Microarray Protocol for Agilent Inkjet-Deposited Presynthesized Oligo Arrays—Aminoallyl Method

AfCS Procedure Protocol PP00000184

Version 1, 10/20/03

The following procedure details the preparation of fluorescently labeled target samples (aminoallyl method) and hybridization of these samples to a microarray of Agilent inkjet-deposited presynthesized oligonucleotides. The procedure requires a minimum of 3 μg of purified total RNA as starting material.

cDNA Synthesis

1. Add 3 μg of total RNA in nuclease-free water to a 1.5-ml RNase-free tube in a volume less than 11 μl, add 1 μl of T7 oligo dT primer, and adjust total reaction volume to 12 μl with nuclease-free water.
2. Incubate at 70 °C for 10 min.
3. Briefly centrifuge at 10,000 rpm for 30 sec and place on ice for at least 5 min.
4. Prepare and place on ice an 8 μl/reaction mixture containing the following in the order listed: 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 SuperScript reverse transcriptase.
5. Add 8 μl of the mixture to each sample.
6. Incubate at 42 °C for 1 hr.
7. Briefly centrifuge at 10,000 rpm for 30 sec 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 5 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 10 U/μl T4 DNA polymerase.
11. Mix well and 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. Briefly centrifuge at 10,000 rpm for 30 sec 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 160 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 5 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 500 μl of –20 °C 100% ethanol.

22. Mix well by pipetting gently 4 to 5 times.
23. Centrifuge immediately at 12,000 x g for 20 min at room temperature.
24. Remove supernatant and wash pellet with 500 µl of –20 °C 80% ethanol.
25. Centrifuge at 12,000 x g for 5 min at room temperature.
26. Remove supernatant; be careful not to disturb pellet.
27. Wash pellet with 500 µl of –20 °C 100% ethanol.
28. Centrifuge at 12,000 x g for 5 min at room temperature.
29. Remove supernatant; be careful not to disturb pellet.
30. Air dry pellet for approximately 10 min at room temperature.
31. Resuspend dried pellet in 15 µl of nuclease-free water. At this stage, the cDNA can be stored at –20 °C.

Aminoallyl-UTP (aaUTP) Antisense RNA (aRNA) Synthesis

32. Place the RNA polymerase enzyme mix from the 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 and/or contamination of material.
35. Add to the 15 µl of cDNA template the following, in the order listed: 4 µl of 75 mM ATP solution; 4 µl of 75 mM CTP solution; 4 µl of 75 mM GTP solution; 2 µl of 75 mM UTP solution; 4 µl of 10X reaction buffer; 3 µl of 50 mM aaUTP; and 4 µl of enzyme mix.
36. Mix well by pipetting gently 4 to 5 times.
37. Incubate at 37 °C for 6 hr.
38. Add 2 µl of DNase I and mix well by pipetting gently 4 to 5 times.
39. Incubate at 37 °C for 15 min.

aRNA Cleanup and Quantification

40. Add 60 µl of nuclease-free water to the MEGAscript reaction.
41. Add 350 µl of RLT buffer from RNeasy Mini Kit.
42. Mix well by pipetting gently 4 to 5 times.
43. Add 250 µl of 100% ethanol and mix well by pipetting gently 4 to 5 times. Do not centrifuge.
44. Apply sample to an RNeasy mini spin column sitting in a 2-ml collection tube and centrifuge at ≥8000 x g for 15 sec.
45. Transfer column to a fresh collection tube, add 500 µl of RPE buffer, and centrifuge at ≥8000 x g for 15 sec.
46. Discard flow-through and return column to same collection tube, add 500 µl of RPE buffer, and centrifuge at maximum speed for 2 min.
47. Transfer column to a fresh 1.5-ml collection tube and pipette 36 µl of elution buffer directly onto column membrane.
48. Centrifuge at ≥8000 x g for 2 min to elute RNA.

49. Dilute 1 μl of eluted RNA with 49 μl of nuclease-free water. Determine the RNA yield by spectrophotometric analysis, applying the convention that 1 OD at 260 nm equals 40 μg/ml RNA.
50. At this point, aRNA can be stored at -80 °C.

Coupling Reaction

51. Mix 10 μg of aRNA target with 2 μl of 0.5 M sodium bicarbonate, pH 9.5, and 10 μl of mono-Cy3 or mono-Cy5 solution, and adjust volume to 20 μl/reaction with nuclease-free water.
52. Incubate at room temperature for 1 hr in the dark.
53. Add 80 μl of nuclease-free water.
54. Add 350 μl of RLT buffer from RNeasy Mini Kit.
55. Mix well by pipetting gently 4 to 5 times.
56. Add 250 μl of 100% ethanol and mix well by pipetting gently 4 to 5 times. Do not centrifuge.
57. Apply sample to an RNeasy mini spin column sitting in a 2-ml collection tube and centrifuge at ≥8000 x g for 15 sec.
58. Transfer column to a fresh collection tube, add 500 μl of RPE buffer and centrifuge at ≥8000 x g for 15 sec.
59. Discard flow-through and return column to same collection tube, add 500 μl of RPE buffer, and centrifuge at maximum speed for 2 min.
60. Transfer column to a fresh 1.5-ml collection tube and pipette 40 μl of elution buffer directly onto column membrane.
61. Centrifuge at ≥8000 x g for 2 min to elute RNA.
62. Dilute 1 μl of eluted RNA with 49 μl of nuclease-free water. Determine the RNA yield by spectrophotometric analysis, applying the convention that 1 OD at 260 nm equals 40 μg/ml RNA.
63. Determine the amount of dye incorporation by measuring the OD at 550 nm (Cy3) and 650 nm (Cy5). Use the formula below to estimate the number of dye molecules incorporated per 1000 nucleotides (nt) of labeled aRNA.

No. dye molecules/1000 nt = (ODdye/OD260)*(9010/dye extinction coefficient)*1000

Absorbance (nm)	Dye type	Extinction coefficient
550	Cy3	150,000
650	Cy5	250,000

Fragmentation

64. Mix 5 μg of labeled aRNA target with 5 μl of 25X fragmentation buffer, 3 μl of deposition control target, and 5 μl of mouse cot-1 DNA, and adjust volume to 125 μl/reaction with nuclease-free water.
65. Incubate at 60 °C for 30 min.
66. Briefly centrifuge at 10,000 rpm for 30 sec and place on ice.
67. Add 130 μl of 2X hybridization buffer to end the fragmentation reaction.

Array Hybridization

68. Wash hybridization chambers and accessories with RNase Zap.
69. Assemble hybridization chamber as follows:
    a. Place slide (active side or bar code facing upward and to the right) onto base of chamber.
    b. Place cleaned plastic backing on top of slide.
    c. Place gasket on top of plastic backing.
    d. Place small stainless steel cover flat on gasket (all screws should be assembled but not fastened tightly).
    e. Hand tighten screws and use a flat-head screwdriver to ensure screws are secure.
    f. Place a clean RNase-free rubber septum into each of the four ports.
    g. Push septa tightly into the ports.
70. Insert a 25-gauge needle completely into one of the two septa. The needle is used as a vent to release air as the hybridization mixture is injected into the chamber.
71. Attach a 25-gauge needle to a clean 1-ml syringe.
72. Draw up the hybridization mixture.
73. Carefully inject the hybridization mixture into the second septum.
74. Incubate chamber on a rotator at 60 °C for 17 hr.

Washing

75. Prepare and warm all washing solutions to their desired temperature 1 hr prior to washing.
76. Submerge the hybridization chamber in 42 °C wash solution B1 and disassemble hybridization chamber with array surface facing up.
77. Immediately transfer the slide into a Coplin jar containing 42 °C wash solution B1 and incubate at 42 °C for 5 min.
78. Transfer the slide into another Coplin jar containing 42 °C wash solution B1 and incubate at 42 °C for 5 min.
79. Transfer the slide into another Coplin jar containing wash solution B2 and incubate at room temperature for 5 min.
80. Transfer the slide into another Coplin jar containing wash solution B3 and incubate at room temperature for 3 min.
81. Repeat step 80.
82. Place slide in centrifuge rack and centrifuge at 950 rpm for 5 min to dry.
83. 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 Oligo; catalog no. 50 OD HPLC T7-$(dT)_{24}$ primer

Superscript II reverse transcriptase (RT), 200 U/μl: Invitrogen; catalog no. 18064014;
includes
- First-strand cDNA buffer, 5X
- Dithiothreitol (DTT), 0.1 M

dNTP mix, 10 mM: Invitrogen; catalog no. 10297018;
includes
- dATP, 10 mM
- dCTP, 10 mM
- dGTP, 10 mM
- dTTP, 10 mM

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. 18021-014

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

Total RNA digestion solution; AfCS Solution Protocol ID PS00000059

Phase lock gel tube, heavy, 0.5 ml: Eppendorf AG; catalog no. 62111-396

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

Ammonium acetate, 7.5 M: Sigma-Aldrich; catalog no. A2706

Ethanol, 100%: Aaper Alcohol; 030801

Ethanol, 80%: AfCS Solution Protocol ID PS00000028

MEGAscript T7 Kit: Ambion; catalog no. 1334;
includes
- 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/μl

aaUTP: Ambion; catalog no. 8437

RNeasy Mini Kit: Qiagen; catalog no. 74104;
includes
- RLT buffer
- RNeasy mini spin columns
- Collection tube, 2 ml
- RPE buffer
- Collection tube, 1.5 ml
- Elution buffer

Sodium bicarbonate, pH 9.5, 0.5 M: AfCS Solution Protocol ID PS00000565

Mono-Cy3 solution: AfCS Solution Protocol ID PS00000567

Mono-Cy5 solution: AfCS Solution Protocol ID PS00000566

In situ Hybridization Kit plus: Agilent Technologies; catalog no. 5184-3568
- Fragmentation buffer, 25X
- Deposition hybridization buffer, 2X

Deposition control target: Operon; catalog no. SP300

Mouse cot-1 DNA, 1 μg/μl: Invitrogen; catalog no. 18440016

Hybridization chamber, 11K/16.2K: Agilent Technologies; catalog no. G2533A

Backings, septa, and gaskets: Agilent Technologies; catalog no. G2533-65002

RNase Zap: Ambion; catalog no. 9780, 9782

Wash solution B1: AfCS Solution Protocol ID PS00000541

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

Wash solution B2: AfCS Solution Protocol ID PS00000542

Wash solution B3: AfCS Solution Protocol ID PS00000543

Slide centrifuge rack: Thermo Shandon; catalog no. 113

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Date: 10/20/03

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