

Microarray Protocol for Agilent Inkjet-Deposited Presynthesized Oligo Arrays

**AfCS Procedure Protocol PP00000179
Version 1, 08/05/03**

The following procedure details the preparation of fluorescently labeled target samples 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 4.5 µl, add 5 µl of T7 promoter primer (from Agilent Fluorescent Linear Amplification Kit), and adjust total reaction volume to 9.5 µl with nuclease-free water.
2. Incubate at 65 °C for 10 min.
3. Briefly centrifuge at about 10,000 rpm for 10 sec and place on ice.
4. Prepare and place on ice a 10.5 µl/reaction mixture containing, in the following order, 4 µl of 5X first-strand reaction buffer; 2 µl of 0.1 M dithiothreitol (DTT); 1 µl of 10 mM dNTP mix; 1 µl of random hexamers; 1 µl of MMLV-RT; 0.5 µl of RNaseOUT; and 1 µl of 0.3% triton X-100.
5. Add 10.5 µl of the mixture to each sample.
6. Incubate at 40 °C for 4 hr.
7. Incubate at 65 °C for 15 min.
8. Place on ice for 5 min.
9. Briefly centrifuge at about 10,000 rpm for 10 sec.

Fluorescent cRNA Synthesis

10. Add 4 µl of cyanine 3-dCTP (6 mM) or 4 µl of cyanine 5-dCTP (4 mM).
11. Prepare and place on ice a 56 µl/reaction mixture containing, in the following order, 20.1 µl of nuclease-free water; 20 µl of 4X transcription buffer; 6 µl of 0.1 M DTT; 8 µl of NTP mix; 0.5 µl of RNaseOUT; 0.6 µl of inorganic pyrophosphatase; and 0.8 µl of T7 RNA polymerase.
12. Add 56 µl of the mixture to each sample.
13. Incubate at 40 °C for 3 hr.

cRNA Precipitation and Cleanup

14. Adjust sample volume to 100 µl (if necessary) with nuclease-free water and add 350 µl RLT buffer from RNeasy Mini Kit.
15. Mix well by pipetting gently 4 to 5 times.
16. Add 250 µl 100% ethanol and mix well by pipetting gently 4 to 5 times. Do not centrifuge.
17. Apply sample to an RNeasy mini spin column sitting in a 2-ml collection tube and centrifuge at ≥8000 x g for 15 sec.
18. Transfer the column to a fresh collection tube, add 500 µl RPE buffer, and centrifuge at ≥8000 x g for 15 sec.

19. Discard flow-through and return column to same collection tube, add 500 μ l RPE buffer, and centrifuge at maximum speed for 2 min to dry the column membrane.
20. Transfer the column to a fresh 1.5-ml collection tube and pipette 14 μ l nuclease-free water directly onto the column membrane.
21. Centrifuge at $\geq 8000 \times g$ for 1 min to elute RNA.

cRNA Quantification

22. Prepare a 1:50 dilution of the labeled cRNA using nuclease-free water (i.e., mix 1 μ l of labeled cRNA with 49 μ l of nuclease-free water).
23. Measure the OD at 260 nm.
24. Calculate concentration by applying the 1 OD at 260 nm equals 40 μ g/ml RNA formula.

Hybridization

25. Combine 10 μ g of cyanine 3-dCTP-labeled cRNA with 10 μ g of cyanine 5-dCTP-labeled cRNA in a total volume of 7.5 μ l.
26. Add 2.5 μ l of mouse Cot-1 DNA, 2.5 μ l of deposition control target SP300, and 12.5 μ l of 2X deposition hybridization buffer.
27. Denature in boiling water for 2 min.
28. Allow sample to cool at room temperature for 2 min.
29. Centrifuge briefly at about 10,000 rpm for 10 sec.
30. Pipette the fluorescently labeled target (25 μ l) onto the slide surface.
31. Place a 24 x 30 mm cover slip on top of the slide; be careful not to generate any air bubbles.
32. Place the slide in a hybridization chamber and submerge the chamber in a 65 $^{\circ}$ C water bath overnight.

Washing

33. Prepare and warm all washing solutions to their desired temperature 1 hr prior to washing.
34. Disassemble hybridization chamber with array surface facing up.
35. Immerse the slide in 100 ml of wash solution B1, warmed to 42 $^{\circ}$ C, in a Coplin jar until the cover slip moves freely away from the slide (approximately 3 min).
36. Remove cover slip with forceps and decant washing solution.
37. Move the slide to another jar filled with 100 ml of wash solution B1, warmed to 42 $^{\circ}$ C, and shake at room temperature for 3 min.
38. Move the slide to another jar filled with 100 ml of room temperature wash solution B2 and shake at room temperature for 3 min.
39. Repeat step 38.
40. Move the slide to another jar filled with 100 ml of room temperature wash solution B3 and shake at room temperature for 3 min.
41. Repeat step 40.
42. Place slide in centrifuge rack and centrifuge at 950 rpm for 5 min to dry.
43. The microarray slide is now ready for scanning.

Reagents and Materials

RNase-free tube, 1.5 ml: VWR Scientific; catalog no. KT749510-1590

Agilent Fluorescent Linear Amplification Kit; Agilent Technologies; catalog no. G2554A; includes

- T7 promoter primer, 100 μ l
- First-strand reaction buffer, 5X, 80 μ l
- Dithiothreitol (DTT), 0.1 M, 160 μ l
- dNTP mix, 10 mM, 20 μ l
- Random hexamers, 200 ng/ μ l, 20 μ l
- MMLV-RT, 200 U/ μ l, 20 μ l
- RNaseOUT, 40 U/ μ l, 20 μ l
- Transcription buffer, 4X, 400 μ l
- NTP mix, 160 μ l
- Inorganic pyrophosphatase, 12 μ l
- T7 RNA polymerase, 2500 U/ μ l, 16 μ l
- Lithium chloride, 4 M, 1.6 ml

Triton X-100, 0.3%: AfCS Solution Protocol ID PS00000544

Cyanine 3-dCTP: PerkinElmer/NEN Life Sciences; catalog no. NEL576

Cyanine 5-dCTP: PerkinElmer/NEN Life Sciences; catalog no. NEL577

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

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

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

Deposition control target: Operon Technologies; catalog no. SP300

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

Cover slips, 24 x 30 mm: VWR Scientific; catalog no. 48396-104

Hybridization chamber: TeleChem International, Inc.; catalog no. AHC

Wash solution B1: AfCS Solution Protocol ID PS00000541

Wash solution B2: AfCS Solution Protocol ID PS00000542

Wash solution B3: AfCS Solution Protocol ID PS00000543

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

Slide centrifuge rack: Thermo Shandon; catalog no. 113

Author: Yun Anna Cao/Sangdun Choi

Date: 08/05/03

Approved: Mel Simon