Genomic DNA Capture-Array Protocol

Steps:

1.

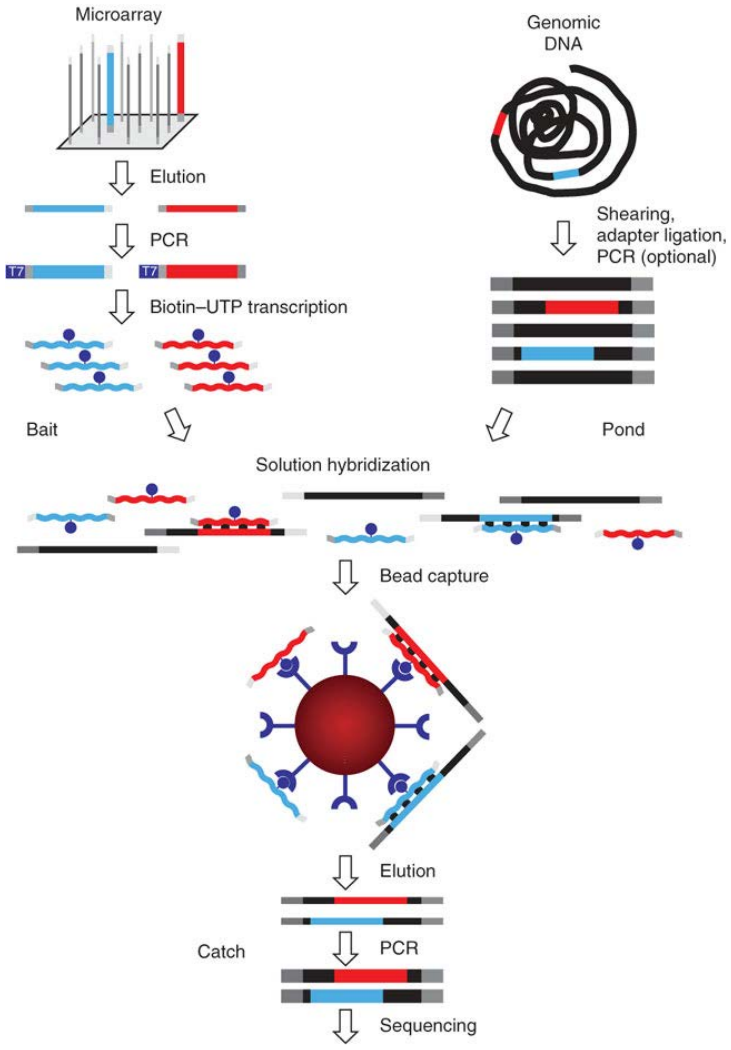
2.

3.

4.

5.

6.



(Gnirke *et al*. 2009*)* Figure 1: Overview of Hybrid selection method. Illustrated are steps involved in the preparation of a complex pool of biotinylated RNA capture probes (bait; top left), whole-genome fragment input library (pond; top right) and hybrid-selected enriched output library (catch; bottom). Two sequencing targets (one for each strand of the genome) and their respective baits are shown in red and blue. Universal adaptor sequences are gray. The excess of single-stranded nonself-complementary RNA (wavy lines) drives the hybridization

Overview of Major Steps in Capture Array Protocol

1. Adaptor-ligated, PCR amplified gDNA libraries are hybridized to RNA baits. This protocol assumes that already have prepared the libraries.
2. Biotin-labelled mRNA baits will be hybridized to sheared, adaptor labelled gDNA at 65˚C for 66 hours in the presence of human Cot-1 DNA, salmon sperm DNA and adapter primer blocking reagents.
3. Next, mRNA-gDNA hybrids will be eluted using streptavidin coated magnetic beads. Genomic DNA capture fragments will then be PCR amplified.

Enrich the Adapter-Modified DNA Fragments by PCR

This protocol uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends, and to amplify the amount of DNA in the library.

The six-base index is introduced into the adapter at the PCR stage. The PCR is performed with three primers that anneal to the ends of the adapters. Two of these primers are used in all sample preparations, while the third contains the index tag that will be used to discriminate between samples after the sequencing is completed.

Note regarding First Amplification of library (preceding hybridization enrichment):

There are two options, add the index during the first PCR, or adding the index during the second PCR (post-capture). Placing the index before capture allows you to capture 2-6 samples at the same time, which makes capture setup cheaper and easier. However, capturing 1 sample at a time is more efficient, and reduces non-specific pulldown due to the primer binding sites (the shorter these sites, the less non-specific pulldown). If we add the index after the adapter ligation and PCR, the primer binding site (on one side) will be half as long, and therefore less likely to pull down non-specific fragments.

First Amplification (index during PCR1):

Reagent	PCR 1 Volume
2x Kapa HiFi Master Mix	25 uL
25µM PE_PCR_Primer_1.0	1.0 uL
0.5µM PE_PCR_Primer_2.0	1.0 uL
25µM Index Primer	1.0 uL
Library DNA	20 uL
Water	2.0uL

First Amplification (no indexing):

Reagent	PCR 1 Volume
2x Kapa HiFi Master Mix	25 uL
25µM PE_PCR_Primer_1.0	1.0 uL
25µM PE_PCR_Primer_2.0	1.0 uL
Library DNA	20 uL
Water	3.0 uL

Primers:

All primers should be HPLC purified. Always order a larger amount of Multiplex PCR Primer 1.0 as I tend to get poor yields back from IDT.

Multiplex PCR Primer 1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACAC GACGCT CTTCCGATCT
Multiplex PCR Primer 2.0	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Index Primer options:

Multi_PE_PCR_Index2	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGA GTTC
Multi_PE_PCR_Index4	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGA GTTC
Multi_PE_PCR_Index5	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGA GTTC
Multi_PE_PCR_Index6	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGA GTTC
Multi_PE_PCR_Index7	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGA GTTC
Multi_PE_PCR_Index12	CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGA GTTC

Consumables Illumina-Supplied

The following consumables are provided in the Paired-End Sample Preparation Kit:

Phusion DNA Polymerase

Ultra Pure Water

The following consumables are provided in the Multiplexing Sample Preparation Oligonucleotide Kit:

PCR Primer InPE 1.0

PCR Primer InPE 2.0

PCR Primer Index 1-12, sequences follow:

PCR Primer, Index 1

5’ CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTC

PCR Primer, Index 2

5’ CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTC

PCR Primer, Index 3

5’ CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTC

PCR Primer, Index 4

5’ CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTC

PCR Primer, Index 5

5’ CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTC

PCR Primer, Index 6

5’ CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTC

PCR Primer, Index 7

5’ CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTC

PCR Primer, Index 8

5’ CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTC

PCR Primer, Index 9

5’ CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTC

PCR Primer, Index 10

5’ CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTC

PCR Primer, Index 11

5’ CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTC

PCR Primer, Index 12

5’ CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTC

Capture Blocking Primers

The blocking primers are just the reverse compliment of the 1.0 and 2.0 primers:

Multi_PE_PCR_BLOCK_1.0	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGT
Multi_PE_PCR_BLOCK_2.0	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC

If you are capturing indexed items, I would add this to the block #3 mixture with a final concentration equal to the rest of the blocks (blocks the 24nt added to the sequence (but it does not block the varying 6nt index):

Multi_PE_Index_Primer_2.0	CAAGCAGAAGACGGCATACGAGAT
Multi_PE_Index_BLOCK_2.0	ATCTCGTATGCCGTCTTCTGCTTG

1. **Hybridization:**
   Separate hybridization reactions are performed for each genomic strand. For each sample, set up two different hybridization enrichments, which will be combined after the washing steps and amplification steps are completed.

Reagent	Contents	Storage
Hybe #1	20x SSPE	Room Temp
Hybe #2	0.5M EDTA	Room Temp
Hybe #3	50x Denhardts	-20˚C
Hybe #4	1% SDS	Room Temp
Block #1 (not supplied)	1ug/μL human Cot-1 DNA	-20˚C

Block #2	1ug/µL Salmon Sperm DNA	-20˚C
Block #3 (not supplied)	(25 µM each) Blocking Primers	-20˚C
RNase inhibitor	Superase-In	-20˚C
3M NaOAc	3M NaOAc	Room Temp
Bead Binding Buffer	1M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA	Room Temp
Wash Buffer #1	1x SSC, 0.1% SDS	Room Temp
Wash Buffer #2	0.1x SSC, 0.1% SDS	Room Temp
Elution Buffer	0.1M NaOH	Room Temp
Neutralization Buffer	1M Tris-HCl pH 7.5	Room Temp
Oligo Bait Library	Biotinylated RNA Baits, 100ng/µL	-80˚C

Blocking primers are complementary to the library preparation adapters/primers. These will be specific to the sequencer (Illumina, 454, Solid, etc.) and the sequencing method (Paired-End, etc.). Blocking Primers MUST be used for Blocking During Hybridization of Capture Oligo Baits with genomic library. Make Blocking Primer solution by mixing equal parts of 100 uM index primer (+ and - strands), thus **4 primers total, each at 25uM concentration. This solution will be used as BLOCK # 3.

Other necessary materials:

1. Nuclease-free water
2. Dynabeads MyOne Streptavidin T1 (Invitrogen #656-01)
3. MinElute PCR Purification Kit
4. Nuclease-Free Thermocycler tubes.
5. Basic wet-Lab equipment: Thermocycler, pipettes and tips, water bath

Sample Preparation:

1. Adjust the volume of 500 ng of prepped DNA library to 3.4 µL. If necessary, reduce the volume of 500 ng of prepped DNA library on a speed-vac concentrator. Adjust the volume with nuclease-free water.
2. Prepare Library Mix. Combine the following in a thermocycler compatible tube:
    a. Prepped Library 3.4 µL
    b. Block #1 2.4 µL
    c. Block #2 2.5 µL

d. Block #3 0.6 µL
Hold at room temperature while continuing below

3. Prepare Hybridization Buffer Mix. Combine the following per capture in nuclease free thermocycler compatible tube:
    a. Hybe #1 25 µL
    b. Hybe #2 1 µL
    c. Hybe #3 10 µL
    d. Hybe #4 13 µL
    Hold at room temperature
4. Perpare Oligo Bait Library Mix. Combine the following per capture in nuclease free thermocycler compatible tube:
    a. Oligo Bait Library 5 µL
    b. RNase inhibitor 1 µL
    Hold at room temperature

Hybridization

Set up the following program on a thermocycler:

Step	Temp	Time, min
1	95°C	5
2	65°C	3
3	65°C	2
4	65°C	forever

1. Transfer the tube containing the Library Mix to the thermocycler and start the program. This will warm the Library Mix up to 95 ˚C for 5 minutes.
2. At the start of step 2 (65 ˚C for three minutes), transfer the Hybridization Buffer Mix to the thermocycler. This will pre-warm the Hybridization Mix.
3. At the start of step 3 (65 ˚C for two minutes), transfer the Oligo Bait Library Mix to the thermocycler and let warm for 2 minutes.
4. At the start of step 4, transfer 13 µL of the Hybridization Buffer Mix and 7 µL of the Library Mix to the tube containing 6 µL of the Oligo Bait Library Mix and mix by pipetting.
5. Keep the hybridization at 65 ˚C for 66 hours. Depending upon the application, samples can be hybridized for 72 hours.

6. Washing and Eluting

1. Prepare magnetic beads by washing with Binding buffer 3 times, then re-suspend in 200 µL of Binding buffer.

2. Mix the 200µL bead solution with the hybridization-capture mix and incubate on a rotator for 30 minutes at room temperature (20 °C).

3. Pellet beads with magnetic separator and remove the supernatant.

4. Re-suspend beads in 500 µL of Wash Buffer #1.

5. Incubate the samples for 15 minutes at room temperature. Separate the beads and buffer on a magnetic separator and remove the supernatant.

6. Mix the beads in pre-warmed (65 °C) 500 µL Wash Buffer #2. Incubate the samples for 10 minutes at 65 °C.

7. Remove wash buffer and repeat step 6 two more times. Remove the wash buffer and continue to step 8.

8. Mix the beads in 50 µL Elution Buffer. Incubate the samples for 10 minutes at room temperature.

9. Separate the beads and buffer on a magnetic separator. Transfer the supernatant to a tube containing 70 µL of Neutralization Buffer.

10. Desalt the capture solution with a Qiagen MinElute PCR purification column, eluting in 34 µL buffer EB.

Post Capture Amplification

QIAquick PCR Purification Kit

Procedure

This protocol assumes 5 µg of DNA input into library prep. If you use 1 µg, adjust the protocol as described in the following table.

Input of DNA to Library Prep	Volume of Purified Library into PCR	Volume of Water	Number of PCR Cycles
5 µg	1 µL	21 µL	18
1 µg	5 µL	17 µL	18

1. Prepare the following PCR reaction mix using the appropriate PCR Index primer for each separate sample:
    a. DNA (1 µl)
    b. Phusion DNA Polymerase (25 µl)
    c. PCR Primer InPE 1.0 (1 µl)

i. 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG ACGCTCTTCCGATCT-3’

d. PCR Primer InPE 2.0 (1 µl)

i. 5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC TGAACCGCTCTTCCGATCT-3’

e. Ultra Pure Water (21 µl)

f. Sample specific PCR Primer Index (1 µl)

The total volume should be 50 µl.

2. Amplify using the following PCR protocol:

a. 30 seconds at 98°C

b. 18 cycles of:

10 seconds at 98°C

30 seconds at 65°C

30 seconds at 72°C

c. 5 minutes at 72°C

d. Hold at 4°C

3. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample solution and concentrate it on one QIAquick column, eluting in 50 µl of QIAGEN Buffer EB.