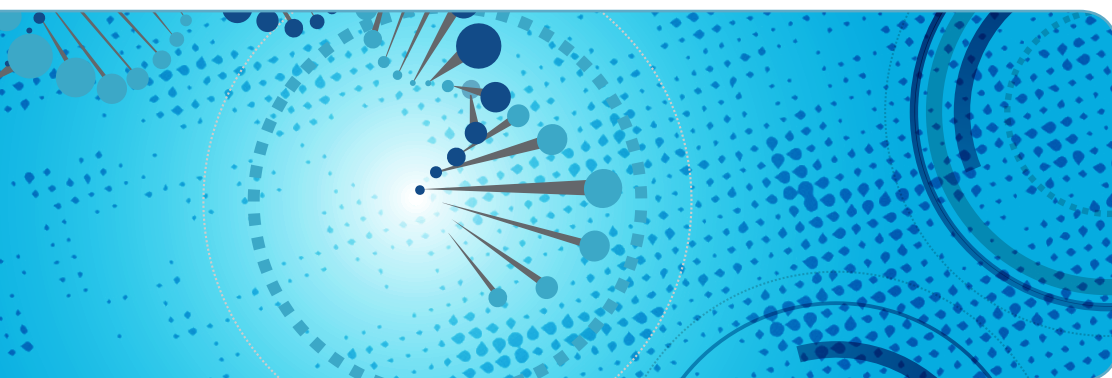


# xGen hybridization capture of DNA libraries

For NGS target enrichment



## Uses IDT's:

- xGen Hybridization and Wash Kit
- xGen Universal Blockers—TS Mix, 10 bp TS Mix, or NXT Mix
- xGen Lockdown Panels and Probe Pools
- xGen Gene Capture Pools
- xGen Library Amplification Primers

## Uses IDT's:

- Illumina platform-compatible libraries

See what more we can do for you at [www.idtdna.com](http://www.idtdna.com).





## Revision history

Document version	Date released	Description of changes
4	May 2019	Updated to specify that xGen Universal Blockers—NXT Mix can bind to Illumina Nextera adapter sequences with 8 bp or 10 bp indexes
3	March 2019	Added Appendix B to describe combining panels; removed instructions for preparing dry xGen Lockdown Probes since they are currently only provided in solution.
2	June 2018	Updated to include 10 bp blockers
1	April 2018	Original version

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# Introduction

This protocol includes the steps necessary for target enrichment of a next generation library prepared from genomic DNA, using xGen Lockdown Panels or Probe Pools. Visit [www.idtdna.com/protocols](http://www.idtdna.com/protocols) to verify that you are using the most current version of this protocol.



**Important!** Do not use this optimized protocol with the previous xGen Lockdown Reagents kit (cat # 1072280 or 1072281) because you will not have sufficient volumes of some required buffers.

## xGen Lockdown Probe Pools

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 Probe Pools can also be used to enhance the performance of existing capture panels by rescuing poorly represented regions, such as areas of high GC content. For more information on combining xGen Lockdown Panels and Probes see [Appendix B](#).

xGen Lockdown Panels are predesigned and inventoried enrichment panels for targeted next generation sequencing. They are typically based on 1X tiling of xGen Lockdown Probes. Several research panels are available for the human genome, including panels for the whole exome, disease-related genes, and sample identification. Visit [www.idtdna.com/LockdownPanels](http://www.idtdna.com/LockdownPanels) for a complete list.

## xGen blockers

xGen Universal Blockers will bind to platform-specific adapter sequences on a designated strand to help prevent non-specific binding, improve the number of reads on target, and increase the depth of enrichment. Universal blockers—TS Mix and Universal blockers—10 bp TS Mix are compatible with ligation-based library prep techniques, such as Illumina's TruSeq™ library kits. Universal blockers—TS Mix is designed for 6- and 8-base, single- and dual-indexing schemes. Universal blockers—10 bp TS Mix is designed for 10-base, dual-indexing schemes. xGen Universal Blockers—NXT Mix binds to Illumina Nextera™ adapter sequences with 8 bp and 10 bp indexes.

## xGen Hybridization and Wash Kit

The components of the xGen Hybridization and Wash Kit have been optimized for the hybridization and wash steps in target capture protocols using xGen Lockdown Probe Pools.



**Note:** The Cot DNA provided with this kit serves to block repetitive elements for human DNA. If you are using a library composed of non-human DNA, consider using mouse Cot or salmon sperm DNA, or contact our NGS technical support group at [applicationsupport@idtdna.com](mailto:applicationsupport@idtdna.com) for additional guidance.



## Input recommendations

### Input for library preparation

This protocol was verified with libraries prepared from third party library preparation kits, including the KAPA® Hyper Prep Kit, and the TruSeq and Nextera DNA Library Prep Kits from Illumina. For optimal results, we recommend using fragmented DNA between 150–350 bp.

### Input for capture

We recommend using 500 ng of each prepared library for hybrid capture. For exome captures, multiplexing has been tested on up to 12 samples (6 µg total DNA) with limited impact on data quality. Using less input for capture can result in higher duplicate rates, lower mean coverage, and poor coverage uniformity.

### Concentrating DNA for hybrid capture

For optimal results, use a SpeedVac™ system (Savant) for concentrating DNA. Although a bead-based concentration system can be used, our testing has found reproducible, though minor, adverse impact on GC bias during bead-based concentration.



**Note:** To multiplex a high quantity of samples, we recommend using a SpeedVac system; however, if you require a quicker turnaround, you may prepare the DNA samples following the instructions in [Appendix A: AMPure XP Bead DNA concentration protocol](#).



## Consumables and equipment

### Consumables from IDT

Item	Description	Catalog #	Storage
xGen Lockdown Probe Pools	Custom— <a href="http://www.idtdna.com/CustomLockdownProbes">www.idtdna.com/CustomLockdownProbes</a> Inventoried— <a href="http://www.idtdna.com/LockdownPanels">www.idtdna.com/LockdownPanels</a>	Varies	–20°C
xGen Hybridization and Wash Kit	xGen Hybridization and Wash Kit, 16 rxn	1080577	
	Box 1		–20°C
	Box 2		4°C
	xGen Hybridization and Wash Kit, 96 rxn	1080584	
	Box 1		–20°C
	Box 2		4°C
Blocking oligos for TruSeq libraries	xGen Universal Blockers—TS Mix, 16 rxn	1075474	
	xGen Universal Blockers—TS Mix, 96 rxn	1075475	
	xGen Universal Blockers—TS Mix, 4 x 96 rxn	1075476	
	xGen Universal Blockers—10 bp TS Mix, 16 rxn	1081100	–20°C
	xGen Universal Blockers—10 bp TS Mix, 96 rxn	1081101	
	xGen Universal Blockers—10 bp TS Mix, 4 x 96 rxn	1081102	
Blocking oligos for Nextera libraries	xGen Universal Blockers—NXT Mix, 16 rxn	1079584	
	xGen Universal Blockers—NXT Mix, 96 rxn	1079585	–20°C
	xGen Universal Blockers—NXT Mix, 4 x 96 rxn	1079586	
xGen Library Amplification Primer	16 rxn	1077675	
	96 rxn	1077676	–20°C
	192 rxn	1077677	
Human Cot DNA	150 µL	1080768	–20°C
	650 µL	1080769	
(Optional) IDTE, pH 8.0	10 x 2 mL	11-01-02-05	room temp
Nuclease-Free Water	10 x 2 mL	11-04-02-01	room temp

Go to [www.idtdna.com/SDS](http://www.idtdna.com/SDS) for safety data sheets (SDSs) and [www.idtdna.com/COA](http://www.idtdna.com/COA) for certificates of analysis (COAs) for IDT products.

### Consumables from other suppliers

Item	Supplier	Catalog #
Ethanol	General laboratory supplier	Varies
Agencourt® AMPure® XP- PCR Purification beads	Beckman-Coulter	A63880
Digital electrophoresis chips	Bio-Rad Experion™ DNA 1K Analysis Kit	700-7107
	Agilent High Sensitivity DNA Kit	5067-4626
	Agilent High Sensitivity D1000 ScreenTape, or equivalent	5067-5584
twin.tec™ 96 Well LoBind PCR Plates, Semi-skirted (if working with multiple samples)	Eppendorf	0030129504
KAPA HiFi HotStart ReadyMix	Kapa Biosystems	KK2601

**Consumables from other suppliers (continued)**

Item	Supplier	Catalog #
Library Quantification Kit – Illumina/Universal	Kapa Biosystems	KK4824
MAXYMum Recovery® Microtubes, 1.7 mL	VWR	22234-046
MAXYMum Recovery PCR Tubes, 0.2 mL flat cap (if following the tube protocol)	VWR	22234-056
Buffer EB (or equivalent: 10 mM Tris-HCl, pH 8.5)	QIAGEN, or general laboratory supplier	19086
Plate protocol: Microseal® B PCR Plate Sealing Film, adhesive, optical	Bio-Rad	MSB1001
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851 or Q32854

**Equipment**

Item	Description
<b>Plate protocol</b>	
Two thermal cyclers	Bio-Rad C/S1000 or T100
Magnet (IDT qualified two options):	
• Magnum™ EX Universal Magnet Plate	Alpaqua (cat # A000380)
• Magnetic Stand-96	Thermo Fisher Scientific (cat # AM10027)
Plate centrifuge	General laboratory supplier
<b>Tube protocol</b>	
Thermal cycler	Bio-Rad C/S1000 or T100
Water bath or heating block	General laboratory supplier
Magnet (IDT qualified two options):	
• DynaMag™-2 Magnet	Thermo Fisher Scientific (cat # 12321D)
• DynaMag-PCR Magnet	Thermo Fisher Scientific (cat # 492025)
<b>Plate and tube protocols</b>	
Microcentrifuge	General laboratory supplier
Vacuum concentrator	Thermo Fisher Scientific SpeedVac™ system (cat # varies) or equivalent
Vortex mixer	General laboratory supplier
qPCR system or fluorescence-based DNA quantitation system, such as Qubit® fluorometer (Thermo Fisher Scientific) for final quantitation of library	Various suppliers
Digital electrophoresis system	Bio-Rad Experion™ Electrophoresis Station (cat #700-7010), Agilent 2100 Electrophoresis Bioanalyzer® system (cat # G2939AA), Agilent 2200 TapeStation® System (cat # G2965AA), or equivalent





## Plate protocol

We recommend performing the plate protocol instead of the tube protocol in most cases, since the plate protocol has demonstrated increased reproducibility between samples and enables higher throughput. Our plate protocol has been developed for a maximum of 4 columns of samples in standard 96-well plate format (32 reactions at a time). We do not recommend running more than 32 samples at a time because the timing and temperature of washes will be impacted. If processing very few samples, you may prefer to use individual tubes over plates. If this is the case for your DNA library, follow the [Tube protocol](#).



**Note:** Using different instruments or equipment other than those specifically qualified for this protocol may require further optimization.

### Guidelines

During the 4 hr incubation, the sample plate needs to be sealed properly, either with adhesive seals or with a plate sealer, to avoid evaporation. Excessive evaporation during hybridization can lead to capture failure.

The duration of hybridization should be kept consistent for all samples within a project. For GC-rich or small panels (<1000 probes), longer hybridization times (up to 16 hr) may improve performance.

### Before you start

Two thermal cyclers, set at different incubation temperatures, are used for hybrid capture in this protocol.

1. Create the following PCR programs:

HYB program (lid set at 100°C)	
95°C	30 sec
65°C	4 hr
65°C	Hold
WASH program (lid set at 70°C*)	
65°C	Hold

\* It is critical to reduce the lid temperature to 70°C for the WASH program.

## 2. Prepare the xGen Lockdown Probes.

- If you received the xGen Lockdown Probes as a hydrated solution:
  - Thaw at room temperature (RT, 15–25°C).
  - Mix thoroughly and centrifuge briefly.
- If you receive Gene Capture Pools (GCPs) in individual wells:
  - Mix 4 µL of each GCP and dry down.
  - Resuspend in 4 µL of Nuclease-Free Water.

For information on how to use these products in combination, see [Appendix B](#).

## Workflow



\* Perform during hybridization reaction

## Perform hybridization reaction



**Note:** To multiplex a high quantity of samples, we recommend using a SpeedVac system; however, if you require a quicker turnaround, prepare the beads following the instructions in [Appendix A: AMPure XP Bead DNA concentration protocol](#).

1. Create the Blocker Master Mix in a tube. Multiply by the number of samples and add a 10% overfill.

Blocker Master Mix component	Volume per reaction (µL)
Human Cot DNA	5
xGen Blocking Oligos based on your library adapters	2

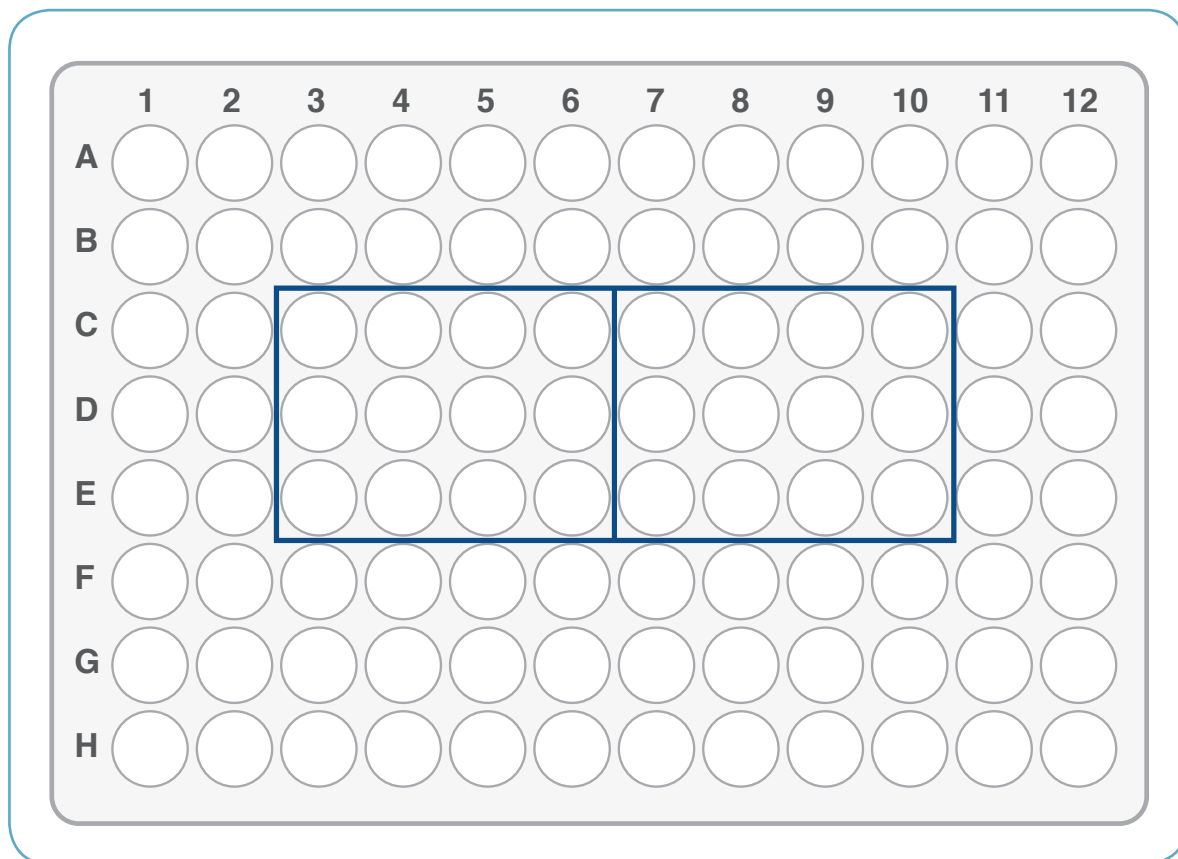
2. Vortex to mix well.
3. Add 7 µL of the Blocker Master Mix to each well of a LoBind plate.
4. Add 500 ng of library to each well containing Blocker Master Mix.  
If multiplexing samples, use 500 ng of each library.



**Note:** We recommend using wells in the middle of the plate. Avoid using wells on the plate edges because evaporation is more likely to occur in the outer rows and columns if the plate is not sealed properly.



**Tip:** Mark the wells that contain DNA before drying down the plate, since they will not be distinguishable from empty wells after dry down.



5. Dry down the mixture in a SpeedVac system.



**Safe stop:** Be sure to seal the sample plate. Store the plate at RT overnight, or –20°C for longer.

6. Thaw all contents of the xGen Hybridization and Wash Kit to 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.

7. Create the Hybridization Master Mix in a tube. Multiply by the number of samples and add a 10% overfill.

Hybridization Master Mix component	Volume per reaction (μL)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
xGen Lockdown Panel or custom probes	4
Nuclease-Free Water*	1.8

\* Do not use water if using an xGen spike-in panel. See [Appendix B](#) for more information.

8. Vortex or pipet the mix to mix well.
9. Add 17 μL of the Hybridization Master Mix to each well of the plate containing dried DNA.



**Note:** If you are combining two panels, see [Appendix B](#) for spike-in volume details.

10. Securely seal the plate with a Microseal B seal.
11. Incubate at least 5 min at room temperature.
12. Vortex the samples, making sure that they are completely mixed.
13. Briefly centrifuge the samples.
14. Place the plate on the thermal cycler and start the HYB program.

## Prepare buffers



**Note:** Before preparing the buffers, remove the Dynabeads® M-270 Streptavidin beads from storage at 4°C. Equilibrate the beads at room temperature at least 30 min before performing the washes.

1. Dilute the following xGen buffers to create 1X working solutions as follows, multiplying by the required number of samples and adding 10% overfill:

Component	Nuclease-Free Water (μL)	Buffer (μL)	Total (μL)
xGen 2X Bead Wash Buffer	150	150	300
xGen 10X Wash Buffer 1	225	25	250
xGen 10X Wash Buffer 2	135	15	150
xGen 10X Wash Buffer 3	135	15	150
xGen 10X Stringent Wash Buffer	270	30	300

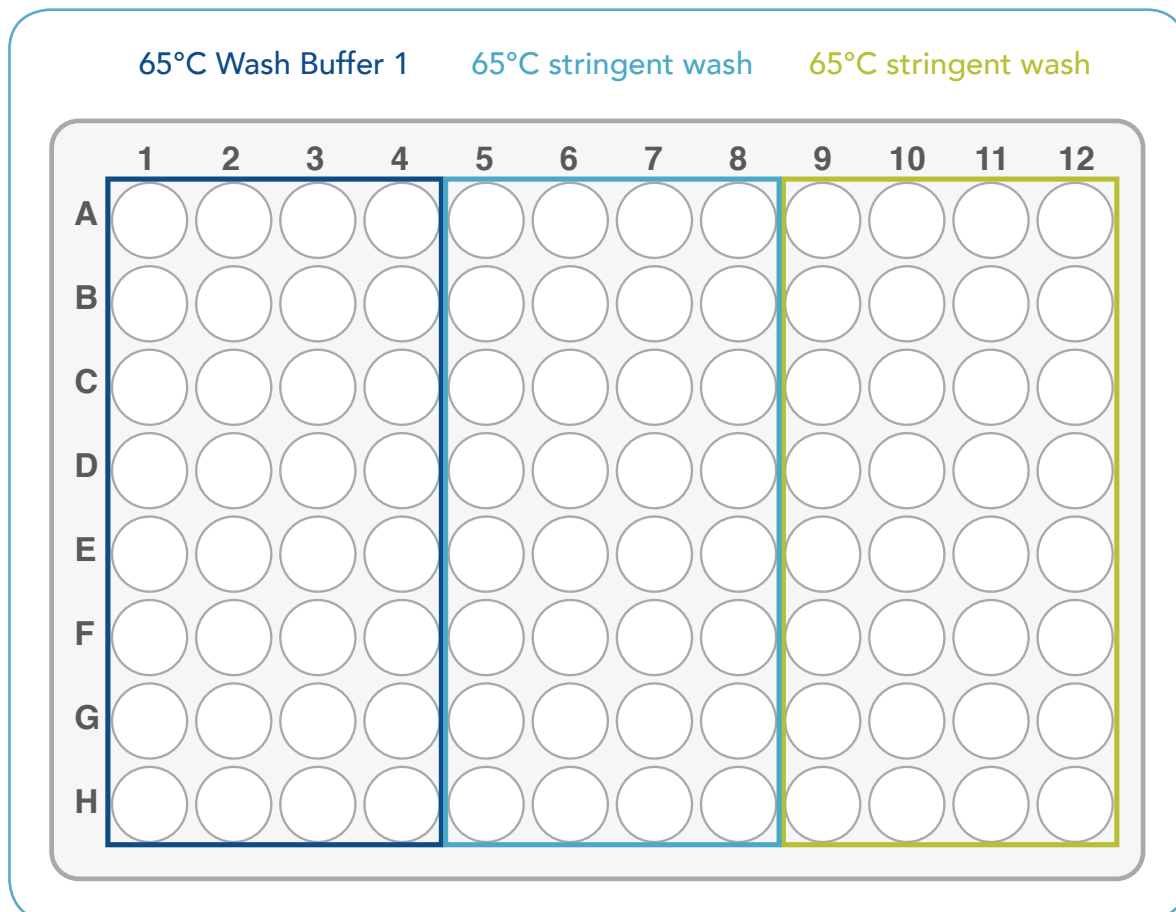


**Note:** If Wash Buffer 1 is cloudy, heat the bottle in a 65°C water bath to allow resuspension.



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

2. Use a fresh PCR plate. For 32 samples, as an example, aliquot and label the plate as follows:
  - Columns 1–4: 110  $\mu$ L of Wash buffer 1
  - Columns 5–8: 160  $\mu$ L of Stringent Wash Buffer
  - Columns 9–12: 160  $\mu$ L of Stringent Wash Buffer



**Important!** Do not discard the remaining Wash Buffer 1. The remaining buffer is needed to perform the **Room temperature washes** later in the protocol.

3. Seal the buffer plate and set aside.
4. In a LoBind tube, make the Bead Resuspension Mix. Multiply by the number of samples and add a 10% overfill.

Bead Resuspension Mix component	Volume per reaction ( $\mu$ L)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
Nuclease-Free Water	5.8


## Wash Streptavidin beads




**Important!** Only perform bead washes with beads that have equilibrated to room temperature.

1. Mix the beads thoroughly by vortexing for 15 sec.
2. Add 50  $\mu$ L of Streptavidin beads to a new PCR plate, filling a well for every sample being captured.
3. Add 100  $\mu$ L of Bead Wash Buffer from [Prepare buffers, step 1](#) to each well, then gently pipet the mix 10 times.
4. Place the plate containing beads on a magnet and allow the beads to fully separate from the supernatant (approximately 1 min).
5. Remove and discard the clear supernatant, ensuring that the beads remain in the well.
6. Remove the plate containing beads from the magnet.
7. Perform the following wash:
  - a. Add 100  $\mu$ L of Bead Wash Buffer to each well containing beads, then gently pipet the mix 10 times.
  - b. Place the plate on the magnet for approximately 1 min, allowing beads to fully separate from the supernatant.
  - c. Carefully remove and discard the clear supernatant.
8. Perform an additional wash by repeating [step 7](#) (above) for a total of 2 washes.
9. Resuspend the beads in 17  $\mu$ L of Bead Resuspension Mix from [Prepare buffers, step 4](#).
10. Mix thoroughly to ensure the beads are not left to dry in the well. If needed, briefly centrifuge the plate containing beads at 25 x g.

## Perform bead capture

 **Important!** If any of the sample accidentally splashes onto the plate seal while vortexing in **Perform bead capture**, briefly and gently centrifuge the plate (10 sec at 25 x g).

1. Start the WASH program in the second thermal cycler to start warming the buffer plate prepared in **Prepare buffers, step 2**. Make sure the lid temperature is set to 70°C for the WASH program.

 **Note:** The buffer plate needs to warm up for **at least** 15 min. We recommend starting incubation at the same time as the bead capture.


2. After the 4 hr incubation is complete, remove the sample plate from the thermal cycler.
3. Once the sample plate has been removed from the instrument, stop the HYB program.
4. Immediately after the HYB program is complete, start the WASH program.

 **Tip:** At this point, both thermal cyclers should be running the WASH program.

5. Using a multichannel pipette and fresh LoBind tips, transfer the fully homogenized beads to the samples.
6. Securely seal the sample plate.
7. Gently vortex the sample plate until it is fully mixed, being careful not to splash onto the plate seal.
8. Place the sample plate in the thermal cycler for 45 min. During incubation, remove the plate every 10–12 min to quickly and gently vortex.

 **Note:** It is safe to place the sample plate in the thermal cycler before the lid temperature has fully cooled to 70°C when starting the incubation.

## Perform washes

 **Important!** Always keep the buffer plate on the thermal cycler during washes. Make sure to reseal the buffer plate in between washes.

When performing the heated washes, keep the buffer plate on the thermal cycler to maintain its set temperature.

### Heated washes

1. After 45 min, remove the sample plate from the thermal cycler.
2. With the buffer plate remaining in the thermal cycler, transfer 100 µL of heated Wash Buffer 1 to each sample and pipet the mix 10 times, being careful to minimize bubble formation.
3. Reseal the buffer plate, then close the lid.



- Place the sample plate on the magnet for 1 min. Remove the supernatant.



**Note:** Due to the supernatant having a high concentration of hybridization buffer and enhancer, use appropriate disposal methods.

- Remove the sample plate from the magnet, then add 150  $\mu$ L of heated Stringent Wash Buffer to each well containing a sample.
- Reseal the buffer plate, then close the lid.
- Pipet the mix 10 times, being careful to minimize bubble formation. Always use fresh pipette tips for each well.
- Securely seal the sample plate, then incubate for 5 min in the thermal cycler.
- Place the sample plate on the magnet for 1 min, then remove the supernatant.
- Remove the sample plate from the magnet, then add 150  $\mu$ L of heated Stringent Wash Buffer from the buffer plate to the sample plate.
- Pipet the mix 10 times, being careful to minimize bubble formation. Securely seal the sample plate, then incubate for 5 min on the thermal cycler.
- Place the sample plate on the magnet for 1 min.

### Room temperature washes



**Important!** To ensure that the beads remain fully resuspended, vigorously mix the samples during the room temperature washes.

- Remove supernatant. Add 150  $\mu$ L of Wash Buffer 1.
- Securely seal the sample plate with a **fresh** seal, then vortex at full-speed thoroughly, until fully resuspended.



**Important!** It is critical to use a new seal at this step to avoid the risk of contamination because there will be some bead splash on the seal.

- Incubate for 2 min while alternating between vortexing for 30 sec and resting for 30 sec, to ensure the mixture remains homogenous.
- Centrifuge the sample plate for 5 sec at 25 x g.



**Important!** This brief centrifugation of the sample plate is a critical step to avoid well-to-well contamination.

- Place the sample plate on the magnet for 1 min, then remove and discard the seal.
- Remove the supernatant, then remove the sample plate from the magnet.
- Add 150  $\mu$ L of Wash Buffer 2, then securely seal the sample plate with a fresh seal and vortex thoroughly until fully resuspended.
- Incubate for 2 min while alternating between vortexing for 30 sec and resting for 30 sec, to ensure the mixture remains homogenous.

9. After the incubation, briefly centrifuge the sample plate (5 sec at 25 x g).
10. After centrifuging, place the sample plate on the magnet for 1 min, then remove and discard the seal.
11. Remove the supernatant, then remove the sample plate from the magnet.
12. Add 150 µL of Wash Buffer 3, then securely seal the sample plate with a fresh seal and vortex thoroughly until fully resuspended.
13. Incubate for 2 min while alternating between vortexing for 30 sec and resting for 30 sec, to ensure the mixture remains homogenous.
14. After the incubation, briefly centrifuge the sample plate (5 sec at 25 x g).
15. After centrifuging, place the sample plate on the magnet for 1 min, then remove and discard the seal.
16. Remove the supernatant.
17. With the sample plate still on the magnet, use fresh pipette tips to ensure that all residual Wash Buffer 3 has been removed, then remove the plate from the magnet.
18. Add 20 µL of Nuclease-Free Water to each capture.
19. Pipet the mix 10 times to resuspend any beads stuck to the side of the well.



**Important!** Do not discard the beads. You will use the entire 20 µL of resuspended beads with captured DNA in [Perform post-capture PCR](#).

## Perform post-capture PCR

1. In a tube, prepare the Amplification Reaction Mix, multiplied by the number of samples on the plate and adding 10% overfill, as follows:

Amplification Reaction Mix component	Volume (µL)
2X KAPA HiFi HotStart ReadyMix	25
xGen Library Amplification Primer	1.25
Nuclease-Free Water	3.75



**Note:** If using a different master mix than KAPA HiFi, the magnesium concentration may need to be optimized for on-bead PCR.

2. Add 30 µL of the Amplification Reaction Mix to each sample for a final reaction volume of 50 µL.
3. Securely seal the sample plate, then gently vortex the plate to thoroughly mix the reaction.
4. Briefly centrifuge the plate.

- Place the plate in a thermal cycler, and run the following program with the lid temperature set to 105°C:

Step	Number of cycles	Temperature (°C)	Time
Polymerase activation	1	98	45 sec
Amplification			
Denaturation	Variable—refer to the Panel table below.	98	15 sec
Annealing		60	30 sec
Extension		72	30 sec
Final extension	1	72	1 min
Hold	1	4	Hold



**Note:** The number of PCR cycles should be optimized per panel size and the number of pooled libraries per capture, to ensure there is enough yield for sequencing.

We recommend starting optimization with the following:

Panel size	1-plex	4-plex	8-plex	12-plex
>100,000 probes (xGen Exome)	10 cycles	8 cycles	7 cycles	6 cycles
10,000–100,000 probes	12 cycles	10 cycles	9 cycles	8 cycles
500–10,000 probes	13 cycles	11 cycles	10 cycles	10 cycles
1–500 probes	14 cycles	12 cycles	11 cycles	11 cycles



**Optional stopping point:** Amplified captures may be stored at 4°C overnight.

## Purify post-capture PCR fragments



**Important!** Ensure Agencourt AMPure XP beads have been equilibrated to room temperature before proceeding.

- Prepare 250 µL of fresh 80% ethanol per sample, multiplied by the number of samples with a 10% overfill.
- Add 75 µL (1.5X volume) of Agencourt AMPure XP beads to each amplified capture.
- After adding the beads, pipet the mix thoroughly and incubate for 5–10 min.
- Place the plate on the magnet until the supernatant is clear (2–5 min).
- Remove the supernatant without disturbing the beads.
- While keeping the plate on the magnet, add 125 µL of 80% ethanol, then incubate for 1 min.
- Remove the ethanol, then repeat another ethanol wash.
- Allow the beads to air dry for 1–3 min. Do not overdry the beads.
- Remove the sample plate from the magnet and elute in 22 µL of Buffer EB, or equivalent (10 mM Tris-Cl, pH 8.5). Mix thoroughly. Alternatively, TE can be used.

10. Incubate for 5 min at room temperature.
11. Place the plate on a magnet until supernatant is clear (1–2 min).
12. Transfer 20  $\mu$ L of eluate to a fresh plate, making sure that no beads are carried over.



**Optional stopping point:** Purified PCR fragments may be stored at  $-20^{\circ}\text{C}$  for up to 1 week.

## Validate and quantify library

1. Measure the concentration of the captured library using a fluorescence-based method for DNA quantitation (such as Qubit dsDNA HS Assay kit) or qPCR.



**Note:** Knowing the concentration of your captured library helps ensure your library is within the detection limits of the chip or tape (as measured in **step 2**, below) for use on 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 the Agilent 2200 TapeStation system using a DNA tape or other similar system).

## Perform sequencing

Perform sequencing according to the instructions for your Illumina instrument.



## Tube protocol

This protocol has been developed for a maximum of 6 capture reactions using individual tubes. If you are using plates for your DNA library, use the [Plate protocol](#).



**Note:** Despite the similar workflow, using the plate protocol reduces sample variability when compared to the tube protocol.



**Tip:** Using alternative instruments or equipment not validated in this protocol may require optimization.

## Guidelines

During the 4 hr incubation, the tube needs to be sealed properly to avoid evaporation. Excessive evaporation during the hybridization can lead to capture failure.

The duration of hybridization should be kept consistent for all samples within a project. For GC-rich or small panels (<1000 probes), longer hybridization times (up to 16 hr) may improve performance.

## Before you start

Two cycling programs, set at different incubation temperatures, are used for hybridization capture in this protocol.

1. Create the following PCR programs:

HYB program (lid set at 100°C)	
95°C	30 sec
65°C	4 hr
65°C	Hold
WASH program (lid set at 70°C*)	
65°C	Hold

\* It is critical to reduce the lid temperature to 70°C for the WASH program.

## 2. Prepare the xGen Lockdown Probes.

- If you received the xGen Lockdown Probes as a hydrated solution:
  - Thaw at room temperature (15–25°C).
  - Mix thoroughly and centrifuge briefly.
- If you receive Gene Capture Pools in individual wells:
  - Mix 4 µL of each GCP and dry down.
  - Resuspend in 4 µL of Nuclease-Free Water.


For information on how to use these products in combination, see [Appendix B](#).

## Workflow



\* Perform during hybridization reaction

## Perform hybridization reaction

 **Note:** To multiplex a high quantity of samples, we recommend using a SpeedVac system; however, if you require a quicker turnaround, prepare the beads following the instructions in [Appendix A: AMPure XP Bead DNA concentration protocol](#).

1. In a 1.7 mL MAXYMum Recovery microtube (low-bind), add the following components:

Blocker component	Volume per reaction (μL)
Human Cot DNA	5
xGen Blocking Oligos based on your library adapters	2

2. Add 500 ng of library to each tube containing Blocker components.  
If multiplexing samples, use 500 ng of each library.
3. Dry down the mixture in a SpeedVac system.



**Safe Stop:** Be sure to seal the sample tube. Store the sample at RT overnight, or –20°C for longer.

4. Thaw all contents of the xGen Hybridization and Wash Kit to 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.

5. Create the Hybridization Master Mix by adding the following components to the tube from [step 2](#) (above).

Hybridization Master Mix component	Volume per reaction (μL)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
xGen Lockdown Panel or custom probes	4
Nuclease-Free Water*	1.8

\* **Do not use water** if using an xGen spike-in panel. See [Appendix B](#) for more information.

6. Pipet the mix, then incubate at room temperature for 5–10 min.
7. Vortex, then briefly centrifuge.
8. Transfer 17 μL of the capture to a low-bind 0.2 mL PCR tube, then briefly centrifuge.



**Note:** If you are combining two panels, see [Appendix B](#) for spike-in volume details.

9. Place the sample tube in the thermal cycler and start the HYB program.

## Prepare buffers



**Note:** Before preparing the buffers, remove the Dynabeads M-270 Streptavidin beads from storage at 4°C. The beads need to be at room temperature for a minimum of 30 min before performing the washes.

1. Dilute the following xGen buffers to create 1X working solutions:

Component	Nuclease-Free Water (μL)	Buffer (μL)	Total (μL)	Storage
xGen 2X Bead Wash Buffer	160	160	320	Keep at room temperature.
xGen 10X Wash Buffer 1	252	28	280	Aliquot 110 μL of the 1X Buffer into a separate tube and heat to 65°C. The remaining solution should be kept at room temperature.
xGen 10X Wash Buffer 2	144	16	160	Keep at room temperature.
xGen 10X Wash Buffer 3	144	16	160	Keep at room temperature.
xGen 10X Stringent Wash Buffer	288	32	320	Aliquot into two tubes (160 μL each). Heat tubes to 65°C in a water bath or heating block.



**Note:** If Wash Buffer 1 is cloudy, heat the bottle in a 65°C water bath to allow resuspension.



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

2. Prepare the following Bead Resuspension Mix in a low-bind tube:

Bead Resuspension Mix component	Volume per reaction (μL)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
Nuclease-Free Water	5.8



## Wash Streptavidin beads



**Important!** Only perform bead washes with beads that have equilibrated to room temperature.

1. Mix the beads thoroughly by vortexing for 15 sec.
2. Aliquot 50  $\mu$ L of streptavidin beads per capture into a single 1.7 mL low-bind tube. For example, for 1 capture, prepare 50  $\mu$ L of beads and for 2 captures, prepare 100  $\mu$ L of beads.
3. Add 100  $\mu$ L of Bead Wash Buffer per capture. Gently pipet the mix 10 times.
4. Place the tube on a magnetic rack, allowing the beads to fully separate from the supernatant (approximately 1 min).
5. Remove and discard the clear supernatant, ensuring that the beads remain in the tube.
6. Remove the tube from the magnet.
7. Perform the following wash:
  - a. Add 100  $\mu$ L of Bead Wash Buffer per capture, then pipet the mix 10 times.
  - b. Place the tube on a magnetic rack for approximately 1 min, allowing the beads to fully separate from the supernatant.
  - c. Carefully remove and discard the clear supernatant.
8. Perform an additional wash by repeating [step 7](#) (above) for a total of 2 washes.
9. Resuspend the beads in 17  $\mu$ L per capture of Bead Resuspension Mix from [Prepare buffers, step 2](#).
10. Mix thoroughly to ensure that the beads are not left to dry in the tube.  
If needed, briefly centrifuge the tube at 25 x *g*.
11. Aliquot 17  $\mu$ L of resuspended beads into a new low-bind 0.2 mL tube for each capture reaction.

## Perform bead capture

1. Place the 1X Wash Buffer 1 (110  $\mu$ L aliquot) and the 1X Stringent Wash Buffer (both aliquots) in a 65°C water bath for at least 15 min.



**Tip:** The buffers will be used during the **Heated washes**, but we recommend starting this incubation at the same time as the bead capture, so that the buffers will be at the correct temperature when needed.

2. After the 4 hr incubation, take the tube out of the thermal cycler.
3. Once removed, stop the HYB program.
4. Immediately after the HYB program completes, start the WASH program.
5. Transfer 17  $\mu$ L of resuspended streptavidin beads to the 0.2 mL tube containing the sample.
6. Vortex to ensure that sample is fully resuspended. Gently and briefly centrifuge, if needed (10 sec at 25 x g).
7. Place the sample tube in the thermal cycler and set a timer for 45 min.



**Note:** It is safe to place the sample tubes in the thermal cycler before the lid temperature has fully cooled to 70°C when starting the incubation.

8. Every 10–12 min, remove the tube from the thermal cycler and gently vortex to ensure the sample is fully resuspended.
9. At the end of the 45 min, take the sample off the thermal cycler. Proceed immediately to **Heated washes**.

## Perform washes



**Important!** It is critical to ensure that the buffers have reached 65°C in a water bath before starting the **Heated washes**.

### Heated washes

1. Transfer 100  $\mu$ L of heated Wash Buffer 1 to the sample, pipet the mix 10 times, being careful to minimize bubble formation.
2. Place the tube on a magnetic rack for 1 min. Remove the supernatant.



**Note:** Due to the supernatant having a high concentration of hybridization buffer and enhancer, use appropriate disposal methods.



**Tip:** If you do not have a magnetic rack that holds 0.2 mL tubes, transfer the entire reaction to a 1.7 mL tube.

3. Remove the tube from the magnet and add 150  $\mu$ L of heated Stringent Wash Buffer to the sample.
4. Pipet the mix 10 times, being careful to not introduce bubbles.

5. Incubate in the water bath at 65°C for 5 min.
6. Place the sample on the magnet for 1 min. Remove the supernatant.
7. Remove the tube from the magnet and add 150 µL of heated Stringent Wash Buffer to the sample.
8. Pipet the mix 10 times, being careful to not introduce bubbles.
9. Incubate in the water bath at 65°C for 5 min.
10. Place the tube on a magnet for 1 min.

### Room temperature washes



**Important!** To ensure the beads remain fully resuspended, vigorously mix the samples during the room temperature washes.

1. Remove and discard supernatant. Add 150 µL of Wash Buffer 1 equilibrated to room temperature.
2. Vortex thoroughly until fully resuspended.
3. Incubate for 2 min while alternating between vortexing for 30 sec and resting for 30 sec, to ensure the mixture remains homogenous.
4. At the end of the incubation, briefly centrifuge the tube.
5. Place on the magnet for 1 min.
6. Remove the supernatant. Add 150 µL of Wash Buffer 2.
7. Vortex thoroughly until fully resuspended.
8. Incubate for 2 min while alternating between vortexing for 30 sec and resting for 30 sec, to ensure the mixture remains homogenous.
9. At the end of the incubation, briefly centrifuge the tube.
10. Place on the magnet for 1 min.
11. Remove the supernatant. Add 150 µL of Wash Buffer 3.
12. Vortex thoroughly until fully resuspended.
13. Incubate for 2 min while alternating between vortexing for 30 sec and resting for 30 sec, to ensure the mixture remains homogenous.
14. At the end of the incubation, briefly centrifuge the tube.
15. Place the sample tube on the magnet for 1 min.
16. Remove and discard the supernatant.
17. With the sample tube still on the magnet, use a fresh pipette tip to remove residual Wash Buffer 3 from the tube, then remove the tube from the magnet.
18. Add 20 µL of Nuclease-Free Water to each capture.
19. Pipet the mix 10 times to resuspend any beads stuck to the side of the tube.



**Important!** Do not discard the beads. Use the entire 20 µL of resuspended beads with captured DNA in [Perform post-capture PCR](#).

## Perform post-capture PCR


1. If a 1.7 mL tube was used for the washes, transfer the sample to a low-bind 0.2 mL PCR tube.
2. Add the following components to create the Amplification Reaction Mix:

Amplification Reaction Mix component	Volume per reaction (μL)
2X KAPA HiFi HotStart ReadyMix*	25
xGen Library Amplification Primer	1.25
Nuclease-Free Water	3.75

\* If using a PCR master mix other than Kapa HiFi, the magnesium concentration may need to be optimized for on-bead PCR.

3. Place the tube in a thermal cycler, and run the following program with the heated lid set at 105°C:

Step	Number of cycles	Temperature (°C)	Time
Polymerase activation	1	98	45 sec
Amplification			
Denaturation	Variable—refer to the Panel table below.	98	15 sec
Annealing		60	30 sec
Extension		72	30 sec
Final extension	1	72	1 min
Hold	1	4	Hold

 **Note:** The number of PCR cycles should be optimized per panel size and the number of pooled libraries per capture, to ensure there is enough yield for sequencing.

We recommend starting optimization with the following:

Panel size	1-plex	4-plex	8-plex	12-plex
>100,000 probes (xGen Exome)	10 cycles	8 cycles	7 cycles	6 cycles
10,000–100,000 probes	12 cycles	10 cycles	9 cycles	8 cycles
500–10,000 probes	13 cycles	11 cycles	10 cycles	10 cycles
1–500 probes	14 cycles	12 cycles	11 cycles	11 cycles

 **Optional stopping point.** Amplified captures may be stored at 4°C overnight.

## Purify post-capture PCR fragments



**Important!** Ensure Agencourt AMPure XP beads have been equilibrated to room temperature before proceeding.

1. Prepare 250  $\mu\text{L}$  of fresh 80% ethanol per sample, multiplied by the number of samples with a 10% overfill.
2. Add 75  $\mu\text{L}$  (1.5X volume) of Agencourt AMPure XP beads to each amplified capture (transfer to a larger 1.7 mL tube, if needed).
3. After adding the beads, mix thoroughly and incubate for 5–10 min.
4. Place the sample tube on a magnet until the supernatant is clear (2–5 min).
5. Remove supernatant without disturbing the beads.
6. While keeping the tube on the magnet, add 125  $\mu\text{L}$  of 80% ethanol, then incubate for 1 min.
7. Remove the ethanol, then repeat another ethanol wash.
8. Allow the beads to air dry for 1–3 min. Do not overdry the beads.
9. Remove the sample tube from the magnet and elute in 22  $\mu\text{L}$  of Buffer EB, or equivalent (10mM Tris-Cl, pH 8.5). Mix thoroughly. Alternatively, TE can be used.
10. Incubate for 5 min at room temperature.
11. Place the tube on a magnet until the supernatant is clear (1–2 min).
12. Transfer 20  $\mu\text{L}$  of eluate to a fresh tube making sure that no beads are carried over.



**Optional stopping point.** Purified PCR fragments may be stored at  $-20^{\circ}\text{C}$  for up to 1 week.

## Validate and quantify library

1. Measure the concentration of the captured library using a fluorescence-based method for DNA quantitation (such as Qubit dsDNA HS Assay kit) or qPCR.



**Note:** Knowing the concentration of your captured library helps ensure your library is within the detection limits of the chip or tape (as measured in [step 2](#), below) for use on 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 the Agilent 2200 TapeStation system using a DNA tape or other similar system).

## Perform sequencing

Perform sequencing according to the instructions for your Illumina instrument.



## Appendix A

### AMPure XP Bead DNA concentration protocol (Optional)



**Important!** This protocol requires 7.5  $\mu$ L of Human Cot DNA. To order additional Cot DNA, go to the [Cot DNA ordering page](#).

1. Add 500 ng of library to the sample well. If multiplexing, pool 500 ng of each library into the sample well (maximum of 12 samples).



**Note:** This could be a large volume requiring either 1.7 mL tubes, or a deep well plate.

2. Add 7.5  $\mu$ L of Human Cot DNA.
3. Add 1.8X volume of AMPure XP beads.
4. If using plates, securely seal the plate with a Microseal B seal.
5. Vortex thoroughly to mix. If using plates, adjust the settings to prevent any splashing onto the seal.
6. Incubate for 10 min at room temperature.
7. Incubate the plate or tube on the magnet for at least 2 min or until supernatant is clear.
8. Remove and discard the supernatant. Keeping the tube on the magnet, add 80% ethanol to cover the surface of the beads. Incubate for 30 sec without disturbing the beads.
9. Remove and discard the supernatant, then repeat another ethanol wash.
10. Allow the beads to air dry for approximately 2 min. Do not overdry.
11. Add these components to the tube to make the Hybridization Reaction Mix:

Hybridization Reaction Mix components	Volume per reaction ( $\mu$ L)
xGen 2X Hybridization Buffer	9.5
xGen Hybridization Buffer Enhancer	3
xGen Blocking Oligos based on your library adapter	2
xGen Lockdown Panels or custom probes	4.5
<b>Total</b>	<b>19</b>



**Note:** The Hybridization Reaction Mix elutes the DNA from the AMPureXP beads.

12. Vortex to mix. Ensure that the beads are fully resuspended.
13. Incubate for 5 min at room temperature.
14. After incubation, place on a magnet for 5–10 min or until the supernatant is clear.

15. Transfer 17  $\mu$ L of the supernatant to the sample plate, or tube, where the hybridization will occur.



**Important!** Make sure to avoid bead carryover during the transfer process.

Proceed to [Perform hybridization reaction step 13, page 12](#) for plate captures, or [step 8, page 23](#) for tube captures immediately after the sample DNA is ready.





## Appendix B

### Combining xGen Lockdown Panels and Probes

Combine panels at equimolar amounts to achieve uniform coverage. When combining panels (during **Perform hybridization reaction**), prepare the Hybridization Master Mix based on the recommendations in Table 1. Multiply by the number of samples, then add a 10% overfill (See **Table 2**).



**Tip:** For more information or assistance with your specific experimental design, contact our technical support group at [applicationsupport@idtdna.com](mailto:applicationsupport@idtdna.com).

Table 1. Spike-in volumes when combining two panels

		Spike-in panel types							
		Gene Capture Pools		Lockdown Probe Panel $\geq 4000$ probes		Lockdown Probe Panel $< 4000$ probes		xGen Spike-in panel*	
		Volume		Volume		Volume		Volume	
		Panel	Spike-in	Panel	Spike-in	Panel	Spike-in	Panel	Spike-in
Main panel types	Gene Capture Pools	3 $\mu$ L	3 $\mu$ L	4 $\mu$ L	2 $\mu$ L	4 $\mu$ L	1 $\mu$ L	4 $\mu$ L	2 $\mu$ L
	Lockdown Probe Panel $\geq 4000$ probes	N/A	N/A	3 $\mu$ L	3 $\mu$ L	4 $\mu$ L	2 $\mu$ L	4 $\mu$ L	2 $\mu$ L
	Lockdown Probe Panel $< 4000$ probes	N/A	N/A	N/A	N/A	3 $\mu$ L	3 $\mu$ L	4 $\mu$ L	2 $\mu$ L

\* Inventoried spike-in panels (e.g., xGen CNV Backbone Panel—Tech Access, Human mtDNA Research Panel, or Human ID Panel)

Table 2. Volumes per component of the Hybridization Master Mix

Hybridization Master Mix components	Volume per reaction ( $\mu$ L)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
Main panel	See <b>Table 1</b>
Spike-in panel	See <b>Table 1</b>
Nuclease-Free Water	0–0.8 (only if needed*)
<b>Total volume*</b>	<b>17–17.2 <math>\mu</math>L</b>

\* Depending on the spike-in panel used, your volume could be less than 17  $\mu$ L; if so, add a small amount of water to reach the total recommended volume (17–17.2  $\mu$ L).

## xGen hybridization capture of DNA libraries

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