ArrayControl™ Spots and Spikes

(Cat #AM1780, AM1781)

Instruction Manual

Manual1780M Revision C **Revision Date: July 16, 2007**

For research use only. Not for use in diagnostic procedures.

Literature Citation When describing a procedure for publication using this product, we would appreciate that you refer to it as the ArrayControl™ Kit.

If a paper that cites one of Ambion's products is published in a research journal, the author(s) may receive a free Ambion T-shirt by sending in the completed form at the back of this instruction manual, along with a copy of the paper.

Warranty and Liability Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in "Limited Warranty for Consumables," which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption "EXCLUSIONS, CONDITIONS, EXCEPTIONS, AND LIMITATIONS" in the full warranty statement. Please contact Applied Biosystems if you have any questions about our warranties or would like information about post-warranty support.

Ambion/AB Trademarks Applied Biosystems, AB (Design), Ambion, MEGAscript and RETROscript are registered trademarks, and ArrayControls, MEGAclear, MessageAmp, NucAway, Poly(A)Purist, and SlideHyb are trademarks of Applera Corporation or its subsidiaries in the US and/or certain other countries.

All other trademarks are the sole property of their respective owners.

I. Introduction

A. Product Description

Microarray analysis can be an extremely powerful tool in gene expression profiling because it can provide data about the expression of hundreds or thousands of genes in a single experiment. The ArrayControl™ Spots and Spikes for glass array hybridization are a set of 8 complementary nucleic acids designed for interpretation and validation of microarray hybridization data. ArrayControl ***Sense Oligo Spots*** are spotted or printed onto microarrays along with the experimental sequences. The ArrayControl ***RNA Spikes*** are templates for synthesis of labeled cDNA that will hybridize with the ArrayControl Spots. The RNA Spikes can be mixed with experimental RNA samples before labeling so that both experimental sequences and the RNA Spikes are labeled simultaneously. Upon hybridization to a glass microarray, the labeled cDNA mixture will hybridize with nucleic acids on the microarray, producing experimental data as well as control data. The hybridization data from the ArrayControl Spots and Spikes provides a basis for analysis of the experimental data.

The ArrayControl sequences were selected from *E. coli* genes that show no sequence similarity to mammalian genomes, based on database searches. They were then tested experimentally to make sure that they do not cross hybridize to each other, or to human or mouse RNAs.

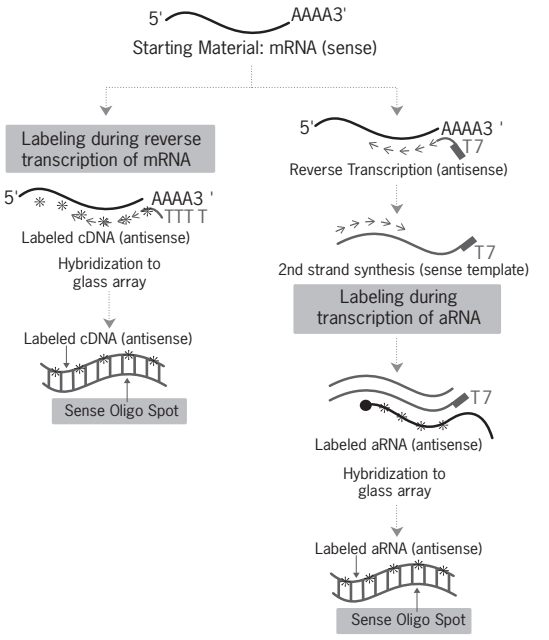
ArrayControl Sense Oligo Spots

The ArrayControl Sense Oligo Spots are a set of 8 sense DNA oligonucleotides (70 base) designed to hybridize with the ArrayControl RNA Spikes. They are designed for use with microarrays made with single-stranded 25–70 nt probes (e.g., oligonucleotides) and samples prepared with a method that labels single-stranded antisense copies of the target (see Figure 1). For example, target prepared with the Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kits will produce single stranded antisense label RNA that will hybridize to the Sense Oligo Spots.

Spotting/printing ArrayControl Sense Oligo Spots onto glass slides

The ArrayControl Sense Oligo Spots have a 5' amino modification to make them compatible for printing on both poly-L lysine (reactive amine) and reactive aldehyde glass slides. Consider including 1 or several Sense Oligo Spots in each sub-array or block, to validate printing reproducibility. Slide to slide printing quality and hybridization variations can be inferred by comparing the hybridization signals from a standardized labeled mix of the ArrayControl RNA Spikes on different slides (see section *III.G. Creating a Labeled Hybridization Standard* on page 15).

Figure 1. Use of ArrayControl Sense Oligo Spots



ArrayControl RNA Spikes

The RNA Spikes are a set of 8 purified RNA transcripts with sequence homology to the corresponding ArrayControl Spot (e.g., cDNA made from RNA Spike 1 will hybridize with Sense Oligo Spot 1). They are 3' poly(A) tailed RNAs supplied at a precise concentration. The sizes and base compositions of each RNA Spike are shown in the table below. This information is provided so that precise normalization of fluorescent signal to RNA Spike length and base composition can be obtained.

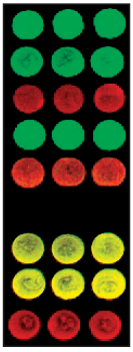
Table 1. RNA Spike Lengths and Base Compositions

RNA Spike	Length	Nucleotide Composition			
		C	U	A	G
1	750 nt	176	199	164	211
2	752 nt	223	177	155	197
3	1000 nt	232	259	166	343
4	1000 nt	235	220	270	275
5	1034 nt	284	231	236	273
6	1250 nt	346	273	279	352
7	1474 nt	411	302	359	402
8	2000 nt	544	427	473	556

Uses of the ArrayControl Spots and Spikes

Using arrays spotted with ArrayControl Sense Oligo Spots, the RNA Spikes are intended to aid in determining the quality of several different aspects of a microarray hybridization experiment, including the following:

- Uniformity and optimization of microarray printing
- As internal indicators for dye normalization
- Defining the limits of sensitivity and selectivity of your microarray system. For instance, what is the lowest amount of an RNA transcript that can be detected at a certain hybridization temperature.
- To define the range of differential expression that can be reliably measured on a microarray.
- As negative controls: by leaving 1 or more of the RNA Spikes out of a labeling reaction, and hybridizing it to a microarray with all 8 of the ArrayControl Spots printed.



	RNA Spike amount added to labeling rxn		cyanine 5/ cyanine 3 ratio*	
RNA Spike	cyanine 5 (pg)	cyanine 3 (pg)	expected	observed
Spike 1	0	500	cyanine 3	0.001
Spike 3	100	500	0.2	0.166
Spike 8	500	0	cyanine 5	397.02
Spike 2	0	500	cyanine 3	0.001
Spike 6	500	100	5.0	5.253
Spike 4	500	500	1.0	1.019
Spike 5	500	500	1.0	1.069
Spike 7	500	0	cyanine 5	448.5
Human RNA	5×10^6	5×10^6	n/a	n/a

* The RNAs labeled with only cyanine 3 or cyanine 5 are expected to have only background levels of the other dye.

Figure 2. Image of Microarray Illustrating the Fluorescent Signals from the ArrayControl Sense Oligo Spots Using Different Ratios of RNA Spikes During Labeling with Cyanine 3 and Cyanine 5 dCTP.

The ArrayControl Sense Oligo Spots were spotted on a glass slide in triplicate. The slide was then used in a dual labeled hybridization containing 2 samples with different amounts of cyanine 3 vs. cyanine 5 labeled RNA Spikes in a background of 5 µg of human total RNA. The table lists the amount of each RNA Spike added to the either the cyanine 3 or cyanine 5 labeling reaction, and the expected and observed ratio of cyanine 5/cyanine 3 hybridization.

B. Kit Components and Storage

Cat #	Amount	Product	Storage
AM1780	10 µL ea.	RNA Spikes 1–8 (100 ng/µL)	below –70°C
	7 mL	TE Buffer (pH 7.0)	–20°C
AM1781	10 µL ea.	Array Control Sense Oligo Spots 1–8 (60 µM)	–20°C

C. Materials Not Provided with the Kit

- Microarray spotting or printing buffer
- Glass microarray slides
- cDNA labeling reagents
- Microarray hybridization reagents
- Microarray analysis equipment

D. Related Products Available from Ambion

MessageAmp™ II aRNA Amplification Kits see our web or print catalog	Ambion offers a full line of MessageAmp II Kits tailored for different array analysis applications. The MessageAmp II Kit offers maximum flexibility; samples can be amplified using either single- or double-round amplification, and the reagent cocktails are configured to accommodate modification. For arrays requiring biotin-labeled samples, Ambion offers the MessageAmp II-Biotin *Enhanced* Single Round aRNA Amplification Kit. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp II Kits which are available with and without Cy™3 and Cy5. Bacterial RNA can be amplified using the MessageAmp II Bacteria RNA Amplification Kit. We also offer the MessageAmp II-96 and Amino Allyl MessageAmp II-96 aRNA Amplification Kits for high throughput applications.
RETROscript® Kit Cat #AM1710	First strand cDNA synthesis kit for RT-PCR. When purchased with SuperTaq™, this kit provides reagents, controls and protocols for reverse transcription and PCR. Both oligo(dT) and random primers for cDNA priming are included, as is RNase inhibitor.
Amino Allyl cDNA Labeling Kit Cat #AM1705	The Amino Allyl cDNA Labeling Kit generates cDNA for secondary fluorescent dye labeling to be used for glass array analysis. It includes all the reagents, except the amine-reactive labeling moiety (e.g. cyanine dyes) for 2-step labeling of cDNA. The reaction produces more labeled cDNA, more efficiently than direct dye incorporation.
NucAway™ Spin Columns Cat #AM10070	Guaranteed RNase- and DNase-free, Ambion's NucAway Spin Columns provide a fast, efficient way to remove unincorporated nucleotides, and to effect buffer exchange after probe synthesis and other reactions.

MEGAscript® Kits Cat #AM1330–AM1338	High yield transcription kits for production of large amounts of RNA. By employing Ambion's novel, patented MEGAscript technology, these kits use concentrations of nucleotides that would normally inhibit the RNA polymerases, resulting in ultra high-yield transcription reactions. Kits are available with T7, SP6, and/or T3 RNA polymerase.
MEGAclear™ Cat #AM1908	MEGAclear purifies RNA from transcription, and other enzymatic reactions yielding high quality RNA free of unincorporated nucleotides, enzymes and buffer components with close to 100% recovery of input RNA.
SlideHyb™ Glass Array Hybridization Buffers and Glass Array Hybridization Cassette see our web or print catalog	There are 3 unique SlideHyb Glass Array Hybridization Buffers; they have identical salt and formamide compositions, but differ in hybridization kinetics and blocking reagents. Ambion also offers the Glass Array Hybridization Cassette for incubation of glass microarray hybridization reactions.

II. Using ArrayControl Spots and Spikes

A. Spotting Suggestions

Each ArrayControl Sense Oligonucleotide Spot is supplied in water at 60 µM. Concentrated printing buffer can be added directly to the Sense Oligo Spot solutions to prepare them for spotting. ***Typical concentrations for spotting oligonucleotides are from 10 µM–1 mM.*** Follow the recommendation of the facility printing the array, or that of the printing device manufacturer regarding printing buffer and DNA concentration.

Array Design or Layout

The 8 ArrayControl Sense Oligo Spots will fill a single column in a 96-well plate. Consider printing replicates of the controls to increase statistical confidence and to monitor any variance across the printed area. For easy visual alignment and identification of a microarray pattern, the control spots can be kept together as a control group. You may also want to include blank features (printing buffer only) as well as additional positive control features in the control group if they are not already present in your design (i.e. genomic DNA, housekeeping genes, etc.).

Array printing protocols on the web

Protocols for printing arrays can be found on the following website (among others):

- http://arrayit.com/Products/Substrates/substrates.html

B. Handling the ArrayControl RNA Spikes

Each tube of ArrayControl RNA Spikes contains 1 µg of RNA (100 ng/µL); when diluted, this is enough for hundreds of RNA Spike additions to sample labeling reactions. Even though the RNA Spikes are supplied nuclease-free, they are highly susceptible to degradation by contaminating RNase. Since they are used in very dilute solutions, even the slightest amount of degradation will be a detriment to their use as microarray standards. Therefore, use the supplied 100 ng/µL RNA Spike stocks only to make dilutions, and keep the remainder at –80°C. Diluted RNA Spikes can be used for 1 week and then should be discarded, particularly if the vial has been frozen and thawed a number of times. Ambion recommends making a series of dilutions in several tubes, and storing them at –80°C. Then, just before a new experiment, simply thaw those RNA Control Spike dilutions that are needed.

Always plan the dilution protocol for each RNA Spike mix ***before*** setting up your labeling reaction. Rushed calculations just before a labeling reaction inevitably lead to errors immortalized in microarray data.

C. Array Control Dilution Series Examples

The RNA Spikes should be diluted for inclusion in labeling or RNA amplification reactions. In a typical 20 µL labeling reaction containing 5–10 µg of sample RNA, Ambion recommends using ***0.5 pg to 5 ng RNA Spikes.*** The RNA Spikes should always be diluted in non-stick tubes using the TE Buffer supplied. Non-stick tubes are recommended to prevent the RNA from binding to the sides of the tubes. Below are 3 different dilution protocols to address different standardization concerns. These can be used in various combinations and are provided as a reference only.

IMPORTANT

Diluted RNA Spike solutions can be stored for a week at –20˚C or they can be divided into several tubes and stored at –80˚C until needed. Do not freeze/thaw dilutions more than twice.

1. Dilution of individual RNA Spikes: instructions for making 3 different working solutions of each individual RNA Spike.
2. Preparation of a single solution with a mixed concentration of the RNA Spikes.
3. Preparation of a mixed concentration RNA Spike solution for use at different ratios.

1. Dilution of individual RNA Spikes

To include only a few of the 8 RNA Spikes in a labeling reaction it is convenient to have a set of individual RNA Spikes at useful concentrations. Below is an example for such dilutions. These solutions can also be used to make the mixed concentrations in sections II.C.2 and 3.

a. Thaw RNA Spikes on ice.

Once thawed, vortex the tube to mix thoroughly, and centrifuge briefly to collect the contents in the bottom of the tube.

b. Label 3 sets of tubes with the RNA Spike that will be diluted (1–8) and the concentrations: 1 ng/µL, 100 pg/µL, and 10 pg/µL.

c. Make the dilutions as follows:

Concentration	TE Buffer	RNA Spike (or dilution)
1 ng/µL	198 µL	2 µL undiluted RNA Spike
100 pg/µL	45 µL	5 µL 1 ng/µL RNA Spike
10 pg/µL	45 µL	5 µL 100 pg/µL RNA Spike

Mix each dilution by closing the tube and vortexing for ~30 seconds, then centrifuge to collect contents at the bottom of tube before removing an aliquot for the subsequent dilution.

2. Single RNA Spike mixture with a range of concentrations

This RNA Spike mixture contains a range of concentrations that can be added to an RNA sample before labeling so that both sample RNA and RNA Spikes will be labeled simultaneously. Ambion recommends using 1–2 µL of this mix in a 20 µL cDNA labeling reaction with 5–20 µg of total RNA. This protocol can be modified to have RNA Spike concentrations that serve as an optimal control for your microarray system (for example see step c on page 9).

a. Set up 4 tubes for dilutions and dilute RNA Spikes in TE Buffer as follows:

i. Using the RNA Spikes supplied with the kit (100 ng/µL), make a 10 ng/µL solution of RNA Spikes 1+2 and RNA Spikes 3+4 in 2 separate tubes.

2 µL RNA Spike 1	2 µL RNA Spike 3
2 µL RNA Spike 2	2 µL RNA Spike 4
16 µL TE Buffer	16 µL TE Buffer
10 ng/µL RNA Spikes 1+2	10 ng/µL RNA Spikes 3+4

Mix well by closing the tubes and vortexing for ~30 sec; centrifuge to collect contents at the bottom of tubes.

ii. Using the RNA Spikes supplied with the kit (100 ng/µL), make a 1 ng/µL solution of RNA Spikes 5+6 and RNA Spikes 7+8 in 2 separate tubes.

2 µL RNA Spike 5	2 µL RNA Spike 7
2 µL RNA Spike 6	2 µL RNA Spike 8
196 µL TE Buffer	196 µL TE Buffer
1 ng/µL RNA Spikes 5+6	1 ng/µL RNA Spikes 7+8

Mix well by closing the tube and vortexing for ~30 sec; centrifuge to collect contents at the bottom of tube.

b. In a nuclease-free tube, make the mixed concentration RNA Spike solution from the dilutions in steps a.i and ii:

Amount	Component	Final conc.
170 µL	TE Buffer	
10 µL	RNA Spikes 1+2 (10 ng/µL)	500 pg/µL
5 µL	RNA Spikes 3+4 (10 ng/µL)	250 pg/µL
10 µL	RNA Spikes 5+6 (1 ng/µL)	50 pg/µL
5 µL	RNA Spikes 7+8 (1 ng/µL)	25 pg/µL

Mix well by closing the tube and vortexing for ~30 seconds; centrifuge to collect contents at the bottom of tube.

c. Other uses for the mixed concentration RNA Spikes:

- A 1:10 dilution of the mix (10 µL in 90 µL of TE Buffer) will make a more dilute mix that is useful for determining the lower limits of detection (2.5–50 pg RNA Spikes per labeling reaction).
- Alternatively, you can use a 1:2, 1:5 or 1:10 dilution of the above mix in one labeling reaction (e.g., cyanine 3) and a 1X amount in the other (e.g., cyanine 5) as an way of assessing fluorescent intensity ratios of 2 dyes over a 20 fold concentration range. This is an alternative to (3) below.

3. Mixed concentration RNA Spikes at different ratios

The RNA Spikes can be used in dual color array analysis for ratio analysis and dynamic range determination at the same time. This section describes the set-up of 4 RNA Spike mixes containing the different RNA Spikes at different ratios and concentrations. Aliquots of 2 of the mixes would be added to 1 dye labeling reaction (e.g., with cyanine 3), and aliquots of the other 2 mixes would be added to the other dye labeling reaction (e.g., with cyanine 5). The 2 nucleic acid labeling reactions (including the labeled RNA Spikes) are then mixed, purified, and used for microarray hybridization. After signal normalization, the ratio of the fluorescent intensities from each Control Spot should reflect the ratio of the amount of RNA Spike added to each labeling reaction.

- Table 2 (below) shows the amount of each RNA Spike that will be present in the final mixture.
- Figure 3 (below) is an overview of the dilution procedure.
- Detailed instructions start with step 3.a on page 9.

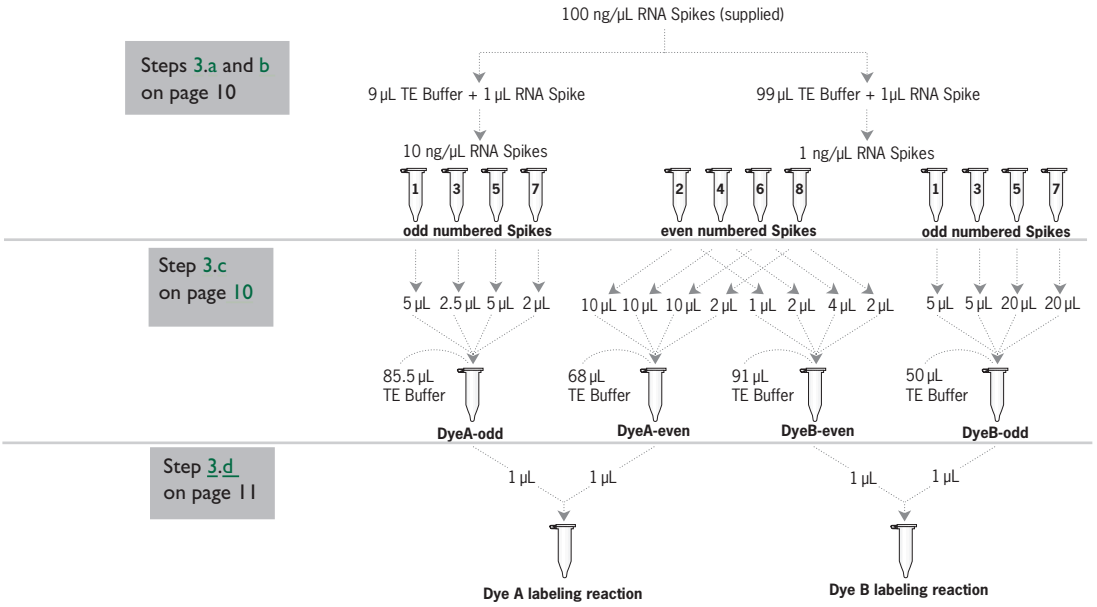
Table 2. Final Amount of each RNA Spike in "Mixed Concentration RNA Spikes at Different Ratios"

Ratio (e.g. cyanine 3: cyanine 5)	Odd numbered RNA Spikes (pg:pg)	Even Numbered RNA Spikes (pg:pg)
10:1	500:50 Spike 1	100:10 Spike 2
5:1	250:50 Spike 3	100:20 Spike 4
2.5:1	500:200 Spike 5	100:40 Spike 6
1:1	200:200 Spike 7	20:20 Spike 8

a. In separate tubes, make 10 µL of a 10 ng/µL solution of each of the odd-numbered RNA Spikes.

The RNA Spikes are supplied at 100 ng/µL, so mix 1 µL RNA Spike with 9 µL TE Buffer. Make 10 ng/µL solutions for RNA Spikes 1, 3, 5, and 7. Mix well by closing the tube and vortexing for ~30 seconds; centrifuge briefly to collect contents at the bottom of the tubes.

Figure 3. Overview of Preparing Mixed Concentration RNA Spikes at Different Ratios



b. In separate tubes, make 100 µL of a 1 ng/µL solution of each RNA Spike

Mix 1 µL RNA Spike with 99 µL TE Buffer. Mix well by closing the tube and vortexing for ~30 seconds; centrifuge briefly to collect contents at the bottom of the tubes.

c. Make the 4 RNA Spike mixtures that will be added to labeling reactions

In 4 separate tubes add the indicated amounts of TE Buffer and diluted RNA Spikes following the table below. Mix well by closing the tube and vortexing for ~30 seconds; centrifuge briefly to collect contents at the bottom of the tubes.

	10 ng/µL RNA Spikes (odd numbered)				1 ng/µL RNA Spikes (even numbered)				1 ng/µL RNA Spikes (odd numbered)				
	1	3	5	7	2	4	6	8	1	3	5	7	TE Buffer
Dye A-odd	5 µL	2.5 µL	5 µL	2 µL	—	—	—	—	—	—	—	—	85.5 µL
Dye A-even	—	—	—	—	10 µL	10 µL	10 µL	2 µL	—	—	—	—	68 µL
Dye B-odd	—	—	—	—	—	—	—	—	5 µL	5 µL	20 µL	20 µL	50 µL
Dye B-even	—	—	—	—	1 µL	2 µL	4 µL	2 µL	—	—	—	—	91 µL

d. Using the mixtures in an array experiment

- Add 1 µL of the ***Dye A-odd*** and 1 µL of the ***Dye A-even*** RNA Spikes to 1 dye labeling reaction (e.g. cyanine 3).
- Add 1 µL of the ***Dye B-odd*** and 1 µL of the ***Dye B-even*** RNA Spikes to the other dye labeling reaction (e.g. cyanine 5).

III. ArrayControl Applications

A. Using ArrayControl Sense Oligo Spots for Printing Optimization

ArrayControl Sense Oligo Spots are extremely useful for optimization of microarray production, among their possible applications are the following:

- Identify the optimal DNA concentration for printing
- Verify that DNA carryover is not occurring during printing
- Check printing uniformity

Finding the best DNA concentration for spotting/printing arrays

The ArrayControl Sense Oligo Spots are precisely quantified and can be used as standards (with labeled cDNA made from the ArrayControl Spikes) to identify the optimal DNA spotting concentration for your microarray spotting device and glass substrate. A typical experiment would consist of spotting identical volumes of several ArrayControl Sense Oligo Spots diluted to 0.1–1.0 µg/µL in the recommended printing/spotting buffer. The array would then be hybridized with labeled cDNA made from ArrayControl RNA Spikes corresponding to the Spots on the array. The hybridization signal should increase with increasing DNA concentration up to the point where the glass is saturated with DNA and the hybridization signal plateaus. The optimal DNA spotting concentration is the one that produces the highest hybridization signal.

Checking for DNA carryover during microarray production

To evaluate carryover of DNA between pin washes, dry down an aliquot of Sense Oligo Spots, and then resuspend it to the desired concentration in 1X printing buffer (e.g., ~10X the concentration that will normally be used to make arrays). Print part of an array using the high concentration of Sense Oligo Spots as the first DNA deposited on the slide, and use printing buffer alone for subsequent spots. To evaluate whether the pin washes are adequate between spots, hybridize the test array with labeled cDNA made from the corresponding ArrayControl Spike. If signal is seen only from spots printed with a high concentration of ArrayControl Spot DNA, and not in subsequent spots, then it can be assumed the wash protocol is sufficient for a lower working concentration during microarray manufacturing.

Checking printing uniformity

Uniformity of microarray printing can be assayed by spotting replicates of the Sense Oligo Spots in various locations on the microarray. Then hybridize the array with labeled cDNA made from a mixture of RNA Spikes and experimental RNA. The hybridization signal from the Sense Oligo Spots as well as the background signal from each of these spots would be expected to be identical for an ideal microarray.

B. Production of Test Arrays

The Sense Oligo Spots can also be used to make test arrays. Test arrays are useful for evaluating new procedures such as cDNA labeling, RNA amplification, and hybridization and wash reagents. A test array printed with several house keeping genes and the 8 Sense Oligo Spots can economize resources compared to printing a high-density microarray for protocol optimization. Test arrays can also be used to determine the optimal printing parameters as discussed above.

C. Determining Detection and Sensitivity Limits of a Microarray System

Each microarray system (i.e. RNA sample, cDNA labeling method and fluorescent dyes, the microarray itself, hybridization parameters, and scanning instrument) has a different limit of detection and sensitivity. This limit is expressed as the lowest amount of input RNA or cDNA that gives a reproducibly detectable signal above background. You may already know the approximate detection and sensitivity limits for your system, but ArrayControl Spots and Spikes can be used to rigorously demonstrate them. The detection limit will be affected by the type [total or poly(A) RNA] and amount of RNA labeled for microarray hybridization. Use a "typical" amount of sample RNA to determine the detection limit for your system. In other words, if you use 5 µg of total RNA for labeling and hybridization, then use 5 µg of total RNA when investigating the sensitivity of the ArrayControl RNA Spikes in your system. There are basically 2 ways to evaluate the limit of detection, and they may yield different results:

a. Titrating RNA Spikes into a labeling reaction before hybridization.

   This method will identify a detection limit using controls in a complex mixture during both labeling and hybridization.

b. Labeling a single or a few RNA Spikes alone (in the absence of sample RNA).

   This method eliminates the possible competition between control RNA and experimental RNA for reaction components during labeling. The labeled control(s) is purified and quantified, then used in hybridizations at known amounts to determine the limit of detection in the microarray system.

D. Determining the Dynamic Range of a Microarray System

The dynamic range of a microarray system is the range of input labeled nucleic acid (mass or fluorescent intensity) that produces a linear hybridization signal. This value is important because relative differences in fluorescence are valid only when both readings fall within the dynamic range of the system.

Ambion recommends trying a range from 0.5–50,000 pg RNA Spikes with your RNA sample. Because the RNA Control Spikes vary in length, their respective fluorescent signal will be proportional to their length when equal molar amounts of each Spike are used. If equal molar amounts of each Spike are labeled, the longest transcripts will contain the greatest number of labeled nucleotides and hence should generate more signal than a shorter transcript. There will not be a ***perfect*** correlation between RNA Spike length and fluorescent signal because the RNA Spike sequences are different, thus each will have slightly different levels of label incorporation relative to length, and different hybridization kinetics. For very accurate measurements it may be useful to normalize the RNA Spikes with respect to size and base composition (see Table 1 on page 2.)

When dual colored labeling methods are used, the dynamic range for each fluorophor can be different. An important metric for ratio analyses is the determination of the upper and lower limits of fluorescence intensity that produce accurate data. As fluorescence intensity approaches the lower limits of detection, ratio analyses are less predictable. Likewise, when signals are near the upper limit, the ratio observed may not be accurate. The RNA Spikes can be used to determine the limits for accurate ratio analysis.

E. Normalization Coefficient(s)

Because there are differences in the incorporation efficiencies of different dye labeled nucleotides, and quantum yields of the different fluorophors typically vary, direct comparisons of differentially labeled samples require normalization. To normalize the observed ratios of the 2 fluorescent signals, calculate the correction coefficient using the mean intensity for all features in an array. There are several different principles to consider when calculating a normalization coefficient (Tseng et al. 2001, Kalocasi et al. 2001). A discussion of these approaches is beyond the scope of this Instruction Manual, but the ArrayControl system can be useful for testing the different approaches.

F. Negative Control

Add a negative control to any experiment that includes an array with all 8 ArrayControl Sense Oligo Spots by simply omitting 1 or 2 RNA Spikes from a labeling reaction containing experimental RNA and/or most of the 8 RNA Spikes. The fluorescent intensity of the hybridization from the corresponding Sense Oligo Spot on the microarray should be at or even below background ("black holes") unless there is a problem with background or spot deposition.

G. Creating a Labeled Hybridization Standard

The ArrayControl RNA Spikes can be labeled separately or as a mix in a labeling reaction that does not include an experimental RNA sample. This can be useful for creating a single uniform hybridization standard, or positive control, that can be included in microarray hybridizations (with or without other RNA Spikes).

For example, set up 2 separate 20 µL reverse transcription reactions that contain 25 ng of any 2 ArrayControl RNA Spikes. Include cyanine 5 in one labeling reaction, and include cyanine 3 in the other. After the RNA Spikes are labeled, mix them together and purify them using the standard clean-up step used in your laboratory (e.g., NucAway™ Spin Columns). Measure the A_{260} and dilute the mixture to an appropriate concentration for hybridization (typically ~1 ng/µL). An identical amount of this dual labeled hybridization control can be used with each microarray experiment. Any variation in the hybridization signal between microarray images can be attributed to the hybridization, the microarray, or post hybridization handling (e.g., changes in temperature, buffer, wash, microarray quality).

IV. Troubleshooting

A. None of the Sense Oligo Spots Fluoresce

If none of the Sense Oligo Spots on a microarray hybridized with labeled cDNA made from the ArrayControl Spikes produce a hybridization signal, then consider the following suggestions.

Label cDNA with oligo(dT) or random primers only

Make sure your labeling protocol does not use a mix of gene specific primers for creating labeled cDNA. The RNA Spikes should be labeled with oligo(dT) or random primers since they may not hybridize with gene specific primers.

Use 0.5 pg to 5 ng of RNA Spikes in cDNA labeling reactions

Using less than 0.5 pg of RNA Spikes may produce a hybridization signal below the useful limit of detection.

B. ArrayControl Sense Oligo Spots are Much More Intense than Array Gene Features

This will be observed when the concentrations of the RNA Spikes are well above the average amount of mRNA used in the cDNA labeling reaction. It is convenient to have at least 1 "very bright" control for reference. Do this by adding a relatively large mass amount of that RNA Spike to the cDNA labeling reaction. Use lower concentrations of the other 7 RNA Spikes so that their hybridization signals are closer to, or mimic the fluorescent intensity ranges represented in the microarray.

C. One or More Sense Oligo Spots Do Not Fluoresce

If some of the Sense Oligo Spots show the expected hybridization signal, but 1 or more Spots on the same microarray do not show any hybridization signal, then consider the following troubleshooting suggestions.

Error was made in printing/spotting Sense Oligo Spots or in pipetting RNA Spikes

Though it seems obvious, the most likely reason for one or more of the Sense Oligo Spots to fail to fluoresce is either an accidental failure to print one of the Spots on the array, or a pipetting error or accidental omission of an RNA Spike from one of the dilution steps.

Too little Sense Oligo Spot was spotted

As the lower limit of detection is approached the signal to noise ratio will be reduced. For most arrays this limit is about 1.0–2.5 pg per spot. Some array systems are more or less sensitive but if you are using this amount of RNA Spikes then you may be approaching the limits of detection.

Sense Oligo Spot is degraded

Although unlikely, it is possible that 1 or more of the Spots is degraded.

RNA Spike is degraded

If one or more Sense Oligo Spots do not fluoresce, it is possible that the corresponding RNA Spike used in the cDNA labeling reaction was degraded. The RNA controls can be analyzed on an Agilent 2100 bioanalyzer. The RNA should be intact and represented by a single distinct peak of the appropriate size.

V. Appendix

A. Experimental RNA for Use in Microarray Hybridization

Labeled DNA for microarray hybridizations can be prepared from several different RNA sources, including total RNA, poly(A) selected RNA and amplified antisense RNA (aRNA). The amount of RNA (including RNA Spikes) needed should be determined empirically with several test experiments.

Total RNA

Total RNA is typically used in the range of 5–20 µg. The exact amount of mRNA in a total RNA sample varies, depending on a number of factors that include cell type or tissues used, metabolic status of cells during isolation, and most importantly the integrity of the RNA during isolation and storage. The expected percentage of mRNA in a total RNA sample is 2–5%. A 5 µg sample of high quality total RNA should contain 100–250 ng of mRNA.

Poly(A) selected RNA

Poly(A) selected RNA has a much higher ratio of mRNA to rRNA than total RNA, but the exact ratio will depend on the enrichment and isolation procedure in addition to the factors mentioned above. Differences in mRNA:rRNA ratios will effect the true amount of mRNA used in a labeling reaction. For example if two 1 µg poly(A) RNA samples are labeled, but 1 sample contains about 13% rRNA and the other sample has only 3% rRNA, there will be a 10% difference in the actual amount of labeled mRNA. This can complicate normalization and analysis, particularly for genes that are expressed at levels bordering the detection level. One way to determine the amount of rRNA in mRNA sample is to inspect the sample on an Agilent 2100 bioanalyzer.

Amplified RNA

RNA samples can be amplified by in vitro transcribing double-stranded cDNA; this is done when very little RNA is available (Van Gelder 1990). Ambion's MessageAmp™ II aRNA Amplification Kit can be used to amplify insufficient RNA samples. Diluted RNA Spikes can be added to RNA samples before amplification to monitor RNA amplification efficiencies and reproducibility. In this application, 1–100 pg of RNA Spikes would typically be used with 1–5 µg of total RNA to be amplified. ArrayControl RNA Spikes can also be used to determine the sensitivity and amplification efficiency of a given procedure (Madison et al. 1998).

B. References

Hughes TR, Mao, Jones AR, Burchard J, Marton MJ, Shannon KW, Lefkowitz SM, Ziman M, Schelter JM, Meyer MR, et al. (2001) Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nature Biotechnology* **19:** 342–347.

Kalocasi P, Shams S (2001) Use of bioinformatics in arrays. *Methods Mol Biol* **170:** 223–236.

Kane MD, Jatkoe TA, Stumpf CR, Lu J, Thomas JD, and Madore SJ(2000) Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays. *Nucleic Acids Res* **28:** 4552–4557.

Lee M-LT, Kuo FC, Whitmore GA, Sklar J (2000) Importance of replication in microarray gene expression studies: Statistical methods and evidence from repetitive cDNA hybridizations. *Proc Natl Acad Sci USA* **97:** 9834–9839.

Madison RD, Robinson GA (1998) Lambda RNA Internal Standards Quantify Sensitivity and Amplification Efficiency of Mammalian Gene Expression Profiling. *BioTechniques* **25(3)**:504–514.

Tseng GC, Oh MK, Rohlin L, Liao JC, Wong WH (2001) Issues in cDNA microarray analysis: quality filtering, channel normalization, models of variations and assessment of gene effects. *Nucleic Acids Res* **29(12):** 2549–2557.

Van Gelder, RN, von Xastrow, ME, Yool, A, Dement, DC, Barchas, JD, Eberwine, JH (1990) Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc Natl Acad Sci USA* **87:** 1663–1667.

Yue H, Eastman PS, Wang BB, Minor J, Doctolero MH, Nuttall RL, Stack R, Becker JW, Montgomery JR, Vainer M, Johnston R (2001) An evaluation of the performance of cDNA microarrays for detecting changes in global mRNA expression. *Nucleic Acids Res* **29(8):**E41–1.

C. Array Control Kit Specifications

Contents:

Cat #	Amount	Product	Storage
AM1780	10 µL ea.	RNA Spikes 1–8	below –70°C
	7 mL	TE Buffer (pH 7.0)	–20°C
AM1781	10 µL ea.	Array Control Sense Oligo Spots 1–8	–20°C

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.

- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

D. Quality Control

Functional Testing

ArrayControl Sense Oligo Spots are deposited onto glass slides and are hybridized with cyanine 3 or cyanine 5 labeled cDNA or RNA made from the ArrayControl RNA Spikes to verify that a hybridization signal can be detected.

Nuclease testing

Each component is tested in the following nuclease assays:

RNase activity

Meets or exceeds specification when a sample is incubated with ^{32}P-labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity

Meets or exceeds specification when a sample is incubated for 14–16 hr with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

Meets or exceeds specification when a sample is incubated for 14–16 hr with 40 ng ^{32}P-labeled *Sau*3A fragments of pUC19 and analyzed by PAGE.