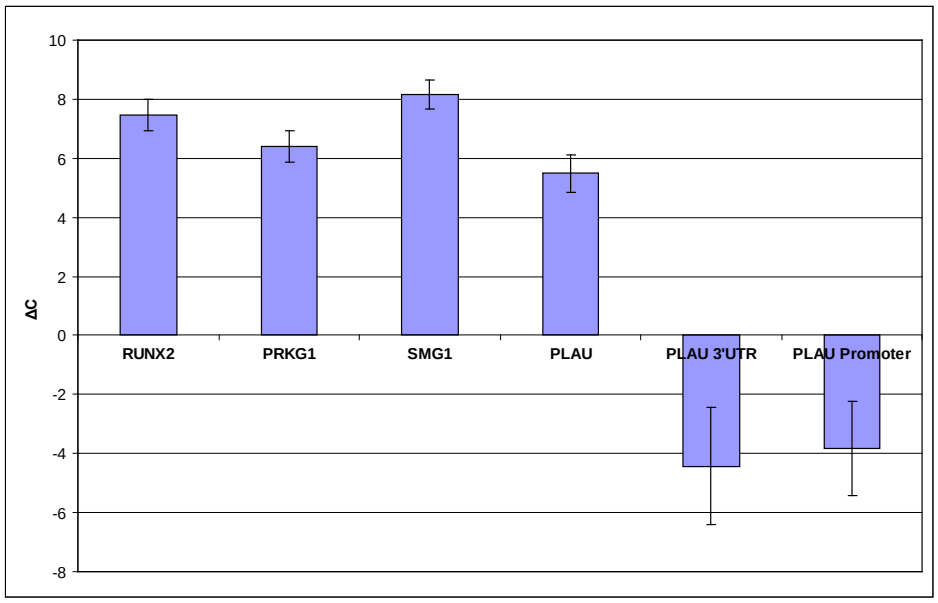


Table 1: Example qPCR results—Table format

	Positive Control loci				Neg Control Loci	
	RUNX2	PRKG1	SMG1	PLAU	PLAU 3'UTR	PLAU Promoter
Average ΔCT (n >25)	7.5	6.4	8.1	5.5	-4.4	-3.8
Std Dev (n >25)	0.5	0.5	0.5	0.6	2.0	1.6

These measurements were generated from exome capture libraries prepared from HuRef (NS12911) and NA12878 genomic DNA samples.

Note: If the value for any one of the test loci is significantly lower, it does not mean that enrichment failed but may be due to other variables such as genomic polymorphisms and other variables that could affect qPCR performance.

Proceed to SOLiD® sequencing

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

Data Analysis

BED files

Reference BED files are available for download from **www.invitrogen.com** for the primary targeted sequences of the human genome assembly. Files are provided for hg18 and hg19 assemblies.

Visit **www.invitrogen.com** and search for **TargetSeq BED hg18** or **TargetSeq BED hg19**. Links to these files are also provided on the product page for the TargetSeq™ Exome Enrichment System.

BED files can be viewed using the UCSC Genome Browser and/or used as the reference file for BioScope™ or LifeScope™ Genomic Analysis Software analysis of TargetSeq™ sequencing data.

Appendix

Technical Support

Web Resources

Visit the Invitrogen website at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
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Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (**www.invitrogen.com**).

Corporate Headquarters:
5791 Van Allen Way
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_support@invitrogen.com

Japanese Headquarters:
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: **jpinfo@invitrogen.com**

European Headquarters:
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
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E-mail: **eurotech@invitrogen.com**

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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.invitrogen.com/support or email techsupport@invitrogen.com

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