

Transcript Profiling by Microarray Analysis—Agilent
AfCS Procedure Protocol PP00000019
Version 1, 1/11/02

The following procedure details the preparation of fluorescently labeled target samples and hybridization of these samples to a microarray of Agilent inkjet-deposited cDNAs. 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 in nuclease-free water 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 70 °C for 10 min.
3. Centrifuge briefly and place on ice.
4. Add 4 µl of 5X first strand cDNA buffer, 2 µl of 0.1 M dithiothreitol (DTT), 1 µl of 10 mM dNTP mix, and 1 µl of 200 U/µl SuperScript II RT.
5. Mix well by pipetting gently 4 to 5 times.
6. Incubate at 42 °C for 1 hr.
7. Centrifuge briefly and place on ice.

Second Strand cDNA Synthesis

8. Add to the first strand cDNA synthesis reaction, 30 µl of 5X second strand buffer; 3 µl of 10 mM dNTP mix; 1 µl of 10 U/µl E. coli DNA ligase; 4 µl of 10 U/µl E. coli DNA polymerase I; 1 µl of 2 U/µl RNase H; and 91 µl of nuclease-free water.
9. Incubate at 16 °C for 2 hr.
10. Add 2 µl of 5 U/µl T4 DNA polymerase.
11. Incubate at 16 °C for 5 min.
12. Add 7.5 µl of total RNA digestion solution.
13. Incubate at 65 °C for 10 min.
14. Centrifuge briefly and place on ice.

cDNA Cleanup and Precipitation

15. Prepare a Phase Lock Gel tube by centrifuging at 12,000 x g for 30 sec.
16. Add 162 µl of 25:24:1 phenol:chloroform:isoamyl alcohol to the synthesized cDNA.
17. Mix well by pipetting gently 4 to 5 times.
18. Transfer the entire cDNA mixture to the Phase Lock Gel tube.
19. Centrifuge at 12,000 x g for 3 min.
20. Transfer the aqueous upper phase to a fresh 1.5-ml RNase-free tube.
21. Add 75 µl of 7.5 M ammonium acetate and 375 µl of -20 °C 100% ethanol.
22. Mix well by pipetting gently 4 to 5 times.
23. Centrifuge immediately at 14,000 x g for 20 min at room temperature.
24. Remove supernatant and wash pellet with 0.5 ml -20 °C 80% ethanol.
25. Centrifuge at 14,000 x g for 5 min at room temperature.
26. Remove the supernatant, being careful not to disturb the pellet, which may be loose.

27. Wash pellet with 0.5 ml of $-20\text{ }^{\circ}\text{C}$ 100% ethanol.
28. Centrifuge at 14,000 x g for 5 min at room temperature.
29. Remove the supernatant, again being careful not to disturb the pellet.
30. Air-dry the pellet for approximately 10 min.
31. Resuspend the dried pellet in 16 μl of nuclease-free water. At this stage, the cDNA can be stored at $-20\text{ }^{\circ}\text{C}$.

In vitro Transcription

32. Place the RNA polymerase enzyme mix from the Ambion MEGAscript T7 Kit on ice.
33. Vortex the 10X reaction buffer and 4 ribonucleotide solutions until they are completely thawed. Place the ribonucleotides on ice and the 10X reaction buffer at room temperature.
34. Centrifuge all reagents briefly prior to assembling the reaction to prevent loss of material.
35. Mix the reaction components at room temperature in the following order: 4 μl of ATP solution; 4 μl of CTP solution; 4 μl of GTP solution; 4 μl of UTP solution; 4 μl of 10X reaction buffer; 16 μl of cDNA template; and 4 μl of enzyme mix.
36. Mix well by pipetting gently 4 to 5 times.
37. Incubate at $37\text{ }^{\circ}\text{C}$ for 4 hr.
38. Add 1 μl of 2 U/ μl DNase I and mix well by pipetting gently 4 to 5 times.
39. Incubate at $37\text{ }^{\circ}\text{C}$ for 15 min.

Cleanup and Quantification of in vitro Transcribed RNA

40. Add 60 μl of nuclease-free water to the MEGAscript reaction.
41. Prepare a Phase Lock Gel tube by centrifuging at 12,000 x g for 30 sec.
42. Add 100 μl of 25:24:1 phenol:chloroform:isoamyl alcohol to the synthesized cRNA.
43. Mix well by pipetting gently 4 to 5 times.
44. Transfer the entire cRNA mixture to the Phase Lock Gel tube.
45. Centrifuge at 12,000 x g for 3 min.
46. Transfer the aqueous upper phase to a fresh 1.5-ml tube.
47. Adjust sample volume to 100 μl (if necessary) with nuclease-free water and add 350 μl of RLT buffer from RNeasy Mini Kit.
48. Mix well by pipetting gently 4 to 5 times.
49. Add 250 μl of 100% ethanol and mix well by pipetting gently 4 to 5 times. Do not centrifuge.
50. Apply sample to an RNeasy mini spin column sitting in a 2-ml collection tube and centrifuge at 8,000 x g for 15 sec.
51. Transfer the column to a fresh collection tube, add 500 μl of RPE buffer, and centrifuge at 8,000 x g for 15 sec.
52. Discard flow-through and return column to same collection tube, add 500 μl of RPE buffer, and centrifuge at maximum speed for 2 min to dry the column membrane.
53. Transfer the column to a fresh 1.5-ml collection tube and pipette 30 μl of nuclease-free water directly onto the column membrane.

54. Centrifuge at 8,000 x g for 1 min to elute RNA.
55. Remove 0.5 μ l of eluted RNA to a fresh tube and add 49.5 μ l of nuclease-free water. Determine the RNA yield by spectrophotometric analysis, applying the convention that 1 OD at 260 nm equals 40 μ g/ml RNA.

Fluorescent Labeling of the Target Samples

56. Mix 3 to 10 μ g of in vitro transcribed antisense RNA target (for both reference and test samples) and 2 μ l of 3 μ g/ μ l random primers and adjust volume to 14 μ l/reaction with nuclease-free water.
57. Incubate at 70 °C for 10 min and place on ice.
58. Add 6 μ l of 5X first strand cDNA buffer; 3 μ l of 0.1 M DTT; 0.6 μ l of 50X dNTP (low dTTP) mix (final nucleotide concentrations: 500 μ M of dATP/dCTP/dGTP, 200 μ M of dTTP); 1.4 μ l of nuclease-free water; 3 μ l of Cy5-dUTP (or Cy3-dUTP); and 2 μ l of 200 U/ μ l SuperScript II RT.
59. Mix well by pipetting gently 4 to 5 times.
60. Incubate at 42 °C for 2 hr.
61. Add 500 μ l of Tris-EDTA buffer, pH 8.0 (T₁₀E₁), and transfer the mixture to a Microcon-30 filter.
62. Centrifuge at 10,000 x g for 9 min at room temperature. Check volume of concentrated solution and, if necessary, centrifuge further to obtain a final volume of 10 to 20 μ l.
63. Place a fresh tube on top of filter unit, invert and centrifuge at 10,000 x g for 2 min to recover target.
64. Mix recovered Cy3 (reference) and Cy5 (test) labeled targets in a Microcon-30 filter containing 500 μ l T₁₀E₁.
65. Centrifuge at 10,000 x g for 9 min at room temperature. Check volume of concentrated solution and, if necessary, centrifuge further to obtain a final volume of 7.5 μ l.
66. Place a fresh tube on top of filter unit, invert, and centrifuge at 10,000 x g for 2 min to recover target.
67. Transfer 7.5 μ l of target to a fresh tube and add 2.5 μ l of deposition control target, 2.5 μ l of 1 μ g/ μ l mouse Cot-1 DNA, and 12.5 μ l of 2X deposition hybridization buffer.
68. Heat the target preparation at 98 °C for 2 min.
69. Centrifuge at 10,000 x g for 2 min to collect condensation.

Array Hybridization

70. Pipette the fluorescently labeled target (25 μ l) onto the slide surface.
71. Place a 24 x 30 mm coverslip on top of the slide, being careful not to generate any air bubbles.
72. Place the slide in a hybridization chamber and submerge the chamber in a 60 °C waterbath overnight.

Array Post-hybridization Washing

73. Disassemble hybridization chamber with array surface facing up.

74. Immerse the slide in 100 ml of wash solution A1 at 42 °C in a Coplin jar until the coverslip moves freely away from the slide (approximately 3 min).
75. Remove coverslip with forceps and decant wash solution.
76. Add 100 ml of wash solution A1 and incubate at room temperature for 3 min.
77. Decant wash solution, add 100 ml of wash solution A2, and incubate at room temperature for 2 min.
78. Repeat step 77.
79. Decant final wash solution, place slide in centrifuge rack, and centrifuge at 110 x g for 5 min to dry.
80. Microarray slide is now ready for scanning.

Reagents and Materials

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

T7-(dT)₂₄ primer: Genset Oligos; catalog no. 50 OD HPLC T7-(dT)₂₄ primer
[5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)₂₄]

Nuclease-free water: Ambion; catalog no. 9930

First strand cDNA buffer, 5X: Invitrogen; catalog no. 18064014, part no. Y00146

Dithiothreitol (DTT), 0.1 M: Invitrogen; catalog no. 15508013

dNTP mix, 10 mM: AfCS Solution Protocol ID PS00000022

SuperScript II RNase H Reverse Transcriptase (SuperScript II RT), 200 U/μl:
Invitrogen; catalog no. 18064014

Second strand buffer, 5X: Invitrogen; catalog no. 10812014

E. coli DNA ligase, 5 U/μl: New England Biolabs; catalog no. M0205L

E. coli DNA polymerase I, 10 U/μl: New England Biolabs; catalog no. M0209L

RNase H, 2 U/μl: Invitrogen; catalog no. 18021014

T4 DNA polymerase, 5 U/μl: Invitrogen; catalog no. 18005025

Total RNA digestion solution: AfCS Solution Protocol ID PS00000059

Phase Lock Gel tube: Eppendorf AG; catalog no. 0032 005.055

Phenol:chloroform:isoamyl alcohol, 25:24:1: Invitrogen; catalog no. 15593031

Ammonium acetate, 7.5 M: AfCS Solution Protocol ID PS00000017

Ethanol, 100%: Aaper Alcohol; catalog no. Ethyl Alcohol, 200

Ethanol, 80%: AfCS Solution Protocol ID PS00000028

MEGAscript T7 Kit: Ambion; catalog no. 1334

Reaction buffer, 10X

RNA polymerase enzyme mix

ATP solution, 75 mM

CTP solution, 75 mM

GTP solution, 75 mM

UTP solution, 75 mM

DNase I, 2 U/ μ l

RNeasy Mini Kit: Qiagen; catalog no. 74104

RLT buffer

RNeasy mini spin columns

Collection tube, 2 ml

RPE buffer

Collection tube, 1.5 ml

Random primers: Invitrogen; catalog no. 48190011

dNTP (low dTTP) mix, 50X: AfCS Solution Protocol ID PS00000023

Cy5-dUTP: Amersham Biosciences; catalog no. PA55022

Cy3-dUTP: Amersham Biosciences; catalog no. PA53022

Tris-EDTA buffer, pH 8.0, 10 mM, 1 mM ($T_{10}E_1$, pH 8.0): AfCS Solution Protocol ID PS00000065

Microcon-30 filter: Amicon; catalog no. 42410

Deposition control target: Operon; catalog no. SP300

Mouse Cot-1 DNA, 1 μ g/ μ l: Invitrogen; catalog no. 18440016

Deposition hybridization buffer, 2X: Agilent Technologies; catalog no. G2558A

Coverslips, 24 x 30 mm: VWR Scientific; catalog no. 48393-092

Hybridization chamber: ArrayIt; catalog no. AHC

Wash solution A1: AfCS Solution Protocol ID PS00000070

Coplin staining jar: VWR Scientific; catalog no. 25460-000

Wash solution A2: AfCS Solution Protocol ID PS00000071

Slide centrifuge rack: Thermo Shandon; catalog no. 113

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