# xGen™ hybridization capture of DNA libraries

## For NGS target enrichment

**Uses these IDT products:**

- xGen Predesigned Hybridization Capture Panels
- xGen Custom Hybridization Capture Panels
- xGen Hybridization and Wash v2 Kit:
  - xGen Hybridization and Wash v2 Reagents
  - xGen Hybridization and Wash v2 Beads
- xGen Universal Blockers
- xGen Library Amplification Primer Mix

**Generates:**

- Illumina® platform-compatible libraries

# REVISION HISTORY

| Version | Release date | Description of changes |
|---|---|---|
| 8 | May 2024 | Clarification to vortex and drying instructions. |
| 7 | February 2023 | Updated product name |
| 6 | August 2022 | Updated product names; updated PCR steps to refer to xGen 2x HiFi PCR mix |
| 5 | December 2021 | Updated product names |
| 4 | May 2019 | Updated to specify that xGen Universal Blocker 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 |

# Table of contents

# OVERVIEW

This protocol includes the steps necessary for target enrichment of a next generation sequencing library prepared from DNA or RNA, using xGen™ Hyb Capture 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 unintentional hybridization between library molecules.
2. Performing hybridization using one of the many options of the xGen Predesigned Hybridization Capture Panel or an xGen Custom Hybridization Capture Panel and the xGen Hybridization and Wash v2 Reagents and Beads.
3. Perform bead capture of the hybridized mixture.
4. Perform post-capture washes.
5. Post-capture PCR to amplify the library for sequencing.

After preparing an NGS library using one of the methods described in the Input recommendations section, this procedure describes the process for using the IDT xGen Universal Blockers to prevent off-target fragments from annealing to the intended target sequence via adapter-to-adapter hybridization. The type of xGen Universal Blocker depends upon your chosen method of adapter ligation and/or sequencing instrument that will be used. IDT offers a variety of different options that can be reviewed in the Consumables and equipment section.

Once the adapters have been blocked, the protocol describes the conditions necessary for hybridization of one of the xGen Predesigned Hybridization Capture Panels or one of the xGen Custom Hybridization Capture Panels. These panels contain capture probes that were 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 v2 Kit, which includes two products: xGen Hybridization and Wash v2 Reagents and xGen Hybridization and Wash v2 Beads. Both components are compatible with any of the IDT xGen Hyb Panels. There are instructions to perform hybridization capture either in plates or tubes depending on the number of targeted sequencing experiments that you will be performing.

After the probes are hybridized to the target fragments, the procedure for separating the desired DNA targets from the off-target fragments is presented. Since each probe has a 5′ biotin modification, this procedure describes the use of streptavidin-coated magnetic beads to capture the probe and targeted DNA duplexes. Post-capture washes are performed to further remove non-specifically bound DNA. IDT provides a selection of compatible suppliers of the necessary reagents and magnetic stands in the Consumables and equipment section.

After removal of the non-specific DNA from the sample, the procedure for post-capture PCR is described using a PCR master mix with the IDT xGen Library Amplification Primer Mix. IDT offers suggestions for the number of cycles for amplification based on the number of probes in the panel as a starting point to create enough DNA in the final captured library.

## Target capture workflow

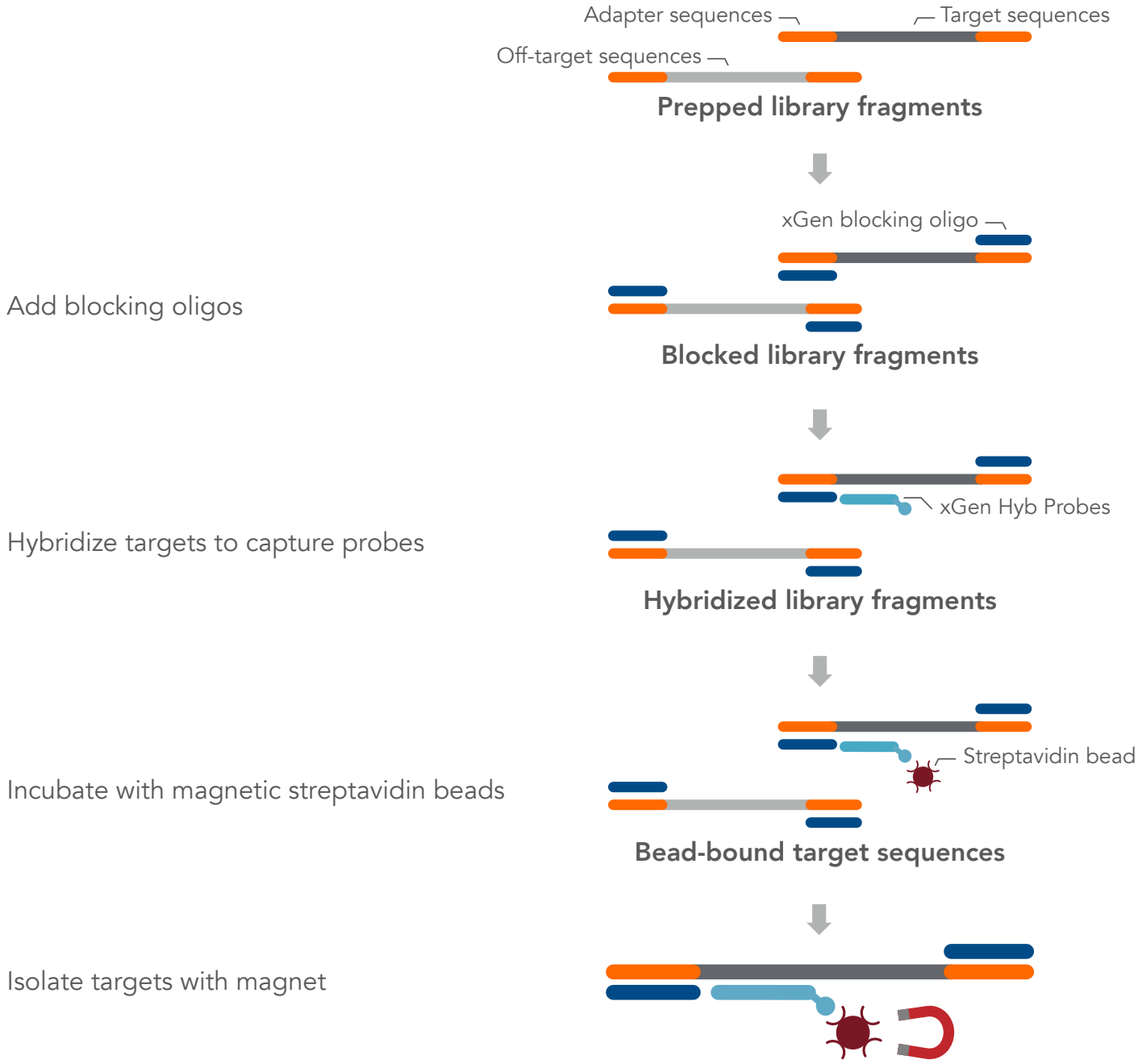



**Figure 1. Desired prepared library fragments are separated from off-target fragments using hybridization capture.** First, xGen Universal Blockers are mixed with prepared library fragments to prevent adapter-to-adapter hybridization. Blocked library fragments are then annealed to the 5′ biotinylated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

# INPUT RECOMMENDATIONS

## Input for library preparation

This protocol was developed with libraries prepared from multiple library preparation kits, including the xGen cfDNA & FFPE DNA Library Prep v2 MC Kit, xGen DNA Library Prep Kits, and third-party kits. 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 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 hyb capture

For optimal results, use a SpeedVac® system (Savant) for concentrating DNA.

**Note:** To multiplex a high quantity of samples, we recommend using a SpeedVac® system; however, if you require a quicker turnaround, you may also consider preparing the DNA samples following the instructions in Appendix A: AMPure XP Bead DNA concentration protocol.

**Important:** The AMPure XP Bead DNA concentration protocol (Appendix A) requires 7.5 µL of Human Cot DNA. To order additional, go to the xGen Hybridization Capture Core Reagents webpage.

# CONSUMABLES AND EQUIPMENT

## Consumables—IDT

| Item | Description | Catalog # | Storage |
|---|---|---|---|
| xGen Hyb Panels | Custom<br>Predesigned | Varies | –20°C |
| xGen Hybridization and Wash v2 Kit | xGen Hybridization and Wash v2 Reagents, 16 rxn | 10010351 | –20°C |
| | xGen Hybridization and Wash v2 Beads, 16 rxn | 10010353 | 4°C |
| | xGen Hybridization and Wash v2 Reagents, 96 rxn | 10010352 | –20°C |
| | xGen Hybridization and Wash v2 Beads, 96 rxn | 10010354 | 4°C |
| xGen Universal Blockers for TruSeq® libraries | xGen Universal Blockers TS, 16 rxn | 1075474 | –20°C |
| | xGen Universal Blockers TS, 96 rxn | 1075475 | |
| | xGen Universal Blockers TS, 4x96 rxn | 1075476 | |
| | xGen Universal Blockers 10 bp TS, 16 rxn | 1081100 | |
| | xGen Universal Blockers 10 bp TS, 96 rxn | 1081101 | |
| | xGen Universal Blockers 10 bp TS, 4x96 rxn | 1081102 | |
| xGen Universal Blockers for Nextera® libraries | xGen Universal Blockers NXT, 16 rxn | 1079584 | –20°C |
| | xGen Universal Blockers NXT, 96 rxn | 1079585 | |
| | xGen Universal Blockers NXT, 4x96 rxn | 1079586 | |
| xGen Library Amplification Primer Mix | 16 rxn | 1077675 | –20°C |
| | 96 rxn | 1077676 | |
| | 192 rxn | 1077677 | |
| (Optional; additional) 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 for safety data sheets (SDSs) and www.idtdna.com/COA for certificates of analysis (COAs) for IDT products.

## Consumables—Other suppliers

| Item | Supplier | Catalog # |
|---|---|---|
| 200 proof Ethanol | General laboratory supplier | Varies |
| AMPure® XP—PCR Purification beads | Beckman Coulter | A63880 |
| Digital electrophoresis chips | Bio-Rad Experion® DNA 1 K 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 |
| 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 |
| 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 |
|---|---|
| **Plate protocol** | |
| Two thermal cyclers | Bio-Rad C/S1000 or T100 |
| Magnet (IDT recommends two options): | |
| Magnum® EX Universal Magnet Plate | Alpaqua (cat # A000380) |
| Magnetic Stand-96 | Thermo Fisher Scientific (cat # 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 (cat # 12321D) |
| DynaMag-PCR Magnet | Thermo Fisher Scientific (cat # 492025) |
| **Plate and tube protocols** | |
| Microcentrifuge | General laboratory supplier |
| Vacuum concentrator | Thermo Fisher Scientific SpeedVac® system 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 4200 TapeStation® System (cat # G2991BA), or equivalent |

# PLATE PROTOCOL

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). 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 expected results.

## 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 experimental results.

## Before you start

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

1. Create the following PCR programs:

| HYB program (lid set at 100°C) | |
|---|---|
| 95°C | 30 sec |
| 65°C | 4 h |
| 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. 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<br>Dry down DNA ⛔<br>Perform hybridization incubation | Total time: 15 minutes<br>Total time: Variable<br>Total time: 4–16 hours |
| 2 | Prepare buffers | Total time: 15 minutes* |
| 3 | Wash streptavidin beads | Total time: 15 minutes* |
| 4 | Bead capture incubation | Total time: 45 minutes |
| 5 | Perform post-capture washes | Total time: 30 minutes |
| 6 | Perform post-capture PCR ⛔ | Total time: 30 minutes |
| 7 | Post-capture PCR clean up ⛔ | Total time: 30 minutes |

* Perform during hybridization reaction

# Perform hybridization reaction

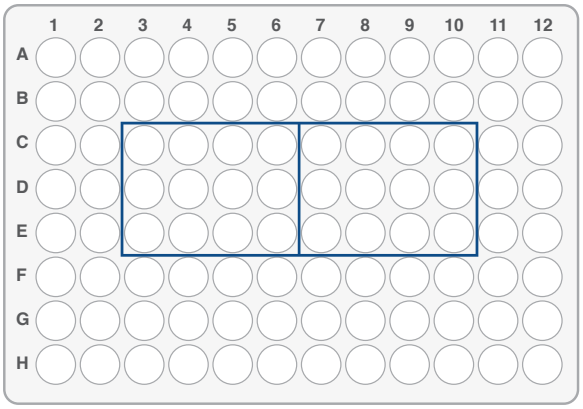
1. Create the Blocker Master Mix in a tube. Multiply by the number of capture reactions being prepared and add a 10% overfill. Each separate capture reaction, including both single and multiplex library captures, equal 1 separate capture reaction.

| Blocker Master Mix components | Volume per reaction (µL) |
|---|---|
| Human Cot DNA | 5 |
| xGen Universal Blockers 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.
5. Vortex to mix well and spin down.

**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.




6. Dry down the mixture in a SpeedVac system. Suggested settings for dry down are 60°C with Vacuum On. Allow to dry for 2hrs or until wells are completely dry.

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

7. Thaw all contents of the xGen Hybridization and Wash v2 Reagents to room temperature and mix thoroughly.

**Note:** Inspect the tube of the xGen 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.

8. Create the Hybridization Master Mix in a 1.5 mL tube. Multiply by the number of samples and add a 10% overfill.

| Hybridization Master Mix components | Volume per reaction (µL) |
|---|---|
| xGen 2X Hybridization Buffer | 8.5 |
| xGen Hybridization Buffer Enhancer | 2.7 |
| xGen Predesigned or Custom Hybridization Capture Panel | 4 |
| Nuclease-Free Water* | 1.8 |

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

9. Vortex or pipette the Hybridization Master Mix to mix well.

10. 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.

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

## Prepare buffers

**Note:** Before preparing the buffers, take out the xGen Hybridization and Wash v2 Beads box, which contains 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.

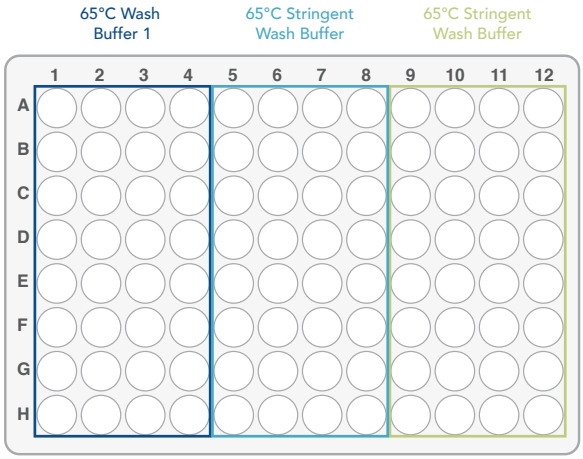
1. Dilute the following buffers to create a 1X working solution as follows, multiplying by the required number of samples:

| 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 | Keep at room temperature until aliquoted into plates |
| 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 | Keep at room temperature until aliquoted into plates |

   **Note:** If the 10X 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 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 by the number of samples and add a 10% overfill.

| 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 |

## Streptavidin bead wash

**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 µL of streptavidin beads to a new PCR plate, filling a well for every sample being captured.
3. Add 100 µL of Bead Wash Buffer from Prepare buffers, step 1 to each well, then gently pipette 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 µL of Bead Wash Buffer to each well containing beads, then gently pipette 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 3 washes.
9. Resuspend the beads in 17 µL 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 pipette mix 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 min. 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, then add 150 µL of heated Stringent Wash Buffer to each well containing a sample.
6. Reseal the buffer plate, then close the lid.
7. Pipette mix 10 times, being careful to minimize bubble formation. Always use fresh pipette tips for each well.
8. Securely seal the sample plate, then incubate for 5 min in the thermal cycler.
9. Place the sample plate on the magnet for 1 min, 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. Pipette mix 10 times, being careful to minimize bubble formation. Securely seal the sample plate, then incubate for 5 min on the thermal cycler.
12. 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.

1. Remove supernatant. 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 min while alternating between vortexing for 30 sec and resting for 30 sec, to ensure the mixture remains homogenous.
4. 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.

5. Place the sample plate on the magnet for 1 min, 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, then securely seal the sample plate with a fresh seal and 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. 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. Pipette 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 components | Volume (µL) |
|---|---|
| xGen 2x HiFi PCR Mix | 25 |
| xGen Library Amplification Primer Mix | 1.25 |
| Nuclease-Free Water | 3.75 |

**Note:** If using a different master mix than xGen 2x 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 program with the lid temperature set to 105°C:

| Step | Number of cycles | Temperature (°C) | Time |
|---|---|---|---|
| Polymerase activation | 1 | 98 | 45 sec |
| Amplification<br>Denaturation<br>Annealing<br>Extension | Variable—refer to the Panel table below. | 98<br>60<br>72 | 15 sec<br>30 sec<br>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<br>(xGen Exome Hyb Panel 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.

## Post-capture PCR clean up

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

1. Prepare 250 µL of fresh 80% ethanol per sample, multiplied by the number of samples with a 10% overfill.
2. Add 75 µL (1.5X volume) of AMPure XP beads to each amplified capture.
3. After adding the beads, pipette mix thoroughly and incubate for 5–10 min.
4. Place the plate on the magnet until the supernatant is clear (2–5 min).
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 min.
7. Remove the ethanol, then repeat another ethanol wash.
8. Allow the beads to air dry for 1–3 min. 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 min at room temperature.
11. Place the plate on a magnet until supernatant is clear (1–2 min).
12. Transfer 20 µL of eluate to a fresh plate, making sure 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 1 K 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. If you are using plates for your DNA library, use the Plate protocol.

**Note:** 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 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 h |
| 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. 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<br>Dry down DNA ⛔<br>Perform hybridization incubation | Total time: 15 minutes<br>Total time: Variable<br>Total time: 4–16 hours |
| 2 | Prepare buffers | Total time: 15 minutes* |
| 3 | Wash streptavidin beads | Total time: 15 minutes* |
| 4 | Bead capture incubation | Total time: 45 minutes |
| 5 | Perform post-capture washes | Total time: 30 minutes |
| 6 | Perform post-capture PCR ⛔ | Total time: 30 minutes |
| 7 | Post-capture PCR clean up ⛔ | Total time: 30 minutes |

* Perform during hybridization reaction

# Perform hybridization reaction

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

| Blocker component | Volume per reaction (µL) |
|---|---|
| xGen Human Cot DNA | 5 |
| xGen Universal Blockers 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 v2 Reagents 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.
5. Create the Hybridization Master Mix by adding the following components to the tube from step 2 (above).

| Hybridization Master Mix components | Volume per reaction (µL) |
|---|---|
| xGen 2X Hybridization Buffer | 8.5 |
| xGen Hybridization Buffer Enhancer | 2.7 |
| xGen Hyb Panel | 4 |
| Nuclease-Free Water* | 1.8 |

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

6. Pipette 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, take out the xGen Hybridization and Wash v2 Beads box, which contains the Dynabeads M270 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 components | Volume per reaction (µL) |
|---|---|
| xGen 2X Hybridization Buffer | 8.5 |
| xGen Hybridization Buffer Enhancer | 2.7 |
| Nuclease-Free Water | 5.8 |

## Streptavidin bead wash

**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 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 and for 2 captures, prepare 100 µL of beads.
3. Add 100 µL of Bead Wash Buffer per capture. Gently pipette 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 µL of Bead Wash Buffer per capture, then pipette 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 3 washes.
9. Resuspend the beads in 17 µL 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 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 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 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 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 µL of heated Wash Buffer 1 to the sample, then pipette 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 µL of heated Stringent Wash Buffer to the sample.
4. Pipette 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. Pipette 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. Pipette 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 components | Volume (µL) |
|---|---|
| xGen 2x HiFi PCR Mix | 25 |
| xGen Library Amplification Primer Mix | 1.25 |
| Nuclease-Free Water | 3.75 |

* If using a PCR master mix other than xGen 2x HiFi PCR Mix, 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<br>Denaturation<br>Annealing<br>Extension | Variable—refer to the Panel table below. | 98<br>60<br>72 | 15 sec<br>30 sec<br>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<br>(xGen Exome Hyb Panel 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.

## Post-capture PCR clean up

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

1. Prepare 250 µL of fresh 80% ethanol per sample, multiplied by the number of samples with a 10% overfill.
2. Add 75 µL (1.5X volume) of 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 µL 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 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 min at room temperature.
11. Place the tube on a magnet until the supernatant is clear (1–2 min).
12. Transfer 20 µL of eluate to a fresh tube making sure 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 1 K 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 Human Cot DNA, go to the xGen Hybridization Capture Core Reagents webpage.

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 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 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 over-dry.
11. Add these components to the tube to make the Hybridization Reaction Mix:

| Reaction Mix components | Volume per reaction (µL) |
|---|---|
| xGen 2X Hybridization Buffer | 9.5 |
| xGen Hybridization Buffer Enhancer | 3 |
| xGen Universal Blockers based on your library adapter | 2 |
| xGen Hyb Panel | 4.5 |
| Total | 19 |

   **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 µL 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 for plate captures, or step 8 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 by the number of samples, then add a 10% overfill (See **Table 2**).

For more information regarding spike-in panels, contact us at **www.idtdna.com/contactus**.

### Table 1. Spike-in volumes when combining two panels

| | | Spike-In Panel | |
|---|---|---|---|
| | | ≤20K probes | >20K probes |
| Main Panel | ≤20K probes | 3 µL Main Panel<br>+<br>3 µL Spike-In Panel | N/A |
| | >20K probes | 4 µL Main Panel<br>+<br>1 µL Spike-In Panel | 3 µL Main Panel<br>+<br>3 µL Spike-In Panel |

### Table 2. Volumes per component of the Hybridization Master Mix

| Hybridization Master Mix components | 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 volume*** | **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 DNA libraries

For more information, go to **www.idtdna.com/ContactUs**

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