High-throughput hybridization capture enrichment of long genomic fragments

For NGS target enrichment

Uses these IDT products*: xGen™ DNA Library Prep MC Kit, xGen Hybridization and Wash v2 Kit (Reagents & Beads), xGen Custom Hyb Panel, Oligos from IDT (Barcoded Adapters & Amplification Primers)

Generates: Enriched and barcoded samples ready for long read sequencing workflow

Overview

Historically, most target enrichment strategies have been limited to short read sequencing. The protocol described here enables targeted enrichment by hybridization capture using a cutting edge long-read sequencing approach. Target enrichment was performed by IDT xGen Custom Hyb Panels (design considerations for long fragments may be necessary—ask your local IDT representative). Enrichment is performed in pools of 4 to 16 samples and the resulting barcoded long fragment DNA pools can then be subject to Third-Generation sequencing workflows (e.g., Pacific Biosciences [PacBio] or Oxford Nanopore). This workflow has been optimized for high-throughput purposes and good per sample economy; it can be conducted in a 96-well plate format and automated.

To assess sample quality, qPCR quantification using xGen Input DNA Quant Primers was used and shown to provide expected results. (Cat. No. 10009856; Contact us for more information about the Input DNA Quantification Assay).

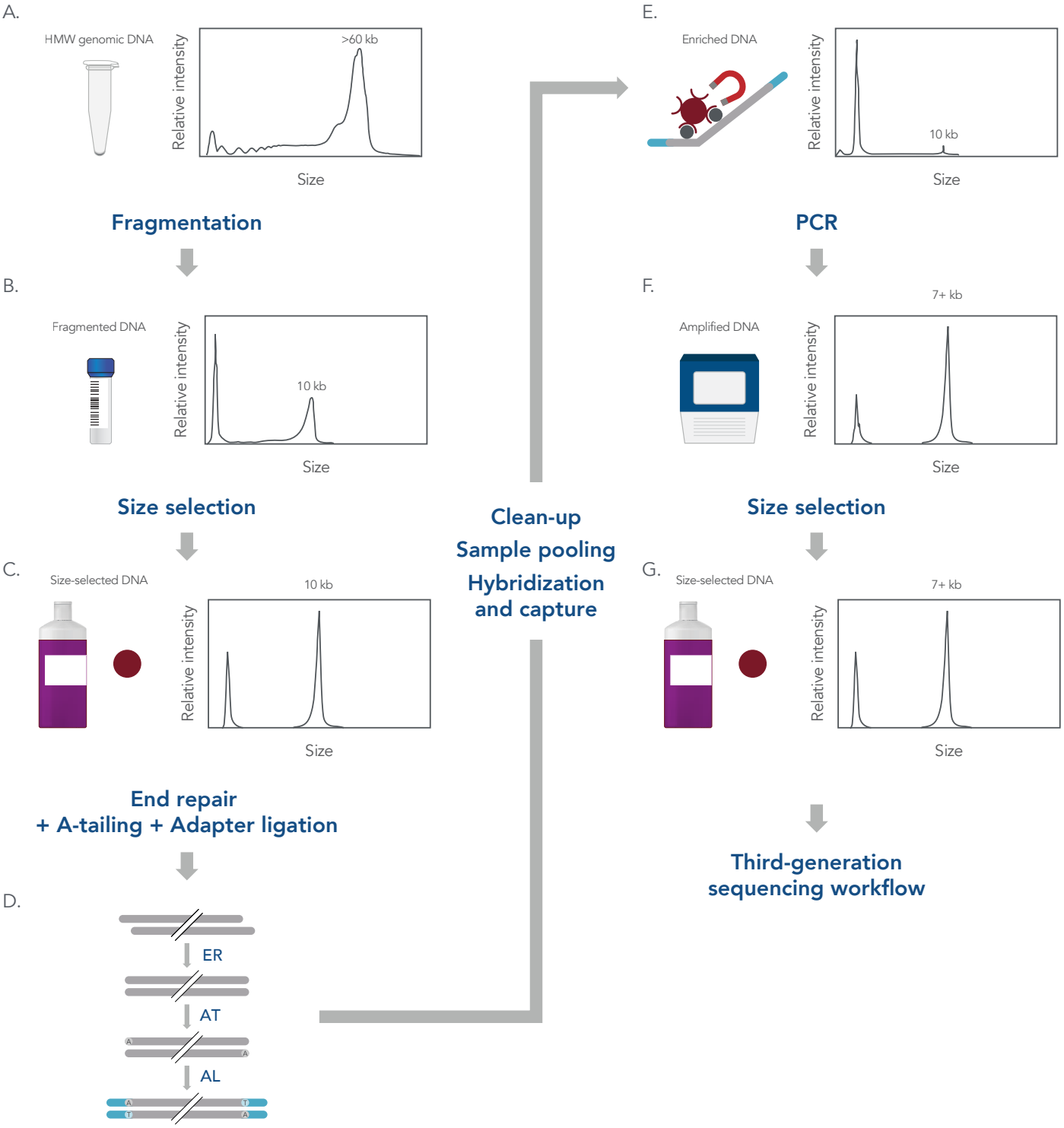
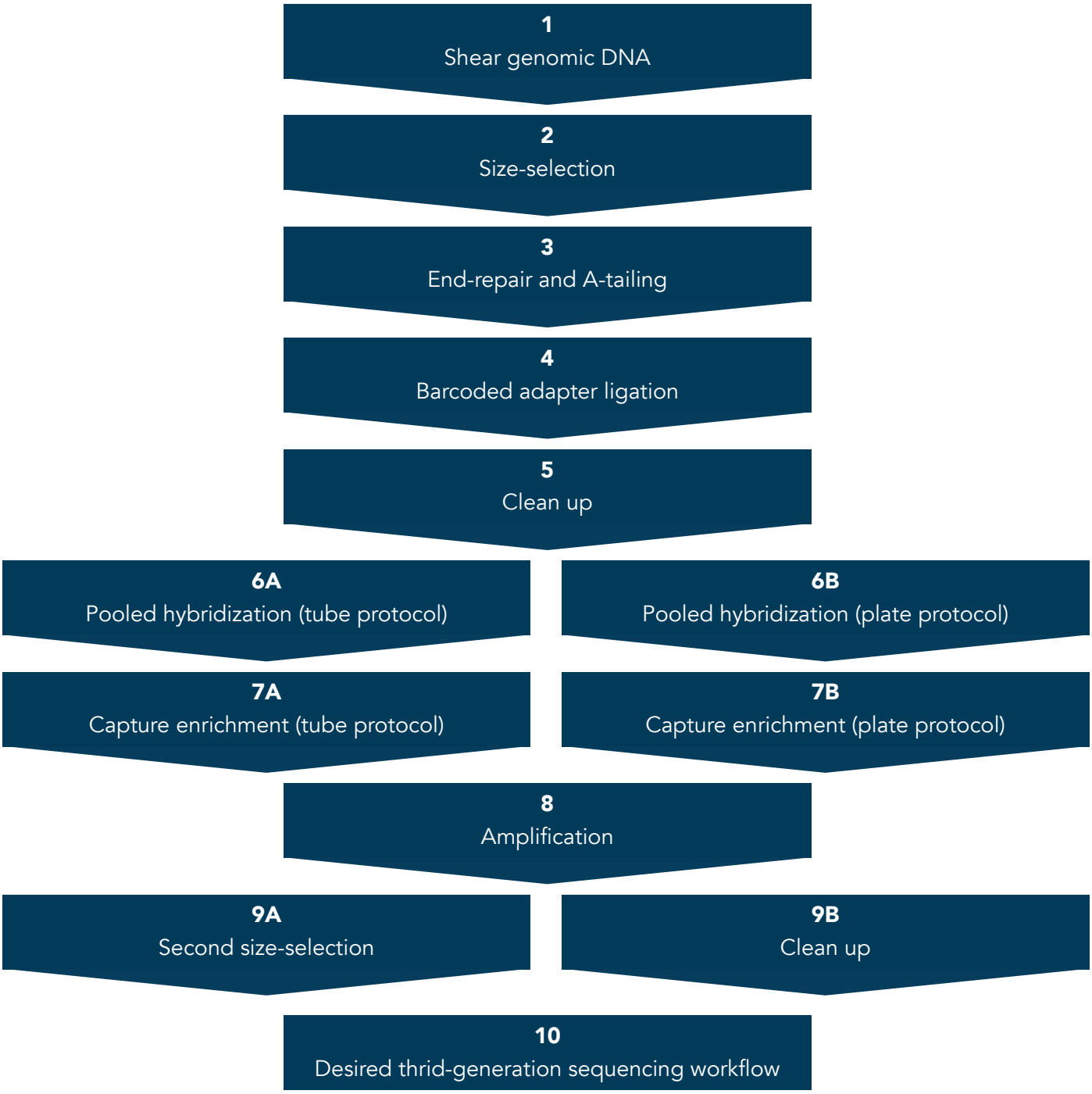


Figure 1. High-throughput hybridization-capture enrichment of long genomic fragments workflow. (**A**) High molecular weight (HMW) genomic DNA is required for the preparation and enrichment of long fragments. (**B**) The HMW DNA is fragmented to ~10 kb using a high-throughput compatible g-TUBE. (**C**) Unwanted smaller fragments are removed by size-selection using specially prepared size-selection beads. (**D**) The size-selected fragments are end-repaired (ER) A-tailed (AT), and adapter ligated (AL) to an adapter sequence, carrying a sample identifying barcode sequence. (**E**) Sample pools are hybridized with the xGen Custom Hyb Panel, captured, and enriched. (**F**) The enriched target fragments get amplified via long-range PCR. (**G**) Amplified enriched fragments are size-selected a second time or cleaned-up for optimal sequencing read length. Then enriched and barcoded sample pools are subjected to the desired Third-Generation Sequencing workflow.

Protocol overview



Consumables and equiment

These kits and consumables are required to perform the long fragment capture.

Consumables—IDT

Kits

Name	Catalog numbers	Comment
xGen DNA Library Prep MC Kit	10009861(16rxn)/ 10009819(96rxn)	One kit reaction per sample
xGen Hybridization and Wash v2 Reagents	10010351(16rxn)/ 10010352(96rxn)	One kit reaction per capture pool
xGen Hybridization and Wash v2 Beads	10010353(16rxn)/ 10010354(96rxn)	One kit reaction per capture pool

Capture panels

Name	Comment
xGen Custom Hyb Panel	Contact IDT experts for design optimization required for long fragment capture. One reaction per capture pool required.

Oligos

The following amplification primers and a variable number of barcoded adapters (depending on the desired sample multiplexing for capture) are required for the preparation. Capital letters represent the barcode sequences used for sample distinction.

BC	Name	Sequence	Synthesis Scale	Purification
01fw	Univ.V3_bc1001_T_overhang	gcagtcgaacatgtagctgactcaggtcacCACATATCAGAGTGCGggtagT	25 nmol	Desalted
01rv	Univ.V3_bc1001_rev_comp	/5phos/ctaccCGCACTCTGATATGTGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
02fw	Univ.V3_bc1002_T_overhang	gcagtcgaacatgtagctgactcaggtcacACACACAGACTGTGAGggtagT	25 nmol	Desalted
02rv	Univ.V3_bc1002_rev_comp	/5phos/ctaccCTCACAGTCTGTGTGTgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
03fw	Univ.V3_bc1003_T_overhang	gcagtcgaacatgtagctgactcaggtcacACACATCTCGTGAGAGggtagT	25 nmol	Desalted
03rv	Univ.V3_bc1003_rev_comp	/5phos/ctaccCTCTCACGAGATGTGTgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
04fw	Univ.V3_bc1004_T_overhang	gcagtcgaacatgtagctgactcaggtcacCACGCACACACGCGCGggtagT	25 nmol	Desalted
04rv	Univ.V3_bc1004_rev_comp	/5phos/ctaccCGCGCGTGTGTGCGTGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
05fw	Univ.V3_bc1005_T_overhang	gcagtcgaacatgtagctgactcaggtcacCACTCGACTCTCGCGTggtagT	25 nmol	Desalted
05rv	Univ.V3_bc1005_rev_comp	/5phos/ctaccACGCGAGAGTCGAGTGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
06fw	Univ.V3_bc1006_T_overhang	gcagtcgaacatgtagctgactcaggtcacCATATATATCAGCTGTggtagT	25 nmol	Desalted

Oligos (continued)

BC	Name	Sequence	Synthesis Scale	Purification
06rv	Univ.V3_bc1006_rev_comp	/5phos/ctaccACAGCTGATATATATGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
07fw	Univ.V3_bc1007_T_overhang	gcagtcgaacatgtagctgactcaggtcacTCTGTATCTCTATGTGggtagT	25 nmol	Desalted
07rv	Univ.V3_bc1007_rev_comp	/5phos/ctaccCACATAGAGATACAGAgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
08fw	Univ.V3_bc1008_T_overhang	gcagtcgaacatgtagctgactcaggtcacACAGTCGAGCGCTGCGggtagT	25 nmol	Desalted
08rv	Univ.V3_bc1008_rev_comp	/5phos/ctaccCGCAGCGCTCGACTGTgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
09fw	Univ.V3_bc1009_T_overhang	gcagtcgaacatgtagctgactcaggtcacACACACGCGAGACAGAggtagT	25 nmol	Desalted
09rv	Univ.V3_bc1009_rev_comp	/5phos/ctaccTCTGTCTCGCGTGTGTgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
10fw	Univ.V3_bc1010_T_overhang	gcagtcgaacatgtagctgactcaggtcacACGCGCTATCTCAGAGggtagT	25 nmol	Desalted
10rv	Univ.V3_bc1010_rev_comp	/5phos/ctaccCTCTGAGATAGCGCGTgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
11fw	Univ.V3_bc1011_T_overhang	gcagtcgaacatgtagctgactcaggtcacCTATACGTATATCTATggtagT	25 nmol	Desalted
11rv	Univ.V3_bc1011_rev_comp	/5phos/ctaccATAGATATACGTATAGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
12fw	Univ.V3_bc1012_T_overhang	gcagtcgaacatgtagctgactcaggtcacACACTAGATCGCGTGTggtagT	25 nmol	Desalted
12rv	Univ.V3_bc1012_rev_comp	/5phos/ctaccACACGCGATCTAGTGTgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
13fw	Univ.V3_bc1013_T_overhang	gcagtcgaacatgtagctgactcaggtcacCTCTCGCATACGCGAGggtagT	25 nmol	Desalted
13rv	Univ.V3_bc1013_rev_comp	/5phos/ctaccCTCGCGTATGCGAGAGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
14fw	Univ.V3_bc1014_T_overhang	gcagtcgaacatgtagctgactcaggtcacCTCACTACGCGCGCGTggtagT	25 nmol	Desalted
14rv	Univ.V3_bc1014_rev_comp	/5phos/ctaccACGCGCGCGTAGTGAGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
15fw	Univ.V3_bc1015_T_overhang	gcagtcgaacatgtagctgactcaggtcacCGCATGACACGTGTGTggtagT	25 nmol	Desalted
15rv	Univ.V3_bc1015_rev_comp	/5phos/ctaccACACACGTGTCATGCGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
16fw	Univ.V3_bc1016_T_overhang	gcagtcgaacatgtagctgactcaggtcacCATAGAGAGATAGTATggtagT	25 nmol	Desalted
16rv	Univ.V3_bc1016_rev_comp	/5phos/ctaccATACTATCTCTCTATGgtgacctgagtcagctacatgttcgactgc	25 nmol	Desalted
—	Amplification primer	/5Phos/gcagtcgaacatgtagctgactcaggtcac	100 nmol DNA Oligo in Tube	LabReady (100 µM)

Consumables—other suppliers

Kits and reagents

Name	Supplier	Catalog number
LA Taq DNA polymerase, hot-start version	Takara	RR042A or RR042B
[Optional for GC-rich targets] LA Taq DNA polymerase with GC buffers	Takara	RR02AG
Lithium Chloride Precipitation Solution, 7.5 M	Various suppliers	Varies
Polyethyleneglycol 8000 (50% w/v)	Various suppliers	Varies
Elution Buffer, Buffer EB (or equivalent: 10 mM Tris-HCl, pH 8.5)	Qiagen, or general laboratory supplier	19086
10X Adapter Buffer (1M NaCl 0.1M Tris, pH 7.5)	Various suppliers	Varies

Other consumables

Name	Supplier	Catalog number
g-TUBE	Covaris	520079 or 520104
2 mL low-bind tubes	Various suppliers	Varies
Low-bind 1.5 mL tubes or 96-deep-well plates (min. 800 µL volume per well)	Various suppliers	Varies
Low-bind 0.2 mL PCR tubes or 96-well plates	Various suppliers	Varies
Low-bind 0.2 mL 8-strip PCR tubes (tube protocol)	Various suppliers	Varies
Microseal® B PCR Plate Sealing Film, adhesive, optical	Bio-Rad	MSB1001
Aerosol resistant tips and pipette ranging from 1–1000 µL	Various suppliers	Varies
200–proof/absolute ethanol	Various suppliers	Varies
Nuclease-free water	Various suppliers	Varies
Fluorometric assays for library quantification (Qubit dsDNA HS/BR or equivalent)	Various suppliers	Varies
TapeStation Genomic DNA ScreenTape assay or equivalent for fragment size determination	Agilent	Varies
Agencourt® AMPure® XP-PCR Purification beads	Beckman Coulter	A63880

Equipment

Name	Supplier	Catalog number
1.5 ml tube and plate magnets for individual tubes and plates	Permagen	MSR812
Fluorometer (Qubit®) or similar input DNA quantification instrument	Various suppliers	MSP750
TapeStation 2200 for fragment size determination	Agilent	Varies
1.5 mL to 200 µL tube adapter (for tube protocol)	Various suppliers	Varies
Microcentrifuge	Various suppliers	Varies
Plate centrifuge	Various suppliers	Varies
Programmable thermocycler (one required for tube protocol, two required for plate protocol)	Various suppliers	Varies
ThermoMixer with 1.5 mL heat block or equivalent	Eppendorf	Varies
Vortex	Various suppliers	Varies
Vacuum concentrator	Various suppliers	Varies
Plate shakers compatible with deep well plates and PCR plates	Various suppliers	Varies

Guidelines

Reagent preparation

Before the enrichment of long genomic fragments can be started, barcoded adapters and size-selection beads need to be prepared (section Reagent Preparation). Even if these reagents have been previously prepared, check whether sufficient quantities are available. Make sure the size-selection beads are not older than three months.

Storage and usage recommendations

Upon receipt, store the reagents according to the respective reagent's/kit's storage recommendation.

Avoid cross-contamination

To reduce the risk of sample cross-contamination, physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed. Follow the instructions below to avoid cross-contamination:

- Clean lab areas using 0.5% sodium hypochlorite (10% bleach).
- Use barrier pipette tips to avoid exposure to potential contaminants.
- Always change tips between each sample.

General handling

Working with high molecular weight DNA requires some handling adaptation. Vigorous pipetting involving small bore pipette tips and extensive vortexing should be avoided wherever possible to preclude from unnecessarily fragmenting the input DNA.

Size-selection beads

The preparation of size-selection beads has been tested with Agencourt AMPure© XP Beads (Beckman Coulter). If different beads are used, solutions and conditions for DNA binding may differ.

Consider the information below in context of the prepared size-selection beads:

- Exact pipetting is crucial for precise size-selection, inaccurate pipetting may lead to suboptimal size-selection and results.
- Size-selection beads shall be stored at +2–8°C. Do not freeze size-selection beads.
- Do not use the size-selection beads for longer than three months after preparation.

Third-Generation sequencing compatibility

This enrichment has been tested with and is compatible to the following Third-Generation Sequencing approaches:

- The low DNA input workflow using the PacBio SMRTbell® Express Template Prep Kit (#102-088-900, enabling HiFi read sequencing). In this workflow, the sample shearing step can be skipped, and the enriched and barcoded fragments can directly be subjected to the "Remove Single-Strand Overhangs" step. We recommend performing the nuclease treatment in the protocol.
- The whole genome workflow of the Oxford Nanopore Ligation Sequencing Kit V14 (#SQK-LSK114, enabling duplex base calling and Q20+ read accuracy).

Input DNA considerations

Input DNA qualification

DNA used for long fragment capture must be of excellent quality and high molecular weight. DNA that is already fragmented cannot be used as input for this protocol. We recommend a thorough initial quality control to determine sample eligibility. Samples with a significant portion (>30%) of fragments smaller than 10,000 bp should not be prepared. Moreover, pooling and balancing of barcoded samples should account for different length differences that result from sample quality. Optimally, samples of similar fragment size are pooled together (if sample barcodes are still unique). If not possible then median fragment length should be used to optimize the equimolar ratio for sample pooling.

The recommended minimum DNA input quantity (1.5 µg) refers to the fragments ≥10,000 bp, for samples with fragments below this threshold, we recommend increasing the total input amount, respectively.

For optimization of sample compatibility, magnetic bead-based extraction protocols such as the Qiagen MagAttract HMW DNA Kit (manual) or the PerkinElmer chemagic DNA Blood 10k Kit (automatable) is recommended.

Input DNA quantification

- Determine dsDNA concentration using Qubit, or a similar fluorometric method for measuring the double-stranded, adaptable DNA content of your sample.
- A range of total DNA input amounts ranging from 1.5–5 µg have provided expected results with this kit. We recommend a total initial input amount of 3 µg per sample.

Concentrating DNA for hyb capture

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

Hybridization

During the overnight (minimum 8 hours) incubation, the sample tubes or plates need to be sealed properly with appropriate caps, 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 (<100 kb target size), longer hybridization times (up to 24 hours) may improve experimental results.

We recommend for moderate number (≤6) of sample pools performing the capture and wash in 1.5 µL low-bind tubes (tube protocol), for higher sample pool numbers low-bind PCR plate (plate protocol).

Sample pooling for hybridization

Sample pooling requires careful consideration: differences in coverage within and sequencing reads among samples should be addressed using a high enough safety margin. The sample multiplexing strategy should therefore be adjusted according to the expected output of the long read sequencing technique, the desired mean coverage, and the target size.

$$\text{Number of samples per pool} = \frac{\text{SMRT or flow cell output [bp]} * 70\% \text{ On-target rate}}{\text{Desired mean target coverage x target size [bp] x (1 + Safety margin)}}$$

For optimal results, 200 ng or greater per sample should be used for pooled hybridization. We recommend the total amount of pooled DNA for hybridization not be <800 ng (or four samples) or >4 µg. In addition to DNA quantity, sample fragment sizes should be considered for equimolar pooling.

Amplification

The enriched DNA fragments are split into two pools for long-range PCR amplification via one of three methods.

1. For target sequences of low to moderate GC-content, we recommend performing two reactions with LA Taq DNA Polymerase Hot-Start Version.
2. For extremely high GC-content targets, we recommend both pools to be PCR reactions using the TaKaRa LA Taq DNA Polymerase with GC Buffer approach.
3. For enrichment of target loci with high GC-content, we recommend performing one of each of the above PCR reactions.

Notes:

- This protocol is readily automatable. Contact us if you require additional reagent overage volume or would like to learn about our custom packaging options.
- While IDT does not supply automated liquid handling instruments or consumables, our automation team collaborates with automation solution providers and customers to develop automated scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Contact us for more information.

Reagent preparation

Anneal Barcoded Adapters

Sequences for barcoded adapters can be found above in the **Consumables and equipment** section. For each barcoded adapter (1–16) used for pooling the annealing reaction must be conducted in a separate tube. The resulting volume (22 µL barcoded adapter) is sufficient for 4 reactions. In the case that larger amounts of barcoded adapters are required the reaction can be scaled up accordingly. For optimal use, prepare the required number of barcoded adapters in low-bind 8-Strip PCR tubes or a PCR plate such that the adapters can easily be transferred to the samples using a multichannel pipette.

1. Single-stranded linear barcoded adapters must be annealed to a final concentration of 10 µM prior to ligation.
2. Dilute the barcoded adapters to 100 µM in water.
3. Prepare the following reactions:

Component	Stock Conc.	Volume (µL)
10X Adapter buffer (1M NaCl 0.1M Tris, pH 7.5)	10x	2.2
Barcoded adapter (forward – T_overhang)	100 µM	2.2
Barcoded adapter (reverse – complement)	100 µM	2.2
Nuclease-free water		15.4
Total:		**22**

4. Incubate in a thermocycler with the following thermal profile:

Step	Temperature (°C)	Time
1	80	2 minutes
2	Ramp from 80 to 25	Ramp at Δ0.1°C per second
3	25	1 second
4	4	Hold

5. Place on ice until ready to use. Store annealed linear barcoded adapters at –20°C for long term storage. Prior to use, thaw at room temperature and mix by vortexing.

Preparation of size-selection beads

Size-selection beads are prepared using Agencourt AMPure XP Beads (Beckman Coulter). Note that a protocol specific buffer swap is required. This custom preparation results in a total of 10 mL of size-selection beads, which is sufficient for preparing approximately 50 samples (126 µL required per sample and 245 µL per enrichment pool +10% overage). If larger amounts of size-selection beads are required, the reaction can be scaled up accordingly.

Size-selection beads are stable at +2–8°C for approximately 3 months.

1. Add 2000 µL of Ampure XP beads into each of three 2 mL tubes (2000 uL into each tube for a total of 6000 µL).
2. Quickly spin down the solution in the tubes by balancing the tubes against each other.
3. Place the tubes on the magnetic stand until the solution is clear.
4. In the meantime, prepare 10 mL of the size-selection bead buffer in a new 15 mL tube as follows:
   a. Pipette 4.9 mL nuclease-free water into the 15 mL tube.
   b. Add 100 µL 1M Tris-HCl, pH 8.5.
   c. Add 1 mL of 7.5M LiCl.
   d. Add 4 mL of 50% PEG 8000.
   e. Vortex for 30 sec.
5. When the solution on the magnetic stand (step 3) is clear completely remove and discard the supernatant.
6. Add 1 mL of nuclease-free water to each tube.
7. Vortex until all beads are in solution. Quickly spin down the tubes.
8. Put back on the magnetic stand. Remove the supernatant once the solution is clear.
9. Repeat wash (steps 5–8) with nuclease-free water for a total of three washes. After the supernatant has been removed, proceed to step 10 immediately and do not let the beads dry.
10. Add 1 mL size-selection bead buffer (step 4) to the washed beads in all three tubes.
11. Remove the tube from the magnetic stand and resuspend the beads by alternating pipette mixing and vortexing until fully homogenized.
12. Transfer the size-selection buffer solution containing the beads to the remaining bead buffer in the 15 mL tube and vortex until all beads are homogeneously dissolved.

Protocol

Shear genomic DNA

In this section, you will need the following:

- Covaris g-TUBEs
- Nuclease-free water

Important: Depending on the individual microcentrifuge used the acceleration characteristics might differ which may require adjustments to the centrifugation speed. This protocol was tested using the Eppendorf Centrifuge 5424. Before shearing large sample batches with a different centrifuge, testing is recommended to assess whether the specified centrifugation speed results in the desired 9–11 kb fragment peaks.

1. Dilute 1.5–5 µg of HMW genomic DNA (gDNA) to 155 µL total volume in nuclease-free water in a Covaris g-TUBE. Do not use buffer for dilution.
2. Firmly close the g-TUBE's cap.
3. Briefly mix by inverting the g-TUBE several times.
4. Centrifuge the g-TUBEs at 1700 rcf for 5 minutes.
5. After centrifugation, check whether liquid is left g-TUBE's upper chambers. If this is the case, without turning the g-TUBE, repeat centrifugation at 1700 rcf for one additional minute and repeat this step.
6. Turn around the g-TUBE and centrifuge a second time at 1700 rcf for 5 minutes (total of two centrifugation steps at 1700 rcf for 5 minutes).
7. After centrifugation, check whether liquid is left in the g-TUBE's upper chambers. If this is the case, without turning the g-TUBE, repeat centrifugation at 1700 rcf for one additional minute and repeat this step.
8. Turn around the g-TUBE and centrifuge at 3400 rcf for 60 seconds.
9. After centrifugation, check whether liquid is left g-TUBE's upper chambers. If this is the case, without turning the g-TUBE, repeat centrifugation at 3400 rcf for an additional 30 seconds and repeat this step.
10. Turn around the g-TUBE and repeat steps 8 and 9 until the sample has been centrifuged for a total of 4 times at 3400 rcf for one minute. Always check that all liquid has left the upper chamber.
11. Recover 144 µL sample from the g-TUBE's cap and transfer to a low-bind 1.5 mL tube or a well of a deep well plate. The exactness of the recovery volume is crucial for the following steps.

    Optional stopping point: The sheared DNA can be stored for up to 24 hours at 4°C or at –20°C for two weeks.

Size-selection

In this section you need the size-selection beads and freshly prepared 80% ethanol. Refer to section **Reagent preparation** and subsection **Preparation of size-selection beads** for preparation.

In this section you will need:

a. Size-selection beads
b. Freshly prepared 80% (v/v) ethanol
c. Elution Buffer (Tris-HCL Buffer 1 M, pH 8.5)
d. Reagents for Agilent TapeStation gDNA or equivalent to determine fragment size distribution in the range of 1–12 kb.

Important:

- Make sure that size-selection beads and Elution Buffer have been equilibrated to room temperature before proceeding. Directly before using, mix the size-selection beads well by vortexing until the solution appears homogenous. All size-selection steps should be performed at room temperature.
- Exact pipetting is crucial for precise size selection, inaccurate pipetting may lead to suboptimal size selection and results.

1. Add 126 µL of size-selection beads (0.875X volume ratio) to the sheared fragments from the previous step (exactly 144 µL). Precise pipetting volumes are critical for successful size selection.

   Tube: mix bead/DNA solution by carefully pipetting up and down until solution is homogenous.

   Plate: shake the plate at 1400 for 2 minutes.

2. Allow the DNA to bind to the size-selection beads for 15 minutes at room temperature. By gently shaking or mixing, make sure that the beads stay dissolved homogeneously throughout the incubation time.
3. If necessary, spin down the tubes or plate to collect the beads.
4. Place the tubes or plate in a magnetic bead rack until the beads are collected to the side of the tubes or plate wells and the solution appears clear. Then remove and discard supernatant.
5. Wash beads with freshly prepared 80% ethanol:
   a. Do not remove the tubes or plate from the magnetic rack.
   b. While on the magnetic rack, slowly dispense 1 mL (tube) or 700 µL (deep well plate) of 80% ethanol against tube/well wall opposite of the beads. **Do not** resuspend the beads.
   c. After 30 seconds, remove and discard the 80% ethanol.
6. Repeat **step 5** once.
7. Spin down the tubes/plate to collect the remaining liquid.
8. Check for any remaining droplets in the tubes/wells. If droplets are present, repeat **step 7**.
9. Put the tubes/plate back on the magnetic rack. Pipette off any remaining liquid.
10. Allow beads to air-dry for approximately 60–120 seconds.
11. Add 52 µL Elution Buffer to the tubes/plate wells containing the beads.

    Tube: pipette up and down slowly to mix until beads are uniformly resuspended.

    Plate: shake the plate at 1400 for 2 minutes. Check if all beads are homogenously resuspended. If not, repeat this step. Spin down the plate to collect the beads.

12. Elute the DNA by letting the mix stand at room temperature for 5 minutes. Alternatively, the yield can be increased by incubating at 37°C for 5 minutes.
13. Place the tubes/plate back on the magnetic bead rack. Wait for approximately 2 minutes until the solution appears clear.
14. Transfer 50 µL of the supernatant containing the size-selected fragments to a new tube or to a new well of a 200 µL PCR plate.
15. Discard the beads.
16. Optional: Verify your DNA amount and fragment size using a TapeStation (gDNA Screen Tape) or equivalent.

Optional stopping point: The size selected DNA can be stored for up to 24 hours at 4°C or at –20°C for two weeks. However, it is recommended to proceed directly to the End-repair and A-tailing section.

End-repair and A-tailing

In this section, you will need the following:

- xGen DNA Library Prep MC Kit

Note: Thaw enzyme reagents on ice for at least 10 minutes prior to use and pipette to mix, non-enzyme reagents can be thawed at room temperature and mixed by vortexing.

Thermocycler conditions for ERAT program:

Lid kept at 70°C/ Reaction volume 60 µL:

- Hold @ 4°C
- 30 minutes @ 20°C
- 30 minutes @ 65°C
- Hold @ 4°C

1. Set up a thermocycler and start the ERAT program with the heated lid set to 70°C (a heated lid is required).
2. Prepare the End-Repair Master Mix on ice:

Component	Volume for 1 reaction (µL)	Volume for ___ reactions (+overage)
Low EDTA TE	3	
• Buffer E1	4.7	
• Enzyme E2	1	
• Enzyme E3	1	
• Enzyme E4	0.3	
End-Repair Master Mix:	**10**	

Component	Volume for 1 reaction (µL)
End-Repair Master Mix:	10
Size-selected DNA:	50
Total reaction volume:	**60**

3. Thoroughly mix the End-Repair Master Mix by moderate vortexing for 5 seconds.
4. Add 10 µL of the premixed End-Repair Master Mix to the 50 µL size-selected DNA.

   Tube: mix by slowly pipetting up and down.

   Plate: mix by shaking at 1400 for 2 minutes, then spin down briefly.
5. Incubate the reaction in the preset thermocycler and advance the ERAT program to the 30 minutes @ 20°C step.
6. Before completion of the thermocycler program, prepare the master mix for the barcoded adapter ligation step (following section). The samples should be kept at 4°C for no more than one hour.

   Important: Proceed to the adapter ligation step within one hour to avoid a loss in yield.

Barcoded adapter ligation

In this section you will need the following:

- xGen DNA Library Prep MC Kit
- Annealed Barcoded Adapters (10 µM)

Refer to section **Reagent preparation** and subsection **Anneal Barcoded Adapters** for preparation. Make sure that Barcoded Adapters used are unique for all samples pooled in the subsequent hybridization step. Non-unique barcodes will cause inability to differentiate between all samples with the same barcode.

Thermocycler conditions for ADALIG program:

Lid heating off / Reaction volume 90 µL:

- Hold @ 20°C
- 15 minutes @ 20°C
- Hold @ 4°C

1. Set up a thermocycler and start the ADALIG program with the heated lid turned off.
2. Prepare the Barcoded Adapter Ligation Master Mix on ice:

Component	Volume for 1 reaction (µL)	Volume for ___ reactions (+overage)
• Buffer L1	18	
• Enzyme L2	6	
Barcoded Adapter Ligation Master Mix:	**24**	

Component	Volume for 1 reaction (µL)
Barcoded Adapter Ligation Master Mix:	24
End-repaired DNA:	60
Barcoded adapter (Unique barcode per sample pool!):	5
Total reaction volume:	**89**

3. Thoroughly mix the Barcoded Adapter Ligation Master Mix by moderate vortexing for 5 seconds.

   Important: Make sure that Barcoded Adapters used are unique for all samples that will be pooled together in the subsequent hybridization step.

   Tip: To increase throughput Barcoded Adapters can be transferred using a multichannel pipette.

4. Add 5 µL of the annealed Barcoded Adapter to the end-repaired DNA.
5. Note down the Barcoded Adapter that was added to each sample.
6. Using a 100 µL pipette, add 24 µL of the premixed Barcoded Adapter Ligation Master Mix to the end-repaired DNA.

   Tube: after adding the Barcoded Adapter Ligation Master Mix change the pipette volume to 80 µL and mix by carefully pipetting up and down until homogeneous.

   Plate: after adding the Barcoded Adapter Ligation Master Mix change the multichannel pipette volume to 80 µL and mix by carefully pipetting up and down until homogeneous.

7. Incubate the reaction in a thermocycler with the heated lid turned OFF and advance the ADALIG program to the 15 minutes @ 20°C step.
8. Proceed immediately to the Clean-up. The samples should be kept at 4°C for no more than one hour.

   Important: Immediately proceed to the Clean-up step.

Clean-up

In this section you will need the following:

- Agencourt AMPure XP beads, or equivalent
- Freshly prepared 80% (v/v) ethanol
- Elution Buffer (Tris-HCL Buffer 1 M, pH 8.5)

Important: Make sure the beads and the Elution Buffer are at room temperature before you begin this procedure.

Note: This clean-up is conducted using the original Agencourt AMPure XP beads, do not use the size-selection beads.

1. Add 45 µL volume of AMPure XP beads (0.5X) to the adapter-ligated DNA.

   Tube: mix bead/DNA solution by carefully pipetting up and down until solution is homogenous.

   Plate: shake the plate at 1400 for 2 minutes.

2. Allow the DNA to bind to the AMPure XP beads for 7 minutes at room temperature. By gently shaking or mixing, make sure that the beads stay dissolved homogeneously throughout the incubation time.
3. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
4. Remove and discard the supernatant, using a clean pipette tip, without disturbing the pellet.

5. Wash beads with freshly prepared 80% ethanol.
   a. Do not remove the tubes or plate from the magnetic rack.
   b. While on the magnetic rack, slowly dispense 1 mL (tube) or 180 µL (PCR plate) of 80% ethanol against tube/well wall opposite to the beads. Do not resuspend the beads.
   c. After 30 seconds, remove and discard the 80% ethanol.
6. Repeat step 5 once.
7. Pulse-spin the samples, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
8. Check for any remaining droplets in the tubes/wells. If droplets are present, repeat step 7.
9. Allow beads to air-dry for approximately 60–120 seconds. Do not over-dry the beads.
10. Add 22 µL Elution Buffer to the tubes/plate wells containing the beads.
11. Pipette carefully up and down to mix until the beads are homogeneously resuspended.
12. Elute the DNA by incubating at room temperature for 5 minutes. Alternatively, the yield can be increased by incubating at 37°C for 5 minutes.
13. Spin the tubes/plate down to collect the beads, then place the tubes/plate back on the magnetic bead rack. Wait for approximately 2 minutes until the solution appears clear.
14. Transfer 20 µL of the supernatant containing the cleaned-up DNA to a new tube or to a new well of a 200 µL PCR plate.
15. Discard the beads.
16. Verify your DNA concentration and amount using a Qubit quantification assay. Alternatively other fluorometric quantifications assays in a 96-well format can be used.

Optional stopping point: The cleaned-up and adapter-ligated DNA can be stored for up to 24 hours at 4°C or at –20°C for two weeks.

Pooled hybridization (tube protocol)

The tube protocol is recommended for 6 or less hybridization pools. For a higher number of sample pools, please jump to section Pooled Hybridization (Plate Protocol).

In this section, you will need the following:

- IDT xGen Hybridization and Wash v2 Reagents
- IDT xGen Custom Hyb Panel
- Amplification primer (IDT custom oligo, 100 µM)

Note: Thaw all required reagents of the xGen Hybridization and Wash v2 Reagents, the xGen Custom Hyb Panel, and the Amplification Primer at room temperature (15–25°C). Mix thoroughly and centrifuge briefly.

Thermocycler conditions for HYB program:

Lid kept at 100°C / Reaction volume 30 µL:

- 7 minutes @ 95°C
- Hold @ 65°C

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.

1. Multiplex up to sixteen samples (with unique barcoded adapters) with 200 ng or greater each to a total sample DNA mass of 0.8–4 µg per sample pool in a new 200 µL low-bind PCR tube (of an 8-strip). Please further consider the Sample pooling for hybridization subsection in the Guidelines section of this protocol to optimize your sample multiplexing strategy.
2. Add 5 µL human COT DNA (1 mg/mL, contained in IDT xGen Hybridization and Wash v2 Reagents) each to the PCR tubes containing the sample pools.
3. Add 8 µL of 100 µM Amplification Primer (IDT custom oligo) each to the PCR tubes containing the sample pools and Human COT DNA.
4. Dry the sample pools in a vacuum concentrator (Speed Vac). Do not apply heat. For drying down in a 200 µL PCR tube format, use a 1.5 mL to 200 µL tube adapter.

   Optional stopping point: Be sure to seal the sample pool tubes. Store the samples at RT overnight, or –20°C for longer.

5. Create the Hybridization Master Mix in a 1.5 mL tube (buffer and enhancer contained in IDT xGen Hybridization and Wash v2 Reagents box):

Component	Stock Conc.	Volume for 1 reaction (µL)	Volume for ___ reactions (+overage)
Nuclease-free water		3	
xGen 2X Hybridization Buffer	2x	11	
xGen Hybridization Buffer Enhancer		4	
Total		18	

6. Vortex or pipette the Hybridization Master Mix to mix well.
7. Add 18 µL of the Hybridization Master Mix to each tube containing the dried sample pools.
8. Close the tubes and incubate at room temperature for 5–10 minutes.
9. After incubation, carefully pipette at least 10 times to mix.
10. Briefly centrifuge the samples.
11. Place the sample tube in the thermocycler and start the HYB program.
12. After the 95°C step of the program is completed, add 4 µL of the IDT xGen Custom Hyb Panel to each sample pool.
13. Immediately after adding the IDT xGen Custom Hyb Panel, with a pipette set to 18 µL, carefully pipette up and down 10 times. Try not to introduce air bubbles.
14. Incubate the tube in a thermocycler at 65°C overnight but for at least 8 hours. Incubation for up to 24 h is possible. Longer incubation times are especially recommended for GC-rich targets. The thermocycler's heated lid should be turned on and set to maintain 100°C.

Important: During the incubation, the sample tubes need to be sealed properly to avoid evaporation. Excessive evaporation during hybridization can lead to capture failure.

Capture Enrichment (Tube Protocol)

The tube protocol is recommended for 6 or less hybridization pools. For a higher number of sample pools please jump to section Capture Enrichment (Plate Protocol).

In this section, you will need the following:

- IDT xGen Hybridization and Wash v2 Reagents
- IDT xGen Hybridization and Wash v2 Beads

Important: Before performing the Capture Enrichment (Tube Protocol), for a new Eppendorf ThermoMixer or equivalent, using a calibrated thermometer, validate that a 1.5 mL test tube incubated at 65°C maintains the temperature. If a discrepancy between the temperature set and the temperature measured is observed adjust the setting accordingly.

Notes:

- Before preparing the buffers, take out the xGen Hybridization and Wash v2 Beads, 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 minutes before performing the washes. **Do not** use beads other than Dynabeads M270.
- When disposing the supernatants from the heated wash steps containing high concentration of hybridization buffer and enhancer, use appropriate disposal methods.
- If Wash Buffer 1 is cloudy, heat the bottle in a 65°C water bath to allow resuspension.

Prepare buffers

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

Component	Nuclease-free water (µL)	Buffer volume (µL)	Total volume (µL)	Water for ___ pools	Buffer for ___ pools
2x Bead Wash Buffer	160	160	320		
10x Wash Buffer 1	252	28	280		
10x Wash Buffer 2	144	16	160		
10x Wash Buffer 3	144	16	160		
10x Stringent Wash Buffer	288	32	320		

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

2. Preheat the following wash buffers to 65°C in an Eppendorf ThermoMixer. All other buffers should be kept at room temperature:
   a. Aliquot 110 µL of the 1X Wash Buffer 1 into a separate tube and heat to 65°C in an Eppendorf ThermoMixer. The remaining volume of Wash Buffer 1 should be kept at room temperature.
   b. Aliquot 1X Stringent Wash Buffer into two tubes (160 µL each). Heat both tubes to 65°C in an Eppendorf ThermoMixer.
   c. Incubate the heated buffers for at least 15 minutes at 65°C before performing the heated washes.

Tip: The heated fraction of 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.

3. Prepare the following Bead Resuspension Mix in a 1.5 mL tube and heat to 65°C in an Eppendorf ThermoMixer:

Component	Stock Conc.	Volume (µL)	Volume for ___ pools
Nuclease-Free Water		6.5	
xGen 2X Hybridization Buffer	2x	8.5	
xGen Hybridization Buffer Enhancer		2	
Total		17	

Prepare capture beads

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

1. Mix the beads thoroughly by vortexing for 15 sec.
2. Aliquot 60 µL of streptavidin beads per capture into a single 1.5 mL low-bind tube. For example, for 1 capture, prepare 60 µL of beads and for 2 captures, prepare 120 µL of beads. Enough beads for up to six captures can be prepared in a single tube.
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 minute).
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 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 17 µL of Bead Resuspension Mix per capture from Prepare buffers, step 3.
10. Mix thoroughly so that the beads are not left to dry in the tube. If needed, briefly centrifuge the tube at 25 x g.
11. Split 17 µL portions of the homogeneous Bead Resuspension Mix per capture to new 1.5 mL low-bind tubes.
12. Incubate the 1.5 mL low-bind tubes containing the Bead Resuspension Mix at 65°C for 3 minutes.

Capture of hybridized probes

Important:

- It is critical that the buffers have reached 65°C in a water bath before starting the heated washes.
- A quick transfer of the hybridized pool (at 65°C) into the 1.5 mL tube containing the capture beads is critical for the capture efficiency. Try to avoid any delay while transferring and make sure a temperature of 65°C for the hybridized pool and the 1.5 mL tube containing the beads is constantly maintained until the room temperature wash steps are reached.

1. After at least 8 hours hybridization, while keeping the PCR tubes incubated at 65°C in the thermocycler, quickly transfer the hybridized pool with a pipette set to 24 µL to the heated 1.5 mL tube containing the capture beads. Do not pipette to mix. After transferring immediately return the 1.5 mL tube to the Eppendorf ThermoMixer at 65°C.
2. When all hybridization pools are transferred, mix by shaking on the Eppendorf ThermoMixer at 1200 rpm for 1 minute.
3. Incubate in the Eppendorf ThermoMixer at 65°C for 45 minutes using the interval mix function mix by shaking every 10 minutes at 1200 rpm for 1 minute.

Heated washes

Important: It is critical ensure that buffers are 65°C in the Eppendorf ThermoMixer before starting the heated washes.

Tip: For up to 6 sample pools, we recommend performing the washes in parallel: during the incubation time of the first pool the next pool can be washed, etc. When the last sample pool is put to incubation, the next round of washing for the first pool can be performed.

1. Turn off the interval mix function, but leave the temperature at 65°C.
2. Leaving the sample pools incubated at 65°C, add 100 µL of heated Wash Buffer 1 to each sample pool.
3. Mix the samples with heated Wash Buffer 1 by tapping the tube until homogeneous. Quickly spin down if necessary.
4. Perform wash with heated 1X Stringent Wash Buffer:
   a. Place the tube on a magnetic rack. As soon as the solution appears clear, remove the supernatant.
   b. Immediately, remove the tube from the magnet and add 150 µL of heated Stringent Wash Buffer to the sample.
   c. Mix the sample by tapping the tube until homogeneous. Quickly spin down if necessary.
   d. Incubate in the Eppendorf ThermoMix at 65°C for 5 minutes.
5. Repeat steps 4 a to d for a total of two washes with heated Stringent Wash Buffer.

Room temperature washes

1. Place the tube on a magnet for 1 minutes.
2. Remove and discard supernatant. Add 150 µL of Wash Buffer 1 equilibrated to room temperature.
3. Mix the sample by tapping the tube until homogeneous. Briefly centrifuge the tube.
4. Carefully transfer the sample pool to a new 1.5 mL tube using a 1 mL pipette tip. This step helps to reduce the off-target reads.
5. Incubate for 2 minutes. By tapping the tube to mix, make sure that the beads stay dissolved homogeneously throughout the incubation time.
6. At the end of the incubation, briefly centrifuge the tube.
7. Place on the magnet for 1 minute.
8. Remove the supernatant. Add 150 µL of Wash Buffer 2.
9. Mix the sample by tapping the tube until homogeneous.
10. Incubate for 2 minutes. By tapping the tube to mix, make sure that the beads stay dissolved homogeneously throughout the incubation time.
11. At the end of the incubation, briefly centrifuge the tube.
12. Place on the magnet for 1 minute.
13. Remove the supernatant. Add 150 µL of Wash Buffer 3.
14. Mix the sample by tapping the tube until homogeneous.
15. Incubate for 2 minutes. By tapping the tube to mix, make sure that the beads stay dissolved homogeneously throughout the incubation time.
16. At the end of the incubation, briefly centrifuge the tube.
17. Place on the magnet for 1 minute.
18. Remove and discard the supernatant.
19. Briefly centrifuge the tube.
20. Place on the magnet and use a fresh pipette tip to remove residual Wash Buffer 3 from the tube, then remove the tube from the magnet. Immediately go to the next step, **do not** let the beads dry.
21. Add 50 µL of nuclease-free water to each sample pool.
22. Carefully pipette mix 10 times to resuspend any beads stuck to the side of the tube.
23. Put the beads onto ice until used in the amplification step.

Important: Do not discard the beads. Use the entire 50 µL including the resuspended beads with captured DNA in the amplification reaction.

Proceed with section 8. Amplification.

Pooled Hybridization (Plate Protocol)

The plate protocol is recommended for more than 6 sample pools. For a smaller number of sample pools please jump to section Pooled Hybridization (Tube Protocol).

The plate protocol has been developed for a maximum of 4 columns of samples in standard 96-well plate format (32 reactions at a time). We do not recommend running more than 32 samples at a time because the timing and temperature of washes may be impacted.

In this section, you will need the following:

- IDT xGen Hybridization and Wash v2 Reagents
- IDT xGen Custom Hyb Panel
- Amplification primer (IDT custom oligo, 100 µM)

Note: Thaw all required reagents of the xGen Hybridization and Wash v2 Reagents, the xGen Custom Hyb Panel, and the Amplification Primer at room temperature (15–25°C). Mix thoroughly and centrifuge briefly.

Thermocycler conditions for HYB program:

Lid kept at 100°C / Reaction volume 25 µL:

- 7 minutes @ 95°C
- Hold @ 65°C

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.

1. Prepare the Blocker Master Mix in a tube.

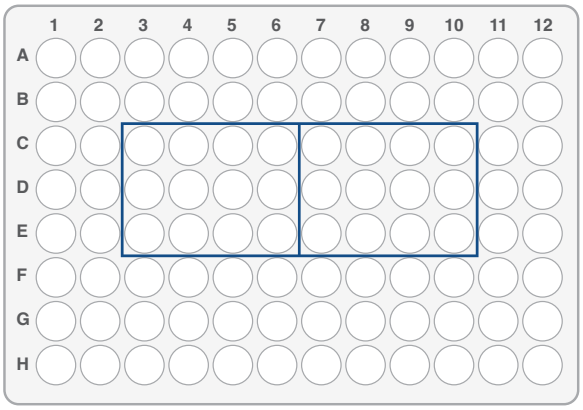
Component	Volume for 1 reaction (µL)	Volume for ___ pools
Human COT DNA (1 mg/mL, contained in IDT xGen Hybridization and Wash Kit v2 Reagents box)	5	
100 µM Amplification Primer (IDT custom oligo)	8	
Total	13	

2. Vortex to mix well.

3. Multiplex up to sixteen samples (with unique barcoded adapters) with 200 ng or greater each to a total sample DNA mass of 0.8–4 µg per pool in a new low-bind PCR-plate well. Please further consider the Sample pooling for hybridization sections in the Guidelines section of this protocol to optimize your sample multiplexing strategy.

   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.



4. Add 13 µL of the Blocker Master Mix to the low-bind PCR tube containing the pooled samples.
5. Dry the pools in a vacuum concentrator (Speed Vac). Do not apply heat.

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

6. Create the Hybridization Master Mix in a 1.5 mL tube (buffer and enhancer contained in IDT xGen Hybridization and Wash v2 Reagents box):

Component	Stock Conc.	Volume (µL)	Volume for ___ pools
Nuclease-Free Water		3	
xGen 2X Hybridization Buffer	2x	11	
xGen Hybridization Buffer Enhancer		4	
Total		18	

7. Vortex or pipette mix the Hybridization Master Mix to mix well.
8. Add 18 µL of the Hybridization Master Mix to each well of the plate containing dried DNA.
9. Securely seal the plate with a Microseal B seal.
10. Incubate at room temperature for 5–10 minutes.
11. Carefully pipette up and down to mix after incubation at least 10 times.
12. Briefly centrifuge the samples.

    Important: for later steps it is important that the thermocycler used for hybridization is easily accessible and not located on a narrow shelf. It is best to move the thermocycler to a freely accessible work bench prior to hybridization.

13. Place the sample plate in the thermocycler and start the HYB program.
14. After the 95°C step of the program is completed, add 4 µL of the IDT xGen Custom Hyb Panel to each plate well containing sample pools using a multichannel pipette.
15. Immediately after adding the IDT xGen Custom Hyb Panel, with another multichannel pipette set to 20 µL carefully pipette up and down to mix at least 10 times. Try not to introduce air bubbles.
16. Incubate the tube in a thermocycler at 65°C overnight but for at least 8 hours. Incubation for up to 24 h is possible. Longer incubation times are especially recommended for GC-rich targets. The thermocycler's heated lid should be turned on and set to maintain 100°C.

Important: During the incubation, the sample tubes need to be sealed properly to avoid evaporation. Excessive evaporation during hybridization can lead to capture failure.

Capture Enrichment (Plate Protocol)

The plate protocol is recommended for more than 6 sample pools. For a smaller number of sample pools please jump to section Capture Enrichment (Tube Protocol).

The plate protocol has been developed for a maximum of 4 columns of samples in standard 96-well plate format (32 reactions at a time). We do not recommend running more than 32 samples at a time because the timing and temperature of washes may be impacted.

In this section, you will need the following:

- IDT xGen Hybridization and Wash v2 Reagents
- IDT xGen Hybridization and Wash v2 Beads

Thermocycler conditions for WASH program:

- Lid kept at 70°C*
- Hold @ 65°C

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

Important:

- For performing the Capture enrichment (plate protocol) a second thermocycler running the WASH program is required. Before you start, make sure this thermocycler is easily accessible and not located on a narrow shelf.
- When mixing the sample pools by pipetting, it is important to mix gently and slowly. Do not use pipette tips smaller than 100 µL to mix or transfer the samples, as their small bores would introduce shear forces to the sample DNA.

Notes:

- Before preparing the buffers, take out the xGen Hybridization and Wash v2 Beads, 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 minutes before performing the washes. **Do not** use beads other than Dynabeads M270.
- When disposing the supernatants from the heated wash steps containing high concentration of hybridization buffer and enhancer, use appropriate disposal methods.
- If Wash Buffer 1 is cloudy, heat the bottle in a 65°C water bath to allow resuspension.

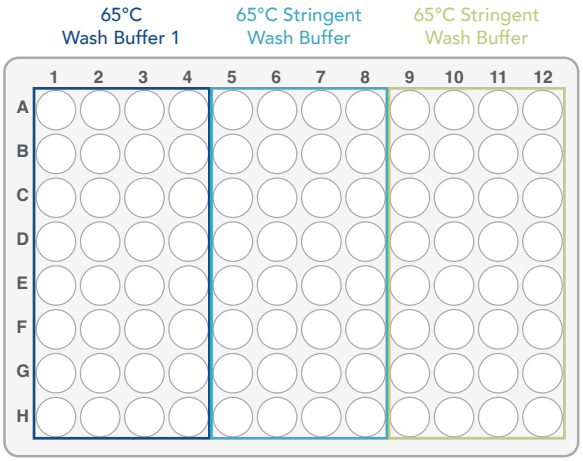
Prepare buffers

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

Component	Nuclease-free water (µL)	Buffer volume (µL)	Total volume (µL)	Water for ___ pools	Buffer for ___ pools
2x Bead Wash Buffer	160	160	320		
10x Wash Buffer 1	252	28	280		
10x Wash Buffer 2	144	16	160		
10x Wash Buffer 3	144	16	160		
10x Stringent Wash Buffer	288	32	320		

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

2. Use a fresh PCR plate to prepare the buffer plate. For 32 samples, as an example, aliquot and label the plate as follows:
   a. Columns 1-4: 110 µL of Wash Buffer 1
   b. Columns 5-8: 160 µL of Stringent Wash Buffer
   c. 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. Prepare the following Bead Resuspension Mix in a 1.5 mL low-bind tube and heat to 65°C in an Eppendorf ThermoMixer or water bath:

Component	Stock Conc.	Volume (µL)	Volume for ___ pools
Nuclease-Free Water		6.5	
xGen 2X Hybridization Buffer	2x	8.5	
xGen Hybridization Buffer Enhancer		2	
Total		17	

Prepare capture beads

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

1. Mix the beads thoroughly by vortexing for 15 sec.
2. Add 60 µL of streptavidin beads to a new PCR plate, filling a well for every sample pool captured.
3. Add 100 µL of Bead Wash Buffer from Prepare buffers, step 1 to each well, then gently pipette 10 times to mix.
4. Place the plate containing beads on a magnet and 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. Perform the following wash:
   a. Add 100 µL of Bead Wash Buffer to each well containing beads, then gently pipette 10 times to mix.
   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** a to c (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 so the beads are not left to dry in the well. If needed, briefly centrifuge the plate containing beads at 25 x g.
11. Place the plate containing the beads on the second thermocycler and start the WASH program. Make sure the lid temperature is set to 70°C for the WASH program.
12. Incubate the plate for 3 minutes at 65°C.

Capture of hybridized probes

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).
- A quick transfer of the Bead Resuspension Mix (at 65°C) into the plate containing the sample pool is critical for the capture efficiency. Try to avoid any delay while transferring and make sure a temperature of 65°C for the hybridized pool is constantly maintained until the room temperature wash steps are reached.

Note: The buffer plate needs to warm up for at least 15 minutes before the heated washes. We recommend starting the incubation of the buffer plate at 65°C as soon as all beads have been transferred to the sample pools.

1. After at least 8 hours hybridization, while keeping both PCR plates incubated at 65°C in the thermocyclers with their lids open, quickly transfer the fully homogenized beads to the sample pools using a multichannel pipette set to 17 µL. **Do not** pipette mix the sample pools with the beads.
2. Securely seal the sample pool plate.
3. Discard the empty plate that contained the Bead Resuspension Mix.
4. Take the sample pool plate out of the thermocycler and carefully mix by vortexing on the lowest possible intensity until all wells are fully mixed.
5. When mixed, immediately incubate the sample pool plate in the second thermocycler that already runs the WASH program. Close the lid.
6. Stop the HYB program of the first thermocycler and immediately start the WASH program. After WASH has started, incubate the buffer plate in the first thermocycler. Close the lid.
7. Incubate the sample pool plate in the second thermocycler for 45 minutes. During incubation, remove the plate every 10–12 minutes to quickly and gently vortex on the lowest possible intensity.

Heated washes

Important:

- It is critical that the buffer plate has reached 65°C in the thermocycler before starting the heated washes.
- Always keep the buffer plate on the thermocycler during washes. Make sure to reseal the buffer plate and close the thermocycler's lid in between washes.

1. After 45 minutes, while keeping both PCR plates incubated at 65°C in the thermocyclers with their lids open, transfer 100 µL of heated Wash Buffer 1 to each plate well containing a sample pool. Very slowly pipette 10 times to mix, being careful to minimize bubble formation.
2. Reseal the buffer plate, then close the lid.
3. Perform wash with heated 1X Stringent Wash Buffer:
   a. Remove the sample pool plate from the thermocycler and place on the magnet for 1 minute. Remove the supernatant.
   b. Remove the sample pool plate from the magnet, then add 150 µL of heated Stringent Wash Buffer to each plate well containing a sample.
   c. Immediately, return the sample pool plate to the thermocycler and very slowly pipette 10 times to mix.
   d. Seal the sample pool plate, close the lid and incubate for 5 minutes.
4. Repeat steps 3 a to d for a total of two washes with the heated 1X Stringent Wash Buffer.

Room temperature washes

1. Place the sample pool plate on a magnet for 1 minute.
2. Remove and discard supernatant. Add 150 µL of Wash Buffer 1 equilibrated to room temperature. Very slowly pipette 10 times to mix, being careful to minimize bubble formation. Transfer the sample pools to a new plate well. This step helps to reduce the off-target rate.
3. Incubate for 2 minutes.
4. Centrifuge the sample plate for 5 sec at 25 x g.
5. Place the sample plate on the magnet for 1 minute, then remove and discard the seal.
6. Remove and discard supernatant. Add 150 µL of Wash Buffer 2 equilibrated to room temperature. Very slowly pipette 10 times to mix, being careful to minimize bubble formation.
7. Incubate for 2 minutes.
8. Centrifuge the sample plate for 5 sec at 25 x g.
9. Place the sample plate on the magnet for 1 minute, then remove and discard the seal.
10. Remove and discard supernatant. Add 150 µL of Wash Buffer 3 equilibrated to room temperature. Very slowly pipette 10 times to mix, being careful to minimize bubble formation.
11. Incubate for 2 minutes.
12. Centrifuge the sample plate for 5 sec at 25 x g.
13. After centrifuging, place the sample plate on the magnet for 1 minute, then remove and discard the seal.
14. Remove the supernatant.

15. With the sample plate still on the magnet, use fresh pipette tips to be sure that all residual Wash Buffer 3 has been removed, then remove the plate from the magnet. Immediately go to the next step, **do not** let the beads dry.
16. Add 50 µL of nuclease-free water to each capture.
17. Very slowly pipette 10 times to resuspend the beads.
18. Put the sample pool plate onto ice until used in the amplification step.

Important: Do not discard the beads. Use the entire 50 µL including the resuspended beads with captured DNA in the amplification reaction.

Proceed with section 8. Amplification.

Amplification

In this section, you will need the following:

- Takara LA Taq DNA Polymerase Hot-Start Version
- [Optional] Takara Taq DNA Polymerase GC-buffer Version
- 100 µM Amplification Primer (IDT custom oligo)
- Nuclease-free water

Note: For GC-rich target sequences we recommend performing the long-range PCR with the optional TakaRa LA Taq DNA Polymerase with GC Buffer. Please refer to the subsection Amplification in the Guidelines section of this protocol.

For each sample pool, prepare a total of two reactions of 100 µL long range PCR reaction volume. Prepare either Takara LA Taq DNA Polymerase Hot-Start Version or optional TaKaRa LA Taq DNA Polymerase with GC Buffer reactions.

1. For the required number of reactions, prepare (a) master mix(es) in (a) 1.5 mL tube(s). From the master mix(es), set up the following amplification reactions in low-bind PCR tubes or plates. Keep on ice:

Takara LA Taq DNA Polymerase Hot-Start Version (for low to medium target GC-content)

Component	Volume for 1 reaction (µL)	Volume for ___ pools
Water	48	
10X LA PCR Buffer	10	
2.5 mM each dNTPs	12	
Amplification Primer (100 µM, IDT custom oligo)	4.4	
LA Taq HS Polymerase	0.6	
Total:	**75**	
Resuspended beads with captured sample pool	25 (split recovered 50 µL into halves to perform two reactions)	

OPTIONAL: TaKaRa LA Taq DNA Polymerase with GC Buffer (for high target GC-content)

Component	Volume for 1 reaction (µL)	Volume for ___ pools
Water	8	
2X LA PCR GC Buffer I	50	
2.5 mM each dNTPs	12	
Amplification Primer (100 µM, IDT custom oligo)	4.4	
LA Taq Polymerase	0.6	
Total:	**75**	
Resuspended beads with captured sample pool	25 (split recovered 50 µL into halves to perform two reactions)	

2. Pipette mix well by slowly pipetting up and down 10 times.
3. Place the tube in a thermocycler, and run the following program(s) with the heated lid set at 105°C:

Takara LA Taq DNA Polymerase Hot-Start Version

Step	Temperature (°C)	Time	Cycles
1	95	2 minutes	
2	95	20 seconds	Variable - refer to the target size table below.
3	62	15 seconds	
4	68	12 minutes	
5	68	7 minutes	
6	4	Hold	

OPTIONAL: TaKaRa LA Taq DNA Polymerase with GC Buffer

Step	Temperature (°C)	Time	Cycles
1	95	2 minutes	
2	98	20 seconds	Variable - refer to the target size table below.
3	62	15 seconds	
4	68	15 minutes	
5	68	7 minutes	
6	4	Hold	

Note: The number of PCR cycles should be optimized per target size and the number of multiplexed samples per captured sample pool, so there is enough yield for the subsequent workflow. Overamplification negatively impacts sequencing quality and should be avoided.

We recommend starting optimization with the following:

Target size	4–6 plex	7–12-plex	12–16-plex
>100 Mb	19	-*	-*
50–100 Mb	21	20	19
10–50 Mb	23	22	21
1–10 Mb	24	23	22
500–1000 kb	26	25	24
100–500 kb	27	26	25
50–100 kb	29	28	27
<50 kb	-*	-*	28

* Not recommended

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

Tip: Amplification can be performed overnight. The amplification product can be stored for up to 24 hours in the hold at 4°C steps of the long-range PCR programs.

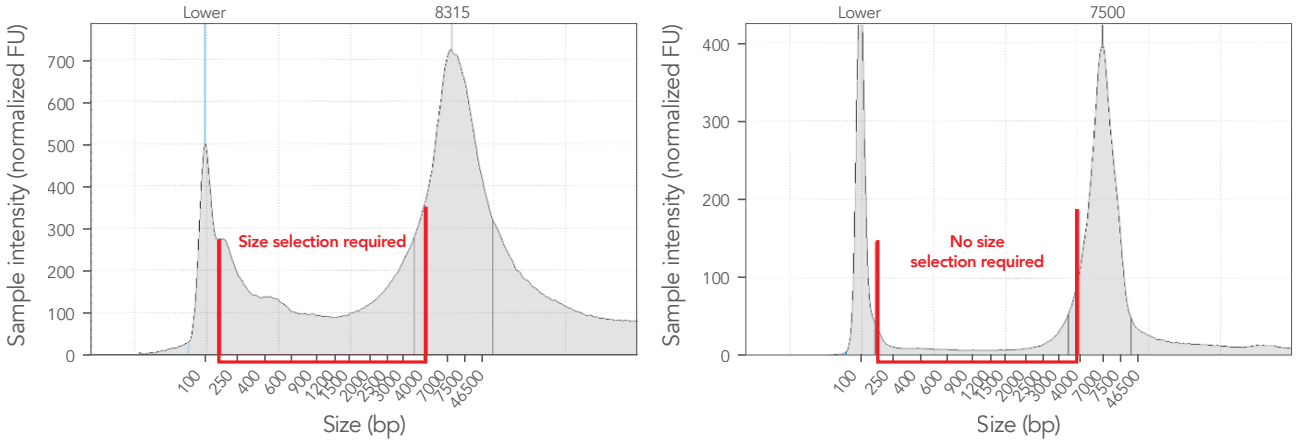
4. After amplification, verify your DNA amount and fragment size using a TapeStation (gDNA Screen Tape) or equivalent. Take a sample for analysis from the PCR reactions (without clean-up).

Note: The fragment size distribution of the amplification product is dependent on the initial sample quality and the number of PCR cycles. If a significant number of fragments below 4,000 bp are present, a second size-selection using the size-selection beads is recommended, otherwise a clean-up is sufficient.

Based on the DNA fragment size distribution and concentration decide:

a. When the average concentration is below 8 ng/µL (or the total mass in both PCR reactions less than 1600 ng total), perform an appropriate number of additional cycles.

b. When a) is not applicable and a significant portion of fragments are below the target size range, a second size selection instead of the clean-up should be performed (see depiction below). Is this the case, go to section 9a. Second Size-Selection.



c. When both a) and b) are not applicable, no size selection is required, and the PCR can be cleaned up according to 9b. Clean-Up.

Second size selection

In this section you will need:

- Size selection beads
- Freshly prepared 80% (v/v) ethanol
- Elution Buffer (Tris-HCL Buffer 1 M, pH 8.5)

Refer to section Reagent preparation and subsection Preparation of size-selection beads for preparation.
The second size-selection is performed in case a significant share of fragments below 4,000 bp are present in the amplified enriched sample pool—jump to section 9b. Clean-Up if this is not the case.

Important:

- Make sure size-selection beads and Elution Buffer have been equilibrated to room temperature before proceeding. Directly before using, mix the size-selection beads well by vortexing until the solution appears homogenous. All size-selection steps should be performed at room temperature.
- Exact pipetting is crucial for precise size selection, inaccurate pipetting may lead to suboptimal size selection and results.

Note: The 0.875x size-selection bead volume ratio will select the samples with a cutoff at approximately 4500 bp. If the amplification product is smaller than this (e.g., for extremely GC-rich targets), the volume ratio needs to be increased.

1. Pool both 100 µL PCR reactions per amplified sample pool into a 1.5 mL low-bind tube or into a well of a deep well plate.
2. Add 90 µL nuclease-free water to the pooled reactions.

3. Prefill a second 1.5 mL low-bind tube or a second deep well plate with exactly 245 µL (0.875x volume ratio) size-selection beads.
4. Transfer exactly 280 µL of the pooled PCR reactions into the prepared wells containing the size-selection beads. If the volume of the pooled PCR reactions is smaller add an appropriate amount of nuclease-free water to the pooled reactions before transferring. The exact 0.875x volume ratio of size-selection beads to pooled PCR reactions is crucial for a reliable size selection.

   Tube: Mix bead/PCR reactions by slowly pipetting up and down until solution is homogenous.

   Plate: Shake the plate at 1400 for 2 minutes.
5. Allow the DNA to bind to the size-selection beads for 15 minutes at room temperature. By gently shaking or mixing, make sure that the beads stay dissolved homogeneously throughout the incubation time.
6. If necessary, spin down the deep well plate/tubes to collect the beads.
7. Place the tubes or plate in a magnetic bead rack until the beads are collected to the side of the tubes or plate wells and the solution appears clear. Then remove and discard supernatant.
8. Wash beads with freshly prepared 80% ethanol:
   a. Do not remove the tubes or plate from the magnetic rack.
   b. While on the magnetic rack, slowly dispense 1 mL (tube) or 700 µL (deep well plate) of 80% ethanol against tube/well wall opposite to the beads. Do not resuspend the beads.
   c. After 30 seconds, remove and discard the 80% ethanol.
9. Repeat step 8 once.
10. Spin down the tubes/plate to collect the remaining liquid.
11. Check for any remaining droplets in the tubes/wells. If droplets are present, repeat step 10.
12. Put the tubes/plate back on the magnetic rack. Pipette off any remaining liquid.
13. Allow beads to air-dry for approximately 60–120 seconds.
14. Add 47 µL Elution Buffer buffer to the tubes/plate wells containing the beads.

    Tube: pipette up and down slowly to mix until beads are uniformly resuspended.

    Plate: shake the plate at 1400 for 2 minutes. Check if all beads are homogenously resuspended. If not, repeat this step. Spin down the plate to collect the beads.
15. Elute the DNA by letting the mix stand at room temperature for 5 minutes. Alternatively, the yield can be increased by incubating at 37°C for 5 minutes.
16. Place the tubes/plate back on the magnetic bead rack. Wait for approximately 2 minutes until the solution appears clear.
17. Transfer 45 µL of the supernatant containing the enriched sample pools to a new low-bind tube or to a well of a new 200 µL low-bind PCR plate.
18. Discard the beads.
19. Verify your DNA amount and fragment size using a TapeStation (gDNA Screen Tape) or equivalent.

Proceed with desired Third-Generation Sequencing workflow using the enriched sample pool as an input. For compatibility and interfaces refer to subsection Third-Generation Sequencing in the Guidelines section of this protocol.

Safe stopping point: The enriched sample pools can be stored for up to 24 hours at 4°C or at –20°C for four weeks.

Clean-Up

The clean-up is performed in case no significant share of fragments below 4,000 bp is present in the amplified enriched sample pool–jump to section 9a. Second size selection if this is not the case.

In this section you will need the following:

- Agencourt AMPure XP beads, or equivalent
- Freshly prepared 80% (v/v) ethanol
- Elution Buffer (Tris-HCL Buffer 1 M, pH 8.5)

Important: Make sure Agencourt AMPure XP beads and Elution Buffer have been equilibrated to room temperature before proceeding. Directly before using, mix the Agencourt AMPure XP beads well by vortexing until the solution appears homogenous. All size-selection steps should be performed at room temperature.

1. Pool both 100 µL PCR reactions per amplified sample pool into a 1.5 mL low-bind tube or into a well of a deep well plate.
2. Add 90 µL volume of AMPure PB beads (0.45X).

   Tube: mix bead/DNA solution by carefully pipetting up and down until solution is homogenous.

   Plate: shake the plate at 1400 for 2 minutes.
3. Allow the DNA to bind to the AMPure XP beads for 7 minutes at room temperature. By gently shaking or mixing, make sure that the beads stay dissolved homogeneously throughout the incubation time.
4. Pulse-spin the samples. Place the sample tubes/plate on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
5. Remove and discard the supernatant, using a clean pipette tip, without disturbing the pellet.
6. Wash beads with freshly prepared 80% ethanol.
   a. Do not remove the tubes or plate from the magnetic rack.
   b. While on the magnetic rack, slowly dispense 1 mL (tube) or 700 µL (deep well plate) of 80% ethanol against tube/well wall opposite to the beads. **Do not** resuspend the beads.
   c. After 30 seconds, remove and discard the 80% ethanol.
7. Repeat step 6 once.
8. Pulse-spin the samples, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Check for any remaining droplets in the tubes/plate wells. If droplets are present, repeat step 8.
10. Allow beads to air-dry for approximately 60–120 seconds. Do not over-dry the beads.
11. Add 47 µL Elution Buffer to the tubes/plate wells containing the beads.
12. Pipette carefully up and down to mix until beads are homogeneously resuspended.
13. Elute the DNA by incubating at room temperature for 5 minutes. Alternatively, the yield can be increased by incubating at 37°C for 5 minutes.
14. Spin the tubes/plate down to collect the beads, then place the tubes/plate back on the magnetic bead rack. Wait for approximately 2 minutes until the solution appears clear.
15. Transfer 45 µL of the supernatant containing the enriched sample pools to a new low-bind tube or to a well of a new 200 µL low-bind PCR plate.

16. Discard the beads.
17. Verify your DNA amount and fragment size using a TapeStation (gDNA Screen Tape) or equivalent.

Proceed with desired Third-Generation Sequencing workflow using the enriched sample pool as an input. For compatibility and interfaces refer to subsection Third-Generation Sequencing in the Guidelines section of this protocol.

Safe stopping point: The enriched sample pools can be stored for up to 24 hours at 4°C or at –20°C for four weeks.

Appendix A: Troubleshooting

Issue	Possible cause	Time
Library migrates unexpectedly on Bioanalyzer	Over-amplification of library leads to the formation of heteroduplex structures that migrate abnormally.	Reduce the number of PCR cycles to avoid over-amplification.
Incomplete resuspension of beads after ethanol wash during purification steps	Over drying of beads	Continue pipetting the liquid over the beads to break up clumps for complete resuspension. To avoid over drying, resuspend beads immediately after the removal of residual ethanol.
Shortage of enzyme reagents	Pipetting enzymes at –20°C	Place enzyme reagents on ice for 10 minutes before pipetting.
Retention of liquid in pipette tip	Viscous reagents (e.g., Buffer L1) may stick to pipette tip, especially for non-low retention tips.	Pipette up and down several times so all liquid is released from the pipette tip.
Unexpected increase in Barcoded Adapter dimers	• Improper adapter dilution • Improper bead purification	• Use the specified dilution for your input quantity, or further dilute the Barcoded Adapters. • Use the specified bead volume particularly for the post-PCR purification.

High-throughput hybridization capture enrichment of long genomic fragments

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