xGen™ Hybridization and Wash Kit v3 (EAP) for PacBio® Targeted Kinnex™

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REVISION HISTORY

Version	Release date	Description of changes
1	August 2024	Initital release

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WORKFLOW

Step	Description
1	Combine indexed library with Human Cot DNA and PacBio Kinnex Blockers Dry down cDNA-blocker mixture
2	Hybridize to probes
3	Bind probes to beads
4	Wash bead-captured cDNA
5	Perform post-capture PCR
6	Clean up post-capture PCR fragments

CONSUMABLES AND EQUIPMENT

Consumables from IDT

(EAP) – denotes that this product is in development and being provided as part of an Early Access Program

Item*	Volume per reaction (µL)†	Storage (°C)
Human Cot DNA	5	–20
PacBio Kinnex Blocker 1	1	–20
PacBio Kinnex Blocker 2	1	–20
xGen 1X Hybridization Buffer (EAP)	14	–20
Custom Kinnex Hyb Panel	4	–20
xGen 2X Bead Wash Buffer (EAP)	159	4
xGen 1X Stringent Wash Buffer (EAP)	50	4
xGen 1X Wash Buffer I (EAP)	150	4
xGen 1X Wash Buffer II (EAP)	150	4
xGen 2X HiFi PCR Mix (Included in the kit but not used in this protocol)	25	–20
PacBio Kinnex Amplification Primers	1.25	–20
xGen Hybridization and Wash Beads**	50	4

* All reagents should be at room temperature when used, except for xGen 2X HiFi PCR Mix.

† Volume does not include overfill.

** Items purchased separately.

100 nmole DNA Oligos ordered from IDT

Item	Sequence to order
PacBio Kinnex Blocker 1	CTACACGACGCTCTTCCGATCTAC/ideoxyI//ideoxyI//ideoxyI//ideoxyI//ideoxyI//ideoxyI//ideoxyI/AATGAAGTCGCAGGGTTGG/3SpC3/
PacBio Kinnex Blocker 2	AAGCAGTGGTATCAACGCAGAGTACTTTTT/3SpC3/
PacBio Kinnex cDNA Amp Primer 1	CTACACGACGCTCTTCCGATCTAC
PacBio Kinnex cDNA Amp Primer 2	AAGCAGTGGTATCAACGCAGAGT

Consumables—Other suppliers

Item	Supplier	Volume per reaction (µL)*	Catalog #
Nuclease-Free Water	Various	182.75	Varies
Ethanol (dilute to 80%)	Various	400	Varies
Buffer EB (or equivalent: 10 mM Tris-HCl, pH 8.5)	QIAGEN, or general laboratory supplier	22	19086
AMPure® XP Beads	Beckman Coulter	75	A63880
twin.tec™ PCR Plates LoBind® or TempAssure® PCR Flex-Free 8-Tube Strips	Eppendorf USA Scientific		0030129504 1402-4700
Microseal® 'B' PCR Plate Sealing Film, adhesive, optical	Bio-Rad		MSB1001
High Sensitivity D1000 ScreenTape®	Agilent		5067-5584
High Sensitivity D1000 Reagents	Agilent		5067-5585
Qubit® dsDNA HS Assay Kit	Thermo Fisher Scientific		Q32851 or Q32854
KAPA HiFi HotStart ReadyMix (2X)	Roche		KK2601 or KK2602

* Volume does not include overfill.

Equipment

Item	Supplier	Catalog #
T100 Thermal Cycler or Veriti™ 96-Well Fast Thermal Cycler or equivalent	Bio-Rad Applied Biosystems	1861096 4375786
ThermoMixer® C	Eppendorf	5382000023
SmartBlock™ PCR 96	Eppendorf	5306000006
DynaMag®-2 Magnet 96-Well Side Pull Bar Magnet or Magnum EX	Invitrogen Permagen Alpaqua	12321D MSP750 A000380
Plate centrifuge	Various	Varies
Microcentrifuge	Various	Varies
Vacuum concentrator	Various	Varies
Vortex mixer	Various	Varies
Qubit® Fluorometer or Qubit® Flex Fluorometer or equivalent	Thermo Fisher Scientific	Q33238 Q33327
TapeStation® System or equivalent	Agilent	G2991BA

GUIDELINES

Notes: All reagents are shipped on dry ice except for capture beads. Store the xGen Hybridization Buffer at −20°C. Store the wash buffers at 4°C. Confirm the buffers are fully homogeneous (no visible fluid current when pipette mixed) at room temperature (RT) before use.

If the hybridization capture is to be performed on plates, we recommend avoiding wells on the plate edges because evaporation is more likely to occur in the periphery rows and columns.

In general, it is good practice to avoid periphery wells on thermal cyclers. Periphery wells are more likely to perform less consistently.

HYBRIDIZATION INPUT AND TIME RECOMMENDATIONS

This protocol begins after step 3.1 & 3.2 in the PacBio Kinnex Library Prep Protocol "cDNA amplification & clean up".

**For cDNA Kinnex capture panels we recommend an overnight (16 hour) hybridization capture incubation.

xGen Hyb Wash v3 is compatible with 100 ng to 6 µg of total cDNA input per reaction for overnight hybridizations. Depending on application needs, optimum hybridization time can be as short as 1-hour and as long as overnight.

xGen Hyb Wash v3 is compatible with all conditions, including multiplexing, that performed consistently with xGen Hyb Wash v1/v2.

100 ng to 6 µg inputs can be used for short hybridizations, such as 1-hour or 2-hours. For short hybridization times, the lowest (100 ng) and highest (6 µg) total inputs can exhibit higher noise and lower performance than longer hybridization times, especially for very small (< 2 Mb) and very large (>30 Mb) Hyb panels.

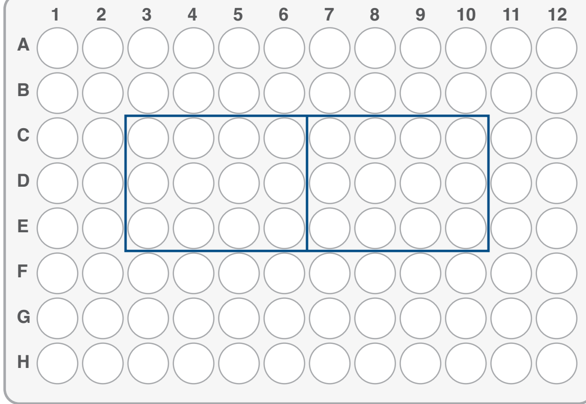
Generally, we recommend not to exceed 2.5 µg total input for 1-hour hybridization time for very large Hyb panels (>30 Mb).

Users are encouraged to explore suitable inputs and hybridization times for specific applications.

PROTOCOL

Prepare cDNA mixture

We recommend avoiding wells on the plate edges and avoiding wells on thermal cycler edges. Mark the wells that contain cDNA before drying down the plate, since they will not be visually distinguishable from empty wells after dry-down.



1. Prepare the Blocker Master Mix in a tube. Multiply by the number of samples and add 10% overfill for each component.

Blocker Master Mix components	Vol per reaction (µL)
Human Cot DNA	5
PacBio Kinnex Blocker 1 (1 µM)	1
PacBio Kinnex Blocker 2 (1 µM)	1
Total	7

2. Vortex to mix well.
3. Add 7 µL of the Blocker Master Mix to each well of a LoBind plate or tube.
4. Add 500 ng of library to each well containing Blocker Master Mix.
   - Do not exceed recommended upper limit per capture reaction described in Hybridization Time and Input Recommendations section of this document
5. Dry down the mixture in a SpeedVac system (or equivalent) according to manufacturer's instructions.

   Optional stopping point: Be sure to seal the sample plate or tube. Store the plate at RT overnight, or −20°C for longer.

6. Proceed to Hybridization.

Hybridization

Inspect xGen Hybridization Buffer. If it does not appear homogeneous or if precipitation is visible, gently warm solution to 45°C and mix until homogeneous. Allow Hybridization Buffer to come back to RT before using.

Note: Hybridization Buffer volume is temperature-sensitive. Vortex and spin down the Hybridization Buffer at room temperature before aliquoting for Master Mix assembly. Draw up and expel Hybridization Buffer very slowly for accurate handling of viscous liquid.

1. Prepare Hybridization Master Mix in a tube. Multiply by the number of samples and add 10% overfill for each component.

Hybridization Master Mix components	Vol per reaction (µL)
xGen 1X Hybridization Buffer	14
Custom Kinnex Hyb Panel	4
Total	18

2. Vortex to mix well.
3. Add 18 µL of the Hybridization Master Mix to each sample well containing dried cDNA and blocker mix.
4. Seal the plate or tube, vortex briefly to mix and spin down.
5. Let the plate or tube sit at RT for 5 min to allow the libraries to resuspend.
6. Incubate the plate or tube in a thermal cycler running the Hybridization program.

Hybridization program		
Block Temperature (°C)	Lid Temperature (°C)	Time
95	105	5 min
70	105	Hold

Samples can be allowed to cool to 23°C (or RT) after specified hyb time. It is not recommended to hold for more than 2 minutes at 23°C (or RT).

Capture bead and buffer preparation

Prepare xGen Hybridization and Wash Beads (capture beads) within 2 hours before use. Allow the capture bead-mix to come to room temperature after removing from 4°C storage. Resuspend the bead-mix by vortex or pipette to complete homogeneity right before aliquoting for bead wash. For individual samples, wash beads in 0.2 mL strip tubes or plate wells. Alternatively, wash beads in bulk in 1.5 mL or 2.0 mL tubes for multiple samples. Draw up and expel the bead mixture very slowly for accurate handling of viscous liquid.

1. Dilute the following buffer to create a 1X working solution as follows: multiply by the required number of samples and add 10% overfill.

Component	Nuclease-Free Water (μL)	Buffer (μL)	Total (μL)
xGen 2X Bead Wash Buffer	159	159	318

2. Add 50 μL of capture bead-mix per sample to a LoBind tube (1.5 mL, 2.0 mL, 0.2 mL strip tube, or plate well).
3. Collect the beads on a magnet and remove the supernatant.
4. Add twice the volume (of bead-mix) of 1X Bead Wash Buffer to the tube. For example, if 500 μL of bead-mix is being washed, use 1 mL of Bead Wash Buffer.
5. Remove from the magnet and briefly vortex the beads to resuspend, then spin down.
6. Collect the beads on a tube magnet and remove the supernatant.
7. Repeat steps 4–6 twice more for a total of 3 washes.
8. Following the last wash, collect the beads on a magnet and remove the supernatant.
9. Remove from magnet and resuspend the beads in 18 μL of 1X Bead Wash Buffer per reaction. Vortex or pipette mix to resuspend. If necessary, quickly spin down without separating beads in solution.

Capture

The samples can be allowed to reach room temperature before addition of the washed beads. Do not hold samples for extended time at room temperature before adding the beads. Before adding the beads to your samples, pipette mix or quickly vortex the washed bead-mix to ensure uniformity.

The ThermoMixer takes time to reach the designated temperature. Turn it on before use and let it come to 23°C or RT before starting capture.

1. Remove samples from thermal cycler and stop Hybridization program.
2. Spin down the plate or tubes briefly to ensure no sample or condensation is left on the seal or tube cap.
3. Ensure washed capture beads are homogenously resuspended immediately before aliquoting.
4. Add 18 μL of resuspended washed capture beads to each sample well at RT and seal plate.
5. Gently vortex the plate or tubes to resuspend the mixture.
6. Mix the plate or tubes on an Eppendorf ThermoMixer C using the Capture program.

Capture program*		
Speed	Temperature	Time
1,200 RPM	23°C	30 min

* Automation mixing method to be determined.

Wash

Inspect the xGen 1X Stringent Wash Buffer and xGen 1X Wash Buffer I. If they do not appear homogeneous or if precipitation is visible, gently warm solution to 45°C and mix until homogeneous. Allow Stringent Wash Buffer and Wash Buffer I to come back to RT before using.

1. After capture, spin down and place the samples on a side pull magnet to collect the beads.
2. Remove and discard the cleared supernatant; make sure not to remove any beads.
3. Use a P20 to remove any residual liquid.
4. Add 50 μL of 1X Stringent Wash Buffer to sample wells and seal plate.
5. Remove from magnet and briefly pulse vortex to completely resuspend the beads, avoiding getting samples on the seal. Quickly spin down the mixture if necessary. If the beads separate or pellet, vortex again and quickly spin down.
6. Place the plate or tube in a thermal cycler and run the Stringent wash program

Stringent Wash		
Temperature (°C)	Lid temperature (°C)	Time
54	105	20 min

After each heated step, samples can be allowed to cool to 23°C or RT. It is not recommended to hold the samples at 23°C or RT for more .than 2 minutes

7. Remove the plate or tubes after Stringent wash incubation.
8. Collect the beads on a magnet. Allow the beads to pellet and solution to clear, and quickly remove the supernatant.
9. Seal the plate or tubes, quickly spin down, and place back onto the magnet.
10. Use a P20 to remove any residual liquid.
11. Remove the samples from the magnet and add 150 μL of 1X Wash Buffer I to sample wells at RT.
12. Pipette mix very slowly—avoid the formation of bubbles—until solution is homogeneous (≥10X) to wash the beads. Seal the plate or tubes and spin down quickly.

    Important: For targeting to work consistently, it is important to spin down after mixing.

13. Collect the beads on a magnet and remove all supernatant.
14. Use a P20 to remove any residual liquid.
15. Remove plate or tubes from magnet and add 150 μL of 1X Wash Buffer II at RT.
16. Pipette mix slowly until solution is homogeneous (≥10X) to wash the beads. Seal the plate or tubes. Handle in a manner to avoid any splashing of the sample. There is no need to spin down unless the sample has splashed on the tube walls or seal.
17. Place the plate or tube in a thermal cycler and run the Wash II program.

Wash II program		
Temperature (°C)	Lid temperature (°C)	Time
60	105	5 min

After each heated step, samples can be allowed to cool to 23°C or RT. It is not recommended to hold the samples at 23°C or RT for more than 2 minutes.

18. Remove samples from the thermal cycler and quickly spin down.
19. Collect the beads on a magnet and remove all supernatant.
20. Use a P20 to remove any residual liquid.
21. Remove from magnet and resuspend beads in 20 μL of nuclease-free water.

Post-capture PCR

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

Amplification Master Mix components	Vol per reaction (µL)
2X KAPA HiFi Hotstart ReadyMix	25
PacBio Kinnex cDNA Amp Primer 1	1
PacBio Kinnex cDNA Amp Primer 2	1
Nuclease-Free Water	3
Total	30

2. Add 30 µL of Amplification Master Mix to each sample for a final reaction volume of 50 µL.

Note: Volume is not additive, so the final reaction volume may not be consistently 50 µL after adding the Amplification Reaction Mix.

3. Securely seal the sample plate or tubes, then gently vortex to thoroughly mix the reaction.
4. Briefly centrifuge the plate or tubes.
5. Place the plate or tubes in a thermal cycler, and run the following program with the lid temperature set to 105°C:

KAPA HiFi PCR Program			
PCR Step	Number of Cycles	Temperature (°C)	Time
Polymerase activation	1	95	3 min
Amplification			
Denaturation	Variable—refer to the panel table below*	98	20 sec
Annealing		60	15 sec
Extension		72	4.5 min
Final extension	1	72	5 min
Hold	1	4	Hold

* The number of PCR cycles should be optimized per panel, input into capture, and hyb time.

We recommend starting optimization with the following:

Panel size	1-plex	4-plex	8-plex	12-plex
>100,000 probes	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
<500 probes	14 cycles	12 cycles	11 cycles	11 cycles

For short hyb (1 hour) with low input (100 ng) on small panels (15,000 probes or less) or multiplex short hybs, it is recommended to increase PCR cycles

Optional stopping point: Store amplified captures per your established internal laboratory procedures.

Post-capture PCR clean up

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

1. Prepare 400 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 or tubes on a magnet until the supernatant is clear (2–5 min).
5. Remove the supernatant without disturbing the beads.
6. While keeping the plate or tubes on the magnet, add 200 µL of 80% ethanol, then incubate for 1 min.
7. Remove the ethanol, then repeat another ethanol wash.
8. After the second ethanol wash, use a P20 to remove any residual ethanol. It is very important to remove all residual ethanol for sample recovery.
9. Allow the beads to air dry for 3 min.

   Important: Do not over-dry the beads.

10. 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.
11. Incubate for 5 min at RT.
12. Place the plate on a magnet until supernatant is clear (1–2 min).
13. 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 Agilent 4200 TapeStation® system using a DNA tape, or other equivalent system).

PacBio Kinnex Library Prep

Perform Kinnex PCR & pooling according to the PacBio protocol on page 13 (step 4).

APPENDIX A

AMPure XP Bead DNA concentration protocol (optional)

1. Add 500 ng of library to the sample well.
   - As low as 100 ng per hyb can be used for some applications.
   - Do not exceed upper limit per capture reaction described in the Hybridization Time and Input section of this document.
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 3 min.

    Important: Do not over-dry.

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

Hybridization Master Mix components*	Vol per reaction (µL)
xGen 1X Hybridization Buffer	14
PacBio Kinnex Blocker 1 (1 µM)	1
PacBio Kinnex Blocker 2 (1 µM)	1
xGen Hyb Panel	4.5
Total	**20.5**

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

12. Remove from magnet and 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 18 µL of the supernatant to the sample plate, or tube, where the hybridization will occur. Make sure to avoid bead carryover during the transfer process.
16. Seal the plate or tube, vortex briefly to mix and spin down.
17. Proceed to **Hybridization step 6**.

DANGER

For hazard statements, refer to section 3 of the SDS.

First aid first aid, refer to section 4 of the SDS.

For PPE recommendations, refer to section 8 of the SDS.

xGen™ HybWash Reagents v3 (EAP) for PacBio Targeted Kinnex

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