xGen™ hybridization capture of Illumina® Nextera™ DNA libraries

For NGS target enrichment

Uses these IDT products*:

- xGen Custom Hybridization Capture Panels
- xGen Hybridization and Wash Kit
- xGen Universal Blockers NXT
- xGen Library Amplification Primer Mix

Generates:

- Illumina platform-compatible libraries

REVISION HISTORY

Version	Release date	Description of changes
6	March 2022	Updated product names
5	June 2021	Updated product names
4	May 2019	Updated to specify that xGen Universal Blockers NXT Mix can bind to Illumina® Nextera™ adapter sequences with 8 or 10 bp indexes
3	March 2019	Added Appendix B to describe combining panels. Removed instructions for rehydrating xGen Custom Hyb Panels which are currently only provided in solution
2	June 2018	Updated to include 10 bp blockers
1	April 2018	Original version

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OVERVIEW

This protocol includes the steps necessary for target enrichment of a next generation sequencing (NGS) library prepared from genomic DNA, using xGen Hybridization Capture Panels (xGen Hyb Panels). Visit 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.

In brief, the steps of this protocol include:

1. Preventing adapter-to-adapter hybridization by adding xGen Universal Blockers NXT.
2. Performing hybridization capture using one of the many options of the xGen Predesigned Hyb Panel or an xGen Custom Hyb Panel and the xGen Hybridization and Wash Kit.
3. Capturing probe and DNA library fragment duplexes with bead-based selection.
4. Post-capture PCR to amplify the library for sequencing.

After preparing an NGS library using one of the methods described in Input recommendations, this procedure describes the process for using the IDT xGen Universal Blockers NXT to prevent off-target fragments from annealing to the intended target sequence via adapter-to-adapter hybridization. xGen Universal Blockers NXT are compatible with the Illumina® DNA Prep (formerly Nextera™) assay.

Once the adapters have been blocked, the procedure describes conditions necessary for hybridization of one of the xGen Predesigned Hyb Panels or one of the xGen Custom Hyb Panels. These panels contain capture probes that are individually synthesized and pooled, which ensures equal representation of each probe in the panel. In addition, xGen Custom Hyb Panels can be used as a spike-in to supplement the target space of one of the predesigned panels. More information on combining panels can be found in Appendix B.

The hybridization procedure presented here is specific to the IDT xGen Hybridization and Wash Kit, which includes components that are compatible with any of the IDT xGen Hyb Panels. This protocol outlines instructions for hybridization reactions done either in plates or tubes, depending on the number of targeted sequencing samples to be performed.

After the probes are hybridized, the desired DNA targets are separated from off-target fragments. Since each probe has a 5′ biotin modification, this protocol describes the use of streptavidin-coated magnetic beads to capture the probe and targeted DNA duplexes. See the Consumables and equipment section, for suggested materials to perform these captures.

After washing the nonspecific DNA from the beads and eluting the desired DNA targets from the beads, the procedure for post-capture PCR using a PCR master mix with the IDT xGen Library Amplification Primer Mix (Figure 1) is described. The number of amplification cycles is based on the number of probes in the panel. The goal is to produce enough DNA in the final captured library for sequencing.

Target capture workflow

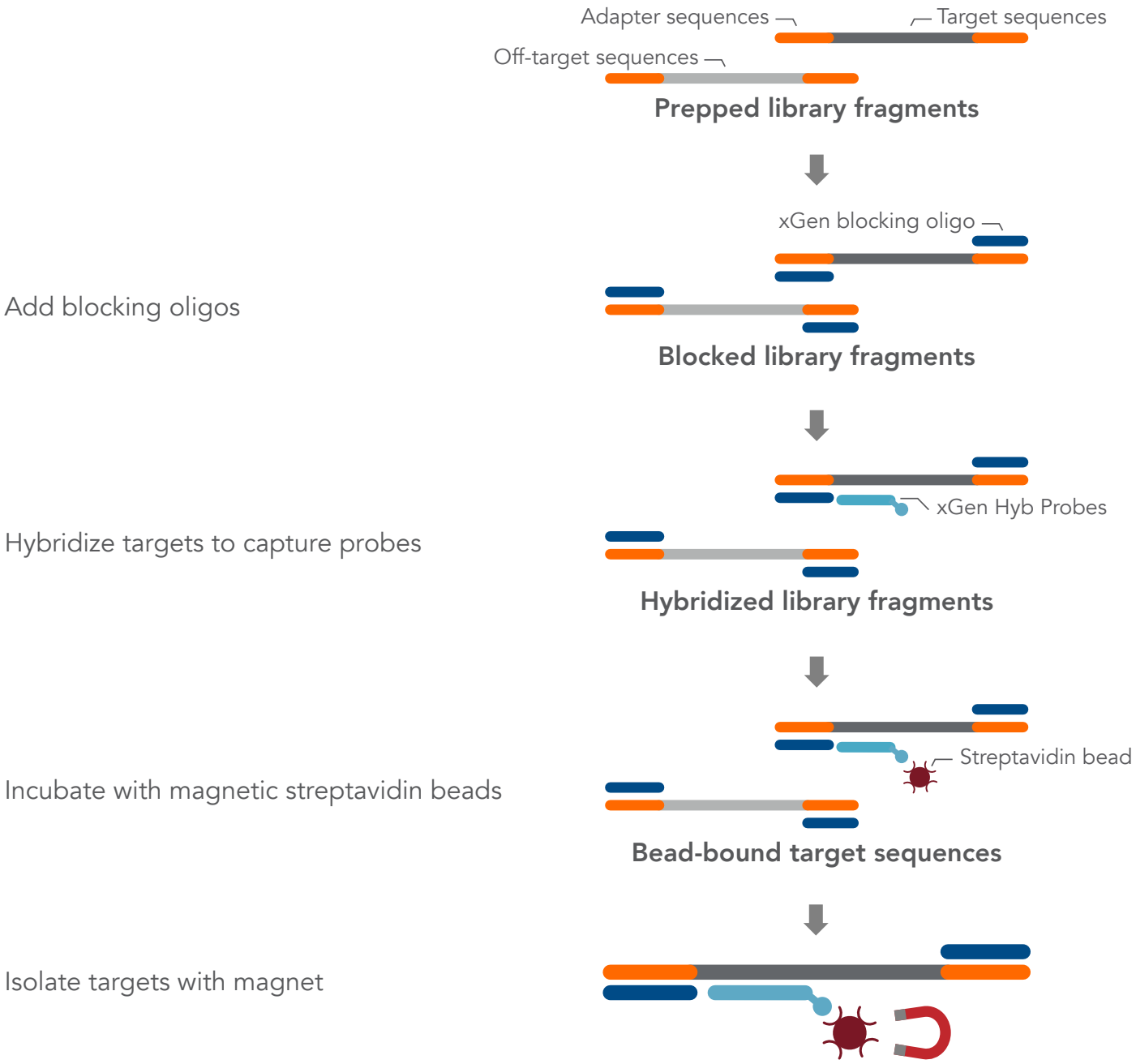


Figure 1. Desired prepared library fragments are separated from off-target fragments using hybridization capture. First, xGen Universal Blockers NXT are mixed with prepared library fragments to prevent adapter-to-adapter hybridization. Blocked library fragments are then annealed to the 5′ biotinylated oligonucleotide probes from an xGen Predesigned Hyb Panel or an xGen Custom Hyb Panel. The probes and fragment duplexes are then separated from the unbound fragments by streptavidin-coated magnetic bead purification. The resulting library contains just the fragments of interest.

INPUT RECOMMENDATIONS

Input for library preparation

This protocol was developed with libraries prepared from the Illumina DNA Prep (formerly Nextera) kit.

Input for capture

We recommend using 500 ng of each prepared library for hybridization capture. For exome captures, multiplexing has been investigated on up to 12 samples (6 µg total DNA) and showed 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

Using a SpeedVac™ system (Savant®) provides a nonbiased method of concentrating DNA samples for hybrid capture. The method is amenable to high quantities of samples for multiplexing applications.

Note: if you require a quicker turnaround, you may also consider preparing the DNA samples following the instructions in Appendix A. This method could produce a slight GC bias in final libraries.

Important This protocol requires 7.5 µL of xGen Human Cot DNA. To order additional Cot DNA, go to the xGen Hybridization Capture Core Reagents page.

CONSUMABLES AND EQUIPMENT

Consumables—IDT

Item	Description	Catalog #	Storage (°C)
xGen Hyb Panels	Custom or predesigned	Varies	–20
	xGen Hybridization and Wash Kit, 16 rxn	1080577	
xGen Hybridization and Wash Kit	Box 1		–20
	Box 2		4
	xGen Hybridization and Wash Kit, 96 rxn	1080584	
	Box 1		–20
	Box 2		4
xGen Universal Blockers for Illumina DNA Prep Kit (Nextera™) libraries	xGen Universal Blockers NXT, 16 rxn	1079584	–20
	xGen Universal Blockers NXT, 96 rxn	1079585	
	xGen Universal Blockers NXT, 4 x 96 rxn	1079586	
xGen Library Amplification Primer Mix	16 rxn	1077675	–20
	96 rxn	1077676	
	192 rxn	1077677	
xGen Human Cot DNA (in case additional quantities are needed)	150 µL	1080768	–20
	650 µL	1080769	
IDTE, pH 8.0 (optional)	10 x 2 mL	11-01-02-05	Room temperature
Nuclease-free water	10 x 2 mL	11-04-02-01	Room temperature

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

Consumables—other suppliers

Item	Description	Catalog #
Ethanol	General laboratory suppler	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 Ready Mix	Kapa Biosystems	KK2601
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	Catalog #
Plate protocol		
Two thermal cyclers	Bio-Rad	C/S1000 or T100
Magnet (IDT qualified two options):		
Magnum™ EX Universal Magnet Plate	Alpaqua	A000380
Magnetic Stand-96	Thermo Fisher Scientific	AM10027
Plate centrifuge	General laboratory supplier	
Tube protocol		
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	12321D
DynaMag™-PCR Magnet	Thermo Fisher Scientific	492025
Plate and tube protocols		
Microcentrifuge	General laboratory supplier	
Vacuum concentrator	SpeedVac System from Thermo Fisher Scientific or equivalent	Varies
Vortex mixer	General laboratory supplier	
qPCR system of fluorescence-based DNA quantitation system	Qubit Fluorometer from Thermo Fisher Scientific or equivalent	
Digital electrophoresis system	Bio-Rad Experion Electrophoresis Station	700-7010
	Agilent 2100 Electrophoresis Bioanalyzer® System	G2939AA
	Agilent 2200 TapeStation® System or equivalent	G2965AA

PLATE PROTOCOL

Our plate protocol has been developed for a maximum of four columns of samples in standard 96-well plate format (Figure 2). The protocol accommodates 32 reactions at a time. Updated, automation-friendly protocols enable various levels of throughput. We do not recommend running more than 32 samples at a time because the timing and temperature of washes may 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: This protocol has been performed with instruments listed in the Equipment table, which have been chosen based on internal testing. Other instruments may not produce the same results and may require further testing for your experimental setup.

Guidelines

During the 4-hour 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 hours) may improve results.

Before you start

1. Two thermal cyclers, set at different incubation temperatures, are used for hybrid capture in this protocol.
   a. Program the first thermocycler according to Table 1 with the lid set at 100°C.

Table 1. HYB program

Step	Number of cycles	Temperature (°C)*	Time
Denaturation	1	95	30 seconds
Hybridization	1	65	4 hours
Hybridization	1	65	∞

* Lid should be set at 100°C.

   b. Program the second thermocycler according to Table 2 with the lid set at 70°C.

Table 2. WASH program

Step	Number of cycles	Temperature (°C)*	Time
Hybridization	1	65	∞

* Reduce the lid temperature to 70°C for the WASH program.

2. Thaw your xGen Hyb Panels at room temperature (RT, 15–25°C). Mix thoroughly and centrifuge briefly.

   For information on how to use xGen Hyb Panels in combination, see Appendix B.

Workflow

1	Combine DNA with blockers Dry down DNA ⛔ Perform hybridization reaction	Total time: 15 minutes Total time: Variable Total time: 4–16 hours
2	Prepare buffers	Total time: 15 minutes*
3	Wash streptavidin beads	Total time: 15 minutes*
4	Perform bead capture	Total time: 45 minutes
5	Perform washes	Total time: 30 minutes
6	Perform post-capture PCR ⛔	Total time: 30 minutes
7	Purify post-capture PCR fragments ⛔	Total time: 30 minutes

* Perform during hybridization reaction

Figure 2. Plate protocol workflow. The full workflow consists of seven steps in total and takes anywhere from 6.5 to 18.5 hours to complete, depending on the duration of the 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.

1. Create the Blocker Master Mix in a tube according to Table 3. Multiply volume by the number of samples and add a 10% overfill.

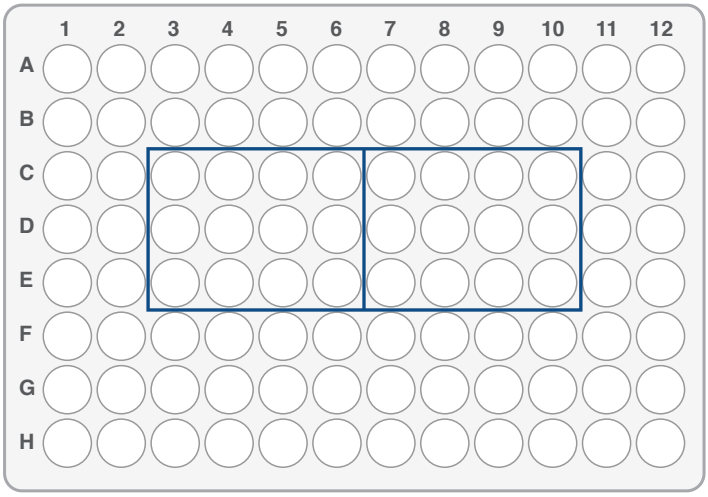
Table 3. Blocker Master Mix

Component	Volume per reaction (µL)
xGen Human Cot DNA	5
xGen Universal Blockers NXT	2
Total	7

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 the library to each well containing Blocker Master Mix. If multiplexing samples, use 500 ng of each library.

Note: 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: 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 at room temperature.

Note: Inspect the xGen 2X Hybridization Buffer tube 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 according to Table 4. Multiply volumes by the number of samples and add a 10% overfill.

Table 4. Hybridization Master Mix

Component	Volume per reaction (µL)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
xGen Predesigned or Custom Hyb Panel	4
Nuclease-free water	1.8
Total	17

* If using an xGen spike-in panel, See Appendix B for more information.

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

Note: If combining two panels, see Appendix B for spike-in volume details.

10. Securely seal the plate with a Microseal® B seal or equivalent.
11. Incubate for at least 5 minutes at room temperature.
12. Vortex the samples, ensuring that they are completely mixed.
13. Briefly centrifuge the samples.
14. Place the plate on the thermal cycler and start the HYB program (see Table 1).

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 for at least 30 minutes before performing the washes.

1. Dilute the following xGen buffers to create 1X working solutions according to Table 5. Multiply volumes by the required number of samples and add 10% overfill.

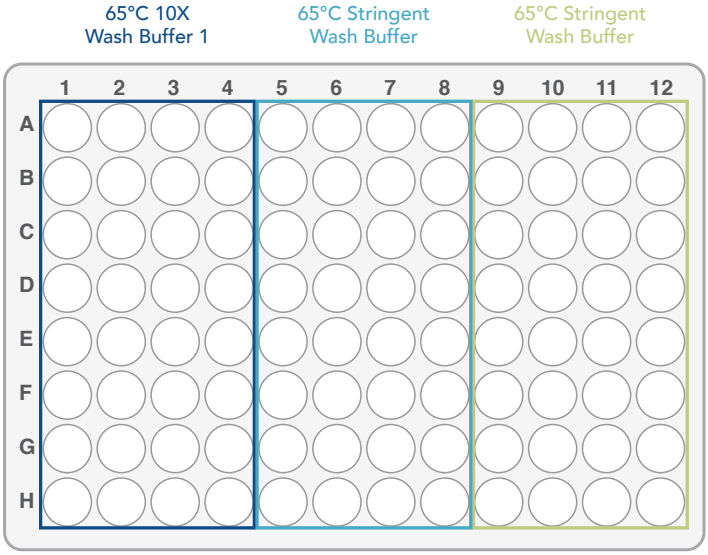
Table 5. xGen wash buffer dilutions

Component	Volume per reaction (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 the 10X Wash Buffer 1 is cloudy, heat the bottle in a 65°C water bath to resuspend the solution.

Tip: Store the 1X working solutions per your established, internal laboratory procedures.

2. Use a fresh PCR plate. For 32 samples, as an example, aliquot and label the plate as follows:
   - **Columns 1–4: 110 µL of Wash buffer 1**
   - **Columns 5–8: 160 µL of Stringent Wash Buffer**
   - **Columns 9–12: 160 µL 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 volumes by the number of samples and add a 10% overfill.

Table 6. 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
Total	17

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 seconds.
2. Add 50 µL of streptavidin beads to a new PCR plate, filling a well for every sample to be captured.
3. Add 100 µL of Bead Wash Buffer from Table 5 to each well, then gently pipet the mix 10 times.
4. Place the plate containing beads on a magnet. Allow the beads to fully separate from the supernatant (approximately 1 minute).
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. Repeat the Bead Wash Buffer wash:
   a. Add 100 µL 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 minute, 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 3 washes.
9. Resuspend the beads in 17 µL of Bead Resuspension Mix from Table 6.
10. Mix thoroughly to ensure the beads do not dry in the well. If needed, briefly centrifuge the plate containing beads at 25 x *g*.

Perform bead capture

Important: If any sample liquid splashes onto the plate seal while vortexing in this section, briefly and gently centrifuge the plate (10 seconds at 25 x *g*).

1. Start the WASH program (Table 2) 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 minutes. We recommend starting incubation at the same time as the bead capture.

2. After the 4-hour 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 each well with sample in the sample plate.
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 minutes. During incubation, remove the plate every 10–12 minutes to quickly and gently vortex.

   Note: The sample plate can be placed 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 minutes, remove the sample plate from the thermal cycler.
2. While the buffer plate is inside the thermal cycler, transfer 100 µL of heated Wash Buffer 1 to each sample. pipet the mixture 10 times, being careful to minimize bubble formation.
3. Reseal the buffer plate, then close the lid.
4. Place the sample plate on the magnet for 1 minute. Remove the supernatant.

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

5. Remove the sample plate from the magnet. Add 150 µL of heated Stringent Wash Buffer to each well containing a sample.
6. Reseal the buffer plate, then close the lid.
7. Pipet the mix 10 times and be careful to minimize bubble formation. Its best to always use fresh pipette tips for each well.
8. Securely seal the sample plate, then incubate for 5 minutes in the thermal cycler.
9. Place the sample plate on the magnet for 1 minute, then remove the supernatant.
10. Remove the sample plate from the magnet, then add 150 µL of heated Stringent Wash Buffer from the buffer plate to the sample plate.
11. Pipet the mix 10 times and be careful to minimize bubble formation. Securely seal the sample plate, then incubate for 5 minutes in the thermal cycler.
12. Place the sample plate on the magnet for 1 minute.

Room temperature washes

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

1. Remove supernatant and add 150 µL of Wash Buffer 1.
2. 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.

3. Incubate for 2 minutes while alternating between vortexing for 30 seconds and resting for 30 seconds, to ensure the mixture remains homogenous.
4. Centrifuge the sample plate for 5 seconds at 25 x g.

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

5. Place the sample plate on the magnet for 1 minute, then remove and discard the seal.
6. Remove the supernatant, then remove the sample plate from the magnet.

7. Add 150 µL of Wash Buffer 2 to each sample well, then securely seal the sample plate with a fresh seal and vortex for 30 seconds at 25 x g, or until each well is fully resuspended.
8. Incubate for 2 minutes while alternating between vortexing for 30 seconds and resting for 30 seconds, to ensure the mixture remains homogenous.
9. After the incubation, briefly centrifuge the sample plate (5 seconds at 25 x g).
10. After centrifuging, place the sample plate on the magnet for 1 minute, 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 minutes while alternating between vortexing for 30 seconds and resting for 30 seconds, to ensure the mixture remains homogenous.
14. After the incubation, briefly centrifuge the sample (5 seconds at 25 x g).
15. After centrifuging, place the sample plate on the magnet for 1 minute, 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. The entire volume of resuspended beads (20 µL) will be used with capture DNA in Perform post-capture PCR.

Perform post-capture PCR

1. In a tube, prepare the Amplification Reaction Mix as in Table 7. Multiply volumes by the number of samples on the plate and add 10% overfill, as follows:

Table 7. Amplification Reaction Mix

Component	Volume per reaction (µL)
2X KAPA HiFi PCR Master Mix	25
xGen Library Amplification Primer Mix	1.25
Nuclease-free water	3.75
Total	**30**

Note: If using a different master mix than KAPA HiFi PCR Mix, 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.

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

Table 8. Post-capture PCR program

Step	Number of cycles	Temperature (°C)*	Time
Polymerase activation	1	98	45 seconds
Denaturation	Variable—Refer to Table 9 below	98	15 seconds
Annealing		60	30 seconds
Extension		72	30 seconds
Final extension	1	72	1 minute
Hold	1	4	∞

* Lid should be set at 105°C.

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

6. We recommend starting with the following and then adjusting for your experimental conditions:

Table 9. Recommended number of cycles based on hyb capture panel size

Panel size	1-plex	4-plex	8-plex	12-plex
>100,000 probes (xGen Exome v2)	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: Store amplified captures per your established, internal laboratory procedures.

Purify post-capture PCR fragments

Important: Ensure Agencourt® AMPure® XP beads have been equilibrated to room temperature before proceeding.

1. Prepare 250 µL of fresh 80% ethanol per sample. Multiply volumes by the number of samples and add a 10% overfill.
2. Add 75 µL (1.5X volume) of Agencourt AMPure XP beads to each amplified capture sample.
3. After adding the beads, pipet the mix thoroughly and incubate for 5–10 minutes.
4. Place the plate on the magnet until the supernatant is clear (2–5 minutes).
5. Remove the supernatant without disturbing the beads.
6. While keeping the plate on the magnet, add 125 µL of 80% ethanol, then incubate for 1 minutes.
7. Remove the ethanol, then **repeat step 6**.
8. Allow the beads to air dry for 1–3 minutes. Do not over-dry the beads.
9. 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 minutes at room temperature.
11. Place the plate on a magnet until the supernatant is clear (1–2 minutes).
12. Transfer 20 µL of eluate to a fresh plate. Ensure that no beads are carried over.

Optional stopping point: Store purified PCR fragments per your established, internal laboratory procedures.

Quantify the 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.
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 equivalent 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 (Figure 3). If using plates for your DNA library, use the Plate protocol.

Using the plate protocol may provide more consistent results due to a variety of variables, including more equivalent treatment of different samples.

Guidelines

During the 4-hour incubation, each tube should be sealed properly 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 hours) may improve performance.

Before you start

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

1. Program the thermocycler according to Table 10 with the lid set at 100°C.

   Table 10. HYB program

Step	Number of cycles	Temperature (°C)*	Time
Denaturation	1	95	30 seconds
Hybridization	1	65	4 hours
Hybridization	1	65	∞

   * Lid should be set at 100°C.

2. Create a second program according to Table 11 with the lid set at 70°C.

   Table 11. WASH program

Step	Number of cycles	Temperature (°C)*	Time
Hybridization	1	65	∞

   * Reduce the lid temperature to 70°C for the WASH program.

3. Thaw xGen Hyb Panels at room temperature (RT, 15–25°C). Mix thoroughly and centrifuge briefly.

   For information on how to use xGen Hyb Panels in combination, see Appendix B.

Workflow

1	Combine DNA with blockers Dry down DNA ⛔ Perform hybridization reaction	Total time: 15 minutes Total time: Variable Total time: 4–16 hours
2	Prepare buffers	Total time: 15 minutes*
3	Wash streptavidin beads	Total time: 15 minutes*
4	Perform bead capture	Total time: 45 minutes
5	Perform washes	Total time: 30 minutes
6	Perform post-capture PCR ⛔	Total time: 30 minutes
7	Purify post-capture PCR fragments ⛔	Total time: 30 minutes

* Perform during hybridization reaction

Figure 3. Tube protocol workflow. The full workflow consists of 7 steps in total and takes anywhere from 6.5 to 18.5 hours to complete, depending on the duration of the 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.

1. In a 1.7 mL MAXYMum Recovery® microtube (low bind), create the Blocker Mix according to Table 12:

Table 12. Blocker Mix

Component	Volume per reaction (µL)
xGen Human Cot DNA	5
xGen Universal Blockers NXT	2
Total	7

2. Add 500 ng of library to each tube containing Blocker Master Mix. 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 per your established, internal laboratory procedures.

4. Thaw all contents of the xGen Hybridization and Wash Kit at room temperature.

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

5. Add the Hybridization Master Mix components (Table 13) to the tube from Step 1 (above).

Table 13. Hybridization Master Mix

Component	Volume per reaction (µL)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
xGen Predesigned or Custom Hyb Panel	4
Nuclease-free water	1.8
Total	17

* 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 minutes.
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 (Table 10).

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 minutes before performing the washes.

1. Dilute the following xGen buffers to create 1X working solutions according to Table 14.

Table 14. xGen wash buffer dilutions

Concentrate	Nuclease-free water (µL)	Buffer (µL)	Total (µL)	Preparation
xGen 2X Bead Wash Buffer	160	160	320	Keep at RT
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 1X solution is kept at RT.
xGen 10X Wash Buffer 2	144	16	160	Keep at RT
xGen 10X Wash Buffer 3	144	16	160	Keep at RT
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 the 10X Wash Buffer 1 is cloudy, heat the bottle in a 65°C water bath to resuspend.

Tip: The 1X working solutions are stable at room temperature (15–25°C) or per your established, internal laboratory procedures.

2. Prepare the following Bead Resuspension Mix in a low bind tube according to Table 15.

Table 15. 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
Total	17

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 seconds.
2. Aliquot 50 µL of streptavidin beads per capture into a single 1.7 mL low bind tube. For example, for 1 capture, prepare 50 µL of beads. For 2 captures, prepare 100 µL of beads.
3. Add 100 µL 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 minute).
5. Remove and discard the clear supernatant, ensuring that the beads remain in the tube.
6. Remove the tube from the magnet.

7. Repeat the following wash:
   a. Add 100 µL of Bead Wash Buffer per capture, then pipet the mix 10 times.
   b. Place the tube on a magnetic rack for approximately 1 minute, 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 3 washes.
9. Resuspend the beads in each sample with 17 µL of the 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 µL 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 µL aliquot) and the 1X Stringent Wash Buffer (both aliquots) in a 65°C water bath. Incubate for at least 15 minutes.

   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 4–16-hour incubation, take the tube(s) 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 µL 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 seconds at 25 x g).
7. Place the sample tube in the thermal cycler and set a timer for 45 minutes.

   Note: Sample tubes can be placed in the thermal cycler before the lid temperature has fully cooled to 70°C when starting the incubation.

8. Every 10–12 minutes, remove the tube from the thermal cycler and gently vortex to ensure the sample is fully resuspended.
9. At the end of the 45 minutes, 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 µL of heated Wash Buffer 1 to the sample and pipet the mix 10 times. Be careful to minimize bubble formation.
2. Place the tube on a magnetic rack for 1 minute. 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 µL of heated Stringent Wash Buffer to the sample.
4. Pipet the mix 10 times, being careful to not introduce bubbles.
5. Incubate each tube in the water bath at 65°C for 5 minutes.
6. Place each tube on the magnet for 1 minute. Remove the supernatant.
7. Remove the tube(s) from the magnet and add 150 µL of heated Stringent Wash Buffer to each sample.
8. Pipet the mix 10 times, being careful to not introduce bubbles.
9. Incubate in the water bath at 65°C for 5 minutes.
10. Place the tube(s) on a magnet for 1 minute.

Room temperature washes

Important: To ensure the beads remain fully resuspended, vigorously mix the samples during the RT washes.

1. Remove and discard supernatant. Add 150 µL of Wash Buffer 1, equilibrated to room temperature, to each sample tube.
2. Vortex thoroughly until fully resuspended.
3. Incubate for 2 minutes while alternating between vortexing for 30 seconds and resting for 30 seconds, to ensure the mixture remains homogenous.
4. At the end of the incubation, briefly centrifuge the tube.
5. Place each tube on the magnet for 1 minute.
6. Remove the supernatant. Add 150 µL of Wash Buffer 2.
7. Vortex thoroughly until fully resuspended.
8. Incubate tube(s) for 2 minutes while alternating between vortexing for 30 seconds and resting for 30 seconds, to ensure the mixture remains homogenous.
9. At the end of the incubation, briefly centrifuge the tube(s).
10. Place on the magnet for 1 minute.
11. Remove the supernatant. Add 150 µL of Wash Buffer 3.
12. Vortex thoroughly until fully resuspended.
13. Incubate for 2 minutes while alternating between vortexing for 30 seconds and resting for 30 seconds, to ensure the mixture remains homogenous.
14. At the end of the incubation, briefly centrifuge each tube.
15. Place each sample tube on the magnet for 1 minute.
16. Remove and discard the supernatant.
17. With the sample tube(s) still on the magnet, use a fresh pipet tip to remove residual Wash Buffer 3 from each tube. Then, remove each 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(s).

    Important: Do not discard the beads. The entire volume of resuspended beads (20 µL) will be used 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 (Table 16).

Table 16. Amplification Reaction Mix

Component	Volume per reaction (µL)
2X KAPA HiFi PCR Master Mix	25
xGen Library Amplification Primer Mix	1.25
Nuclease-free water	3.75
Total	30

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

3. Place the plate in a thermal cycler, and run the following Post-capture PCR program (Table 17) with the lid temperature set to 105°C.

Table 17. Post-capture PCR program

Step	Number of cycles	Temperature (°C)*	Time
Polymerase activation	1	98	45 seconds
Denaturation	Variable—Refer to Table 18 below	98	15 seconds
Annealing		60	30 seconds
Extension		72	30 seconds
Final extension	1	72	1 minute
Hold	1	4	∞

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

4. We recommend starting with the following and then adjusting for your experimental conditions:

Table 18. Recommended number of cycles based on hyb capture panel size

Panel size	1-plex	4-plex	8-plex	12-plex
>100,000 probes (xGen Exome v2)	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: Store amplified captures per your established internal laboratory procedures.

Purify post-capture PCR fragments

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

1. Prepare 250 µL of fresh 80% ethanol per sample. Multiply volumes by the number of samples and add a 10% overfill.
2. Add 75 µL (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 minutes.
4. Place the sample tube on a magnet until the supernatant is clear (2–5 minutes).
5. Remove supernatant without disturbing the beads.
6. While keeping the tube on the magnet, add 125 µL of 80% ethanol, then incubate for 1 minute.
7. Remove the ethanol, then repeat another ethanol wash.
8. Allow the beads to air dry for 1–3 minutes. Do not over-dry the beads.
9. Remove the sample tube 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 minutes at room temperature.
11. Place the tube on a magnet until the supernatant is clear (1–2 minutes).
12. Transfer 20 µL of eluate to a fresh tube. Ensure that no beads are carried over.

Optional stopping point: Store purified PCR fragments per your established internal laboratory procedures.

Confirm 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.
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 equivalent 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 µL of Human Cot DNA. To order additional Cot DNA, visit the xGen 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, which may require either 1.7 mL tubes or a deep well plate.

2. Add 7.5 µL 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 minutes at room temperature.
7. Incubate the plate or tube on the magnet for at least 2 minutes or until supernatant is clear.
8. Remove and discard the supernatant. While the sample is on the magnet, add 80% ethanol to cover the surface of the beads. Incubate for 30 seconds without disturbing the beads.
9. Remove and discard the supernatant, then repeat another ethanol wash.
10. After discarding the supernatant, allow the beads to air dry for approximately 2 minutes. Do not over-dry.
11. Add these components to the tube to make the Hybridization Reaction Mix (Table 19).

Table 19. Hybridization reaction mix.

Component	Volume per reaction (µL)
xGen 2X Hybridization Buffer	9.5
xGen Hybridization Buffer Enhancer	3
xGen Universal Blocker NXT	2
xGen Hyb Panel	4.5
Total	19

Note: The Hybridization Reaction Mix elutes the DNA from the AMPure XP beads.

12. Vortex to mix. Ensure that the beads are fully resuspended.
13. Incubate for 5 minutes at RT.
14. After incubation, place on a magnet for 5–10 minutes or until the supernatant is clear.
15. Transfer 17 µL of the supernatant to the sample plate, or tube, where the hybridization will occur.

    Important: Ensure beads are not carried over during the transfer process.

Proceed to Perform hybridization reaction step 13, page 13 for plate captures, or step 8, page 22 for tube captures immediately after the sample DNA is ready.

APPENDIX B

Combining xGen Hyb Panels

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 volumes by the number of samples and add a 10% overfill (See Table 2).

For more information regarding spike-in panels, contact our technical support group at applicationsupport@idtdna.com.

Table 1. Combining panels for hybridization

	Volume per reaction (µL)			
	xGen Custom Hyb Panel <20,000 probes		xGen Custom Hyb Panel >20,000 probes	
	Volume		Volume	
	Backbone	Spike-in	Backbone	Spike-in
xGen Custom Hyb Panel <20,000 probes	3 µL	3 µL	NA	NA
xGen Custom Hyb Panel >20,000 probes	4 µL	1 µL	3 µL	3 µL

Table 2. Hybridization Master Mix for combined hyb capture panels.

Component	Volume per reaction (µL)
xGen 2X Hybridization Buffer	8.5
xGen Hybridization Buffer Enhancer	2.7
Main panel	See Table 1
Spike-in panel	See Table 1
Nuclease-free water	0–0.8 (only if needed*)
Total	17–17.2 µL

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

xGen™ hybridization capture of Illumina® Nextera™ DNA libraries

Technical support: applicationsupport@idtdna.com

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