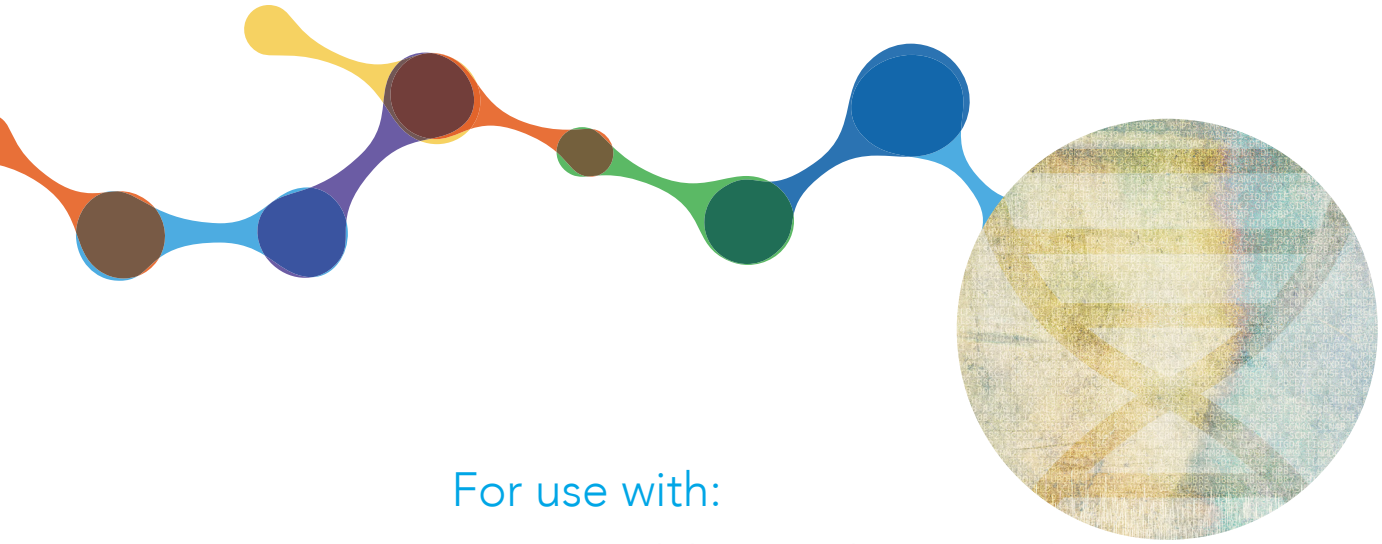
Hybridization capture of DNA libraries using xGen® Lockdown® Probes and Reagents



For use with:

- xGen® Lockdown® Probes or Panels
- xGen® Lockdown® Reagents
- Illumina® adapter-ligated libraries or Ion Torrent™ libraries

Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents

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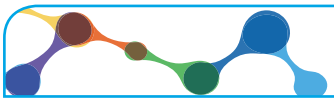


Introduction

This protocol includes the steps necessary for hybridization of xGen® Lockdown® Probes or Panels with a library (i.e., Illumina® single-index or dual-index adapter-ligated library or Ion Torrent™ library) prepared from genomic DNA and for target enrichment by PCR before sequencing. If using an alternate platform, contact xgen@idtdna.com for advice about PCR enrichment.

xGen Lockdown Probes or Panels

xGen Lockdown Probes are individually synthesized, 5'-biotinylated oligos for target capture applications in next generation sequencing. These probes are useful for creating custom capture panels that can be optimized, expanded, and combined with other panels. xGen Lockdown Probes can also be used to enhance the performance of existing capture panels, rescuing poorly represented regions, such as areas of high GC content. If you plan to use xGen Lockdown Probes for spike-ins 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.

xGen Lockdown Panels are stocked enrichment panels for targeted next generation sequencing and are typically based on 1X tiling of xGen Lockdown Probes. We currently offer research panels targeting the human exome, as well as acute myeloid leukemia, pan-cancer, and inherited diseases genes.

xGen Blocking Oligos

xGen Universal Blocking Oligos bind to platform-specific adapter sequences on a designated strand (usually the inverse of the synthetic adapter) to help prevent non-specific binding, improve the number of reads on-target, and increase the depth of enrichment.

xGen Lockdown Reagents

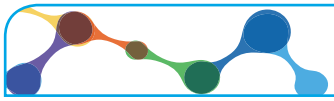
The components of the xGen Lockdown Reagents have been optimized for the hybridization and wash steps in target capture protocols using xGen Lockdown Probes and Panels.

Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents

Protocol overview

Protocol step		Approximate time
A	Prepare capture probes and blocking oligos	45 minutes
B	Combine and dry blocking oligos, Cot-1, and genomic DNA library	
STOP		
C	Hybridize capture probes with the library	4 hours
D–E	Prepare buffers and streptavidin beads	1.75 hours
F	Bind hybridized targets to streptavidin beads	
G	Wash streptavidin beads to remove unbound DNA	
H	Perform PCR enrichment	
STOP		
I	Purify postcapture PCR fragments	2.5 hours
STOP		
J	Validate and quantify library	
STOP		
Ready for sample preparation and sequencing		**Total: 9 hours**

STOP Optional stopping points



Reagents, kits, and equipment

Oligos and reagents from IDT

	Size	Storage conditions	Ordering information
Target capture			
xGen® Lockdown® Probes	Varies	–20°C*	www.idtdna.com/xGen
xGen® Lockdown® Panels	16 or 96 rxns	–20°C*	
xGen® Universal Blocking Oligos	Varies	–20°C*	
xGen® Lockdown® Reagents	16 or 96 rxns	–20°C	
Custom DNA oligonucleotides			
Illumina® P5 Primer: AATGATACGGCGACCACCGA	Varies	–20°C	www.idtdna.com Custom DNA Oligos page
Illumina® P7 Primer: CAAGCAGAAGACGGCATACGA	Varies	–20°C	
Ion Torrent™ Primer A: CCATCTCATCCCTGCGTGTC	Varies	–20°C	
Ion Torrent™ Primer P1: CCACTACGCCTCCGCTTTCCTCTCTATG	Varies	–20°C	
Reagents			
IDTE pH 8.0 (1X TE Solution)	10 × 2 mL	Room temp. (15–25°C)	Cat #11-01-02-05
Nuclease-Free Water	10 × 2 mL	Room temp. (15–25°C)	Cat #11-04-02-01

* See resuspension and storage instructions at www.idtdna.com/protocols.

Safety data sheets (SDSs) and certificates of analysis (COAs) for the xGen Lockdown Reagents may be obtained by emailing xgen@idtdna.com. For SDSs and COAs for other IDT products, go to www.idtdna.com.

Reagents, kits, and equipment

Additional materials and equipment

Materials	Ordering information
>80% Ethanol	General laboratory supplier
Agencourt® AMPure® XP – PCR Purification beads	Beckman-Coulter, Cat #A63880
Digital electrophoresis chips	Bio-Rad Experion™ DNA 1K Analysis Kit, Cat #700-7107; Agilent High Sensitivity DNA Kit, Cat #5067-4626; Agilent High Sensitivity D1000 ScreenTape, Cat #5067-5584; or equivalent
Dynabeads® M-270 Streptavidin	Life Technologies, Cat #65305
Invitrogen™ Human Cot-1 DNA®	Life Technologies, 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® Microtubes, 1.7 mL	VWR, Cat #22234-046
MAXYmum Recovery® PCR Tubes, 0.2 mL flat cap	VWR, Cat #22234-056
Qiagen® Buffer EB (or equivalent: 10 mM Tris-Cl, pH 8.5)	Qiagen, Cat #19086 (or general laboratory supplier)
(Optional) Qubit® Assay Tubes	Life Technologies, Cat #Q32856
(Optional) Qubit® dsDNA HS Assay Kit	Life Technologies, Cat #Q32851
Equipment	**Ordering information**
96-Well and 384-well thermal cyclers	General laboratory supplier
Digital electrophoresis system	Bio-Rad Experion™ Electrophoresis Station Cat #700-7010; Agilent 2100 Electrophoresis Bioanalyzer, Cat #G2939AA; Agilent 2200 TapeStation, Cat #G2965AA; or equivalent
Magnetic separation rack	NEB 6-tube separation rack, Cat #S1506S; Life Technologies 16-tube DynaMag™-2 Magnet, Cat #12321D; Diagenode DiaMag02 magnetic rack, Cat #B04000001; or equivalent
Microcentrifuge	General laboratory supplier
(Optional) Qubit® 3.0 Fluorometer	Life Technologies, Cat #Q33216
Vacuum concentrator or oven	General laboratory supplier
Vortex mixer	General laboratory supplier
Water bath or heating block	General laboratory supplier

A

Prepare capture probes and blocking oligos

xGen Lockdown Probes

If you received the xGen Lockdown Probes as a hydrated solution:

1. Thaw at room temperature (15–25°C).
2. Mix thoroughly and briefly spin down.

If you received the xGen Lockdown Probes dry:

Resuspend in IDTE pH 8.0 to a final concentration of 0.75 pmol/µL.

If the concentration of your capture probe pool is <0.75 pmol/µL, we recommend the following:

1. Dry the portion of material for your capture.
2. Resuspend in nuclease-free water to a final concentration of 0.75 pmol/µL.

For additional support regarding resuspension of Lockdown Probes pools, visit www.idtdna.com/xgen ► **xGen Lockdown Probes** ► **Support** tab ► expand **Number of Reactions and Resuspension Volumes**.

xGen Universal Blocking Oligos

Resuspend xGen Universal Blocking Oligos in IDTE pH 8.0 to a final concentration of 1 rxn/µL (1X final concentration).

For additional support regarding resuspension of xGen Blocking Oligos, visit www.idtdna.com/xgen ► **xGen Blocking Oligos** ► **Support** tab ► view the **Resuspension Instructions**.

B

Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents

Combine and dry blocking oligos, Cot-1, and the genomic DNA library

1. Based on your library type, mix the following in a low-bind 1.7 mL PCR tube (for example, MAXYmum Recovery® tube):

	Illumina® LT-adapter ligated libraries	Illumina® HT-adapter ligated libraries	Ion Torrent™ libraries
Pooled, barcoded library	500 ng	500 ng	500 ng
Cot-1 DNA	5 µg	5 µg	5 µg
xGen® Universal Blocking Oligo (1)	1 µL (TS-p5)	1 µL (TS HT-i5)†	1 µL (IT-P1)
xGen® Universal Blocking Oligo (2)	X µL (TS-p7, 6 nt)*	1 µL (TS HT-i7)†	1 µL (IT-A)‡
xGen® Universal Blocking Oligo (3)	Y µL (TS-p7, 8 nt)*	—	—

* **Important:** If you are using a combination of 6 nt (adapters 1–12) and 8 nt (adapters 13–27) barcoded LT adapters (for example, if you are using TruSeq LT adapters), use the following formulas to determine the fractions of 6 nt (X) and 8 nt (Y) blocking oligos you need:

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

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

$$X + Y = 1$$

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

‡ If the A adapter does not contain a barcode sequence, use 1 µL of xGen Standard Blocking Oligos (that is, the IT A Blocker).

2. Dry the contents of the tube using a vacuum concentrator (for example, SpeedVac® System or a similar evaporator device) set at 70°C or lower.

STOP **Optional stopping point:** After drying, tubes can be stored overnight at room temperature (15–25°C).

C

Hybridize DNA capture probes with the library

1. Thaw all xGen Lockdown Reagents buffers at room temperature.

   Note: Inspect the tube of 2X Hybridization Buffer for crystallization of salts. If crystals are present, heat the tube at 65°C, shaking intermittently, until the buffer is completely solubilized; this may require heating for several hours.

2. Add the following to the tube from Step B.2, and incubate at room temperature for 5–10 min:

8.5 µL	xGen 2X Hybridization Buffer
2.7 µL	xGen Hybridization Buffer Enhancer
1.8 µL	Nuclease-Free Water

3. Pipette up and down to mix, and transfer to a low-bind 0.2 mL PCR tube (for example, MAXYmum Recovery® tube).

4. Incubate in a thermal cycler at 95°C for 10 min.

5. Remove samples from thermal cycler and immediately add 4 µL of the xGen Lockdown Probe pool.

   Note: Final volume will be 17 µL.

6. Vortex and briefly spin down.

7. Incubate samples in a thermal cycler at 65°C (with the heated lid at 75°C) for 4 hr.

   Note: The 65°C hybridization temperature improves the percentage of on-target capture.

D

Prepare wash buffers

1. For a single capture reaction, dilute the following xGen buffers to create 1X working solutions as follows:

   Note: The 1X working solutions are stable at room temperature (15–25°C) for up to 4 weeks.

	Concentrated buffer (µL)	Nuclease-free water (µL)
xGen 2X Bead Wash Buffer	250	250
xGen 10X Wash Buffer I*	30	270
xGen 10X Wash Buffer II	20	180
xGen 10X Wash Buffer III	20	180
xGen 10X Stringent Wash Buffer	40	360

* If necessary, heat xGen 10X Wash Buffer I in a 65°C water bath or heating block to resuspend particulates.

D

Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents

2. Prepare aliquots of Wash Buffer I and Stringent Wash Buffer from Step D.1, and store at the temperature specified in the following table:

Buffer	Volumes of 1X working solution for each capture	Temperature for 1X working solution
Wash Buffer I	100 µL	65°C*
	200 µL	room temp (15–25°C)
Stringent Wash Buffer	400 µL	65°C*

* **Important:** Preheat buffers in a 65°C water bath for at least 2 hours before use in Step G.

3. Keep the remaining 1X buffers at room temperature.

E

Prepare the streptavidin beads

Important: Beads should be prepared immediately before use. Do not allow beads to dry out.

1. Equilibrate Dynabeads® M-270 Streptavidin beads at room temperature for approximately 30 min before use.

Important: We do not recommend using alternative streptavidin magnetic beads, because many of these have delivered significantly reduced capture yields.

2. Mix the beads thoroughly by vortexing for 15 sec.

3. Aliquot 100 µL of beads per capture into a single 1.7 mL low-bind tube.

   For example: for 1 capture, prepare 100 µL of beads and for 2 captures, prepare 200 µL of beads. For more than 6 captures, you will need more than one tube.

4. Place the tube in a magnetic separation rack (magnetic rack), allowing beads to fully separate from the supernatant.

5. Remove and discard the clear supernatant, ensuring that the beads remain in the tube.

E

6. Perform the following wash:
   a. Add 200 µL of 1X Bead Wash Buffer per capture, and vortex for 10 sec.
   b. Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.
   c. Carefully remove and discard the clear supernatant.
7. Perform a second wash by repeating Step E.6.
8. Add 100 µL of 1X Bead Wash Buffer per capture (refer to Step E.3) and vortex.
9. Transfer 100 µL of the resuspended beads into a new 0.2 mL low-bind tube for each capture reaction.
10. Place the tube in a magnetic rack, allowing beads to fully separate from the supernatant.
11. Carefully remove and discard the clear supernatant.

Note: Small amounts of residual Bead Wash Buffer will not interfere with downstream binding of the DNA to the beads.

Important: Proceed immediately to the next section, **Bind hybridized target to the streptavidin beads.**

F

Bind hybridized target to the streptavidin beads

1. Transfer the hybridization samples (from Step C.7) to the tube containing prepared beads (from Step E.11).
2. Mix thoroughly by pipetting up and down 10 times.
3. Bind the DNA to the beads by placing the tube into a thermal cycler set to 65°C (with the heated lid at 75°C) for 45 min.
4. Every 12 min during the 65°C incubation, vortex the tubes for 3 sec to ensure that the beads remain in suspension.

G

Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents

Wash streptavidin beads to remove unbound DNA

Note: Use the 1X wash buffers from Step D.

1) Perform 65°C washes.

1. Add 100 µL preheated 1X Wash Buffer I to the tube from Step F.4.
2. **Important:** Vortex briefly, and spin to collect contents at the bottom of the tube.
3. Transfer the mixture to a new low-bind 1.7 mL tube.
4. **Important:** Vortex briefly.
5. Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.
6. Pipet and discard the supernatant, which contains unbound DNA.
7. Perform the following wash:
   a. Add 200 µL of preheated 1X Stringent Wash Buffer, and slowly pipet up and down 10 times.
   Important: Do not create bubbles during pipetting.
   b. Incubate in a water bath at 65°C for 5 min.
   c. Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.
   d. Pipet and discard the supernatant, which contains unbound DNA.
8. Repeat Step 7.

2) Perform room temperature washes.

1. Add 200 µL of room temperature 1X Wash Buffer I and vortex for 2 min.
2. Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.
3. Pipet and discard the supernatant.
4. Add 200 µL of room temperature 1X Wash Buffer II and vortex for 1 min.
5. Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.
6. Pipet and discard the supernatant.
7. Add 200 µL of room temperature 1X Wash Buffer III and vortex for 30 sec.
8. Place the tube in the magnetic rack, allowing beads to fully separate from the supernatant.
9. Pipet and discard the supernatant.

G

3) Resuspend beads.

1. Remove the tube containing the beads with captured DNA from the magnetic rack.
2. Add 20 µL of Nuclease-Free Water to the beads.
3. Pipet up and down 10 times and ensure any beads stuck to the side of the tube have been resuspended.

Important: Do not discard the beads. Use the entire 20 µL of resuspended beads with captured DNA in Step H.

H

Perform final, postcapture PCR enrichment

1. Based on your library type, prepare the PCR mix in 0.2 mL low-bind PCR tubes as follows:

For Illumina® libraries		For Ion Torrent™ libraries	
2X KAPA HiFi HotStart ReadyMix	25 µL	2X KAPA HiFi HotStart ReadyMix	25 µL
10 µM Illumina P5 primer	2.5 µL	10 µM Ion Torrent A primer	2.5 µL
10 µM Illumina P7 primer	2.5 µL	10 µM Ion Torrent P1 primer	2.5 µL
Beads with captured DNA (from Step G.3.2)	20 µL	Beads with captured DNA (from Step G.3.2)	20 µL
Total volume	**50 µL**	**Total volume**	**50 µL**

2. Briefly vortex and spin the PCR mix, but ensure that the beads remain in solution.
3. Place the PCR tube in the thermal cycler, and run the following program with the heated lid set at 105°C:

	Number of cycles	Temperature (°C)	Time
Polymerase activation	1	98	45 sec
Amplification	12		
Denaturation		98	15 sec
Annealing		60	30 sec
Extension		72	30 sec
Final extension	1	72	1 min
Hold	1	4	Hold

Cycling conditions recommended by Kapa Biosystems.

STOP

Optional stopping point: PCR-enriched captures may be stored at 4°C overnight.

I

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Purify postcapture PCR fragments

1. Add 75 µL (1.5X volume) of Agencourt® AMPure® XP beads to each PCR-enriched capture.
2. Follow the binding and washing steps in the Agencourt AMPure protocol, **except use 80% ethanol for the washes**.
3. Elute in 22 µL of Qiagen® Buffer EB or equivalent (10 mM Tris-Cl, pH 8.5).
4. Transfer 20 µL of eluted product to a fresh 1.7 mL low-bind tube, ensuring no beads are carried over.

Optional stopping point: Purified PCR fragments may be stored at –20°C for up to 1 week.

J

Validate and quantify library

1. (Optional) Measure the concentration of the captured library using a Qubit® Fluorometer and the Qubit dsDNA HS Assay Kit.

   Note: This can be done to ensure that the concentration of the captured library is within the detection limits of the chip or tape used in Step J.2 (below) for your digital electrophoresis system.
2. Measure the average fragment length of the captured library on a digital electrophoresis system (e.g., the BioRad Experion™ System using a DNA 1K chip, the Agilent 2100 Bioanalyzer using a high sensitivity DNA chip, or Agilent 2200 TapeStation using a DNA tape).
3. Quantify libraries using the appropriate KAPA Library Quantification Kit, as directed by the manufacturer.

Optional stopping point: Library may be stored at –20°C overnight.

Sequencing

Perform sequencing according to the instructions for your specific platform.

- For Illumina libraries: Use the calculated concentration of undiluted library stock (from Step J.3) to prepare the library for sequencing.
- For Ion Torrent libraries: Use the calculated concentration of undiluted library stock (from Step J.3) to prepare the library for emulsion PCR.

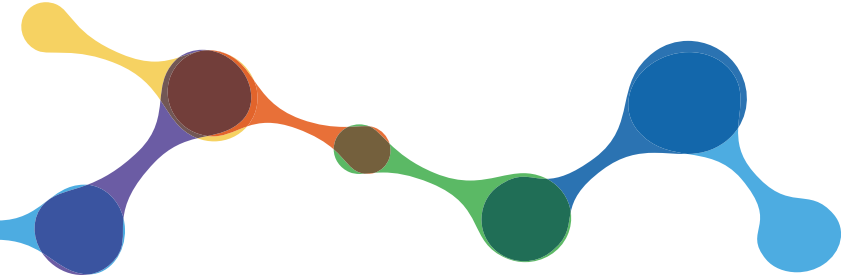
Hybridization capture of DNA libraries using xGen Lockdown Probes and Reagents

Protocol revision history

Version	Date released	Description of changes
1.0	September 2015	Original protocol
2.0	March 2016	Included instructions for solubilizing 2X Hybridization Buffer. Removed requirement to work quickly at Step G.1. Added instructional detail to bead resuspension steps. Removed KAPA library quantification tables.

Tech support:

xgen@idtdna.com



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