xGen® Lockdown® Probes
xGen® Lockdown® Panels

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

Version 2.0

Products Available from IDT

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

Additional Materials Required

95% or 100% Ethanol	General lab supplier
Agencourt® AMPure® XP - PCR Purification beads	Beckman-Coulter, cat. no. A63880
Digital Electrophoresis Chips	Such as Bio-Rad Experion™ DNA 1K Analysis Kit, cat. no. 700-7107 or Agilent High Sensitivity DNA Kit, cat. no. 5067-4626
Dynabeads M-270 Streptavidin	Life Technologies, cat. no. 65305
Human Cot-1 DNA	Invitrogen, cat. no. 15279-011
KAPA HiFi HotStart ReadyMix	Kapa Biosystems, cat. no. KK2601
Library Quantification Kit – Illumina/Universal	Kapa Biosystems, cat. no. KK4824
Library Quantification Kit – Ion Torrent/Universal	Kapa Biosystems, cat. no. KK4827
MAXYmum Recovery® Microtube, 1.7 mL	VWR, cat. no. 22234-046
MAXYmum Recovery® PCR Tubes, 0.2 mL flat cap	VWR, cat. no. 22234-056
Qubit® Assay Tubes	Life Technologies, cat. no. Q32856
Qubit® dsDNA HS Assay Kit	Life Technologies, cat. no. Q32851
SeqCap® EZ Hybridization and Wash Kits (24 or 96 reactions)	Roche NimbleGen, cat. no. 05634261001 or 05634253001

Required Equipment

96-Well and 384-Well Thermal Cyclers	General lab supplier
Digital Electrophoresis System	Such as Bio-Rad Experion™ Electrophoresis Station cat. No. 700-7010 or Agilent 2100 Electrophoresis Bioanalyzer, cat. no. G2939AA
Magnetic Separation Rack	Such as NEB 6-tube separation rack, cat. no. S1506S; or Life Technologies 16-tube DynaMag™-2 Magnet, cat. no. 12321D
Microcentrifuge	General lab supplier
Qubit® 2.0 Fluorometer	Life Technologies, cat. no. 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.

Before you start

a. Hydrate the dried down pool of Lockdown® Probes to 1.5 pmol/µL in IDTE pH 8.0. If a capture probe pool at a lower concentration is used, IDT recommends drying down a portion of the material and rehydrating in water to 1.5 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".

b. Hydrate xGen® Blocking Oligos to 1 nmol/µL in IDTE pH 8.0 (which is 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".

c. 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 now recommends a higher hybridization temperature of 65°C. The higher 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.

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

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

   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 (6 nt)
   Y µL xGen® Universal Blocking Oligo – TS-p7 (8 nt)

*For Illumina TruSeq HT Libraries**

500 ng Illumina TruSeq® HT Library
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 7.5 µL Nimblegen 2X Hybridization buffer (vial 5), 3 µL Nimblegen Hybridization Component A (vial 6), and 2.5 µL Nuclease-free water (this may take up to 10 minutes 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 2 µL Lockdown Probe pool (3 pmol total) to the tube. Vortex and briefly spin down. Final volume should be 15 µL.
f. Incubate hybridization reaction at 65°C (set heated lid at 75°C) for 4 hours.

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 weeks.

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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 hrs 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, *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.
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.

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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 of library on the Experion system 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	$A \times \frac{452}{Avg.\ Fragment\ Length} = W$	W x 1000
Library 1:10,000	B1	B2	B3	B	$B \times \frac{452}{Avg.\ Fragment\ Length} = X$	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	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	$A \times \frac{153}{Avg.\ Fragment\ Length} = W$	W x 1000
Library 1:10,000	B1	B2	B3	B	$B \times \frac{153}{Avg.\ Fragment\ Length} = X$	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 sequencing.

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C. Sequencing

Perform sequencing according to the instructions for your specific platform.

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Appendix A

Probe Supplementation (Spike-In) Protocol v1.0

One of the advantages of xGen® Lockdown® Probes is the ability use them to supplement ("spike in") an existing capture probe set without requiring a new synthesis of the entire pool. The amount of Lockdown Probes to be added can be easily determined using simple calculations. The typical spike in approach is to add an equivalent amount of each of the additional oligos to be spiked in as found in the original pool.

Step-by-Step Guide

1. Calculate the amount in pmol of each probe in the hybridization capture reaction containing the xGen® Lockdown® Panel you want to supplement:

$$\text{pmol per probe} = \frac{\text{pmol used in capture reaction}}{\text{no. of probes in panel}}$$

This step is for calculating the amount in pmol of each probe that is present in the hybridization capture reaction using the pool. E.g., when using the xGen® AML Cancer Panel for target enrichment, the current recommendation is to add 3 pmol of the pool to the libraries being enriched in the hybridization capture reaction. The xGen AML Cancer Panel v1.0 contains 11,743 individual probe sequences; therefore, the effective amount of each probe would be ***3 pmol/11,734 probes = 2.56 x 10^{-4} pmol (0.256 fmol)****.*

2. Calculate the total concentration of spike-in probe needed (pmol/µL, same as µM):

$$\text{Conc.of spike in probe (pmol/µL)} = \frac{\text{no. of pmol per probe (from Step 1)}}{1.0\ \mu L}$$

To add another 200 probes to the xGen AML Cancer Panel described above, you would need ***2.56 x 10-4 pmol (0.256 fmol)*** *of each spike-in probe. The total amount in pmol to be added would be* ***200 probes x 2.56 x 10^{-4} pmol = 0.0512 pmol****. If you want to add 1 µL of the spike-in pool to supplement the main pool in the capture reaction, then the concentration of the spike-in pool would need to be 0.0512 pmol/µL.*

3. Calculate volume required to resuspend dry spike-in pool to 1.5 pmol/µL (1.5 µM):

$$\text{volume (µL)} = \frac{\text{no.of probes x pmol per probe in tube}}{1.5\ \text{pmol/µL}}$$

*A tube of 200 probes delivered as xGen Lockdown Probes Mini pool (at 3 pmol/probe) would contain 600 pmol total probes. To resuspend the pool to 1.5 µM (pmol/µL), you would need to add 400 µL of IDTE, pH 8.0 (***600 pmol/400 µL = 1.5 pmol/µL***).*

4. Prepare diluted spike-in pool. Take 1 µL resuspended spike-in pool (at 1.5 pmol/µL, from **Step 3**) and add IDTE, pH 8.0, to obtain desired spike-in pool probe concentration as calculated in **Step 2**:

$$\text{Final volume of spike in pool} = \frac{1.5\ \text{pmol/µL}}{\text{Conc.of spike in probe needed (from Step 2)}}$$

To obtain the final concentration of 0.0512 pmol/µL needed for the spike in, you would need to take 1 µL of the 1.5 pmol/µL solution (from ***Step 3****) and add IDTE, pH 8.0 to a final volume of 29.3 µL.*

$$M1V1 = M2V2 \qquad (1.5\ \text{pmol/µL})(1\ \text{uL})/0.0512\ \text{pmol/µL} = 29.3\ \mu l$$

5. Perform hybridization capture reaction using **2 µL xGen Lockdown Panel** and **1 µL diluted spike-in pool** (prepared at **Step 4**).

For further information, visit www.idtdna.com/xgen or contact us at xgen@idtdna.com.