

Microarray Protocol for Affymetrix *In Situ* Synthesized Oligo Arrays
AfCS Procedure Protocol PP00000174
Version 1, 08/05/03

The following procedure details the preparation of biotin-labeled target samples and hybridization of these samples to an Affymetrix *in situ* synthesized oligonucleotide GeneChip array. The procedure requires a minimum of 5 µg of purified total RNA as starting material.

First-Strand cDNA Synthesis

1. Add 5 µg of total RNA to a 1.5-ml RNase-free tube, add 1 µl of 100 pmol/µl T7-(dT)₂₄ primer, and adjust volume to 12 µl/reaction with nuclease-free water.
2. Incubate at 65 °C for 10 min.
3. Centrifuge briefly at about 10,000 rpm for 10 sec and place on ice.
4. Add 4 µl of 5X first-strand cDNA buffer, 2 µl of 0.1 M dithiothreitol (DTT), and 1 µl of 10 mM dNTP mix.
5. Incubate at 42 °C for 2 min.
6. Add 1 µl of 200 U/µl SuperScript II RT.
7. Mix well by pipetting.
8. Incubate at 42 °C for 1 hr.
9. Centrifuge briefly at about 10,000 rpm for 10 sec and place on ice.

Second-Strand cDNA Synthesis

10. Add to the first strand cDNA synthesis reaction 30 µl of 5X second-strand reaction buffer, 3 µl of 10 mM dNTP mix, 1 µl of 10 U/µl *E. coli* DNA ligase, 4 µl of 10 U/µl DNA polymerase I, 1 µl of 2 U/µl RNase H, and 91 µl of nuclease-free water.
11. Incubate at 16 °C for 2 hr.
12. Add 2 µl of 5 U/µl T4 DNA polymerase.
13. Incubate at 16 °C for 5 min.
14. Add 10 µl of 0.5 M EDTA and proceed to cleanup and precipitation.

cDNA Cleanup and Precipitation

15. Add 600 µl of cDNA binding buffer (from GeneChip Sample Cleanup Module) to the synthesized cDNA.
16. Apply sample to a cDNA cleanup spin column sitting in a 2-ml collection tube and centrifuge at ≥8000 x g for 1 min.
17. Discard flow-through.
18. Replace the collection tube with another 2-ml collection tube.
19. Add 750 µl of cDNA wash buffer to the spin column and centrifuge at ≥8000 x g for 1 min.
20. Discard flow-through.
21. Centrifuge the spin column at maximum speed for 5 min with the column cap opened.

22. Transfer the column to a fresh 1.5-ml collection tube and pipette 14 μ l cDNA elution buffer directly onto the column membrane.
23. Centrifuge at $\geq 8000 \times g$ for 1 min to elute DNA.
24. At this stage, the cDNA can be stored at $-20 \text{ }^{\circ}\text{C}$.

Biotin-Labeled cRNA Synthesis

25. Add 10 μ l of sample cDNA, 4 μ l of 10X HighYield (HY) reaction buffer, 4 μ l of biotin-labeled ribonucleotides, 4 μ l of DTT, 4 μ l of RNase inhibitor mix, 2 μ l of 20X T7 RNA polymerase, and enough nuclease-free water to provide a final reaction volume of 40 μ l.
26. Briefly mix and centrifuge mixture.
27. Incubate at $37 \text{ }^{\circ}\text{C}$ for 5 hr.
28. Proceed to cleanup and quantification.

Cleanup and Quantification

29. Adjust sample volume to 100 μ l with nuclease-free water and add 350 μ l IVT cRNA binding buffer from the GeneChip Sample Cleanup Module Kit.
30. Mix well by pipetting.
31. Add 250 μ l of 100% ethanol and mix well by pipetting gently 4 to 5 times. Do not centrifuge.
32. Apply sample to an IVT cRNA cleanup spin column sitting in a 2-ml collection tube and centrifuge at $\geq 8000 \times g$ for 15 sec.
33. Transfer the column to a fresh collection tube, add 500 μ l IVT cRNA wash buffer (add ethanol before use), and centrifuge at $\geq 8000 \times g$ for 15 sec.
34. Discard flow-through and return column to same collection tube, add 500 μ l 80% ethanol, and centrifuge at $\geq 8000 \times g$ for 15 sec.
35. Centrifuge the spin column at maximum speed for 5 min with the column cap opened to dry the column membrane.
36. Transfer the column to a fresh 1.5-ml collection tube and pipette 25 μ l nuclease-free water directly onto the column membrane.
37. Centrifuge at $\geq 25,000 \times g$ for 1 min to elute RNA.
38. Dilute 0.5 to 1 μ l of the cRNA with nuclease-free water. Determine the RNA yield by spectrophotometric analysis, applying the convention that 1 OD at 260 nm equals 40 $\mu\text{g/ml}$ RNA. Be sure to check the absorbance at 260 nm and 280 nm and ensure that the A260/A280 ratio is in the 1.9 to 2.1 range.

cRNA Fragmentation

39. Add 8 μ l of 5X fragmentation buffer to 20 μg of cRNA and bring the total volume to 40 μ l with nuclease-free water.
40. Incubate at $94 \text{ }^{\circ}\text{C}$ for 35 min and place on ice.
41. Proceed to hybridization.

Array Hybridization

42. Mix 10 µg of fragmented cRNA with 3.3 µl of 3 nM control oligonucleotide B2, 10 µl of 20X eukaryotic hybridization control, 2 µl of 10 mg/ml herring sperm DNA, 2 µl of 50 mg/ml acetylated BSA, 100 µl of 2X hybridization buffer, and enough nuclease-free water to provide a final volume of 200 µl.
43. Equilibrate the probe array to room temperature before use.
44. Incubate the hybridization mixture at 99 °C for 5 min.
45. Meanwhile, fill the probe array with 160 µl of 1X hybridization buffer and incubate the probe array at 45 °C for 10 min.
46. Incubate the hybridization mixture at 45 °C for 5 min.
47. Centrifuge mixture at full speed for 5 min.
48. Remove the buffer from the probe array and fill it with 200 µl of hybridization mixture.
49. Place the filled array in a rotisserie box heated to 45 °C for 16 hr.

Array Post-Hybridization Washing

50. Prepare all buffers and staining reagents, including nonstringent wash buffer, stringent wash buffer, streptavidin-phycoerythrin (SAPE) stain solution, and antibody solution mix.
51. Turn on the Fluidics Station.
52. From the Microarray Suite, click *Run* and then *Fluidics*.
53. Prime the Fluidics Station by selecting *Protocol* and choosing *Prime* in the drop-down menu.
54. Place or change the nonstringent wash buffer in reservoir A, the stringent wash buffer in reservoir B, and the nuclease-free water in the water reservoir.
55. Click *Run* to begin priming. Close the *Fluidics* window.
56. Create an experiment file for each probe array by clicking *Run* and then *Experiments Info*.
57. Transfer the hybridization mixture from the probe array to a tube.
58. Load the probe array fully with 160 µl of nonstringent wash buffer.
59. From the *Fluidics* window, click on the drop-down menu below *Experiment* and select the correct experiment name; click on the drop-down menu below *Protocol* to select the appropriate protocol.
60. Click *Run* to begin washing and staining.
61. Follow the instructions on the monitor and insert the probe array when prompted.
62. Remove the microcentrifuge tube from the sample holder of the Fluidics Station.
63. Place a microcentrifuge tube containing 600 µl of SAPE stain solution in the sample holder.
64. Watch for instructions on the monitor and when prompted, replace the microcentrifuge tube with a microcentrifuge tube containing 600 µl of antibody solution mix.

65. Watch for instructions on the monitor and when prompted, replace the microcentrifuge tube with another microcentrifuge tube containing 600 μ l of SAPE stain solution.
66. Allow the Fluidics Station to continue until the *EJECT CARTRIDGE* message appears on the monitor, indicating the completion of the protocol.
67. Remove the probe array from the GeneChip Fluidics Station and proceed to scan the probe array using Agilent GeneArray Scanner.

Reagents and Materials

RNase-free tube, 1.5 ml: VWR Scientific; catalog no. 749510-1590

T7-(dT)₂₄ primer: Genset Oligo; Primer sequence:

5'- GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄ - 3'

SuperScript II RT, 200 U/ μ l: Invitrogen Life Technologies; catalog no. 18064014; includes

First-strand cDNA buffer, 5X

Dithiothreitol (DTT), 0.1 M

dNTP mix, 10 mM: Invitrogen Life Technologies; catalog no. 18427013

Second-strand reaction buffer, 5X: Invitrogen Life Technologies; catalog no. 10812014

E. coli DNA ligase, 10 U/ μ l: Invitrogen Life Technologies; catalog no. 18052019

DNA polymerase I, 10 U/ μ l: Invitrogen Life Technologies; catalog no. 18010025

RNase H, 2 U/ μ l: Invitrogen Life Technologies; catalog no. 18021071

T4 DNA polymerase, 5 U/ μ l: Invitrogen Life Technologies; catalog no. 18005025

EDTA, 0.5 M, pH 8.0: Sigma-Aldrich; catalog no. E7889

GeneChip Sample Cleanup Module: Affymetrix; catalog no. 900371; includes

cDNA cleanup spin columns

cDNA binding buffer

cDNA wash buffer, 6 ml concentrate

cDNA elution buffer

IVT cRNA cleanup spin columns

IVT cRNA binding buffer

IVT cRNA wash buffer, 5 ml concentrate

Fragmentation buffer, 5X

Collection tubes, 1.5 ml

Collection tubes, 2 ml

BioArray HighYield RNA Transcript Labeling Kit: Affymetrix; catalog no. 900182; includes

- HighYield (HY) reaction buffer, 10X
- Biotin-labeled ribonucleotides, 10X
- Dithiothreitol (DTT), 10X
- RNase inhibitor mix, 10X
- T7 RNA polymerase, 20X

Ethanol, 100%: Aaper Alcohol; Pure Ethyl Alcohol USP 200 Proof

Ethanol, 80%: AfCS Solution Protocol ID PS00000028

GeneChip Eukaryotic Hybridization Control Kit: Affymetrix; catalog no. 900299
Control oligonucleotide B2, 3 nM
Eukaryotic hybridization control, 20X

Herring sperm DNA: Promega Corporation; catalog no. D1811

Bovine serum albumin (BSA), acetylated, 50 mg/ml: Invitrogen Life Technologies; catalog no. 15561020

Hybridization buffer, 2X: AfCS Solution Protocol ID PS00000545

Hybridization buffer, 1X: AfCS Solution Protocol ID PS00000546

Nonstringent wash buffer: AfCS Solution Protocol ID PS00000549

Stringent wash buffer: AfCS Solution Protocol ID PS00000548

Streptavidin-phycoerythrin stain solution (SAPE stain solution): AfCS Solution Protocol ID PS00000551

Antibody solution mix: AfCS Solution Protocol ID PS00000553

GeneChip Fluidics Station: Affymetrix, Inc.

Agilent GeneArray Scanner: Agilent Technologies

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