Throw Away the Script for Microarrays

Indirect Fluorescent Labeling of Microarray Targets Using ImProm-II™ Reverse Transcriptase

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Abstract

Historically, fluorescent cDNA targets used for microarray hybridization have been produced using direct incorporation of nucleotides coupled to bulky fluorescent dyes such as Cy®3 and Cy®5. These modified nucleotides are not efficiently incorporated by reverse transcriptases. Moreover, reverse transcriptases incorporate different dye-modified dNTPs at different rates. Recently, indirect labeling of cDNA target populations, using aminoallyl-derivatized dUTP incorporation followed by amino-coupling of fluorescent dyes, has been gaining ground on the traditional method of direct incorporation.

ImProm-II™ Reverse Transcriptase offers a cost-effective alternative for indirect labeling of fluorescent cDNA targets for microarray research.

Introduction

Microarrays are typically probed with fluorescently labeled cDNA transcripts. The cDNA probes are reverse transcribed from an RNA template and are labeled by either direct or indirect incorporation of fluorescent nucleotides. Direct incorporation of fluorescently labeled nucleotides has been widely used but can be inconvenient, since reverse transcriptases incorporate differently labeled nucleotides with different efficiencies (1).

A recent development in labeling methods helps to circumvent this problem. The process of indirect labeling allows incorporation of modified nucleotides, commonly 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate (a reactive amine derivative of dUTP) into the reverse transcription reaction. The aminoallyl nucleotide is readily incorporated by both DNA and RNA polymerases (2). A reactive fluorescent dye is then chemically attached to the cDNA transcript in a second reaction.

Here we demonstrate that a new reverse transcriptase, ImProm-II™ Reverse Transcriptase (RT), is an effective and robust enzyme for indirect labeling of cDNA. This was shown by incorporating an aminoallyl-modified nucleotide in a reverse transcription reaction, followed by coupling with a reactive fluorescent dye. The resulting fluorescent cDNA populations from PMA/ionomycin treated and untreated DO11.10 mouse T cell hybridomas were hybridized to 6,800-gene DNA microarrays made with 70mer oligos (Operon) spotted on poly-L-lysine-coated slides. Arrays were manufactured at the J. David Gladstone Institutes Genomics Core Facility. Experiments comparing ImProm-II™ RT to the industry standard, M-MLV H⁻ RT, showed that ImProm-II™ RT is a robust and cost-effective enzyme for the production of indirectly labeled cDNA.

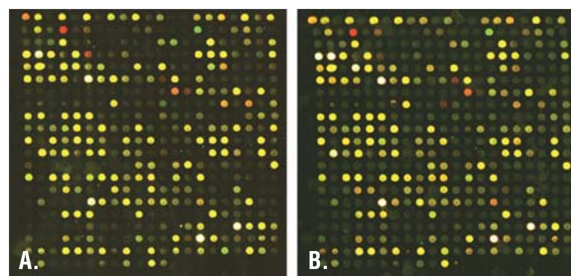

Figure 1. A comparison of typical 21 by 21 feature array blocks hybridized with ImProm-II™- or SuperScript™-II-generated fluorescent cDNA probes. **Panel A:** Array hybridized with ImProm-II™-generated fluorescent cDNA probes. **Panel B:** Array hybridized with SuperScript™-II (M-MLV H⁻)-generated fluorescent cDNA probes. Image brightness was normalized to yield similar absolute background intensity.

The model used for this study was PMA (phorbol 12-myristate 13-acetate) and ionomycin treatment of DO11.10 T cell hybridomas. PMA activates protein kinase C by binding to its diacylglycerol recognition site, while ionomycin mimics the calcium flux resulting from the binding of inositol triphosphate to intracellular receptors. Thus, treatment with PMA/ionomycin is analogous to antigen receptor signaling in T cells. Profiles of differential gene expression in response to antigen receptor signaling in T cells have been previously published (3,4). The goal of these experiments was to confirm (as part of a larger study) that we would observe a profile of differential gene expression substantially similar to the published data using our model system in conjunction with our DNA microarrays and probes prepared using indirect fluorescent cDNA labeling.

Results

Figure 1 shows the fluorescence scan of a typical 21 by 21 feature block from one array hybridized with ImProm-II™-generated fluorescent cDNA probes (Panel A) and another array hybridized with SuperScript™-II (M-MLV H⁻)-generated fluorescent cDNA probes (Panel B). The images have been corrected so that the backgrounds are similar. The features present in both arrays have a nearly identical visual fluorescence intensity and average pixel intensity. The total number of features present, defined as giving fluorescence signals of at least three times background, between the two enzymes and among four experiments (two experiments/enzyme) were also similar (data not shown). The ratios of differential gene expression between untreated and PMA/ionomycin-

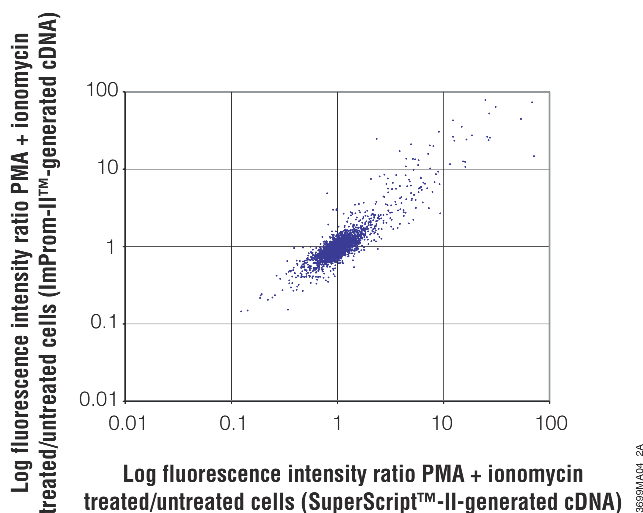

Figure 2. Scatter plot of the log ratios of differential expression of 3,200 genes in untreated vs. PMA + ionomycin-treated DO11.10 cells. Probes generated with ImProm-II™ (y axis) and SuperScript™-II (x axis) Reverse Transcriptases are compared.

treated cells were calculated for the 3,200 genes found to be present in all four data sets using probes generated with either ImProm-II™ or M-MLV H– RT. The differential gene expression profiles observed with PMA/ionomycin treatment from probes prepared using both RTs closely resemble previously published data (3). Also, when these two sets of differential expression ratios are plotted on a scatter plot, it is clear that essentially all of the genes surveyed show similar ratios with each RT (Figure 2).

Conclusion

ImProm-II™ Reverse Transcriptase compares favorably with the industry standard, M-MLV H–, for the production of indirectly labeled fluorescent cDNA targets for microarray hybridization. Gene expression profiles, feature fluorescence intensity, and the number of present features are all comparable between the two enzymes using our model system.

Methods

DO11.10 T cell hybridoma cultures were treated for two hours with 10ng/ml PMA + 0.5mM ionomycin. Control cultures were untreated. RNA was isolated from DO11.10 T cell hybridomas using the single step guanidinium thiocyanate/acid phenol RNA isolation protocol from *Current Protocols in Molecular Biology* (John Wiley and Sons). Twenty micrograms of total RNA were used for each reverse transcription reaction. For reactions using ImProm-II™ RT, the supplied ImProm-II™ Reaction Buffer was used, supplemented with 3M $MgCl_2$. ImProm-II™ Reactions were also supplemented with 2µl RNasin® Ribonuclease Inhibitor. SuperScript™-II reactions were performed according to the manufacturer's recommendations. See summary of reaction conditions in Table 1.

Table 1. Reverse Transcription Reaction Conditions.

Reverse Transcriptase	Total RNA	Oligo $(dT)_{24}$	aa-dUTP/ Nucleotide Mix	Enzyme	Final Volume
ImProm-II™	20µg	5µg	1µl	2µl	40µl
SuperScript™-II	20µg	5µg	1µl	2.5µl	20µl

Aa-dUTP/nucleotide mix consisted of 25mM dATP/GTP/CTP, 15mM aa-dUTP and 10mM dTTP. First strand synthesis was performed using the standard protocol with 2 hours incubation at 42°C.

After first-strand cDNA synthesis, the RNA was hydrolyzed by treatment at 70°C for 10 minutes in 0.2M NaOH. The reactions were neutralized with 0.3M HEPES (pH 7.5) and the cDNA recovered by ethanol precipitation at –20°C for 20 minutes followed by a 10-minute spin at 14,000 × *g*. Pellets were washed once with 75% EtOH containing 2mM sodium bicarbonate (pH 9.0). Pellets were dried, then resuspended in 9.0µl of 50mM bicarbonate (pH 9.0). To this, 2.0µl of reactive Cy®3 or Cy®5 in DMSO (Amersham) was added. The Cy®3 and Cy®5 were previously resuspended from the Amersham Monofunctional Reactive Dye Pack in 12µl of DMSO per tube and dispensed into the probe reactions. After one hour at room temperature in the dark, the coupling was quenched with 1.33M hydroxlamine HCl for five minutes. Cy®3- and Cy®5-labeled probes were combined and purified using a PCR purification kit. The probes were dried down after elution and resuspended in 120µl of Ambion SlideHyb™ #2 buffer with 30% formamide. Slides were hybridized for 19 hours at 42°C, washed, and scanned using an Axon Instruments laser scanner. Differential gene expression ratios were calculated as the ratios of the median pixel fluorescence intensities for each array feature at 532 (Cy®3) and 635nm (Cy®5). Raw ratios were normalized to internal controls to facilitate comparison between arrays.

References

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Ordering Information

Product	Size	Cat.#
ImProm-II™ Reverse Transcription System	100 reactions	A3800
ImProm-II™ Reverse Transcriptase	10 reactions	A3801
	100 reactions	A3802
	500 reactions	A3803

For Laboratory Use.

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