

# Nimblegen Processing of ChIP or DamID DNA on 2.1M Arrays

## Outline

Random primers are annealed to denatured DNA templates and extended by Klenow fragment while fluorescent dyes are incorporated. The labelled samples are then subjected to paired competitive hybridisations on Nimblegen 2.1M tiling arrays.

## Equipment and reagents

- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Bioprime DNA Labeling System (Invitrogen; Cat. No. 18094-011)
- Cy3 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 53021)
- Cy5 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 55021)
- Isopropanol (BDH; Cat. No. 102246L)
- NimbleGen Hybridization Kit, LS (Roche-Nimblegen; Cat. No. 05583934001)
- NimbleGen Wash Buffer Kit (Roche-Nimblegen; Cat. No. 05584507001)
- NimbleGen Hybridization System Accessory Kit (Roche-Nimblegen; Cat. No. 05327695001)
- NimbleGen Mixer HX1 (10) (Roche-Nimblegen; Cat. No. 05223741001)
- Sigma water (Sigma; Cat. No. W4502)
- Sodium Chloride (Sigma; Cat. No. S-3014)
- Hettich micro 20 centrifuge
- Hettich Rotina 35 microtitre plate centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)
- GenePix 4000B Microarray Scanner
- NimbleGen Hybridization System 4

## Procedure

### Making the 10 X low-C dNTP mix:

1. Make a large 10 X low-C dNTP mix for the labelling reaction (5 mM A-,G-,T-dNTPs and 3mM C-dNTP)
  - ◆ 25  $\mu$ l 100mM dNTA
  - ◆ 25  $\mu$ l 100mM dNTT
  - ◆ 25  $\mu$ l 100mM dNTG
  - ◆ 15  $\mu$ l 100mM dNTC
2. Then make up to 500  $\mu$ l using MilliQ water
3. Aliquot the 10 X low-C dNTP mix and then store at -20 °C

### Klenow labelling (Flychip protocol):

The following steps are performed in 200  $\mu$ l PCR tubes and the PCR block. Perform 2 reactions per sample and control.

1. Take 1  $\mu$ g DNA and make up to a total volume of 31.2  $\mu$ l with Sigma water
2. Add 30  $\mu$ l 2.5x Random Primer Reaction Buffer (supplied in the Bioprime Labelling System Kit)
3. Incubate at 100 °C for 5 minutes

4. Snap freeze on ice
5. Mix together the following to make a master mix:
  - ◆ 7.5 µl 10 X low-C dNTP mix
  - ◆ 4.5 µl Cy3 or Cy5 dCTP
  - ◆ 1.8 µl 40U/µl Klenow (supplied in the Bioprime Labelling System Kit)
6. Add 13.8 µl to each sample and mix by pipetting up and down
7. Incubate at 37 °C for 2 to 3 hours
8. Stop the reaction by adding 7.5 µl Stop Buffer (supplied in the Bioprime Labelling System Kit)
9. Combine the Cy3 and Cy5 pairs

### **Precipitation purification (as per Nimblegen protocol):**

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides.

10. Combine the 2 same reactions into 1.5 ml microcentrifuge tubes
11. Add 17.25 µl 5M NaCl-solution
12. Add 165 µl Isopropanol
13. Vortex and incubate for 10 minutes at room temp (dark)
14. Centrifuge for 10 minutes at 13,000 rpm
15. Discard the supernatant and wash the DNA pellet with 500 µl 80% ice-cold ethanol
16. Centrifuge for 2 minutes at 13,000 rpm and remove supernatant
17. Dry the DNA pellet for 5 minutes (dark)

### **Quantify samples (as per Nimblegen protocol):**

18. Rehydrate DNA pellet in 50 µl Sigma water and vortex
19. Briefly centrifuge and incubate for 5 minutes (dark), then vortex and centrifuge again
20. Measure concentration on Nanodrop - as Nucleic acid
21. Combine 34 µg Test Sample with 34 µg Reference Sample into a 1.5 ml tube. (Hybridisation can be performed with as little as 24 µg.)
22. Dry contents in SpeedVac, protect from light
23. Store samples at -20 °C until ready for hybridisation (up to 1 month)

### **Hybridisation to 2.1M arrays (as per Nimblegen protocol):**

The Alignment Oligo provided in the Nimblegen Hybridisation Kit are labeled with Cy dyes, which are sensitive to photobleaching and freeze-thawing. After thawing stock tubes for the first time, aliquot 1.2 µl of the Alignment Oligo into PCR tubes and store at -20 °C (protect from light).

24. Set the Hybridisation System to 42 °C, allow at least 3 hours for the temperature to stabilise
25. Resuspend dried sample pair in 12.3 µl of Sigma water and vortex
26. Prepare Hybridisation Master Mix:
  - ◆ 1.2 µl Alignment Oligo
  - ◆ 11.8 µl Hybridisation Component A
  - ◆ 29.5 µl 2X Hybridisation Buffer
27. Add 31.7 µl of Hybridisation Master Mix to each sample pair
28. Vortex and spin
29. Incubate at 95 °C for 5 minutes in hotblock
30. Incubate at 42 °C for at least 5 minutes or until ready to load sample
31. Vortex and spin before loading

### Prepare Mixers (as per Nimblegen protocol):

32. Remove HX1 mixer from package
33. Open the Precision Mixer Alignment Tool (PMAT, supplied with the Hybridisation System)
34. Push back the plastic spring with a thumb; place the slide in the base of the PMAT so that the barcode is readable facing outward. Make sure the slide is positioned to the rightmost and closest to you; make sure the slide is lying flat against the PMAT. Gently use the AirDuster to remove any particles from the slide
35. Snap the mixer onto the two alignment pins on the lid of the PMAT, with the end of the mixer pointing towards the hinge and the adhesive gasket facing outward
36. Use forceps to remove the backing from the adhesive gasket of the mixer and close the lid of the PMAT
37. Lift the lid by grasping the long ends of the PMAT while applying pressure with a finger through the window in the lid to free the mixer-slide assembly from alignment pins
38. Remove the mixer-slide from the PMAT and place on clean smooth dark surface
39. Rub the Mixer Brayer (supplied in the Hybridisation System Accessory Kit) over the mixer to adhere the gasket and remove any bubbles, starting in the center and rub outwards. Gasket becomes clear when fully adhered to both surfaces
40. Place the mixer-slide in the slide bay of the Hybridisation System

### Load and Hybridise Samples (as per Nimblegen protocol):

41. Using a Gilson P200 slowly load 41  $\mu$ l into the fill port until the sample starts to overflow from the vent port (avoid bubbles, keep pipette tip perpendicular, apply gentle pressure of the tip into the port to ensure a tight seal)
42. Dry sample overflow from the ports, e.g. using a cotton swap
43. Adhere a mixer port seal over both ports and press simultaneously to seal
44. Close the bay clamp
45. Turn on the Mixing Panel, set the mix mode to B, and press the mix button to start mixing. Check indicator light is green for each slide position
46. Hybridise for 20 hours at 42 °C

### Wash Hybridised Arrays (as per Nimblegen protocol):

It is important to proceed through all the washing and drying steps without interruption. We only process two arrays at a time to minimise the time the arrays are stored until scanned.

47. Prewarm 10X Wash Buffer I at 42 °C for a couple of minutes as it can precipitate before use
48. Prepare 250 ml Wash I and for each array 25 ml of Washes I, II and III according to the following table:

Solution	Wash I (250 ml)	Wash I, II and III (25 ml)
RO-water	225 ml	22.5 ml
10X Wash Buffer I, II or III	25 ml	2.5 ml
1M DTT	25 $\mu$ l	2.5 $\mu$ l

49. Prewarm 250 ml of Wash I to 42 °C and pour warm solution into shallow dish, so that the Mixer Disassembly Tool (supplied in the Hybridisation System Accessory Kit) is completely covered
50. Insert Mixer Disassembly Tool into dish and load the mixer-slide assembly into the Tool
51. Carefully peel the mixer off the slide (keeping the Mixer Disassembly Tool flat)
52. Remove the slide from the Tool and agitate the slide for 10-15 seconds in the shallow dish
53. Transfer slide into a slide container that contains Wash I (room temperature)

54. Wash for 2 minutes, shaking the container at least 20 times every 10 seconds
55. Blot off excess buffer on tissue and transfer slide into a slide container that contains Wash II (room temperature)
56. Wash for 1 minute, shaking the container at least 20 times every 10 seconds
57. Blot off excess buffer on tissue and transfer slide into a slide container that contains Wash III (room temperature)
58. Wash for 15 seconds, shaking the container at least 20 times every 10 seconds
59. Transfer the slides to a clean microscope slide box with tissue at the base and centrifuge at 1000 rpm for 5 minutes
60. Proceed immediately to scanning

### **Two-Colour Array Scanning (as per Nimblegen protocol):**

Keep arrays in the dark until you are ready to scan them.

61. Launch the GenePix software 10 minutes before scanning to allow lasers to warm
62. Place the slide in the slide carriage so that the array is facing down and the barcode end is closest to you
63. Open the Hardware Settings dialog box and select the following settings for scanning:
  - ◆ 532 PMT Gain = 650
  - ◆ 635 PMT Gain = 750
  - ◆ Power (%) = 100%
  - ◆ Pixel size ( $\mu\text{m}$ ) = 5
  - ◆ Lines to average = 1
  - ◆ Focus position ( $\mu\text{m}$ ) = 0
64. Press Preview Scan to start a preview scan of the entire slide
65. Then press the Scan Area button:
  - ◆ Move the mouse cursor to the top left of the features on the image
  - ◆ Hold down the mouse cursor and drag a rectangle around the region containing the features
  - ◆ Confirm that all features have been included within the scan area
66. Start a Scan of the array and zoom into a smaller region and adjust the 532 and 635 PMT gains until only few spots remain saturated (saturated spots are displayed white)
67. Zoom into as large a region as possible (exclude areas with high background) and select the Histogram tab:
  - ◆ Make sure that the Wavelength 532 and Wavelength 635 boxes are checked so both wavelength histograms are displayed
  - ◆ Make sure that the Log Axis box is checked
  - ◆ The red and green curves should be superimposed. If the ref curve is above the green, lower the red PMT setting or raise the green PMT setting on the Image tab.
  - ◆ The curve should end above  $1\text{e-}5$  normalized counts at the 65,000 intensity level (saturation)
  - ◆ The histogram graphs only the region of the image viewable on-screen in the image tab
68. After the PMT settings are properly adjusted, stop the current scan (do not save image)
69. Restart the scan
70. Save the images for both channels (single image .tif files) as: NNNNN\_XXXXX, where NNNNN = barcode and XXXXX = user defined text (sample name)

Proceed to NimbleScan data analysis as outlined in the Nimblegen Arrays User's Guide.

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