OpArray™ Protocol

When planning or preparing for an experiment, please read through this protocol completely before proceeding. When conducting your experiment, remove only the OpArrays you plan to use immediately and immediately return the remaining OpArrays to the pouch, leaving the desiccant pack in place. For added protection, OpArrays may be kept in their storage pouch in a desiccator.

OpArray Storage

OpArrays are printed on epoxide slides, pre-processed and supplied ready-to-use.

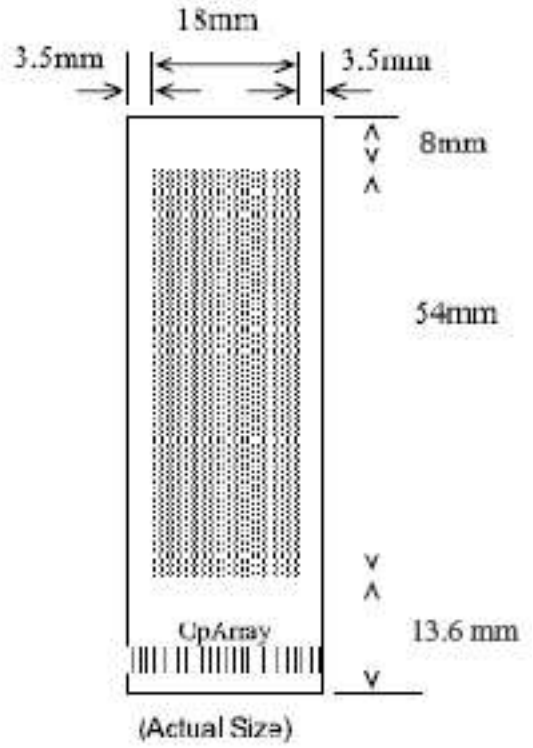
- The microarray slides should be stored in their original container at room temperature in dry conditions (preferably desiccated) and protected from light.
- The slide box containing OpArrays is shipped in a re-sealable storage pouch containing desiccant which is ideal for continued storage.
- Both the storage pouches and slide storage boxes are manufactured from materials that minimize out-gassing and effectively block exposure to foreign contaminants that can elevate background levels in your experiments.
- When properly stored, OpArrays will remain hybridization competent for 4 months or more.

Array Position on OpArray Slides

OpArrays are printed on the same side as the barcode (graphic to the right). The array area is 18mm wide, 54mm long, and is located 8mm from the top of the slide (barcode at bottom). The size and location are compatible with standard 22mm x 60mm cover slips and other equipment for handling microarrays printed on this substrate size. A list of previously tested equipment can be found in Appendix A.

Technical Note: *To reduce possible sources of experimental variation, which may effect experimental results, Microarrays Inc strongly advises OpArray users to gather completed OpArray experimental data by scanning the OpArrays as soon as practical once the experiment is completed. Please schedule your experiments appropriately.*

Degradation of fluorescent dyes is reported post-hybridization; these effects are attributed to a variety of sources. There are several reports regarding reduction of Cy5 signal intensity relative to Cy3 due to the concentration of atmospheric ozone above 5 ppb in the atmosphere. The major consideration in preventing this effect seems to be in the transition from a wet to dry array surface. This protocol is designed to minimize these effects.



Isolation of Total RNA

Total RNA can be isolated employing commonly described procedures (such as Sambrook et al., 2000). However, depending on the organism and tissue type, the method of choice may require different modifications. Microarrays Inc recommends selecting a procedure that is best suited for the tissue type that you are working with. If you are having problems isolating suitable RNA samples, you may wish to refer to the collection of RNA isolation methods described by Farrell (2005).

Total RNA Cleanup

After isolating total RNA using the method of your choice, a subsequent RNA purification helps to remove most of the inhibitory contaminants; such as organic (phenol),and cytosolic (polysaccharide, polyphenols). Microarrays Inc recommends using Qiagen's RNeasy MinElute kit to purify total RNA samples. This method works consistently well for RNA samples isolated from a wide range of tissue types such as brain, kidney, liver, testis, and ovary from human, mouse and rat, *Arabidopsis* seedlings, roots, leaves, flower, Maize leaves, roots, and endosperm, rice leaves and roots, and the leaves of *Medicago truncatula* and *Vitis vinifera* (grape). Additional details concerning the capacity of the columns and other technical details can be found in the Qiagen RNeasy Mini Handbook.

Materials

- RNeasy MinElute Column (Qiagen Cat# 74204)
- Agilent RNA 6000 Nano LabChip Kit
- Refrigerated microcentrifuge
- RNAase-free microfuge tubes and tips
- 100% Ethanol
- DEPC-treated H_2O
- 80% Ethanol (prepared with DEPC treated water)

Method

Note: *Buffer RPE is supplied with the RNeasy MinElute kit as a concentrate. Ensure that ethanol was added prior to use.*

1. Preheat 50 μL RNAase-free DEPC-treated H_2O to 55°C.
2. Adjust sample (10 μg total RNA) to a volume of 100 μL with RNAase-free water.
3. Add 350 μL RLT buffer and mix thoroughly by pipetting.
4. Add 250 μL 100% ethanol to the sample, and mix thoroughly by pipetting.
5. Prepare the RNeasy MinElute spin column for use by placing it in a 2 mL collection tube.
6. Add the sample to the RNeasy MinElute and spin column. Close the tube gently.
7. Centrifuge for 1 minute at 10,000xg.
8. Discard the liquid from the 2 mL collection tube.
9. Pipette 500 μL RPE buffer to the RNeasy MinElute spin column. Close the tube gently.
10. Centrifuge for 1 minute at 10,000xg.
11. Discard the liquid from the 2 mL collection tube.
12. Add 500 μL 80% ethanol to the RNeasy MinElute spin column. Close the tube gently.
13. Centrifuge for 1 minute at 10,000xg.
14. Discard the liquid from the 2 mL collection tube.
15. Close the RNeasy MinElute spin column.
16. Centrifuge the spin column at 10,000xg for 3-5 minutes.
17. Discard the 2 mL collection tube.
18. Transfer the RNeasy MinElute spin column to a new 2 mL or 1.5 mL RNAase-free microfuge tube.

19. Pipette 40 μL DEPC-treated H_2O (pre-heated to 55°C in step 1) directly onto the center of the membrane. Close the tube gently.
20. Incubate at room temperature (RT) for 2 minutes.
21. Centrifuge for 1 minute at maximum speed to elute.
22. Immediately place the RNA solution on ice after elution.
    Note: *Since RNA samples are labile it is important to keep RNA solutions on ice after elution.*
23. Measure the RNA amount using a NanoDrop.
24. Analyze the total RNA quality of your sample using a Bioanalyzer or Experion follow the manufacturer's directions to operate the instrument.

Total RNA Analysis using the Bioanalyzer or Experion

Only samples that have a large percentage of intact RNA will produce a uniform amplification. Degraded samples will produce unpredictable RNA amplification which may cause variation in subsequent microarray hybridization experiments. Therefore, we insist that you check the RNA quality before proceeding to the next step. Microarrays Inc recommends the use of the Bioanalyzer (Agilent Technologies) or Experion (BioRad) to verify the quality of the total RNA sample. Typically, these instruments require very small quantity of RNA for the analysis (100-150 ng/sample).

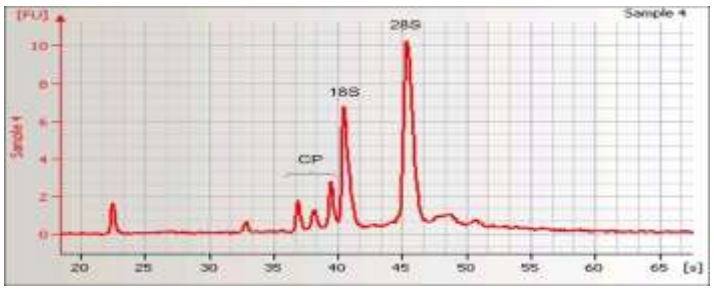


Figure 1. Electropherogram of total RNA from *Arabidopsis* leaf tissue was analyzed using the Bioanalyzer. (CP = Chloroplast RNA)

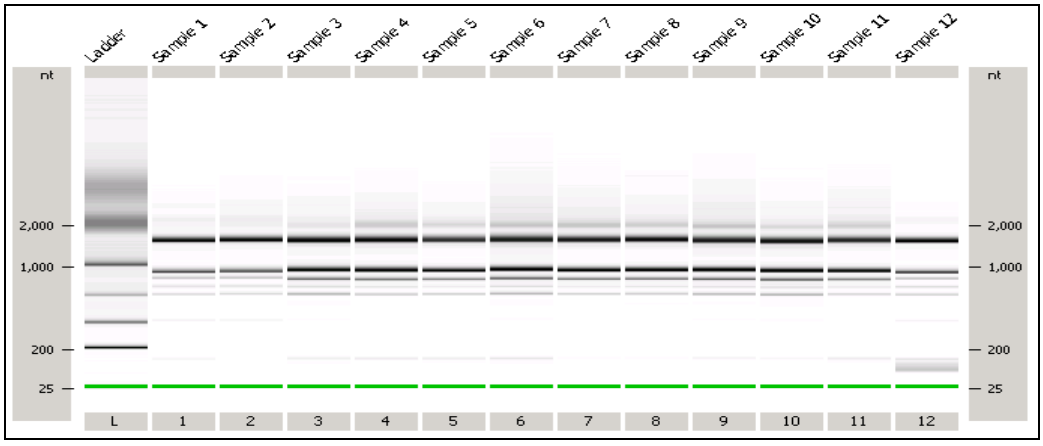


Figure 2. Gel-like image file output from the Bioanalyzer. Sample 12 is the unpurified total RNA, and samples 1-11 are the cleaned-up RNA samples from *Arabidopsis* leaf tissues.

aRNA Amplification

An Overview

A typical microarray experiment requires 30-50 μg total RNA that is equal to ~1 μg polyA$^+$ RNA. This large amount of total RNA requirement makes RNA amplification techniques essential for experiments involving limited amounts of starting material. Most RNA amplification techniques are based on Eberwine methods (van Gelder et al., 1990), employing double-stranded cDNA synthesis using oligo dT primers incorporating one of the T3 or T7 viral promoters, followed by *in vitro* transcription (IVT) as a means to linearly increase the concentration of messenger RNA. The optimized Eberwine method is capable of amplifying mRNA up to ~10^3 fold for one round of amplification, and up to ~10^5 fold for two rounds of amplification (Wang et al., 2000; Baugh et al., 2001). Employing two rounds of RNA amplification, one can successfully perform a microarray experiment using as little as 10 ng of starting RNA, corresponding to the total RNA content of a few isolated cells. Unless it is absolutely required we do not recommend two rounds of RNA amplification as this has been shown to reduce the number of detectable genes by as much as 20%, due to truncation of the 5' complexity of the RNA population (Luzzi et al., 2003).

A variety of alternatives to Eberwine-based methods have been described (Iscove et al., 2002; Aoyagi et al., 2003; Ginsberg and Che, 2002; Seth et al., 2003). The major advantage of the Eberwine RNA amplification method over other methods is attributed to its linear mode of amplification, which helps preserve the relationships between the abundances of different transcripts. Xiang et al. (2003) demonstrated that this linear relationship can be maintained over five cycles of RNA amplification.

In terms of the two major types of microarray platforms, cDNA amplicon and oligonucleotide array elements respectively, certain constraints are placed on the methods of amplification that can be employed. Amplicon-based microarrays are particularly flexible in terms of the targets that can be hybridized, since either strand of the cDNA can be fluorescently labeled. In contrast, hybridization to oligonucleotide-based microarrays is restricted to the use of negative–strand targets. Since the Eberwine-based RNA amplification methods generate only negative strands after the one round of amplification, it is essential to label the aRNA for microarray hybridization. Alternatively, a second round of amplification can be used to generate RNA corresponding to the positive-strand of the gene, which can then be reverse-transcribed to generate a labeled target. Both methods have advantages and disadvantages. Labeling aRNA forces use of riboprobe hybridization methods, which can be challenging under certain conditions; using two rounds of amplification is time consuming and may cause truncation. Microarrays Inc consequently recommends the use of direct labeling of aRNA, rather than second round amplification, unless the extra level of amplification is required. New methods have been recently described for linear aRNA amplification which are specific to the positive strand of the gene (BD Biosciences). Total RNA purified using the Qiagen RNeasy columns performs very well for RNA amplification.

First Strand cDNA Synthesis

Materials

- Aminoallyl Message Amp II kit (Ambion Cat# 1753)
- RNAase-free tips, tubes
- Refrigerated microcentrifuge
- DEPC-treated H_2O
- Thermocycler
- 100% ethanol
- Ice bucket

Method

Note: *The method described below is the recommended protocol by Ambion. However, we noticed that the reaction volume can be reduced by half without affecting the linearity of RNA amplification; e.g. the reaction volume of the first strand synthesis can be reduced to 10 μL, the reaction volume of second strand synthesis to 50 μL and aRNA amplification to 20 μL.*

25. Place up to 1 μg of total RNA (if RNA is limited, 100-500 ng is sufficient) into an RNAase-free microfuge tube.
26. Add 1 μL T7 Oligo(dT) Primer.
    Note: *this is* ***NOT*** *the same as the Oligo(dT) primer used for total RNA target labeling.*
27. Add DEPC-treated H_2O to a final volume of 12 μL.
28. Incubate for 5 minutes at 70°C in a thermocycler.
29. Centrifuge briefly (~5 seconds) to collect sample at bottom of tube.
30. Immediately transfer the tube to ice.
31. Prepare Reverse Transcription Master Mix. It is prudent to include 5% overage to cover pipetting errors.

First Strand Reaction Mix (Single Reaction)

Amount	Component
2 μL	10X First Strand Buffer
1 μL	Ribonuclease Inhibitor
4 μL	dNTP Mix
1 μL	Reverse Transcriptase

32. Mix well by gently pipetting up and down or flicking the tube a few times.
33. Centrifuge briefly (~5 seconds) to collect this master mix at the bottom of tube.
34. Place prepared Reverse Transcription Master Mix on ice.
35. Transfer 8 μL Reverse Transcription Master Mix to each RNA sample from step 30, mix thoroughly by flicking the tube a few times.
36. Incubate the sample tubes at 42°C for 2 hours in a PCR machine with the lid temperature set at 48°C.
37. After the 2 hour incubation, centrifuge the tubes briefly (~5 seconds) to collect the reaction at the bottom of the tube. Place the tubes on ice, and proceed to Second Strand cDNA Synthesis.

Second Strand cDNA Synthesis

Materials

- Aminoallyl Message Amp II kit (Ambion Cat# 1753)
- RNAase-free tips, tubes
- Refrigerated Microfuge centrifuge
- DEPC-treated H_2O
- Thermocycler incubators set at 16°C

Method

38. Work on ice to prepare the Second Strand cDNA Synthesis Mix by adding following ingredients:

    Single Reaction

Amount	Ingredient
63 μL	Nuclease-free Water
10 μL	10X Second Strand Buffer
4 μL	dNTP Mix
2 μL	DNA Polymerase
1 μL	RNAase H

    Note: *When processing more than one sample include ~5% overage to cover pipetting error*.

39. Gently mix by pipetting up and down or by flicking the tube a few times.
40. Centrifuge the tubes briefly (~5 seconds).
41. Add 80 μL second strand mix to 20 μL cDNA sample (step 37).
42. Gently mix by pipetting up and down or by flicking the tube a few times.
43. Centrifuge the tubes briefly (~5 seconds).
44. Incubate the tubes at 16°C for 2 hours, (use either a water bath in a cold room or a thermocycler; **do <u>NOT</u> use a heat block in a 4°C refrigerator because the temperature will fluctuate too much**).
45. After the 2 hour incubation, proceed to cDNA Purification (below), or immediately freeze reactions at –20°C. Do not leave the reactions on ice for long periods of time.

cDNA Purification

Use the cDNA purification kit supplied with the MessageAmp II kit. You can also use other DNA purification systems like Qiaquick PCR purification kit.

The protocol described below is essentially recommended by Ambion with MessageAmp II kit for double stranded cDNA purification.

> ***Note1:*** *Before beginning the cDNA purification, preheat a 10 mL bottle of DEPC-treated H_2O to 55°C.*
> ***Note2:*** *Prepare the aRNA Wash Buffer by adding the appropriate amount of 100% ethanol (ACS grade or better) to the bottle labeled aRNA Wash Buffer. Mix well and mark the label to indicate that the ethanol has been added.*

46. Add 250 μL of cDNA Binding Buffer to each cDNA sample and mix thoroughly by repeated pipetting.
47. Transfer the cDNA sample with added buffer onto the center of the cDNA filter cartridge.

48. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
49. Discard the liquid from the collection tube.
50. Apply 500 μL prepared cDNA Wash Buffer with added ethanol (see note above) to each cDNA filter cartridge.
51. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
52. Discard the liquid from the collection tube.
53. Centrifuge the cDNA filter cartridge for an additional minute to remove trace amounts of ethanol.
54. Transfer cDNA Filter Cartridge to a clean cDNA Elution tube.
55. Apply 10 μL DEPC-treated H_2O preheated to 55°C to the center of filter of the cDNA Filter cartridge.
56. Incubate at room temperature for 2 minutes.
57. Centrifuge at 10,000xg, for at least 1.5 minutes or until the liquid has passed through the filter.
58. Repeat the elution (steps 56-58) with an additional 10 μL DEPC-treated H_2O preheated to 55°C.
59. Discard the filter.
60. Place your double-stranded cDNA sample tube(s) (~18 μL) on ice.

In Vitro Transcription for aRNA Synthesis

An Overview

OpArrays require labeled negative-strand targets for hybridization. Since the first round of amplified aRNAs represents the negative–strand, it is recommended to label the aRNA. aRNA labeling can be done using two methods: (1) direct incorporation of Cy™-dye modified UTP during the process of *in vitro* transcription, or (2) indirect labeling, by incorporating aminoallyl modified UTPs during *in vitro* transcription, followed by mono reactive Cy-dye coupling. The OpArray protocol takes advantage of the second approach. Aminoallyl UTP (aaUTP) does not contain a bulky side chain modification, which allows ~100% replacement of the UTP with aaUTP during RNA synthesis. Microarrays Inc recommends using a 1:1 ratio of UTP:aaUTP.

61. Prepare the IVT Reaction Master Mix by adding the reagents in the following order:

Single Reaction

Amount	Ingredient
2 μL	aaUTP Solution (50 mM)
12 μL	ATP, CTP, GTP Mix (25 mM)
2 μL	UTP Solution (50 mM)
4 μL	T7 10X Reaction Buffer
4 μL	T7 Enzyme Mix

Note: *When processing more than one sample include ~5% overage to cover pipetting error*.

62. Add 24 μL IVT Reaction mix to 16 μL double-stranded cDNA sample.
63. Mix the reaction mix by pipetting up and down several times.
64. Centrifuge at 3,000xg for 30 seconds.

65. Incubate the tube for 4-14 hours at 37°C in a PCR machine with lid temperature set at 40°C.
66. After incubation, stop the reaction by adding 60 μL DEPC-treated H_2O to each sample, bringing the final volume to 100 μL.
67. Mix thoroughly by gentle vortexing.
68. Proceed to aRNA Purification or store the reaction tubes at -80°C.

aRNA Purification

Materials

- Aminoallyl Message Amp II kit (Ambion Cat# 1753)
- RNAase-free tips, tubes
- Refrigerated Microfuge centrifuge
- 100% EtOH
- DEPC-treated H_2O

Method

Become familiar with the steps before proceeding and have all reagents ready to use. This procedure will remove all the unincorporated nucleotides from the aRNA.

69. Add 350 μL aRNA Binding Buffer to each aRNA sample, mix by pipetting up and down three times.
70. Add 250 μL ACS grade 100% ethanol to each aRNA sample.
71. Mix by pipetting the mixture up and down three times.
72. Quickly transfer the sample onto the center of the filter in the aRNA spin column.
73. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
74. Discard the liquid from the collection tube.
75. Replace the aRNA filter cartridge back into the aRNA collection tube.
76. Apply 650 μL Wash Buffer to each aRNA filter cartridge.
77. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
78. Discard the liquid from the collection tube.
79. Centrifuge the aRNA filter cartridge for an additional 3 minutes to remove trace amounts of Wash Buffer.
80. Transfer filter cartridge(s) to a fresh aRNA collection tube.
81. Add 100 μL DEPC-treated H_2O to 55°C to the center of the filter.
82. Incubate at room temperature for 2 minutes.
83. After incubation, centrifuge at 10,000xg, for at least 1.5 minutes or until the liquid has passed through the filter.
84. Discard the aRNA filter.
85. Place your aRNA sample tube(s) (~100 μL volume) on ice.

Quantitation
Determine the concentration of aRNA using either the NanoDrop or a conventional spectrophotometer. In general, you can expect a yield of ~40-60 μg amplified aa-aRNA from 1 μg total RNA as starting material, and 80-120 μg of aa-aRNA from 2 μg of total RNA as starting material. If the amplification is significantly less, it is likely that either inhibitory contaminants present in the total RNA sample or there may have been partial degradation of the starting RNA sample or traces of washing buffer (ethanol) in the cDNA from step 60.

Sample Preparation

86. Prepare an aliquot for Bioanalyzer (5 μL of aRNA 150-200 ng/μL concentration).
87. Aliquot 4 μg aRNA in 1.5 mL microfuge tube (if aRNA is limited, as little as 1 μg should be enough).
88. Completely dry the sample using a Speedvac centrifuge set at 45°C or lower.
89. Store the remaining aRNAs samples at -80°C for further use.

aRNA Analysis using the Bioanalyzer
Follow the procedure recommended by the manufacturer for analysis of the RNA samples. In general, 1 μL of RNA sample (100-200 ng/μL) is sufficient for analysis using a Nano chip.

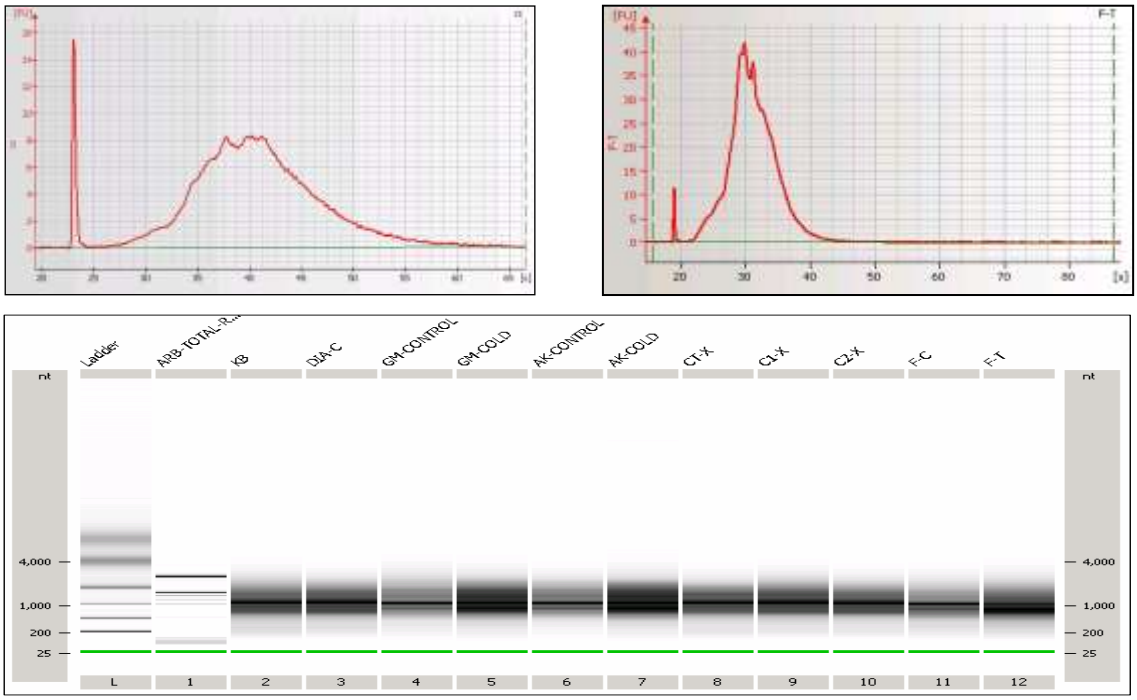


Figure 3. Typical results of aRNA Analysis using the Bioanalyzer.

Coupling of AA-aRNA to the Cy Dye Ester

Materials

- Cy3 Monoreactive dye (Amersham Pharmacia; Cat# PA23001)
- Cy5 Monoreactive dye (Amersham Pharmacia; Cat# PA25001)
- DMSO (Sigma; Cat# D8418)
- Hydroxylamine (Sigma Cat# 159417)
- 0.2 M Sodium Carbonate Buffer

Note: *Typically, the Sodium Bicarbonate Buffer is supplied with the Aminoallyl Message Amp II kit (Ambion Cat# 1753). If the buffer is not provided with the kit follow the procedure described below to make RNAase-free 0.2 M Sodium Bicarbonate buffer.*

Preparation of 0.2 M Sodium Carbonate Buffer: pH 9.0.

- Solution I: Dissolve 0.84g NaHCO3 in 50 mL DEPC H_2O in a disposable sterile Falcon tube.
- Solution II: Dissolve 1.05g Na2CO3, in 50 mL DEPC H_2O in a disposable sterile Falcon tube.
- Mix 45 mL of Solution I and 2.75 of mL Solution II in a disposable sterile Falcon tube.
- Check the pH by aliquoting 5 mL into a new 15 mL Falcon tube, if needed adjust the pH by adding appropriate amount of Sol-I or Sol-II. Never check the pH directly in the stock solution as that is a common source of RNAase contamination.
- Aliquot 0.2 mL into RNAase-free tubes, store at -20°C. Discard the tube after use or 24 hours, whichever comes first.

Preparation of Cy3 and Cy5 Monoreactive Dye

These dyes are supplied in 5 aliquots; the content of each tube is sufficient for at least 4-5 labeling reactions.

Preparation of Cy-dyes Prior to Reaction

- Dissolve the entire contents of a single tube in 27 μL DMSO by flicking the tube several times.
- Leave at RT for at least 30 minutes protected from light.
- Centrifuge at 1,000xg for 30 seconds to collect the dye at the bottom of the tube. The dye is now ready for use, but can be stored at -20°C for up to one month. Protect the dye from light by wrapping with aluminum foil.

Method

90. Dissolve the dried aRNA sample in 5 μL 0.2 M NaHCO3 buffer (make sure the aRNA completely dissolved in to the solution by pipetting the buffer along the sides of the tube).
91. Incubate the tube at RT for at least 20 minutes.
92. Add 5 μL Cy3 or Cy5 (in DMSO) to each aRNA sample.
93. Mix thoroughly by flicking the tube several times.
94. Centrifuge the tube at 1,000xg for 30 seconds.
95. Cover each tube with aluminum foil or otherwise protect the samples from light exposure.
96. Incubate the aRNA dye mixture at RT for 2 hours.

Post Dye-Coupling aRNA Purification

Materials

RNeasy MinElute column (Qiagen Cat# 74204)

- RNAase-free tips and tubes
- Refrigerated centrifuge
- Hydroxylamine (Sigma Cat# 159417)

Preparation of 4 M Hydroxylamine:

- Dissolve 2.7g of Hydroxylamine Hydrochloride salt in 7 mL DEPC treated water.
- Bring the solution to 10 mL total volume.
- Aliquot the 4 M hydroxylamine solution into 100 μL aliquots in RNAase-free microfuge tubes.
- Store excess tubes at -20°C.

Quenching Reaction

This *optional* step involves quenching any unreacted Cy-dye by adding an excess of primary amines.

97. Add 4.5 μL 4 M hydroxylamine into the dye coupling reaction.
98. Mix well.
99. Incubate for 15 minutes in the dark at RT.

Removal of Unincorporated Dye

Note: *The Qiagen RNeasy MinElute column is used for this purpose. Become familiar with the steps before proceeding and have all reagents ready-to-use. RPE buffer is supplied as a concentrate; ensure that ethanol is added before use. Work through this procedure to remove all the unincorporated dye.*

100. Adjust sample volume to 100 μL with DEPC-treated H_2O.
101. Add 350 μL of RLT (kit) buffer.
102. Mix thoroughly.
103. Add 250 μL 100% ethanol to the sample.
104. Mix thoroughly by pipetting.
105. Apply the sample to an RNeasy MinElute Spin Column in a 2 mL collection tube.
106. Close the tube gently.
107. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
108. Discard the liquid from the collection tube.
109. Pipette 500 μL RPE buffer onto the spin column.
110. Close the tube gently.
111. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
112. Discard the liquid from the collection tube.
113. Add 500 μL of 80% ethanol to the RNeasy MinElute Spin Column.
114. Close the tube gently.
115. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
116. Discard the liquid from the collection tube.

117. Centrifuge at 10,000xg, for at least 1 minute or until the liquid has passed through the filter.
118. Transfer the spin column to a new microfuge tube.
119. Apply 20 μL warm DEPC water (55°C) to the center of the spin column.
120. Close the tube gently.
121. Incubate at RT for 2 minutes.
122. Centrifuge at 12,000xg for 1 minute.
123. Repeat the elution with an additional 20 μL of warm DEPC water (55°C).

Measure the amount of dye incorporated into aRNA using a NanoDrop or conventional spectrophotometer.

Estimation of Dye Incorporation

Overview

The Beer-Lambert equation is used to correlate the calculated absorbance with concentration: **A = E * b * c** where **A** is the absorbance represented in absorbance units, **E** is the wavelength-dependent molar absorption coefficient (or extinction coefficient) with units of liter/mol-cm, **b** is the path length in cm, and **c** is the analyte concentration in moles/liter or molarity (**M**).

Dye Incorporation

Use the table below to estimate the amount of dye incorporation. The NanoDrop instrument contains built-in software (the Microarray Concentration module) that uses the general form of the Beer-Lambert equation to automatically calculate the fluorescent dye concentrations.

Table of Extinction Coefficients for Typical Dyes Used in Microarray Experiments

Dye Type	Extinction Coefficient (liter/mol-cm)	Measurement Wavelength (nm)
Cy3	150000	550
Cy5	250000	650
Alexa Fluor 488	71000	495
Alexa Fluor 546	104000	556
Alexa Fluor 555	150000	555
Alexa Fluor 594	73000	590
Alexa Fluor 647	239000	650
Alexa Fluor 660	132000	663
Cy3.5	150000	581
Cy5.5	250000	675

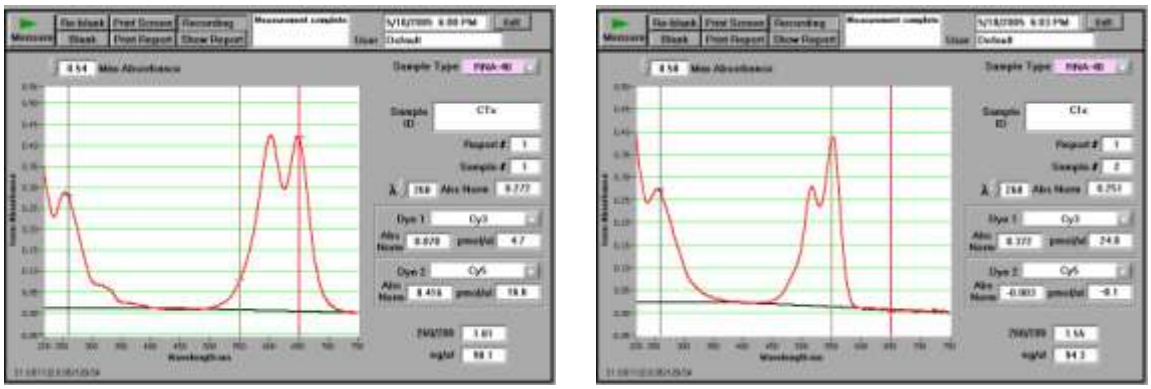


Figure 4. Estimation of Cy3 and Cy5 incorporation into aRNA targets using the NanoDrop spectrophotometer.

Note: *If NanoDrop is not available, a spectrophotometer may be used to measure dye incorporation. Incorporation for Cy5 is measured as its absorption at 650 nm, and incorporation of Cy3 at 550 nm. To calculate the amount of dye incorporated in your sample please use following web links.*
http://www.pangloss.com/seidel/Protocols/percent_inc.html
http://www.prontosystems.com/technical_support/calculator/#ResultsView

Microarray Hybridization

Materials

- OpArray Hybridization Buffer kit (OPHYB-1)
- Microscope slide staining dish (Ted Pella Cat# 21078-1) or mBox (Erie scientific, Cat# BX-IM-20ERIE)
- Microscope slide holders (Ted Pella Cat# 21078)
- 10 mL disposable pipette
- Sterile measuring cylinder
- Extra-deep Hybridization Cassette (Telechem International Cat# AHCXD)
- LifterSlip™ (Erie Scientific Company Cat# 24X601-2-4733)
- 50 mL Falcon tubes
- Incubator set to 42°C (Boekel Shake N Bake Hybridization Oven)
- Sterile diH_2O

OpArrays are always printed on the same side as the barcode on the slide. The array area is 18mm wide, 54mm long, and is located 9mm from the top of the slide (barcode at bottom). OpArray slides are supplied in a pre-processed, ready-to-use format.

Pre-Hybridization

If you have a centrifuge equipped with a microplate centrifugation, use mBox (Erie scientific, Cat# BX-IM-20ERIE) or Microscope slide holder to dry the slides, this will speed up the process and produce less variation between the slides.

Note: *Do not allow the slides to dry during the pre-hybridization and washing procedure.*

Caution note: *mBox may require more buffer than described in the procedure.*

124. Pre-warm an appropriate volume of OpArray Pre-Hyb Solution to 42°C by placing the pre-hyb buffer in a 42°C incubator for 30 minutes.
125. Using a large enough volume of buffer to ensure that all slides are completely covered, incubate slides in OpArray Pre-Hyb Solution for 60 minutes at 42°C.
126. During the pre-hybridization step, prepare Wash Solution 1 by diluting OpArray Wash B 1:40 with diH_2O. Prepare 50 mL per slide (i.e. 1.25 mL OpArray Wash B, add diH_2O to 50 mL).
127. Wash slides for 5 minutes in Wash Solution 1 at 20-25°C.
128. Immediately transfer the slides to a container with sterile DH_2O, and rinse for 30 seconds. Repeat this step for at least two times.
129. Dry the slides using a nitrogen or oil free air jet or dry by centrifugation at 200xg for 5 minutes using a microplate rotor. If microplate rotor is not available use 50 mL Falcon tubes for drying.
130. Preparation of 50 mL Falcon tube for slide drying.
    a. Pack bottom of 50 mL plastic disposable centrifuge tube with Kimwipes.
    b. Using forceps, carefully place slide into tube with label at the bottom.
    c. Spin the slides at 200xg for 5 minutes, if any liquid remains on the slide, repeat for an additional 5 minutes.

Hybridization

Note: *Excessive amount of labeled target causes precipitation of Cy3, leading to very high background on the microarray. Therefore we recommend using a maximum of 0.8 pmol Cy5-labeled target/ μL of hybridization solution and 0.8 pmol of Cy3-abeled target/ μL hybridization solution.*

131. Mix 2.5 μL of Cy5-labeled targets (40 pmol), 2.5 μL of Cy3-labeled targets (40 pmol) and 45 μL of OpArray Hyb Buffer.
    Note: *The OpArray Hyb Buffer can be diluted to a maximum of 90% [v/v] therefore if necessary, concentrate the labeled targets using a speedvac. Store the tubes in ice until the next step.*
132. Rinse the Hybridization Cassette with sterile diH_2O and dry thoroughly.
133. Make sure the flexible rubber gasket is seated evenly in gasket channel.
134. Add 15 μL sterile diH_2O to the lower groove inside the cassette chamber.
135. Insert the OpArray into the cassette chamber, DNA side up (barcode side up).
136. Place the LifterSlip over the microarray slide.
137. Make sure the white stripe of the LifterSlip is at the lower side and properly positioned on the slide surface.
138. Denature labeled target solution from Line 131 by incubating in the tube at 65°C for 5 minutes.
139. Apply the labeled target solution directly onto the slides or place on ice immediately.
140. Apply the denatured target solution slowly to one end of the LifterSlip and let it disperse across the OpArray surface.
141. Quickly place the clear plastic cassette lid on top of the cassette chamber.
142. Apply downward pressure and manually tighten (clockwise) the four sealing screws.
143. Check all four screws again to confirm a tight seal.
144. Place the cassette into a hybridization oven set at 42°C.
145. Hybridize for 14-16 hours.

Post-Hybridization Washing

***Note**: Do not allow the slides to dry during the hybridization and washing procedure. Wash Solutions 2, 3, and 4 should be prepared in advance. Use at least 50 mL solution per slide.*

146. Prepare all post-hybridization washing solutions:

    Wash Solution 2:
    - 50 mL OpArray Wash A
    - 25 mL OpArray Wash B
    - Bring Wash Solution 2 final volume to 500 mL with sterile diH_2O

    Wash Solution 3:
    - 50 mL OpArray Wash A
    - Bring Wash Solution 3 final volume to 500 mL with sterile diH_2O

    Wash Solution 4:
    - 5 mL OpArray Wash A
    - Bring Wash Solution 4 final volume to 500 mL with sterile diH_2O

147. Pre-warm the Wash Solution 2 to 42°C.
148. After hybridization, remove cassette from the hyb oven and manually loosen the four sealing screws (counterclockwise) to remove the lid.
149. Place the hybridized OpArray slides into a slide rack removing them from the cassette chamber using forceps.
150. Place the slide tray containing the OpArrays into a dish with Wash Solution 2 at 42°C for 10 minutes.
151. Transfer the slide tray into a dish with Wash Solution 3.
152. Incubate at 20-25°C (RT) for 10 minutes.
153. Transfer the slide tray into a dish with Wash Solution 4.
154. Incubate at RT for 5 minutes.
155. Transfer the slide tray into another dish with fresh Wash Solution 4.
156. Incubate at RT for 5 minutes.
157. Dry the slides by either using an oil-free nitrogen (or air) jet or by centrifugation (200xg for 5 minutes).
158. Protect the array from light, dust, and abrasion of the array surface. Scan as soon as practical.

***Note:** There are several reports regarding reduction of Cy5 signal due to the atmospheric ozone above 5 ppb. The Ozone induced Cy5 degradation is prudent particularly in the summer months. We advise taking appropriate preventive measures such as increasing the Nitrogen in the microarray lab, or setting up appropriate AIR filtration system to eliminate the Ozone. As a general practice we also recommend completing the scanning as soon as the washing process is completed.*

Scanning of the Microarray Slides

Please follow all manufacturer recommendations for Microarray scanner operation and maintenance.

- Turn on the scanner at least 20 minutes before the scanning to warm up the scanner.
- Place the slide into the slide cartridge and close the scanner.
- If the slides are hybridized with two color targets, select Cy5 channel (650 nm) and Cy3 channel (532 nm), otherwise select appropriate wavelength.
- If the option is available, select the preview scan, and scan entire slide.
- Examine the image and if the option is available check the histogram.
- Adjust the laser power or PMT settings to obtain ration of 1 between these two channels. If the microarrays hybridized with targets with single color make sure to follow a single setting or similar setting to obtain a consistent result.
- After adjusting the setting to get a ratio of 1 between the Cy5 and Cy3 channel, perform high resolution scanning (usually 10 microns).
- Save a multicolor TIFF image, otherwise save single channel TIFF images.

Feature Extraction

Feature extraction is one of the most critical steps in any microarray experiment. In this step you will convert the optical image to numerical values for comparing the results among samples and for drawing conclusions from your experiment. To facilitate feature extraction with any of the commercially or publicly-available microarray analysis software, Microarrays Inc provides files in the standard *.gal configuration. Other configurations may be available through the Microarrays Inc website (http://www.microarrays.com) or upon request.

Control oligos and empty wells

The AROS and CoRe oligo probe sets come with a variety of positive, negative and spiking control oligos. All OpArrays contain the control oligos which are offered with the specific AROS oligo probe set. Many also include the Stratagene's SpotReport® Alien® Amine Oligo Array Validation System. The mRNA Spikes 1 through 10 can be purchased directly from Stratagene, La Jolla, CA, USA.

In each AROS the wells A1, A2, B1, and B2 are null wells (no synthesis is performed in these positions). You may use these wells in a variety of ways. Most researchers choose to leave them oligo free but they do add spotting buffer to the wells. This serves two purposes- the first allows a quick orientation of the scanned slide based on the empty well pattern, and the second allows you to check if the spotting buffer may have been inadvertently contaminated or if the spotting pins have been rinsed insufficiently. In these cases, you'll experience fluorescence of the 'null wells'.

Some AROS sets also contain the sequence

opHsV04NC000001 TCAGTCGATCGCGACGTACGCTAGAACCTC

This oligo will appear 4 times in each 384 well plate of the AROS set. It provides rapid method for verifying the plate layout during the print run. You may find these oligos useful for your experiments as negative controls.

Review of Quality Control files for each Lot Number
As an added feature to control the sources of variation within and among your experiments, Microarrays Inc provides access to all the print and functional QC files for OpArrays. The Print QC files (a multiple work sheet Excel file, is particularly useful for checking if a particular spot may yield suboptimal results because it did not print clearly in a specific lot. Average OpArrays variation is typically between 0.1% and 0.2% variation in feature morphology; QC parameters require less than 0.5% of features in any lot to exhibit poor morphology.

For each OpArrays lot you are working with, Microarrays Inc suggests that you review the print QC files for the lot to eliminate analysis of missing spots. The size of QC files make them likely to be rejected by email systems, so we cannot send QC files directly by email. OpArrays QC data are available for download at the Microarrays Inc web site.

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Appendix A

There are many models of scanners, hybridization stations, and analysis software which are compatible with Operon OpArray products. The items listed below have been specifically reviewed, however, additional equipment if configured to accept a standard microarray slide will likely be compatible with OpArrays. Please contact Operon Technical Support with any specific questions regarding the OpArrays. See contact information on page 16.

Specifically-Tested Equipment

Compatible Microarray Scanners

- Axon 4000B
- Axon 4100A
- Axon 4200AL
- Agilent DNA Microarray Scanner
- PerkinElmer Scanarray GX
- PerkinElmer Scanarray HT
- PerkinElmer ProScann
- PerkinElmer Scanarray Express
- Telechem International Spotlight-2
- Alpha Innotech NovaRay
- Genomic Solutions UC4 Scanner
- GE Healthcare ArrayWoRx Scanner
- Tecan Microarray Scanner
- Genetix aQuire scanner
- Innopsis Microarray Scanner

Microarray scanners **NOT** compatible with OpArray Slides

- Affymetrix Scanner
- Illumina Bead array Scanner
- ABI 1700 Chemiluminescent Microarray Analyzer

Compatible Automated Hybridization Stations

- MAUI Hyb Station
- ArrayBooster™
- GeneMachines HybStation
- GeneTAC Hybridization Station
- CapitalBio Biomixer
- a-Hyb™ Hybridization Station
- Tecan HS400 Hyb-station

Compatible Manual Hybridization Chambers/Cassettes

- Glass array Hybridization Cassette; Ambion
- International Hybridization Chambers; Telechem