xGen® Target Capture Protocol
Protocol for DNA Probe Hybridization and Target Capture Using an Illumina Library
Version 1.1 (optimized for 500–600 bp fragments for 500-cycle runs on the MiSeq® System)

Products Available from IDT

- xGen® Lockdown® Probes (biotinylated DNA capture probes)
- Custom DNA Primers
- xGen® Blocking Oligos
- Nuclease-free Water

A. Hybridization of DNA Capture Probes to the Genomic DNA Library

This procedure summarizes the steps necessary for hybridization of DNA probes with an Illumina library prepared from genomic DNA. Minor modifications will be necessary to the PCR enrichment step if using an alternative platform.

Before you start

i. Hydrate the dried down xGen Lockdown Probes pool to 3 pmol/µL in IDT E8.0. If a capture probe pool at a lower concentration is used, IDT recommends drying down a portion of the material and rehydrating in nuclease-free water to 3 pmol/µL.

ii. Hydrate the Blocking Oligos to 1 mM in IDT E8.0.

iii. Important: Prepare all of the required buffers as indicated on page 3. Before each use, closely inspect the 2X Hybridization Buffer for precipitates. If there is a precipitate, heat to 55°C for 10 min while assembling the other components.

1. Hybridize xGen Lockdown Probes (or other capture probes) to target.
   a. Combine the following in a low-bind 0.2 mL PCR tube:
      - 3 pmol pooled xGen Lockdown Probes (1.5 pmol/µL)
      - 500 ng Illumina library (dried down, and then resuspended in water to 125 ng/µL)
      - 2 µg Cot-1 DNA (1 µg/µL)
      - 1 mM Blocking Oligo 1
      - 1 mM Blocking Oligo 2
      - Total Volume

   b. Add 10 µL 2X Hybridization Buffer and vortex.
   c. Overlay with 40 µL mineral oil. **DO NOT vortex after overlaying with oil.**
      IMPORTANT: **It is essential that you overlay with mineral oil to avoid significant volume loss.**
   d. Denature the mixture in a thermal cycler at 95°C for 5 min, and then slowly decrease the temperature (0.1°C/sec) to 71°C. **DO NOT use the heated lid of the thermal cycler.**
   e. Incubate at 71°C for 48 hr. This reaction will be used in Step 2.e. (below).

2. Bind hybridized target to streptavidin beads.
   a. Allow Dynabeads® M-270 Streptavidin (Invitrogen) to equilibrate to room temperature for 30 min.
   b. Pipet 50 µL M-270 streptavidin beads into a fresh 1.7 mL low-bind microcentrifuge tube and wash twice with 50 µL 2X Bind and Wash Buffer. Use a pipette to remove the buffer after each wash.
   c. Combine the following to make Bead Resuspension Buffer:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Bind and Wash Buffer</td>
<td>50 µL</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>30 µL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>80 µL</td>
</tr>
</tbody>
</table>

Additional Materials Required

- Agencourt® AMPure® XP - PCR Purification beads: Beckman-Coulter, cat. no. A63880
- Bioanalyzer Chips: Such as Bio-Rad DNA 1K analysis chips, cat. no. 700-7107
- Denhardt’s Solution: General lab supplier
- Dynabeads M-270 Streptavidin: Life Technologies, cat. no. 65305
- EDTA: General lab supplier
- 95% or 100% Ethanol: General lab supplier
- Human Cot-1 DNA: Life Technologies, cat. no. 15279-011
- KAPA HiFi HotStart ReadyMix: Kapa Biosystems, cat. no. KK2601
- Library Quantification Kit - Illumina/Universal: Kapa Biosystems, cat. no. KK4824
- MAXYMum Recovery® Microtube, 1.7 mL: VWR, cat. no. 22234-046
- MAXYMum Recovery® PCR Tubes, 0.2 mL flat cap: VWR, cat. no. 22234-056
- PhiX Control v3: Illumina, cat. no. FC-110-3001
- Qubit® dsDNA HS Assay Kit: Life Technologies, cat. no. Q32851
- Sodium Chloride: General lab supplier
- 10X SSC: General lab supplier
- SDS: General lab supplier
- 1M Sodium Hydroxide: General lab supplier
- 1M Tris-HCl, pH 8.8: General lab supplier
- Tween-20: General lab supplier

i. Hydrate the dried down xGen Lockdown Probes pool to 3 pmol/µL in IDT pH 8.0. If a capture probe pool at a lower concentration is used, IDT recommends drying down a portion of the material and rehydrating in nuclease-free water to 3 pmol/µL.

ii. Hydrate the Blocking Oligos to 1 mM in IDT pH 8.0.

iii. Important: Prepare all of the required buffers as indicated on page 3. Before each use, closely inspect the 2X Hybridization Buffer for precipitates. If there is a precipitate, heat to 55°C for 10 min while assembling the other components.
3. Wash the streptavidin beads to remove unbound DNA.

a. Briefly spin down the tube from step 2.f. (above) and place on a magnetic separation rack. Allow beads to separate from the supernatant. Using a pipette, remove the supernatant containing unbound DNA and discard.

b. Wash the beads with the following solutions, sequentially:
   i. 1000 µL Wash Buffer 1 for 5 min, 71°C with rotation
   ii. 1000 µL Wash Buffer 2 for 5 min, 71°C with rotation
   iii. 1000 µL Wash Buffer 2 for 5 min, 71°C with rotation
   iv. 1000 µL Wash Buffer 2 for 5 min, RT with rotation
   v. 1000 µL Wash Buffer 3 for 30 sec, RT with tube on magnet; keep tube on magnet
      • Ensure that each solution has been pre-equilibrated to the appropriate wash temperature.
      • Add the stated volume of each wash buffer to the tube and place on a bench top tube rotator/nutator mixer for the indicated amount of time.
      • Pulse spin (1–3 sec, 800 x g) to collect the liquid without pelleting the beads.
      • Place tube on magnetic holder to attract the magnetic beads.
      • Remove wash buffer using a pipette.

   IMPORTANT! After the final wash (v), do not remove tube from magnet! Remove ALL of the Wash Buffer 3 from the tube after the final wash. SSC present in the buffer may react with NaOH used in subsequent steps to produce a precipitate that will interfere with downstream manipulations.

c. Add 50 µL 125 mM NaOH (freshly diluted from a more concentrated stock solution) and incubate at RT for 10 min. Vortex every 2 min to keep beads in suspension.

d. Return the tube to the magnet for 1 min. Leave the tube on the magnet while you perform the next steps.

e. Add 50 µL 1 M Tris-HCl, pH 8.8, to a new 1.7 mL low-bind tube.

f. Keeping the tube on the magnet, transfer the supernatant (d) to the tube of 1 M Tris-HCl (e). This neutralizes the NaOH added to the hybridization mixture.

g. Add 1.5X volumes (150 µL) Agencourt® AMPure® XP beads (Beckman-Coulter). Proceed according to the manufacturer’s protocol, eluting in 22 µL Elution Buffer.

h. Transfer 20 µL of eluted product into a fresh 1.7 mL low-bind tube, ensuring no beads are carried over.

B. Post-Capture PCR

The eluate from step A.3.h. above allows retention of a small amount of captured library for troubleshooting, if necessary. Otherwise, the library can be eluted in a smaller volume and the entire amount used in the final PCR enrichment.

1. Perform final PCR enrichment.

   a. Prepare the following reaction mix in a low-bind 0.2 mL PCR tube:

   - 2X KAPA HiFi™ HotStart ReadyMix: 25 µL
   - 10 µM Illumina P5 Primer: 2.5 µL
   - 10 µM Illumina P7 Primer: 2.5 µL
   - Nuclease-Free Water: 4 µL
   - Eluted capture library: 16 µL

   **Total Volume**: 50 µL

   b. Briefly vortex the tube, and then spin briefly in a microcentrifuge to collect the reaction mixture at the bottom of the tube.

   c. Place reactions in a thermal cycler and run the following program:

   - 98°C 5 min
   - 98°C 20 sec
   - 60°C 15 sec
   - 72°C 20 sec
   - 72°C 5 min
   - 4°C Hold

   **14 cycles**

   **Note:** Fewer cycles may be performed depending on the yield of the captured library. We recommend starting at 14 cycles to ensure enough material for loading on the sequencer without over-amplifying and biasing the library.

   d. Purify the fragments using 1.5X volume (75 µL) Agencourt AMPure XP beads. Proceed according to the manufacturer’s protocol, eluting in 22 µL Elution Buffer.

   e. Transfer 20 µL of eluted product into a fresh 1.7 mL low-bind tube, ensuring no beads are carried over.
2. Perform quality control.
   a. Measure the concentration of captured library using a Qubit® Fluorometer (Life Technologies). Ensure the concentration is <50 ng/µL. Dilute, if necessary.
   b. Run 1 µL of library on a bioanalyzer (Bio-Rad Experion™ or Agilent 2100) using a DNA 1K chip. Take note of the average fragment length.
   c. Perform library quantification using the KAPA Library Quantification Kit.
   d. Use the correction factor below to determine molarity of the library (insert the appropriate dilution factor and average fragment length from bioanalyzer results):

<table>
<thead>
<tr>
<th>Library Name</th>
<th>Concentrations of Triplicate Data Points (pM, calculated by qPCR instrument)</th>
<th>Average Concentration (pM)</th>
<th>Size-Adjusted Concentration (pM)</th>
<th>Concentration of Undiluted Library Stock (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1000</td>
<td>A1, A2, A3</td>
<td>A</td>
<td>A x \frac{4S2}{\text{Avg. Fragment Length}} = W</td>
<td>W x 1000</td>
</tr>
</tbody>
</table>

Table reproduced from Library Quantification Kit – Illumina/Universal (Kapa Biosystems, Woburn, MA, USA)

C. Sequencing
   a. Prepare a 2 nM solution of the captured library (in nuclease-free water) for use in the denaturing reaction for the MiSeq® System. The library will be sequenced at a final concentration of 7 pM.
   b. Combine the following in a 1.7 mL low-bind tube:
      - 2 nM captured library solution: 3.5 µL
      - 20 pM PhiX Control: 3.5 µL
      - 0.2 M NaOH: 3.5 µL
      Pipet the mixture up and down to mix, and then incubate at room temperature for 5 min.
   c. Add 989.5 µL Hyb Buffer (provided in MiSeq reagents kit, box 1 of 2) to neutralize.
   d. Add 600 µL neutralized library to the sequencing cartridge, and then sequence on the Illumina MiSeq System.

Buffer Compositions

- **2X Bind and Wash Buffer**
  - 10 mM Tris-HCl, pH 7.5
  - 2 M NaCl
  - 1 mM EDTA
  - 0.1% Tween-20

- **Wash Buffer 1: 1X SSC/0.1% SDS**
  - 150 mM NaCl
  - 15 mM Sodium citrate
  - (adjust to pH 7.0 with HCl)
  - 0.1% SDS

- **Wash Buffer 2: 0.1X SSC/0.1% SDS**
  - 15 mM NaCl
  - 1.5 mM Sodium citrate
  - (adjust to pH 7.0 with HCl)
  - 0.1% SDS

- **Wash Buffer 3: 0.2X SSC**
  - 30 mM NaCl
  - 3 mM Sodium citrate
  - (adjust to pH 7.0 with HCl)

- **2X Hybridization Buffer**
  - 0.50 M Sodium phosphate buffer, pH 7.0
  - 1% SDS
  - 2 mM EDTA
  - 2X SSC
  - 4X Denhardt’s Solution

- **Elution Buffer**
  - 10 mM Tris-Cl, pH 8.5

Primer Sequences

- **Illumina P5**: AATGATACGGCGACCACCGA
- **Illumina P7**: CAAGCAGAAGACGCACGAC