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Generating Cy3 or Cy5-labeled cDNA Probe from Amplified aRNA for Microarray Hybridizations Using A Direct Incorporation Method

PROTOCOL #3

Abstract

Linear amplification of RNA is a technique by which microgram amounts of amplified antisense RNA (aRNA) can be generated from small amounts of samples, enabling high-throughput gene expression analysis of these samples using platforms such as microarrays. This application note describes a protocol for fluorescent labeling of aRNA generated using the RiboAmp® RNA Amplification Kit, for probing cDNA microarrays.

Introduction

Microarrays provide a powerful tool for conducting gene expression studies at the whole genome level. However, microarrays require microgram amounts of labeled nucleic acids for hybridization.[1,2] When sample amounts are very limited, such as pure cell populations procured using Laser Capture Microdissection (LCM; Arcturus), fine needle aspirates, or small biopsies, microgram amounts of RNA are often not available.3 Hence techniques, such as linear amplification, have become important to amplify messenger RNA (mRNA) from limited amounts of total cellular RNA for the generation of adequate amounts of material for hybridization. Linear amplification of RNA, using the RiboAmp RNA Amplification Kit (Arcturus), has proven to be a valuable tool in generating microgram amounts of material, sufficient for high-throughput gene expression assays such as microarrays.

The protocol described here may be used to prepare Cyanine 3- or Cyanine 5-labeled cDNA from aRNA generated using the RiboAmp RNA Amplification Kit for hybridization to cDNA microarrays. This protocol provides labeled probe of sense orientation from 5-10 micrograms of aRNA, a sufficient quantity for replicate hybridizations on cDNA microarrays.

NOTE: The probes are typically not used for oligonucleotide arrays, since the targets on such arrays are also generally in the sense orientation.

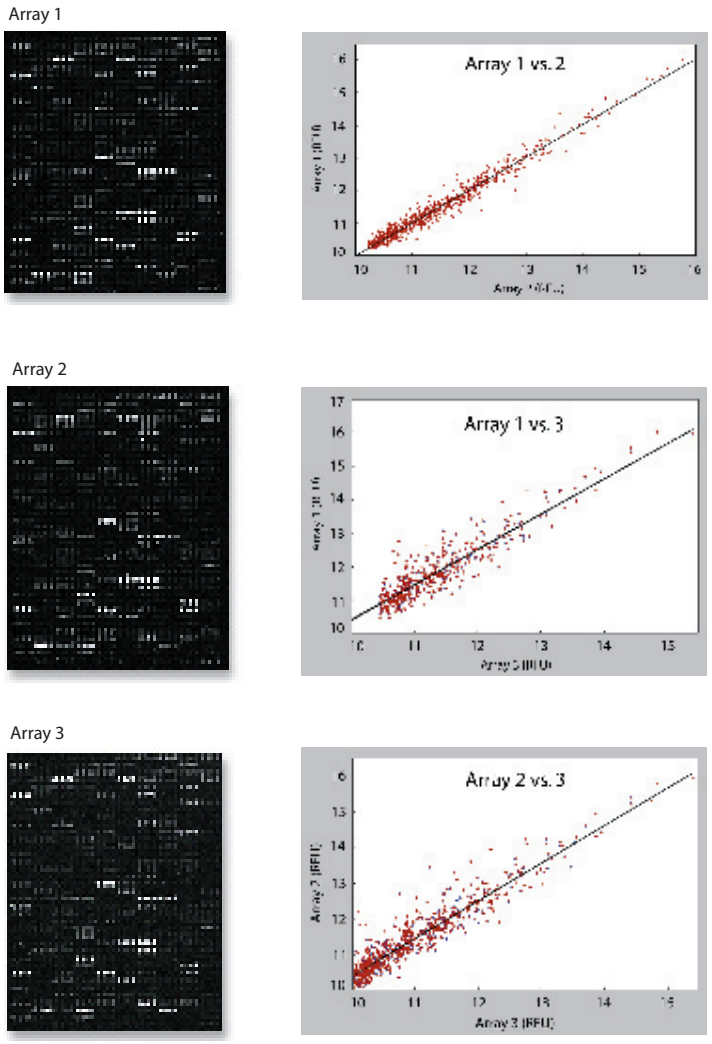

Figure 1. Microarray analysis of three independent amplifications. Microarray images of independent hybridizations of samples generated from three independent amplifications and labeling of mouse testis cell line (TM3) total cellular RNA using the RiboAmp RNA Amplification Kit . Labeling was performed following the protocol described here, and arrays were scanned on a GenePix 4000 scanner (Axon Instruments). Intensity scatter plots of pair-wise array comparisons are shown, with Pearson correlation coefficient, R, reported against them.

Reagents

This protocol requires the following reagents:

- ❑ RiboAmp® RNA Amplification Kit (Arcturus, Cat. # KIT0201)
- ❑ RNase AWAY® (Invitrogen, Cat. # 10328-011)
- ❑ Cy3 labeled dUTP (Amersham, Cat. # PA53022)
- ❑ Cy5 labeled dUTP (Amersham, Cat. # PA55022)
- ❑ RNAsin® Ribonuclease Inhibitor (Promega, Cat. # N2515)
- ❑ SuperScript™ II RT and Buffer (Invitrogen, Cat. # 18064-071)
- ❑ Nuclease Free Water (Invitrogen, Cat. # 10977-023)
- ❑ Rnase H (Invitrogen, Cat. # 18021-071)
- ❑ Random Hexamer (Operon, custom-made)
- ❑ QiaQuick® PCR Purification Kit (Operon, Cat. # 28106)
- ❑ dNTP Set (dATP, dCTP, dGTP, dTTP), 4 x 25 µmol (Amersham, Cat. # 27-2035-01)

Related product:

- ❑ PicoPure® RNA Isolation Kit (Arcturus, Cat. # KIT0202)

Equipment and Labware

The following laboratory materials are also required to complete the protocol:

- ❑ Disposable gloves
- ❑ Ice or cold block (4°C)
- ❑ Nuclease-free pipette tips
- ❑ Kimwipes™ or similar lint-free towels
- ❑ Thermal cycler with heated lid
- ❑ Microcentrifuge for 2.0 ml and 0.65 ml tubes (Eppendorf 5415D or similar)
- ❑ Pipettor: 1000µl, 200µl, 100µl, 20µl, 10µl
- ❑ 2.0 ml and 0.65 ml RNase-free microcentrifuge tubes

RNase-free Technique

RNase contamination will cause experimental failure. Minimize RNase contamination by adhering to the following recommendations throughout your experiment:

- Wear disposable gloves and change them frequently.
- Use RNase-free solutions, glassware and plastic ware.
- Wash scalpels, tweezers and forceps with detergent and bake at 210°C for four hours before use.
- Use RNase AWAY® (Invitrogen) according to the manufacturer's instructions on the horizontal staining rack and any other surfaces that may come in contact with the sample.

Thermal Cycler Programming

Program a thermal cycler with a heated lid as follows:

Heated lid:	Yes
70°C	10 minutes
4°C	hold
27°C	10 minutes
37°C	2 hours
4°C	hold
37°C	20 minutes
4°C	hold

Special Considerations

Frequent freeze-thawing may accelerate RNA degradation. For best results, store RNA at –70°C until needed.

Special Considerations (continued)

Bring all components except enzymes to room temperature just prior to use. Make sure each reagent is completely thawed, then mix well and spin down before dispensing. Dispensing partially thawed reagents may result in reaction mixtures with incorrect concentrations of components. Place reagents on ice or cold block, or refreeze immediately after dispensing. Do not leave reagents at room temperature for a long time.

Briefly mix and spin down sample(s) following incubation steps to maintain proper volumes and concentrations of reagents and to ensure that all nucleic acid templates are mixed with reaction components.

Cy5 and Cy3 conjugated nucleotides, and probe prepared with these reagents, should be protected from extended exposure to light. Addition of nucleotides to the reaction and probe purification should be done in low-light conditions.

Method

Step	Procedure
1.	Take 5-10 µg of amplified aRNA and adjust the volume to 22 µl with nuclease-free water. ⚠ **If the volume is greater than 22 µl, concentrate it down to 22 µl using a vacuum concentrator, paying attention to not completely dry down the aRNA sample.**
2.	Add 2 µl of 5 mg/ml random hexamer.
3.	Mix well by flicking, then briefly spin down by centrifugation.
4.	Heat the tube to 70°C for 10 minutes, then 4°C for 2 minutes in the thermal cycler.
5.	During incubation, prepare the First Strand Master Mix as described here.
6.	When incubation is complete, mix the tube well by flicking, and then briefly spin down by centrifugation.
7.	Add 26 µl of the above First Stand Master Mix to each reaction tube.
8.	Mix well by flicking, and then briefly spin down by centrifugation.
9.	Incubate at 27°C for 10 minutes, followed by 37°C for 2 hours in the thermal cycler.
10.	Treat with 2 units of RNase H for 20 minutes at 37°C in the thermal cycler.
11.	Immediately proceed to PCR product purification using QiaQuick® PCR Purification Kit: Pre-treat the columns placed in collection tube by incubating 100 µl of QiaQuick® PB buffer for 5 minutes, and then centrifuge at 13200 rpm (or full speed on a 5415C Eppendorf Centrifuge) for 1 minute.
12.	Add 260 µl of QiaQuick® PB buffer to the sample tube.
13.	Mix well by flicking, and then briefly spin down by centrifugation.
14.	Load the sample onto the pre-treated columns. Centrifuge at 6000 rpm for 1 minute. ⚠ **After centrifuging, make sure that the entire sample has passed through the column, and that the column is completely dry. If not, centrifuge for an additional 1 minute at 6000 rpm. When dry, the column will be visibly pink (Cy3) or blue (Cy5) if the reaction was successful.**

First Strand Master Mix - 1x	
First Strand Buffer	10 µl
0.1 M DTT	5 µl
25 mM d(AGC)TP	1 µl
1 mM dUTP-Cy3 or Cy5	2 µl
1 mM dTTP	2 µl
RNasin®	2 µl
Superscript™ II RT	4 µl
Total	**26 µl**

15.	Discard flowthrough. Place the column into the same collection tube.
16.	Wash with 750 µl of QiaQuick® PE buffer. Centrifuge at 13200 rpm for 1 minute.
17.	Discard flowthrough. Place the column back into the same collection tube.
18.	Centrifuge at 13200 rpm for an additional 2 minutes to remove residual wash solution.
19.	Place the column into a clean 2 ml microcentrifuge tube.
20.	Add 50 µl of nuclease-free water, pH 8.5, directly onto the column membrane. Incubate for 3–5 minutes, and then centrifuge at maximum speed for 1 minute. ⚠ **If the labeling reaction was successful, the eluate should be visibly pink (Cy3) or blue (Cy5) in color.**
21.	If the column still shows residual probe, add another 30 µl of nuclease-free water, pH 8.5, directly onto the column membrane, incubate for 1 minute, and centrifuge at maximum speed for 1 minute.

Results

We have demonstrated excellent reproducibility of microarray data from labeled probe generated using the above method described here. Three independent amplifications of total cellular RNA from mouse testis cell line (TM3) were performed using the RiboAmp RNA Amplification Kit, labeled with Cy5-dUTP using the protocol described here, and hybridized onto three 9000-element mouse cDNA arrays. Microarray images and scatter plots (Figure 1) comparing the three independent amplifications demonstrate very good reproducibility and high correlations in microarray data with R values ≥0.969. These arrays exhibited an excellent dynamic range of intensities, about 2-3 logs in magnitude, alluding to the high quality of the labeling and hybridization process.

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