

Oligo Array CGH (Maui Hyb System)

I. Reagents

A. Extraction

1. Phase Lock Gel, Heavy 15ml tube (Eppendorf, #0032 005.250)
2. Phenol/Chloroform/Isoamyl Alcohol (Invitrogen, #15593-031)

B. DNA Preparation

1. GenomiPhi DNA Amplification Kit, 100 reactions (Amersham, #25-6600-01)
2. Human Genomic DNA (Promega, Male #G1471, Female #G1521)
3. Lambda DNA/HindIII (Invitrogen, #15612-013)
4. QIAquick PCR Purification Kit, (Qiagen, #28106)
5. Guanidine HCl (Invitrogen, #15502-016)
6. QuantiTect SYBR Green PCR Master Mix (Qiagen #204143)
7. MgCl₂, 25 mM (Roche #1 699 113)
8. Alu PCR Primers: Yd6-forward 5'-GAGATCGAGACCACGGTGAAA-3'
Yd6-reverse 5'-TTTGAGACGGAGTCTCGTT-3'

C. Probe Preparation

1. Ultrapure dNTP set (Amersham, #27-2035-01)
2. Bioprime DNA Labeling System (Invitrogen, #18094-011)
3. Cy5-dUTP (Amersham, #PA55022)
4. Cy3-dUTP (Amersham, #PA53022)
5. 2X In situ Hybridization Buffer (Agilent, #5185-5973)
6. CoT 1 DNA (Invitrogen, #15279-011)
7. Random 25-mer: 5'-NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-3'

II. Genomic DNA Extraction

A. Whole tissue

100 ml Digestion Buffer:

- 2 ml NaCl (5M)
- 1 ml Tris-HCl (1M, pH 8.0)
- 5 ml EDTA (0.5M, pH 8.0)
- 5 ml SDS (10%)
- 87 ml dH₂O

- Store at room temperature. Immediately before use, add proteinase K to conc. of 0.1 mg/ml.

1. An appropriate starting amount of tissue is 200–1000 mg. Thinly slice frozen tissue or macerate fresh tissue with scalpel to minimize large pieces.
2. Suspend in 1.2 ml digestion buffer per 100 mg tissue.

B. Cell culture

1. Scrape cells and suspend in 10 ml cold PBS. Centrifuge 5 min at 500 xg, discard supernatant.
2. Resuspend in 10 ml cold PBS, centrifuge and discard supernatant.
3. For 3×10^7 cells resuspend cells in 0.3 ml digestion buffer. For larger numbers of cells use 1 ml digestion buffer per 10^8 cells.

C. Cell lysis and DNA purification

1. Place samples in tightly capped 15 ml tubes. Incubate horizontally with shaking at 50°C for 12-18 hr.
2. Transfer lysate to a 15 ml Phase Lock Gel tube. Add an equal volume of phenol/chloroform/isoamyl alcohol and vortex vigorously 15 sec.
3. Centrifuge at max. speed for 5 min. to separate phases.
4. Add an equal volume of chloroform and vortex vigorously 15 sec.

5. Centrifuge at max. speed for 5 min. to separate phases.
6. Transfer supernatant to new 15 ml Falcon tube. Add 10% volume 3M Sodium Acetate, pH 5.2 and vortex. Add 2 volumes 100% EtOH, invert vigorously.
7. Spool out DNA on flame sealed glass pipet and transfer to fresh 1.5 ml microcentrifuge tube containing 750 μ l, 70% EtOH.
8. Spool out DNA on flame sealed glass pipet and adhere to side of fresh 1.5 ml microcentrifuge tube. Allow to dry thoroughly, approx. 15 min.
9. Add 100-200 μ l TE. Flick tube to dislodge dried pellet. Allow to rehydrate undisturbed overnight at 4°C.

III. Genomic DNA Preparation

A. Amplification

1. Dilute aliquot of DNA to 40 ng/ μ l. For each experimental sample, one normal reference sample will also be prepared using Human Genomic DNA (male or female) as template.
2. Mix 1 μ l diluted DNA with 9 μ l of Genomiphi Sample Buffer. Heat to 95°C for 3 min. Cool to 4°C on ice.
3. For each amplification reaction, prepare master mix combining 9 μ l of Genomiphi Reaction Buffer with 1 μ l of phi29 enzyme on ice (prepare immediately before use). Add 10 μ l to each denatured template on ice, vortex, transfer to a 30°C thermocycler block and incubate for 18hr.
4. Following amplification, heat at 65°C for 10 min., cool to 4°C.

B. Purification

1. For each sample, prepare master mix containing 20 μ l dH₂O and 4 μ l 3M Sodium Acetate (pH 5.2). Add 24 μ l to each amplification and vortex.
2. Add 100 μ l EtOH and vortex to precipitate (do not chill!!).
3. Centrifuge in a microcentrifuge at RT for 15 min. at 12k rpm.
4. Remove supernatant, and add approx. 300 μ l 70% EtOH.
5. Centrifuge in a microcentrifuge at RT for 1 min. at 12k rpm.
6. Discard supernatant and aspirate to dry.
7. Air-dry open tubes for 5 min.
8. Resuspend in 22 μ l 10 mM Tris (pH 7.5)

IV. Probe Quantitation

A. Total DNA

1. Prepare standard stocks of Lamba-HindIII digest (Invitrogen, #) at a conc. of 1000, 500, 200, 100 & 50 ng/ μ l.
2. Dilute 1 μ l of all samples in 39 μ l PCR grade H₂O (save for qPCR), then dilute again taking 5 μ l of the 1:40 dilution and mixing with 10 μ l TE.
3. Dilute 1 μ l of standard stocks in 119 μ l TE.
4. Mix 40 μ l Pico Green in 10 ml TE.
5. Pipette 1 μ l of diluted standards and samples to triplicate wells. Add 100 μ l of Pico Green/TE and mix by pipetting.
6. Read plate on fluorimeter @ 485nm Ex/520 Em.

B. Human DNA

qPCR Master Mix (per sample):

- 25 μ l QuantiTect SYBR Green PCR Master Mix
- 8 μ l dH₂O
- 1 μ l Yd6 Forward Primer (100mM)
- 1 μ l Yd6 Reverse Primer (100mM)
- 5 μ l MgCl₂ (25 mM)

1. Prepare standards by diluting Human Total Genomic DNA to a conc. of 20, 10, 5, 2 & 1 ng/ μ l.

2. Pipette 10 μ l of standards and diluted samples (prepared in step IV.A.2 above) to duplicate wells. Add 40 μ l of qPCR Master Mix and mix by pipetting.
3. Run qPCR on ABI7900 with the following program:

Stage 1 (1 cycle):	95°C for 15 min.
Stage 2 (45 cycles):	94°C for 15 sec.
	61°C for 1 min.
Stage 3 (1 cycle):	95°C for 15 sec.
	60°C for 15 sec.
	95°C for 15 sec.

IV. Probe Preparation

A. Labeling

10X dNTP low T Mix:

12 μ l 100 mM dATP
 12 μ l 100 mM dGTP
 12 μ l 100 mM dCTP
 6 μ l 100 mM dTTP
 958 μ l of TE, pH 8.0

Labeling Master Mix (prepare for each sample):

5 μ l 10X dNTP low T Mix
 3 μ l Cy5-dUTP or Cy3-dUTP
 1 μ l Klenow Fragment

1. Pipette 5-10 μ g DNA into PCR tube and qs to 21 μ l with dH₂O (use equal amounts of experimental DNA and normal reference DNA for labeled Cy3 and Cy5 probe pairs).
2. Add 20 μ l 2.5X random primer mix, vortex.
3. Denature at 98°C for 5 min. and place on ice.
4. Add 9 μ l Labeling Master Mix, vortex.
5. Incubate in thermocycler at 37°C for 2 hrs.

B. Purification

1. Add 10 μ l 3M Sodium Acetate (pH 5.2) to probe, vortex.
2. Pipet 300 μ l buffer PB to top of QIAquick PCR Purification column.
3. Transfer probe to column and mix with PB buffer by pipetting. Centrifuge at max. speed for 1 min.
4. Return filtrate to column and repeat centrifugation.
5. Discard filtrate. Add 500 μ l 35% Guanidine HCl (aqueous) to column. Centrifuge at max. speed for 1 min.
6. Discard filtrate. Add 750 μ l buffer PE to column. Centrifuge at max. speed for 1 min.
7. Discard filtrate. Return column to waste collection tube and centrifuge again at max. speed for 2 min.
8. Transfer column to fresh 1.5 ml microcentrifuge tube and elute by pipetting 50 μ l buffer EB (warmed to 50°C) to center of QIAquick membrane and allowing to stand for 4 min.
9. Centrifuge at max. speed for 1 min. Pipette additional 50 μ l buffer EB (warmed to 50°C) to center of QIAquick membrane and allow to stand for 1 min.
10. Centrifuge at max. speed for 1 min.

C. Probe Prep

2x Hybridization Buffer
 500 μ l Formamide
 500 μ l 20x SSC
 ----warm to 42°C,

add 40 ul 10% SDS and keep at 42°C until required----

1. Combine the labeled Cy3 and Cy5 probe pairs, concentrate in the speedvac to 18ul
2. Add 5 µl CoT 1 (10 µg/µl), 2 ul Yeast tRNA (50ug/ul) and 25 ul 2x Formamide hybridization buffer.
3. Denature at 95°C for 5 min, incubate at 42°C for 30 min, and then apply to microarray.

D. Slide Prep

Slide pre-hyb Buffer (combine in Coplin Jar in order listed)

37.8 ml dH₂O

15 ml 20X SSC

6 ml 10% BSA

1.2 ml 10% SDS

----warm in water bath at 42°C until required----

1. While probe is blocking, put slides in Pre-hyb Buffer for 30-60 min at 42°C.
2. Transfer slides to a rack and wash with dH₂O 2X, agitating in staining dish for 1 min.
3. Transfer rack to new staining dish and cover with 100% isopropanol, agitate for 1 min
4. Centrifuge dry at 1200 rpm for 3 min (use slides withing 30 min.)

E. Hybridization

1. Centrifuge probe briefly, pipette full volume to microarray and hybridize in Bio-Micro Maui hybstation at 45°C for 17 hrs (minimum).

F. Slide washing

Prepare a jar with wash buffer #1 and a rack inside for temporary keeping slides

Wash #1 4 min

25 ml 20X SSC

5 ml 10% SDS

--- fill dH₂O to 1000 ml ---

Wash #2 & #3 4 min each

25 ml 20X SSC

--- fill dH₂O to 1000 ml ---

Wash #4 4 min

5 ml 20X SSC

--- fill dH₂O to 1000 ml ---

G. Scanning with Agilent scanner.