Research Report

Maximization of Signal Derived from cDNA Microarrays

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INTRODUCTION

Interest in DNA microarrays has been increasing steadily over the past few years, mainly in research for identifying genes related to disease states and targets for clinical intervention (5,8,9). More recently, microarrays have been recognized as a tool for characterizing compounds based on their gene expression pattern or "signature", and, as such, they are being incorporated into the pharmaceutical drug development process (2). The difficulty in distinguishing true differences in gene expression due to experimental variability has been recognized (4), and yet, very little has been done to address this issue. We have sought to improve the quality of our results by optimizing our cDNA microarray system. The aim was to increase the signal intensity and dynamic range while maintaining (or reducing) the variability, thus improving the signal-to-noise ratio of the system.

We have identified previously that, in our cDNA microarray system, the probe preparation step is a major contributor to overall experimental variability (10). This reaction step comprises the enzyme-mediated reverse transcription of mRNA and concomitant incorporation of fluorescently labeled nucleotides. We have examined this complex source of variability in detail using a factorial approach to assess the effect of each individual component on the overall variation and also of the interactions between component settings. The components of interest were RNA type, age and type of fluorescent label, dNTP age, and enzyme type.

Our current method for probe preparation uses total RNA as the starting

material. Many researchers prefer to use purified poly(A) mRNA (3), which requires a further extraction process but presumably improves the efficiency of reverse transcription. We investigated if the quality of hybridizations obtained using poly(A) mRNA warranted the extra purification step.

Speed, fidelity, and the ability to incorporate labeled nucleotides are the most important criteria for reverse transcriptase enzymes. Previous work has demonstrated that the ability of DNA polymerases to incorporate cyanine-labeled dUTP varied markedly (2–3-fold) between thermostable enzymes made by different manufacturers (7), and this is also likely to be the case for transcriptases. SUPERScript™ II (Life Technologies, Rockville, MD, USA) is not significantly inhibited by ribosomal and tRNA and thus is effective for synthesizing cDNA from total RNA. It is not thermophilic but can be used for temperatures up to 50°C. Although not developed specifically for incorporation of fluorescently labeled dNTPs, it is one of the most popular transcriptases used for microarray work (3,8). The possible advantages of an alternative enzyme are higher rates of fluorescence incorporation, longer transcripts, and faster reaction times. Faster reaction time is certainly true for thermophilic enzymes, which have the added benefit of synthesis through stable secondary RNA structure. The reverse transcriptase chosen for testing was the thermophilic rTth. This enzyme has not been developed or tested by the manufacturers for incorporation of fluorescently labeled dNTPs, but we wished to determine whether it would be a useful alternative to SUPERScript II.

ABSTRACT

Microarray technology is a powerful tool for generating expression data on a large number of genes simultaneously. However, as for any assay, it must be reproducible to give confidence in the results. Using a classical statistical method—the factorial design of experiments—we have assessed the effects of different experimental factors in our system. Significant effects on signal were seen when the standard components were substituted with a different enzyme, fluorescent label, or RNA purification method. This has led to the implementation of an improved procedure that maximizes signal without affecting the variability of the system, thus increasing the signal-to-noise ratio. In addition, we were able to quantify the variability between microarrays and replicates within microarrays.

A 2-h incubation time is standard when using SUPERSCRIPT II for probe preparation (3); however, the processivity of SUPERSCRIPT II is approximately 250 nucleotides/min, and an incubation of 50 min is recommended by the manufacturers for long transcripts (generally there is a good yield of transcripts greater than 7 kb). Thus, despite the steric problems encountered with incorporating dyes, it seems unlikely that much additional yield is obtained after a 1-h incubation. We wished to reduce the time needed for the probe preparation step; hence, the necessity of an extra hour's incubation time was investigated.

The physiochemical properties of fluorescent dyes are important in the choice of labels for cDNA microarrays. The cyanine dyes are often considered the most popular dyes for use with microarrays (5,8). They are water soluble, pH insensitive, and are not prone to aggregation (6). Alternative dyes such as the Alexa Fluor™ (Molecular Probes, Leiden, The Netherlands) range have recently become commercially available, and these may offer a number of advantages over the cyanine dyes, including enhanced fluorescence, improved photostability, and pH insensitivity between pH 4.0 and 10.0 (7). Potential improvements in signal were investigated by comparing Cy3™ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with Alexa Fluor 546-14-dUTP™.

Another important attribute of fluorescent dyes is their stability. Deterioration can occur during storage (or even use), and this can have a profound ef-

fect on reproducibility; thus, the age of the dye was examined. Likewise, age and storage conditions (e.g., freeze-thaw cycles) may affect dNTP stability, and this issue was also addressed.

MATERIALS AND METHODS

Sample Preparation

Liver tissue from untreated Sprague Dawley rats was used. Total RNA was extracted from samples of approximately 100 mg tissue using Bio/RNA-Xcell™ (BioGene, Cambs. UK). The RNA purity was assessed using an A_{260/280} measurement and those with a reading greater than 1.75 were pooled. All experiments were conducted using the same RNA pool. Poly(A) mRNA was prepared from 10 µg total RNA (per sample) using mRNA Oligo [dT]30 kit (BIO101, Vista, CA, USA).

cDNA Probe Preparation

All reagents (with the exception of enzymes) were aliquoted and stored at -20°C in the dark. The aliquots were discarded after one use to obviate any changes due to freeze-thaw cycles. RNA samples (10 µg total RNA or mRNA isolated from 10 µg total RNA) were used for the synthesis of cDNA probes. The SUPERSCRIPT Preamplification system (Life Technologies) was used essentially as described in the protocol, except for the changes detailed below. Anchored oligonucleotide d(T)₂₁ (Cambio, Cambridge, UK) was used as

a primer. The reverse transcriptase buffer and MnCl₂ supplied in the GeneAmp® thermostable rTth reverse transcriptase RNA PCR kit (PE Biosystems, Foster City, CA, USA) were substituted when using the thermophilic rTth DNA polymerase. Incubation times were 5 min at 68°C for 2.5 U rTth, 60 min at 39.5°C for 400 U SUPERSCRIPT II RNase H reverse transcriptase enzyme or 60 min for 200 U SUPERSCRIPT II, followed by a further addition of 200 U and a further 60-min incubation at 39.5°C. Three settings were given to incubation time: 1 and 2 h for SUPERSCRIPT II and 5 min for rTth, because the thermophilic enzyme requires a significantly shorter reaction time. The 500-µM dNTP mixture contained a 1:7 ratio of labeled to unlabeled nucleotide; either Alexa Fluor 546-14-dUTP (Molecular Probes) or dCTP-labeled Cy3 (1 µL each).

RNase H and RNase A (Life Technologies) (2 U each) were added to the mixture, which was incubated at 39.5°C for 20 min before removing unincorporated dye using QIAquick™ PCR purification kits (Qiagen, Düsseldorf, Germany).

DNA Microarrays

DNA microarrays were spotted by Biotechnology and Genetics, SmithKline Beecham, and contained 1248 duplicate spots (comprising 1129 different PCR products and 119 "empty" spots) derived from I.M.A.G.E. Consortium clones (ATCC, Manassas, VA, USA). PCR products in isothiocyanate were spotted onto silanated glass using a prototype spotter (Amersham Pharmacia Biotech). Two "spotsets" were spotted onto each microarray slide (Figure 1). The probe was prepared separately for each microarray slide so that, for each experiment, there was one replicate at the probe making level and, for each probe preparation, there was one replicate at the spotset level.

Hybridization and Imaging

Microarray slides were prepared by washing in isopropanol, followed by boiling water. Microarrays were sealed in hybridization chambers (fabricated in-house) and 400 µL denatured probe

Figure 1. Microarray (slide) layout.

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[in 5× standard saline citrate (SSC) buffer, 0.1× SDS) was introduced into the entry port. Following hybridization at 42°C for 16 h in a rotating hybridization oven (Techne, Cambridge, UK), microarrays were washed once for 5 min in 2× SSC and 0.1% SDS, followed by two 5-min washes in 0.5× SSC and 0.1% SSC, at room temperature. Microarrays were imaged using a Generation II Molecular Dynamics™ scanner (Amersham Pharmacia Biotech), and images were analyzed using customized software (Gel-Pro Analyzer™ v. 3.0; Media Cybernetics, Silver Spring, MD, USA).

Design and Analysis of the Factorial Experiment

The components of the probe preparation step were condensed into six factors: (i) the type of fluorescent label, (ii) the age of the fluorescent label, (iii) the type of enzyme, (iv) the age of the dNTPs, (v) the incubation time (for transcription), and (vi) the use of total RNA or poly(A) mRNA. Changing one variable at a time, while holding all others constant, is an unreliable and inefficient process that does not provide information on synergistic or antagonistic effects of factor combinations. Hence, a factorial experiment was designed (using Design Expert®, Statease, Minneapolis, MN, USA) in accordance with statistical theory (1), and the factor settings were varied simultaneously.

For six factors at two levels, a total of 64 runs would cover all possible combinations. To investigate microarray-to-microarray variability within each factor combination, a half-fraction was planned, that is, a set of 32 factor combinations, each with two microarrays (or replicates), that still allows estimation of all main factor effects and all two-factor interactions. (An “effect” is the difference between the observed signals at the two factor settings.)

Further consideration revealed that this simple balanced design was not possible because the levels of one factor (incubation time) needed to be defined differently at each level of another factor (enzyme type). Statistically, this implies that incubation is “nested within” enzyme type. However, balance in the design is not lost (because

incubation time takes one value for one level of enzyme type and two values for the other level of enzyme type), and the analysis remains relatively straightforward. This means that the “main effect” of incubation time cannot be estimated; it is only reasonable to examine the effect of incubation time in relation to particular values of enzyme type. See Table 1 for factor settings.

Analysis of variance (ANOVA) was used to deconstruct the results into effects corresponding to each factor separately (with the exception of incubation, as previously described) and two-factor interactions. Two ANOVAs were constructed, one examining the effect of factor setting on total microarray signal [the ANOVA was carried out on the logarithm (base 10) of the spotset totals] and the other examining the effect on variability *between* microarrays (the ANOVA was carried out on the log₁₀ of the sum of the squared differences between replicate microarrays).

RESULTS AND DISCUSSION

We performed a direct comparison of a number of hybridization methods, measured the variability in the system, and identified the factor combinations that maximize total signal on the microarrays.

Effect of Probe Preparation Components on Signal Intensity

ANOVA of total spot intensities reveals that the label type, RNA type, and, in particular, the enzyme type have the greatest effect on signal intensity (see Table 2). The effects of dNTP age and dye age on signal intensity were less important. This result was perhaps as expected since substituting a component is likely to have more impact than a progressive effect such as aging.

The effects of the most important factors are given in Table 3. The optimal conditions for high total signal intensity are SUPERScript II, Alexa, and total RNA. The signal from the rTth experiments was particularly low. The spots, although much fainter, were in a similar pattern to those obtained using the standard protocol ($r = 0.65$). This indicated that transcription occurred and that the

paucity of signal was a result of either inefficient incorporation of nucleotide or limited strand synthesis.

The conditions for rTth transcription were not optimized, and the incubation time for synthesis was very short. Further work is necessary to establish if the use of thermophilic reverse transcriptases is a viable alternative and whether or not it provides longer transcripts and improved processivity.

Results regarding fluorescent label indicate that the use of the Alexa dye gives a significantly higher signal than the use of Cy3. On average, this signal was nearly threefold higher when using Alexa (Table 3). The effect on transcription using a dUTP-labeled fluorophore, rather than dCTP, was not examined but would be of interest in future studies.

In our hands, hybridizations from mRNA, extracted from total RNA, gave unacceptably low signal intensities and indicates that, although mRNA may give rise to improved hybridizations under certain conditions, the amount of starting material required is greater. This is a serious limitation when tissue or sample is scarce.

There was one statistically significant interaction, between RNA type and enzyme type, as displayed in Table 4. This shows that the effect of switching from poly(A) mRNA to total RNA is negligible for thermophilic rTth enzyme but multiplies the average signal by a factor of 2.67 for SUPERScript II. The interaction can equivalently be described in terms of different multiplying factors for the enzyme effect for the two types of RNA—the effect of switching from the rTth to SUPERScript II, while using total RNA, multiplies the average signal by 6.50; the same enzyme switch, while using poly(A) mRNA, will increase the signal by a factor of 2.84. Due to losses during the purification process, it is likely that the appropriate template is present in lower concentrations in the poly(A) mRNA preparation than the total RNA (albeit in a purer form), so, when using the SUPERScript II enzyme, the template is a limiting factor. When using rTth, little time was allowed for transcription (only 5 min) so that presumably the transcription was limited by time and not by the concen-

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tration of the template. This would explain why the method of RNA purification has little effect when using rTth.

Effect of Probe Preparation Components on Signal Variation between Microarrays

The second ANOVA was designed to show the effect of probe-making components on the variance between replicate microarrays—estimated by the squared difference between replicate measurements. None of the effects showed a significant effect on the between-microarray variability ($P > 0.10$). This means that, to choose the protocol with the highest signal-to-noise ratio, the between-microarray variability was not a significant factor and that the best choice is based on signal strength alone.

Optimal Component Settings

The difference between the method currently used in our laboratory and the optimal probe preparation method identified by our analysis is that Alexa should be used rather than Cy3. This difference gives a 2.7-fold increase in the total signal. We currently employ the SUPERSCRIPT II enzyme in preference to rTth, and our experiment confirms this choice: with total RNA, SUPERSCRIPT II provides a 6.5-fold increase in signal over rTth (see Table 4). To our knowledge, the use of Alexa Fluor label for microarrays has not been published previously, but these results demonstrate that it increases signal intensity without affecting variability. With regard to enzyme type, rTth has also not been examined previously for this application, but with further optimization it may prove useful due to its thermophilic properties.

Variation and Dynamic Range

Referring to Table 2, it can be seen that the variance (residual variation) between replicate microarrays is tenfold higher than the variance between duplicate spotsets (within a microarray). This illustrates the variation that occurs as a result of probe preparation, microarray slide variation, and the hybridization environment; even when the probe

Table 1. The Settings of the Six Factors

Factor	Setting A	Setting B
Age of fluorescent label	1–2 weeks	1–2 months
Type of fluorescent label	Alexa-labeled dUTP	Cy3-labeled dCTP
Type of enzyme	thermophilic rTth	SUPERSCRIPT II
Age of dNTP	1–2 weeks	6 months
Type of RNA preparation	Total RNA	Poly(A) mRNA
Transcription incubation time ^a	2 h	1 h

^aWhen enzyme is setting A, incubation time is C (5 min).

Table 2. ANOVA: Effect of Probe Preparation Components on Signal ($\log_{10}$ of the Spotset Totals)

Source of Variation	Degrees of Freedom	Mean Square	F-Statistic	P-Value
between microarrays				
label type	1	6.1052	77.65	<0.001
RNA type	1	1.9534	24.84	<0.001
dNTP age	1	0.1341	1.71	0.198
label age	1	0.0403	0.51	0.477
enzyme type	1	12.8586	163.54	<0.001
label type:RNA	1	0.2470	3.14	0.083
label type:dNTP	1	0.0825	1.05	0.311
RNA:dNTP	1	0.1446	1.84	0.182
label type:label age	1	0.0399	0.51	0.480
RNA:label age	1	0.0088	0.11	0.739
dNTP:label age	1	0.0145	0.18	0.670
label type:enzyme	1	0.0384	0.49	0.488
RNA:enzyme	1	1.0325	13.13	<0.001
dNTP:enzyme	1	0.0528	0.67	0.417
label age:enzyme	1	0.0005	0.01	0.934
enzyme:incub	1	0.0006	0.01	0.929
Residual	47	0.0786	10.47	
within microarrays (spotsets)	64	0.0075		
Total	127			

A colon (:) denotes an interaction between two factors.

preparation method and RNA sample is constant. This is an important result, as it demonstrates that, to determine gene expression changes with greater certainty, it is more important to increase the number of replicate microarrays than to increase the number of replicate spotsets within the microarray.

An added benefit from this analysis is that our laboratory has a quantified

estimate of the size of the between-microarray standard error. Since precision increases with the square root of sample size, this enables the calculation of the number of replicate microarrays needed to reduce the standard error of the average to whatever level would be required in our future experimentation. Using four microarray replicates halves the estimated standard error, but dimin-

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Table 3. The Effect of Components on Signal, Ignoring Interactions

Factor	Mean on Log Scale		Multiplicative Effect on Natural Scale (A/B)
	Setting A	Setting B	
label type	8.517	8.080	2.73
RNA type	8.422	8.175	1.76
enzyme type	7.982	8.616	0.23

Table 4. Interaction Effects between RNA Type and Enzyme Type

	Mean on Log Scale		Multiplicative Effect on Natural Scale (A/B)
	RNA Type A	RNA Type B	
Enzyme type A	8.016	7.948	1.17
Enzyme type B	8.829	8.402	2.67
Multiplicative effect on natural scale (B/A)	6.50	2.84	

ishing returns, in terms of improved reproducibility, are achieved after seven replicates.

The dynamic range is the ratio between the maximum and minimum signal (above noise) on a microarray. The dynamic range (i.e., the ratio between the signal intensities of the individual genes) is important in differentiating quantitative changes in gene expression. It is worth noting that, in these experiments, while maximizing signal using the optimal conditions, the dynamic range was also increased (results not shown).

This factorial experiment was useful in identifying the factor settings that maximize signal intensity between microarrays. The ANOVA decomposition provides estimates of between-microarray and within-microarray variability. This enabled the calculation of the number of microarrays necessary for statistical interpretation. As the number of replicates increases, it is possible to attach more confidence to results. In our system, seven replicates appear to give an acceptable level of reproducibility. These findings raise doubts regarding the validity of results from single, or even duplicate, microarrays for semi-quantitative analysis of gene expression. This finding reiterates the importance of replicates, as noted by Wittes et al. (11).

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