Rapid Protocol for DNA Probe Hybridization and Target Capture Using an Illumina TruSeq® or Ion Torrent Library

Version 2.1 (See Protocol Revision History, below, for updates to document)

Spike-In Applications: If you plan to use xGen® Lockdown® Probes for Spike-In into existing probe sets or panels, please contact our NGS technical support group at xgen@idtdna.com who will provide tailored recommendations for your specific experimental design.

Products Available from IDT

xGen® Lockdown® Probes (biotinylated DNA capture probes)	**Custom DNA Oligonucleotides**
xGen® Lockdown® Panels	Illumina P5 Primer: AATGATACGGCGACCACCGA
xGen® Blocking Oligos	Illumina P7 Primer: CAAGCAGAAGACGGCATACGA
Nuclease-free Water	Ion Torrent Primer A: CCATCTCATCCCTGCGTGTC
IDTE pH 8.0 (1X TE Solution)	Ion Torrent Primer P1: CCTCTCTATGGGCAGTCGGTGAT

Additional Materials Required

95% or 100% ethanol	General lab supplier
Agencourt® AMPure® XP - PCR Purification beads	Beckman-Coulter, Cat #A63880
Digital electrophoresis chips	Such as Bio-Rad Experion™ DNA 1K Analysis Kit, Cat # 700-7107 or Agilent High Sensitivity DNA Kit, Cat # 5067-4626
Dynabeads® M-270 Streptavidin	Life Technologies, Cat # 65305
Human Cot-1 DNA®	Invitrogen, Cat # 15279-011
KAPA HiFi HotStart ReadyMix	Kapa Biosystems, Cat #. KK2601
Library Quantification Kit – Illumina/Universal	Kapa Biosystems, Cat #KK4824
Library Quantification Kit – Ion Torrent/Universal	Kapa Biosystems, Cat #KK4827
MAXYmum Recovery® Microtube, 1.7 mL	VWR, Cat #22234-046
MAXYmum Recovery® PCR Tubes, 0.2 mL flat cap	VWR, Cat #22234-056
Qubit® Assay Tubes	Life Technologies, Cat #Q32856
Qubit® dsDNA HS Assay Kit	Life Technologies, Cat #Q32851
SeqCap® EZ Hybridization and Wash Kits (24 or 96 reactions)	Roche NimbleGen, Cat #05634261001 or Cat #05634253001

Required Equipment

96-Well and 384-Well Thermal Cyclers	General lab supplier
Digital Electrophoresis System	Such as Bio-Rad Experion™ Electrophoresis Station Cat #700-7010 or Agilent 2100 Electrophoresis Bioanalyzer, Cat #G2939AA
Magnetic Separation Rack	Such as NEB 6-tube separation rack, Cat #S1506S; Life Technologies 16-tube DynaMag™-2 Magnet, Cat #12321D, or Diagenode DiaMag02 magnetic rack, Cat #B04000001.
Microcentrifuge	General lab supplier
Qubit® 2.0 Fluorometer	Life Technologies, Cat #Q32866
Vortex Mixer	General lab supplier
Water Bath	General lab supplier

A. Hybridization of DNA Capture Probes to the Genomic DNA Library

This procedure summarizes the steps necessary for hybridization of xGen Lockdown Probes with an Illumina TruSeq or Ion Torrent library prepared from genomic DNA. Minor modifications to the PCR enrichment step will be necessary if using an alternate platform.

Note: Minor changes have been made to volumes used in this v2.1 protocol. Please review the entire protocol before beginning.

Before you start:

a. **If you received the xGen Lockdown Probes as a hydrated solution**, thaw (ice not required), mix thoroughly, and then briefly spin down. The pool is then ready for use as directed in this protocol.
b. **If you received xGen Lockdown Probes dry**, hydrate the pool of xGen Lockdown Probes to 0.75 pmol/µL in IDTE pH 8.0. If a capture probe pool of a lower concentration is used, IDT recommends drying down the portion of material you will use for your capture, and rehydrating in water to 0.75 pmol/µL. For additional support regarding resuspension of Lockdown Probes pools, visit http://www.idtdna.com/pages/products/nextgen/target-capture/xgen-lockdown-probes, click "Support", and expand "Number of Reactions and Resuspension Volumes".
c. Hydrate xGen Blocking Oligos to 1 nmol/µL in IDTE pH 8.0 (equivalent to 1X concentration). For additional support regarding resuspension of xGen Blocking Oligos, visit http://www.idtdna.com/pages/products/nextgen/target-capture/xgen-blocking-oligos, click "Support", and view "Resuspension Instructions".
d. If using xGen Universal Blocking Oligos instead of index-specific blocking oligos, use 1 µL (equivalent to 1 reaction volume) of each blocking oligo.

1. **Hybridize xGen Lockdown Probes to target.** *Note: IDT recommends a hybridization temperature of 65°C. This high temperature improves the percentage of on-target capture.*

   a. Combine the following in a low-bind 1.7 mL PCR tube:

   For Illumina TruSeq LT Libraries

   Important: If you are using a combination of 6 nt (adapters 1–12) and 8 nt (adapters 13–27) barcoded TruSeq LT adapters, use the formulas below to determine the fractions of 6 nt (X) and 8 nt (Y) blocking oligos you will need. X + Y must equal 1.

   $$X = \frac{number\ of\ libraries\ with\ adapters\ 1\text{–}12}{total\ number\ of\ barcoded\ libraries}$$

   $$Y = \frac{number\ of\ libraries\ with\ adapters\ 13\text{–}27}{total\ number\ of\ barcoded\ libraries}$$

   500 ng pooled, barcoded Illumina TruSeq LT Libraries
   5 µg Cot-1 DNA
   1 µL xGen Universal Blocking Oligo – TS-p5
   X µL xGen Universal Blocking Oligo – TS-p7 (6nt)
   Y µL xGen Universal Blocking Oligo – TS-p7 (8nt)

For Illumina TruSeq HT Libraries*

500 ng pooled, barcoded Illumina TruSeq® HT Libraries
5 µg Cot-1 DNA
1 µL xGen Universal Blocking Oligo TS HT-i5
1 µL xGen Universal Blocking Oligo TS HT-i7

***** No calculations are necessary for determining the required amounts of TruSeq HT blocking oligos because the lengths of the barcoded regions are fixed.

For Ion Torrent Libraries**

500 ng pooled, barcoded Ion Torrent libraries
5 µg Cot-1 DNA
1 µL xGen Universal Blocking Oligo – IT-P1
1 µL xGen Universal Blocking Oligo – IT-A*

**Assumes that the A adapter contains a barcode sequence. If the A adapter does not contain a barcode sequence, use xGen Standard Blocking Oligos.

b. Dry down the contents of the tube completely using a SpeedVac or a similar evaporator device.
c. Resuspend in 8.5 µL Nimblegen 2X Hybridization buffer (vial 5), 3.4 µL Nimblegen Hybridization Component A (vial 6), and 1.1 µL Nuclease-free water (this may take up to 10 min to go into solution).
d. Transfer resuspended material to a 0.2 mL PCR tube and incubate in a thermal cycler at 95°C for 10 min.
e. Add 4 µL xGen Lockdown Probe pool to the tube. Vortex and briefly spin down. Final volume should be 17.0 µL.
f. Incubate hybridization reaction at 65°C (set heated lid at 75°C) for 4 hr.

2. Wash and Recover Captured DNA.

Important: The temperature of the water bath **must** remain at 65°C. The temperatures displayed on many water baths are often imprecise; therefore, we recommend that you place an external, calibrated thermometer in the water bath to verify and maintain the correct temperature.

I. Prepare Sequence Capture and Bead Wash Buffers.

a. Dilute 10X Wash Buffers (I, II, III, and Stringent) and 2.5X Bead Wash Buffer to create 1X working solutions.

Concentrated Buffer	Volume Required (µL)	Add Nuclease-Free Water (µL)	Final Volume of 1X Buffer* (µL)
10X Wash Buffer I	30	270	300
10X Wash Buffer II	20	180	200
10X Wash Buffer III	20	180	200
10X Stringent Wash Buffer	40	360	400
2.5X Bead Wash Buffer	200	300	500

* Volumes are for a single experiment. Store 1X buffers at room temperature (15–25°C) for up to 2 wk.

b. For each capture reaction, preheat the following wash buffers to 65°C in a water bath†:
   400 µL 1X Stringent Wash Buffer
   100 µL 1X Wash Buffer I
   † Equilibrate buffers at 65°C for at least 2 hr. before starting wash steps of the captured DNA.

II. Prepare the Streptavidin Dynabeads.

a. Allow Dynabeads M-270 Streptavidin to equilibrate to room temperature for 30 min before use. IDT does not recommend using alternative streptavidin magnetic beads as these have delivered significantly reduced captured yields.
b. Mix the beads thoroughly by vortexing for 15 sec.
c. Aliquot 100 µL streptavidin beads per capture into a single 1.7 mL low-bind tube (i.e., for 1 capture use 100 µL beads, for 2 captures use 200 µL beads, etc.).
d. Place the tube in a magnetic separation rack. Allow the beads to separate from the supernatant. Carefully remove and discard the clear supernatant ensuring that all of the beads remain in the tube.
e. Add 200 µL 1X Bead Wash Buffer per 100 µL beads (measured at Step c). Vortex for 10 sec.
f. Place the tube back in the magnetic rack to bind the beads. Allow the beads to separate from the supernatant. Carefully remove and discard the clear supernatant ensuring that all of the beads remain in the tube.
g. Repeat Steps e–f.
h. After removing the buffer following the second wash, add 1X the original volume of beads of 1X Bead Wash Buffer (i.e., for 100 µL beads, use 100 µL buffer) and resuspend by vortexing.
i. Transfer 100 µL of the resuspended beads into a new 0.2 mL low-bind tube for each capture reaction.
j. Place the tube in a magnetic rack to bind the beads. Allow the beads to separate from the supernatant. Carefully remove and discard the clear supernatant ensuring that all of the beads remain in the tube.

Important: Proceed immediately to the next step, "*III. Bind hybridized target to the streptavidin beads.*" Do not allow the Dynabeads to dry out. Small amounts of residual Bead Wash Buffer will not interfere with downstream binding of the DNA to Dynabeads.

III. Bind hybridized target to the streptavidin beads.

a. Transfer the hybridization samples from A.1.f. to the tube containing prepared streptavidin beads.
b. Mix thoroughly by pipetting up and down 10 times.
c. Place the tube into a thermal cycler set to 65°C for 45 min (set heated lid at 75°C) to bind the DNA to the beads.
d. Vortex the tubes for 3 sec every 15 min to ensure that the beads remain in suspension.

IV. Wash streptavidin beads to remove unbound DNA.

Important: Work quickly to ensure temperature does not drop much below 65°C.

a. Add 100 µL pre-heated 1X Wash Buffer I to the tube and vortex for 10 sec to mix.
b. Transfer the mixture to a fresh low-bind 1.7 mL tube, and vortex briefly.

c. Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant containing unbound DNA and discard.

d. Perform the following wash:

   i. Add 200 µL preheated 1X Stringent Wash Buffer and pipette up and down 10 times to mix. Incubate at 65°C for 5 min.

   ii. Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant containing unbound DNA and discard.

e. Repeat Step d.

f. Add 200 µL room temperature 1X Wash Buffer I and vortex for 2 min to mix.

g. Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant and discard.

h. Add 200 µL room temperature 1X Wash Buffer II and vortex for 1 min to mix.

i. Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant and discard.

j. Add 200 µL room temperature 1X Wash Buffer III and vortex for 30 sec to mix.

k. Place the tube in the magnetic separation rack. Allow the beads to separate from the supernatant. Using a pipette, remove the supernatant and discard.

l. Remove the tube from the magnetic rack and add 20 µL Nuclease-Free Water to resuspend the beads. Mix thoroughly by pipetting up and down 10 times, and ensure any beads stuck to the side of the tube have been resuspended.

B. Post-Capture PCR

1. Perform final PCR enrichment.

a. Prepare the reaction mix.

For Illumina Libraries

2X KAPA HiFi HotStart ReadyMix	25 µL
10 µM Illumina P5 primer	2.5 µL
10 µM Illumina P7 primer	2.5 µL
Beads plus captured DNA	20 µL
Total Volume	**50 µL**

For Ion Torrent Libraries

2X KAPA HiFi HotStart ReadyMix	25 µL
10 µM Ion Torrent A primer	2.5 µL
10 µM Ion Torrent P1 primer	2.5 µL
Beads plus captured DNA	20 µL
Total Volume	**50 µL**

b. Briefly vortex the mixture and quick spin. Place reactions in the thermocycler and run the following program (cycling conditions recommended by Kapa Biosystems):

98°C	45 sec	
98°C	**15 sec**	10–12 cycles
65°C	**30 sec**	
72°C	**30 sec**	
72°C	1 min	
4°C	Hold	

c. Purify the post-capture PCR fragments using 75 µL (1.5X volumes) Agencourt AMPure XP beads, according to the manufacturer's protocol. Elute in 22 µL of IDTE pH 8.0 (1X TE Solution).

d. Transfer 20 µL of eluted product to a fresh low-bind 1.7 mL tube, ensuring no beads are carried over.

2. Validate Library

a. Measure the concentration of captured library using a Qubit Fluorometer (Invitrogen). Ensure the concentration is appropriate for assessment on the Bio-Rad Experion System or Agilent 2100 Bioanalyzer based on the specifications of the chip you are using.

b. Run 1 µL library on the Experion using a DNA 1K chip or the Bioanalyzer using a high sensitivity DNA chip. Take note of the average fragment length.

c. Perform library quantification using the appropriate KAPA Library Quantification Kit.

d. Use the correction factor below to determine molarity of the library (insert the appropriate dilution factor and average fragment length from Experion or Bioanalyzer results):

For Illumina Libraries:

Library	Concentrations of Triplicate Data Points (pM, calculated by qPCR instrument)			Average Concentration of diluted library (pM)	Size-Adjusted Concentration (pM)	Concentration of Undiluted Library Stock (pM)
Library 1:1000	A1	A2	A3	A	$A\ x \frac{452}{Avg.Fragment\ Length} = W$	W x 1000
Library 1:10,000	B1	B2	B3	B	$B\ x \frac{452}{Avg.Fragment\ Length} = X$	X x 10,000

Table reproduced from Library Quantification Kit – Illumina/Universal (Kapa Biosystems, Woburn, MA, USA)

Use the calculated concentration of undiluted library stock to prepare the library for sequencing.

For Ion Torrent Libraries:

Library Dilution	Dilution Factor (triplicate; calculated by qPCR instrument)			Average Dilution Factor	Size-Adjusted Dilution Factor	Dilution Factor of Undiluted Library Stock
Library 1:1000	A1	A2	A3	A	$A\ x \frac{153}{Avg.Fragment\ Length} = W$	W x 1000
Library 1:10,000	B1	B2	B3	B	$B\ x \frac{153}{Avg.Fragment\ Length} = X$	X x 10,000

Table reproduced from Library Quantification Kit – Ion Torrent platform (Kapa Biosystems, Woburn, MA, USA)

Use the calculated concentration of undiluted library stock to prepare the library for emPCR.

C. Sequencing

Perform sequencing according to the instructions for your specific platform.

Protocol Revision History

Version	Date Released	Description of Changes
2.1	November 2014	Product begins shipping wet (already resuspended). Instructions added for use of product received wet as well as dry. In addition, reagent volumes have been changed to reflect further protocol optimization. Spike-In Protocol (former Appendix A) has been removed from this protocol, and is available separately upon request to xgen@idtdna.com.
2.0	January 2014	Change in hybridization temperature from 47°C to 65°C. Change in hybridization time from 48 hr to 4 hr. Added Ion Torrent information. Change in post-capture amplification from off bead to on bead.
1.0	May 2013	Original protocol.