SpotReport Alien cDNA Array Validation System

INSTRUCTION MANUAL

Catalog #252550

Revision A

For In Vitro Use Only

252550-12

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SpotReport Alien cDNA Array Validation System

CONTENTS

Materials Provided **1**
Storage Conditions **1**
Additional Materials Required **2**
Introduction **2**
Description of the Controls Provided **4**
Alien PCR Products and mRNA Spikes 4
Human β-actin gene 5
Additional Controls 5
Evaluating Microarray Data **6**
General Guidelines for Array Preparation 6
Devising a Microarray Printing Scheme 6
Evaluating Microarray Printing Quality 7
Evaluating mRNA Quality 8
Hybridization Specificity 9
Normalizing for Differences in Dye Incorporation and Quantum Yield 10
Quantitating Expected Dye Ratios 11
Signal Linearity and Sensitivity of the Assay 11
Hybridization Consistency within a Microarray 11
Guidelines for Using the Controls Provided **13**
Preparing and Printing PCR Products and Control DNA Samples 13
Incorporating the Alien mRNA in the Fluorescence-Labeling Reaction 14
Appendix **15**
General Considerations for Preparation of Labeled cDNA 15
Using Blocking DNA in the Array Hybridization 16
References **17**
Endnotes **17**
MSDS Information **17**

SpotReport Alien cDNA Array Validation System

MATERIALS PROVIDED

Material provided	Catalog #	Quantity
SpotReport Alien cDNA Array Validation System Alien mRNA [*in vitro* transcribed, polyadenylated $(A)_{17}$ RNA; 10 ng/µl in DEPC-treated H_2O]		
mRNA Spike 1	252561	100 ng
mRNA Spike 2	252562	100 ng
mRNA Spike 3	252563	100 ng
mRNA Spike 4	252564	100 ng
mRNA Spike 5	252565	100 ng
mRNA Spike 6	252566	100 ng
mRNA Spike 7	252567	100 ng
mRNA Spike 8	252568	100 ng
mRNA Spike 9	252569	100 ng
mRNA Spike 10	252570	100 ng
SpotReport Alien cDNA Array Validation System Alien PCR products (lacks polyA tail; lyophilized, ready-to-spot)		
PCR Product 1	252551	10 µg
PCR Product 2	252552	10 µg
PCR Product 3	252553	10 µg
PCR Product 4	252554	10 µg
PCR Product 5	252555	10 µg
PCR Product 6	252556	10 µg
PCR Product 7	252557	10 µg
PCR Product 8	252558	10 µg
PCR Product 9	252559	10 µg
PCR Product 10	252560	10 µg
Human β-actin PCR product[a] (lyophilized)	252151	10 µg
Poly$(dA)_{40–60}$ oligonucleotide (single stranded DNA 40–60 bases in length; lyophilized)	—	0.1 µg
Salmon sperm DNA (lyophilized)	—	1 µg
Human COT-1 DNA® (lyophilized)	—	1 µg
3× SSC buffer (DNase- and RNase- free)	—	5 ml

[a] Human β-actin (540 bp) (GenBank database accession #X63432)

STORAGE CONDITIONS

Alien mRNA Spikes: –80°C
All Other Nucleic Acid Materials: Room temperature until rehydrated. After rehydration store at –20°C
3× SSC Buffer: Room temperature

Revision A

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Additional Materials Required

Microarray printing device
FairPlay Microarray Labeling Kit (Stratagene Catalog #252002 or 252003; optional)

Introduction

An increasing trend in identifying differentially expressed genes is the use of nucleic acid microarrays that contain hundreds or thousands of probe genes.[1] In these experiments, test and reference RNA are converted by reverse transcription into cDNA with labeled nucleotides (target DNA). The labeled test and reference cDNA are then hybridized to genes on microarrays, and after unhybridized cDNA is removed, signal from the hybridized cDNA is detected. Differences in hybridization signals on the microarray correlate with differences in abundance of the mRNA used to synthesize the labeled cDNA.

There are several significant concerns that arise during the evaluation of microarray hybridization data. These concerns include the quality of the microarray printing, the quality of the mRNA used to synthesize labeled test and reference cDNA, the sensitivity of the assay, the hybridization specificity and consistency within a microarray, and the hybridization signal consistency between microarray slides. To evaluate these concerns, the SpotReport Alien cDNA array validation system* provides positive and negative controls that can be printed onto a microarray along with researcher-provided test genes. The key to this system is a set of DNA controls that have an average GC content of 50%, no significant homology to any known nucleic acids and low secondary structure. This set of exogenous nucleic acid controls can be used with any eukaryotic or prokaryotic microarray system.

The SpotReport Alien cDNA array validation system contains ten exogenous alien mRNA spikes that can be added to the labeling reaction along with the experimental mRNA. The hybridization signals detected from the positive and negative control spots on the microarray can be evaluated to determine the quality of both the microarray and the mRNA, the microarray orientation, and the sensitivity, specificity, signal linearity, and consistency of the assay. In addition, the expected dye ratios can be determined and the differences in signal intensities due to the differences in dye incorporation and quantum yield can be normalized. Table I outlines the applications for each of the components in the SpotReport Alien cDNA array validation system.

* Patent pending.

TABLE I

Applications for Each SpotReport System Component

	alien mRNA spikes	alien PCR product	human β-actin PCR product	poly(dA)$_{40-60}$	salmon sperm DNA	human COT-1 DNA®	3× SSC buffer
identifying DNA carryover during microarray printing							✓
visualizing array orientation	✓	✓	✓				
determining mRNA quality	✓	✓	✓				
positive hybridization control	✓	✓	✓				
negative hybridization control		✓		✓	✓	✓	
determining hybridization specificity		✓		✓	✓	✓	✓
optimizing scanner settings	✓	✓					
normalizing for differences in dye incorporation and quantum yield	✓	✓					
quantitating dye ratios	✓	✓					
determining signal linearity and sensitivity	✓	✓					
determining hybridization consistency	✓	✓	✓				

Description of the Controls Provided

Alien PCR Products and mRNA Spikes

The ten exogenous alien PCR products and corresponding mRNA spikes provided in this kit serve as either positive or negative controls in evaluating microarray systems. These sequences were selected because they do not have homology to any known nucleic acid sequences currently in public databases (BLAST analysis with the Stratagene Clone Collections [*www2.stratagene.com*] and NIH sequence[2] databases). Therefore, labeled cDNA generated from the corresponding mRNA spikes are unlikely to hybridize to human, mouse, rat, yeast, or plant genes spotted on microarrays.

Each of the ten alien controls is provided in two forms: PCR products and mRNA spikes. The PCR products are spotted along with researcher-provided experimental DNA onto microarrays. The mRNA are converted to labeled cDNA and hybridized to the corresponding PCR products on the microarrays.

The alien PCR products do not contain a polyA tail. The lack of a polyA tail ensures that hybridization signal is due to specific hybridization between homologous cDNA and not due to hybridization from nonhomologous cDNA containing a polyT track.

When used as a positive control, the alien mRNA is reverse transcribed and labeled in the same reaction as the experimental mRNA. Following the labeling reaction, the alien cDNA is hybridized to the alien PCR products on the microarray. The intensity of the hybridization signal is then used to evaluate the microarray system (Table I).

When used as a negative control, the alien mRNA is not added to the labeling reaction with the experimental mRNA. In the absence of labeled alien cDNA, there should be little or no detectable hybridization signal where the alien PCR products were spotted on the microarray. Some researchers feel that the hybridization signal from these spots is a better indication of the true background signal than the hybridization signal from printing buffer spots. The spotted alien PCR products can therefore be used to determine background signal in the absence of labeled alien cDNA.

Human β-actin gene

The human β-actin gene was chosen as a positive control because it is expressed at relatively high levels in most human tissues. Each human β-actin probe gene spotted on the microarray is therefore expected to generate a significant hybridization signal with most human mRNA-derived labeled cDNA.

Additional Controls

In general, hybridization signals should not be detected from poly(dA)$_{40–60}$, salmon sperm DNA, or human COT-1 DNA® negative control spots. However, the lack of hybridization signal will be dependent upon the components of the hybridization buffer (see *Hybridization Specificity*).

EVALUATING MICROARRAY DATA

The proper use of the positive and negative controls provided in this kit should be considered when devising microarray experiments. In this section, *Evaluating Microarray Data*, aspects of designing microarray experiments and incorporating useful controls are discussed. More specific guidelines on preparing the control DNA and on the use of the mRNA spikes (provided in this kit) can be found in *Guidelines for Using the Controls Provided*.

General Guidelines for Array Preparation

Guidelines for preparing microarrays are available at many sites on the Internet, such as Pat Brown's lab at Stanford University (http://cmgm.stanford.edu/pbrown/). If you are spotting the DNA on slides from a commercial supplier, follow the protocol recommended by the supplier.

Devising a Microarray Printing Scheme

A pattern for printing should be devised such that the control spots are present in all regions of the slide and in sufficient replicate numbers to permit statistical analysis. Spots of probe genes expected to give significant hybridization signals, such as the alien DNA and/or the human β-actin gene, should be placed in a pattern at the perimeter of the array to serve as landmarks so that it is immediately clear when looking at the array that the entire array is present and that it has been in contact with the hybridization solution. Placing positive and negative control spots in the four corners of the array can also provide points of reference when determining microarray orientation (Figure 1).

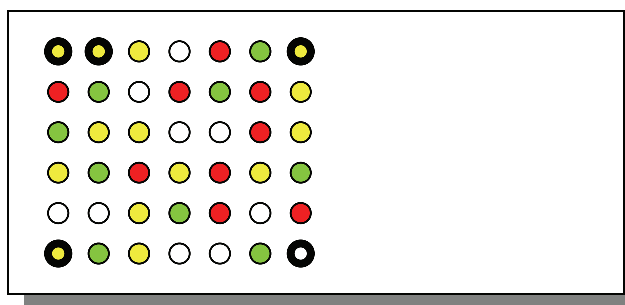

FIGURE 1 Using positive and negative controls (outlined in dark circles) to indicate microarray orientation.

In addition, the alien DNA can be spotted in the first row of the array that is scanned by the microarray scanner. The hybridization signals of these control spots are used to optimize the scanner settings. For example, when the same alien mRNA spikes are added in equal molar amounts into the CY™ 3 and CY™ 5 cDNA labeling reactions, the fluorescence intensities of these spots can be equalized by adjusting the laser/PMT voltages.

Evaluating Microarray Printing Quality

The 3× SSC (or other suitable spotting buffer) negative control spots can be used to evaluate the quality of the microarray printing process. During the printing process, the printing pins pick up DNA from a source plate and print the DNA onto microscope slides at predefined coordinates. The printing pins then move to a wash station where the DNA is removed from the pins before the next DNA sample is collected. This process is repeated until the microarray printing is complete. If the DNA is not completely removed from the printing pins at the wash station, any DNA remaining on the pins will be combined with and printed with the next DNA sample, producing spots printed with the combined DNA. This is commonly referred to as DNA carryover.

DNA carryover during the microarray printing process can be identified by including 3× SSC in two or more wells of the source plate at the time of printing. DNA carryover can be identified by detecting hybridization signal from one or more of the 3× SSC spots. Because it is easier to detect carryover of an experimental DNA that is present in high abundance in the labeled cDNA, we recommend positioning the 3× SSC in a well immediately following the human β-actin PCR product (or any other cDNA known to be of high abundance in the labeled cDNA; Figure 2). If the same printing parameters were used to generate the entire microarray, the detection of hybridization signal from one or more 3× SSC spots indicates that DNA carryover is probably occurring in every DNA spotted during the microarray printing process.

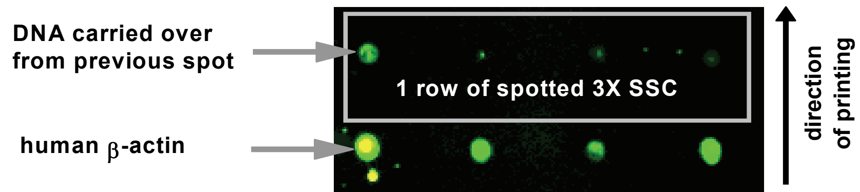

Figure 2 DNA carryover during microarray printing.

Evaluating mRNA Quality

The quality of the experimental mRNA is critical for successful labeled cDNA preparation. The presence of contaminants, such as cellular carbohydrates and proteins, can cause a decrease in labeling efficiency and an increase in background hybridization signal. The guanidinium isothiocyanate method used in the Absolutely RNA RT-PCR miniprep kit[3,4] (Catalog #400800) is ideal for isolating or purifying mRNA for use with microarrays. For general considerations regarding the preparation of fluorescence-labeled cDNA, see *Appendix*.

The quality of the experimental mRNA can be determined by quantitating the hybridization signals of the human β-actin and alien control spots. Labeled human β-actin cDNA is synthesized from experimental human mRNA whereas alien cDNA is synthesized from the alien mRNA spikes provided in the kit. Detection of hybridization signals from both the human β-actin and alien control spots indicates that the experimental human mRNA is of high quality, that the cDNA was efficiently labeled, and that the hybridization was successful. If significant hybridization signals are detected from only the alien control spots, then the quality of the experimental mRNA is poor. If hybridization signals are not detected from either the human β-actin or alien control spots, then one or more parts of the assay (such as the cDNA synthesis/labeling or hybridization) failed. A common cause is when the experimental mRNA contains one or more contaminants, such as RNases, that affected synthesis of the experimental and alien cDNA.

Hybridization Specificity

An important question that arises during the analysis of microarray hybridization signals is whether the hybridization is specific or nonspecific. Hybridization specificity is determined by observing whether or not the labeled cDNA binds to salmon sperm DNA, poly(dA)$_{40–60}$, and human COT-1 DNA® negative control spots. In addition, little or no hybridization signal should be detected when the alien mRNA spikes are used as negative controls (see *Description of the Controls Provided*).

The poly(dA) provided in this kit is single-stranded and 40–60 bases in length. The labeled oligo dT primed cDNA has a polyT tract that can hybridize to the polyA tail in every cDNA that is spotted on the microarray. This would result in a hybridization signal that is based on the presence of a polyA tail in the spotted cDNA and not on the presence of a specific cDNA in the hybridization solution. Because of this, polyA is commonly used in the hybridization buffer to block this undesired hybridization. To verify that this undesired hybridization is not occurring, spot the polyA provided in this kit at the same time the probe genes are spotted. Little to no hybridization signal should be observed from these spots.

Salmon sperm DNA and/or human COT-1 DNA® are commonly used in the hybridization buffer to block nonspecific hybridization and hybridization to repetitive elements, respectively. Effective blocking of these undesired hybridizations can be verified by little to no hybridization signal from the salmon sperm and COT-1 DNA® spots.

3× SSC is commonly used as the spotting buffer for the preparation of DNA microarrays. Hybridization signal from all or most of the spots containing 3× SSC alone indicates that nonspecific hybridization has taken place. Alternatively, hybridization signal from 3× SSC spots that follow detectable cDNA may indicate DNA carryover is occurring (see *Microarray Printing Quality*).

Normalizing for Differences in Dye Incorporation and Quantum Yield

It is well-known that CY 3 and CY 5 fluorescent dyes (Amersham Pharmacia Biotech), the most commonly used dyes incorporated into cDNA for use with microarrays, are incorporated at different levels in reverse transcription reactions and have different quantum yields.[5] This results in a difference in the CY 3 and CY 5 fluorescence intensities even when equal amounts of CY 3- and CY 5-labeled cDNA are present. These differences can be normalized by (1) determining the ratios of the hybridization signal of equal amounts of the CY 3- and CY 5-labeled alien cDNA and then (2) multiplying the values from test or reference cDNA by these ratios (Figure 3).

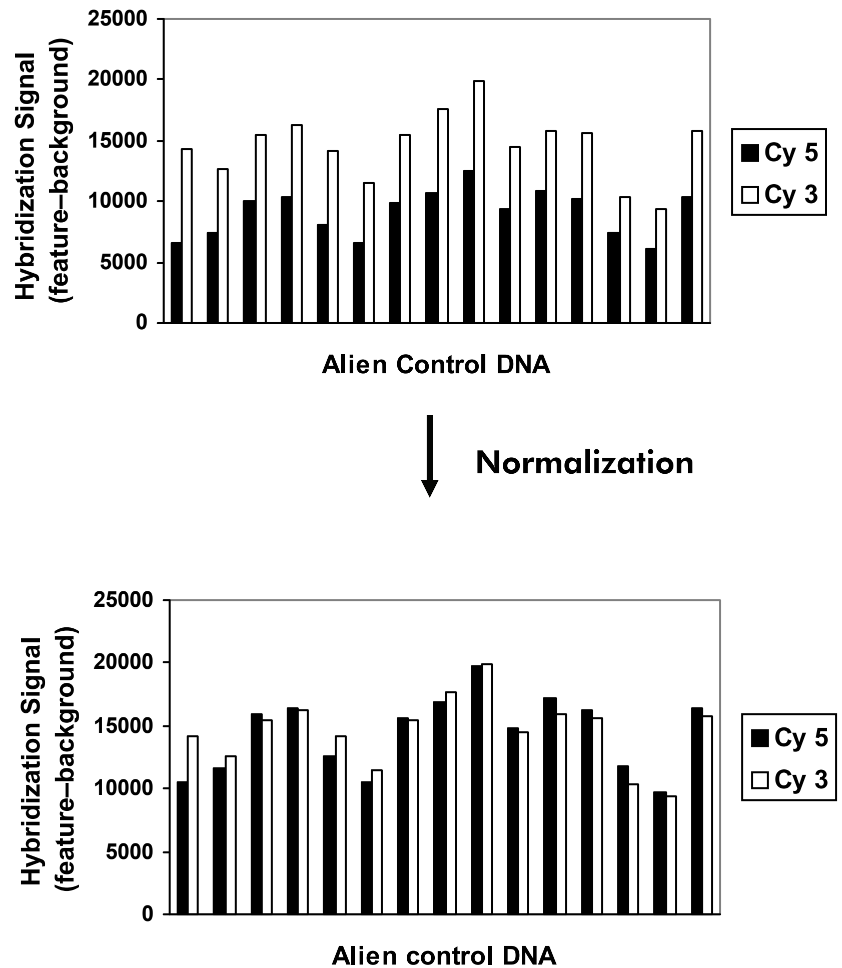

Figure 3 Normalizing hybridization signal for differences in dye incorporation and quantum yield.

The ratios representing the relative expression levels in the test and reference mRNA are calculated after data normalization. Normalizing the data prior to calculating the expression ratios for the test DNA allows for comparisons to be made between different experiments and between different laboratories.

Quantitating Expected Dye Ratios

Because the expression ratio of the spotted test gene is used to determine if the gene is differentially expressed, it is valuable to be able to determine how the expression ratio correlates with the amount of RNA template added to the labeling reaction. The expected dye ratios are determined by simply adding different amounts of the alien mRNA spikes to different dye labeling reactions. For example, add 0.5 and 1.0 nanograms of alien mRNA spike 1 to a CY 3 and CY 5 labeling reaction, respectively, and compare the hybridization signals following hybridization. The dynamic range of the expression ratios can be determined by creating a standard curve.

Signal Linearity and Sensitivity of the Assay

The labeled alien cDNA and spotted DNA are used to determine the signal linearity and sensitivity of the assay. To determine the signal linearity, add different amounts of alien mRNA to test or reference mRNA prior to the cDNA synthesis/labeling reaction. For example, choose amounts that correspond to RNA of high, medium, and low abundances. The relative hybridization signals of the alien cDNA when hybridized to the corresponding alien DNA on the microarray are used to determine the signal linearity.

To determine the sensitivity of the assay, the alien mRNA are added to the cDNA-labeling reaction in decreasing amounts. The sensitivity of the microarray assay is indicated as the lowest amount of alien cDNA detected.

Hybridization Consistency within a Microarray

The consistency of the hybridization signals from different areas of the microarray is a primary concern during the evaluation of microarray data. Factors that can affect the accurate determination of hybridization signals include adequate mixing of the hybridization solution, poor or inconsistent binding of spotted DNA to the slide surface, missing DNA spots, a dirty coverslip, inconsistent or inadequate hybridization temperature, and defects in the microarray surface such as cracks or scratches in the slide coating. The alien and human β-actin controls can be used to identify defective areas within a microarray that should be excluded from further analysis prior to evaluating the overall variation within a microarray using statistics. The number of the alien and human β-actin control spots that must be printed is governed by the type of statistical analysis and the desired confidence limits.

Comparing the hybridization signal of each spot for each type of control can identify defective areas in a microarray that should be excluded from analysis. The hybridization signals of all the spots of each type of control should be similar. The presence of an individual control spot with a hybridization signal that deviates significantly from the norm indicates that the control spot and the experimental spots in its vicinity should be examined to determine whether their hybridization signals can be accurately determined or whether the spots should be excluded from further analysis.

The hybridization consistency of each microarray assay is determined statistically by calculating the average variation of replicates of spotted genes (standard deviation of spot values/mean). The average variation of replicates indicates the amount of variation between multiple spots of the same control DNA. Additional statistical methods for determining experimental variation are available from scientific literature.

GUIDELINES FOR USING THE CONTROLS PROVIDED

Preparing and Printing PCR Products and Control DNA Samples

To prepare microarrays, we resuspend the PCR products in 100 µl of 3× SSC to a final DNA concentration of 0.1 µg/µl. We resuspend the human COT-1 and salmon sperm DNA in 10 µl of 3× SSC to a final DNA concentration of 0.1 µg/µl. We resuspends the poly$(dA)_{40–60}$ in 10 µl of 3× SSC to a final DNA concentration of 0.01 µg/µl. Then, 10 µl of the resuspended PCR products are placed into 10 separate wells of a 384-well source plate. Each PCR product is spotted 10 times per microarray. 10 µl of the resuspended human COT-1, salmon sperm DNA and poly $(dA)_{40–60}$ are placed into single wells of a 384-well source plate. The human COT-1, salmon sperm DNA, and poly $(dA)_{40–60}$ are spotted one time per microarray. The DNA controls and 3× SSC are spotted onto slides using a microarray printing device. The spotted microarrays are then blocked following the slide manufacturer's recommendations.

The number of microarrays that can be printed with the controls provided in this kit is dependent upon the arrayer and protocol that are used. For example, under the following conditions, approximately 1,000 microarray slides can be printed using the DNA provided in the kit.

- the PCR products and other control DNA are prepared as described above
- the pins used to spot the DNA pick up < 0.5 µl of the resuspended DNA per printing
- if the DNA volume is reduced by evaporation during the print run or during storage, that the DNA concentration is adjusted to its original concentration prior to the next print run
- the arrayer can spot 100 slides per printing
- the arrayer can remove DNA from the source plate down to a minimum volume of 5 µl

The microarray slides would be printed 10 times with each of the PCR products and 1 time with each of the other control DNA.

Incorporating the Alien mRNA in the Fluorescence-Labeling Reaction

The amount of alien mRNA spikes to be added to your fluorescence-labeling reaction is dependent upon the goal of your assay. A good starting point is to determine the sensitivity of your assay. For example, add 5 ng, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.04, 0.02 and 0.01 ng of the ten different alien mRNA spikes to 20-100 µg of the test and/or reference total RNA. If less than 100 µg of total RNA is labeled, decrease the amount of alien mRNA added to the labeling reaction to maintain the ratio of alien mRNA to total RNA. The amount of alien mRNA added to the labeling reaction can be increased or decreased as needed. The sensitivity of the assay is indicated by the lowest amount of alien mRNA that is detected following hybridization. The sensitivity of the assay serves as a guideline for the amount of alien mRNA to add in subsequent experiments.

When making dilutions of the alien mRNA, we recommend making the dilutions with DEPC-treated water containing 10 ng/µl of RNase-free yeast tRNA to increase the stability of the low amounts of alien mRNA.

APPENDIX

General Considerations for Preparation of Labeled cDNA

The quantity and quality of the fluorescence-labeled cDNA generated by the labeling protocol is highly dependent on the method used to isolate the RNA and then to prepare and purify the fluorescence-labeled cDNA. The quantity and quality of the fluorescence-labeled cDNA has a significant effect on the experimental results.

- The quality and quantity of the RNA used is critical for successful preparation of labeled cDNA. The presence of cellular lipids, carbohydrates or proteins will significantly increase background fluorescence following hybridization. The presence of genomic DNA will effect cDNA labeling and hybridization efficiency. The OD 260/280 ratio of the RNA must be >1.8. When RNA isolated from mammalian sources is viewed on a denaturing agarose gel, the ribosomal bands (28S and 18S) should appear as two bright bands at approximately 4.5 and 1.9 kilobases. The ratio of intensities of the 28S and 18S bands should be 1.5-2.5:1. Lower ratios may indicate that RNA degradation has occurred and that this RNA may not be suitable for the preparation of labeled cDNA. Additional bands, including low molecular weight bands corresponding to the 5S ribosomal RNA and tRNA may also be visible. The guanidinium isothiocyanate method used in the Absolutely RNA RT-PCR miniprep kit[3,4] is ideal for the isolation of total RNA for use in this application and was used to isolate the RNA used in the development of this kit.

- The presence of EDTA and/or ethanol can inhibit reverse transcriptase activity in the labeling reaction. If the RNA is ethanol precipitated prior to labeling, all ethanol must be removed prior to use in the labeling reaction.

- It is imperative to protect the RNA from any contaminating RNases until the cDNA synthesis is complete. Wear fresh gloves, use newly autoclaved pipet tips, and avoid using pipet tips or microcentrifuge tubes that have been handled without gloves. Ribonuclease A cannot be destroyed by normal autoclaving alone. Baking or DEPC treatment is recommended.

- The quality of the fluorescent dye is critical. The use of partially degraded fluorescent dyes may result in poor cDNA labeling efficiency and a higher fluorescent background following hybridization.

- The fluorescence intensities of CY 3- and CY 5-labeled cDNA hybridized to microarrays under competitive hybridization conditions have a high degree of correlation. If other fluorescent dye pairs are used to label the cDNA, we highly recommend that the same cDNA be labeled with each of the fluorescent dyes, hybridized to a microarray under competitive hybridization conditions, and the Pearson's correlation coefficient between the fluorescence intensities calculated. The ideal correlation is 1.0 and indicates that there is a 1:1 correlation between cDNA labeled with each of the fluorescent dyes. The use of fluorescence-labeled cDNA with a high correlation results in the more accurate identification of differentially expressed genes than the use of fluorescence-labeled cDNA with a low correlation.

- The method used for purification following the cDNA labeling reaction is critical for recovery of the fluorescence-labeled cDNA and removal of the unincorporated fluorescent dye. The guanidinium isothiocyanate method used in the StrataPrep PCR purification kit[6] (Catalog #400771 or 400773) is ideal for the purification of fluorescence-labeled cDNA for use with microarrays.

Using Blocking DNA in the Array Hybridization

Blocking DNA is used in the hybridization mixture to reduce non-specific binding between the fluorescence-labeled cDNA and the surface of the microarray.

- The recommended blocking DNA sources are either human or mouse COT-1 DNA®, yeast tRNA, and poly(dA)$_{40–60}$. If human DNA containing repetitive sequences is spotted on the microarray, use human COT-1 DNA® in the blocking solution. Human COT-1 DNA® is placental DNA that is 50 to 310 bp in size and is enriched for repetitive DNA sequences such as the Alu and Kpn family.[7,8] If mouse DNA containing repetitive sequences is spotted on the microarray, use mouse COT-1 DNA® in the blocking solution. Mouse COT-1 DNA® is mouse DNA that is 50 to 300 bp in size and is enriched for repetitive DNA sequences such as the B1, B2, and L1 family members[9,10] Including human or mouse COT-1 DNA® in the hybridization mixture reduces undesired hybridization between repetitive DNA sequences in the labeled cDNA and probe DNA. Yeast tRNA reduces undesired nonspecific DNA hybridization. Poly(dA) that is 40 to 60 dATP bases in length promotes specific hybridization between the labeled cDNA and probe DNA by reducing hybridization of the polyA sequences in the probe DNA to the polyT tract in the labeled cDNA.

- The amount of blocking DNA to use in the hybridization is dependent on the labeling method used to generate the fluorescence-labeled cDNA, therefore, use the amount recommended in your labeling protocol.

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Endnotes

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MSDS Information

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.