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Microarray results improve significantly as hybridization approaches equilibrium

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Dual-channel long oligonucleotide microarrays are in widespread use. Although much attention has been given to proper experimental design and analysis regarding long oligonucleotide microarrays, relatively little information is available concerning the optimization of protocols. We carried out a series of microarray experiments designed to investigate the effects of different levels of target concentration and hybridization times using a long oligonucleotide library. Based on principles developed from nucleic acid renaturation kinetics studies, we show that increasing the time of hybridization from 18 h to 42 h and 66 h, especially when lower than optimal concentrations of target were used, significantly improved the quality of the microarray results. Longer hybridization times significantly increased the number of spots detected, signal-to-noise ratios, and the number of differentially expressed genes and correlations among replicate arrays. We conclude that at 18 h of incubation, target-to-probe hybridization has not reached equilibrium and that a relatively high proportion of nonspecific hybridization occurs. This result is striking, given that most, if not all, published microarray protocols stipulate 8–24 h for hybridization. Using shorter than optimal hybridization times (i.e., not allowing hybridization to reach equilibrium) has the consequence of underestimating the fold change of differentially expressed genes and of missing less represented sequences.

INTRODUCTION

Major parameters that affect nucleic acid hybridization equilibrium include labeled target concentration and the incubation time allowed for hybridization, both of which are critical because maximum specificity and sensitivity are reached with equilibrium. Typical protocols propose hybridization times of 8–24 h (1) and 16–20 h (2); however, there is little or no experimental data in the literature to justify a given incubation time.

Recent reports have examined different parameters that affect hybridization equilibrium for microarrays. Using a long oligonucleotide platform (60-mer), it was shown that with high complexity targets (i.e., a highly diverse population of RNA), specific target-to-probe hybridization equilibrium took much longer to attain than did nonspecific hybridization equilibrium (3). Increasing the incubation time to approach maximum hybridization substantially improved fluorescent intensities for specific targets. However, other critical criteria that help determine the quality of microarray results, such as differential expression,

signal-to-noise ratios, and correlations among replicates, were not directly assessed. In other studies, using short oligonucleotide platforms (20–30 bases), it was shown that a considerable amount of cross-hybridization occurred under various conditions (4) and that longer hybridization times improved accuracy (5). Our experimental results are in complete agreement with and expand on the findings of these earlier studies (4,5). Using a long oligonucleotide platform, we applied multiple criteria to assess the quality of the microarrays that were carried out under laboratory conditions. We show that as hybridization equilibrium is approached by allowing longer incubation periods and/or approached more rapidly by increasing the concentration of labeled target, microarray quality is significantly improved.

MATERIALS AND METHODS

Microarray Hybridization

Total RNA isolated from mouse (strain Balb/c) heart was purchased

from BD Biosciences Clontech (Palo Alto, CA, USA). The second RNA sample was total RNA isolated and pooled from 5 male and female 1-day-old C57Bl/6J mice as previously described (6). The cDNA synthesis for each of the two RNA samples was carried out in 20 reactions with 20 µg of total RNA per reaction. The resulting cDNA from the 20 reactions was pooled, and amounts proportional to 2.5, 5, 10, and 20 µg of the original template RNA (i.e., 1/20 of the pool for 20 µg, 1/40 for 10 µg, etc.) were removed and labeled with reactive monofunctional cyanine-3 (Cy^{TM}3) and cyanine-5 (Cy5) dyes by an indirect amino allyl labeling method (7) for each RNA sample. The mouse 70-mer oligonucleotide library (Array-Ready Oligo Set^{TM}; Qiagen, Valencia, CA, USA) version 1.1, representing 13,664 known genes, was suspended in 3× standard saline citrate (SSC) at 30 µM and printed at 22°C and 65% relative humidity on aminosilane-coated slides (Cel Associates, Pearland, TX, USA) using a high-speed robotic OmniGrid® machine (GeneMachines, San Carlos, CA, USA) with Stealth SMP3 pins (TeleChem International, Sunnyvale, CA, USA). Spot volumes were 0.5 nL, and spot diameters were 75–85 µm. The oligonucleotides were cross-linked to the slide substrate by exposure to 600 mJ of ultraviolet light (UV). For hybridization, the microarray slides were covered with glass coverslips (Fisher Scientific, Pittsburgh, PA, USA) and placed in humidified hybridization chambers (Corning® Hybridization Chamber; Corning, Acton, MA, USA). The hybridization chambers were placed in a water bath at 48°C for 18 h (1 day), 42 h (2 days), or 66 h (3 days). The slides were placed in a slide rack set in a staining dish and washed in 1× SSC with 0.2% sodium dodecyl sulfate (SDS) for 4 min at 48°C with agitation. The slides were transferred to a staining dish with 0.1× SSC and 0.2% SDS and washed with agitation for 4 min at room temperature and twice for 4 min each in 0.1× SSC at room temperature. The slides were centrifuged at approximately 700× g immediately after washing. Additional details of slide preparation can be found at the University of Cincinnati Genomics and Microarray Laboratory's web site at [http://](http://microarray.uc.edu)

microarray.uc.edu. A total of 36 microarrays were used, each for comparing RNA from adult mouse heart of strain Balb/c versus 1-day-old pup of strain C57Bl/6J. For each of the 12 concentration and hybridization time combinations, we performed three technical replicate arrays; in one array of each triplicate, the dyes were flipped. Imaging and data analyses were carried out as previously described (7).

Data Normalization and Analysis

The data representing raw spot intensities generated by GenePix® Pro version 5.0 software (Axon Instruments, Union City, CA, USA) were analyzed to identify differentially expressed genes. Data normalization was performed in three steps for each microarray separately (7,8). First, channel-specific local background intensities were subtracted from the median intensity of each channel (Cy3 and Cy5). Second, background-adjusted intensities were log-transformed, and the differences (R) and averages (A) of log-transformed values were calculated as $R = \log_2(X1) - \log_2(X2)$ and $A = [\log_2(X1) + \log_2(X2)]/2$, where X1 and X2 denote the Cy5 and Cy3 intensities, respectively, after subtracting local backgrounds. Third, data centering was performed by fitting the array-specific local regression model of R as a function of A. The difference between the observed log ratio and the corresponding fitted value represented the normalized log-transformed gene expression ratio. Normalized log intensities for the two channels were then calculated by adding half of the normalized ratio to A for the Cy5 channel and subtracting half of the normalized ratio from A for the Cy3 channel. The statistical analysis was performed for each gene and for each RNA concentration/hybridization time separately by fitting the following mixed effects linear model (9). $Y_{ijk} = \mu + A_i + S_j + C_k + \epsilon_{ijk}$, where Y_{ijk} corresponds to the normalized log intensity on the i^{th} array ($i = 1, 2, 3$), with the j^{th} treatment combination ($j = 1, 2$), and labeled with the k^{th} dye ($k = 1$ for Cy5 and 2 for Cy3). The μ is the overall mean log intensity, A_i is the effect of the i^{th} array, S_j is the effect of the j^{th} treatment, and

C_k is the effect of the k^{th} dye. Assumptions about model parameters were the same as described by Wolfinger et al. (10), with array effects assumed to be random and treatment and dye effects assumed to be fixed. An additional statistical analysis, referred to as the full analysis, was done using all 36 arrays together (three for each concentration/hybridization time combination) with the same model as described above. Statistical significance of differential expression among RNA samples, after adjusting for array and dye effects, was assessed by calculating P values, and estimates of fold change were calculated. Multiple hypotheses testing adjustment was performed for the full analysis by calculating false discovery rates (FDR; References 11 and 12) and Bonferroni-adjusted P values. Data normalization and statistical analyses were performed using an SAS® statistical software package (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

The number of detected spots and spot intensity values are good predictors of microarray quality (3). Undetected spots were those spots flagged as not found using the default feature identification method of GenePix Pro v5.0 software. Figure 1A shows a plot of the number of detected microarray spots versus three different incubation times for four different total RNA amounts. Figure 1B displays the same data joined as one curve using the following mathematical model of hybridization kinetics:

$$\frac{\partial[TP]}{\partial t} = K_f([P] + [T]) - K_r[TP] \quad [\text{Eq. 1}]$$

in which $[T]$, $[P]$, and $[TP]$ are the concentrations of free target, bound probe, and target-probe complexes, respectively, and K_f and K_r are the rates of hybridization and dissociation, respectively. The solution to the equation (3,13), assuming an excess abundance of probe with respect to target-probe complexes throughout hybridization, is

$$[TP](t) = K_1 \frac{[T]}{k_f/k_r + [T]} [1 - \exp(-t/K_2)] \quad [\text{Eq. 2}]$$

in which

$$K_2 = \frac{1}{k_f([T] + k_f/k_r)}, \quad [\text{Eq. 3}]$$

t is time, $[T]$ signifies the target concentration at time 0, K_1 is the total number of oligonucleotides, and K_2 is the time needed to reach equilibrium. By approximating k_f/k_r as 0.5 and k_f as 1/400 and letting $K_1 = 1$, we graphed this function of RNA concentration and hybridization time versus the number of spots found by the GenePix software. Results were robust with regard to changes in estimates of k_f/k_r as well as for realistic values of K_2 (data not shown). Equation 2 spans from 0 (when time or RNA concentration equals zero) to its asymptotic limit of 1 (as time and RNA concentration approach infinity), which signifies the maximum possible number of bound complexes.

It is evident in Figure 1, A and B, that by increasing the target concentration or by prolonging the hybridization time, the number of detected spots correspondingly increases. The increase in the number of spots was dramatic when a lower amount of labeled target was used. For example, in Figure 1A, for 2.5 μg of total RNA used in target labeling, the increase in the number of detected spots was over 100% from day 1 to day 3, whereas for the remaining total RNA amounts of 5, 10, and 20 μg , the increase was 10%–25%. Furthermore, approximately 95% of the spots on the microarray slide were detected by day 3 for all levels of target concentration. These results and conclusions are in close agreement with those reported by Dai et al. (3) and Bhanot et al. (4).

Microarray spots of low signal intensity are more variable and thus usually of lower quality than microarray spots of higher signal intensity (14). Therefore, a comparison of intensity levels across experimental conditions allows another measure of array quality. A measure of the signal-to-noise ratio was used to determine overall microarray quality, rather than calculated signal intensities, because differences in spot signal intensities depend on both the amount of bound labeled target at each spot and the photomultiplier tube (PMT) values used when scanning (Table 1). Means of signal-to-noise ratios were tested for being significantly different among the combinations of experimental parameters by

using analysis of variance (ANOVA) to model signal-to-noise ratio as a function of the concentration/time combination, including array as a factor in the model, and applying Tukey-Kramer adjustment for multiple comparisons. The signal-to-noise ratios for all combinations of concentration and hybridization times (presented in Table 1) were all significantly different ($P < 0.0001$), with the exception of 10 μg at 2 days versus 3 days ($P = 0.9986$). An increase in the hybridization time and/or concentration of the labeled target resulted in enhanced signal-to-noise ratios, and higher concentrations of labeled target produced a wider spread of intensities. These results are in agreement with results reported for cDNA microarrays, in which labeled target synthesized from 10–50 μg of total RNA was compared (15).

The correlation of samples from dye-flipped replicates was another measure used to determine microarray quality (Figure 1C). The graph of log-transformed background-subtracted intensity correlations shows that increasing the hybridization time from 18 h to 42 h had a marked improvement on intensity correlations, especially for 5 and 10 μg of total RNA. Based on Churchill's (16) expected correlation for separate arrays with different dyes, Pearson's correlation coefficient value of 0.7 was used as the minimum acceptable level of correlation, and for 5 and 10 μg of starting total RNA, the correlations were significantly above this level only for 2 and 3 day hybridization times. Thus, increased hybridization time and target concentration generally improved the correlation values and lowered the variability for spot intensities. In addition, the ratio correlations among dye-flipped arrays improved. There is no expected acceptable level for these correlations because the level of correlation is dependent on the overall differences among RNA samples. The average ratio correlations for starting total RNA concentrations 2.5, 5, 10, and 20 μg were 0.241, 0.482, 0.564, and 0.607, respectively, and the average ratio correlations for 1, 2, and 3 days of hybridization were 0.336, 0.504, and 0.581, respectively.

The ultimate goal in global gene expression microarray experiments is to identify differentially expressed genes.

Table 1. Means of Log Signal-to-Noise Ratios for Different Amounts of Target and Hybridization Incubation Times

Amount of Total RNA for Target (µg)	Hybridization Incubation Period (Days)		
	1	2	3
2.5	-1.16 (± 0.0094)	-0.09 (± 0.0089)	-0.20 (± 0.0088)
5.0	0.05 (± 0.0088)	0.41 (± 0.0088)	0.84 (± 0.0088)
10.0	0.58 (± 0.0088)	1.06 (± 0.0088)	1.05 (± 0.0088)
20.0	1.13 (± 0.0088)	1.54 (± 0.0089)	1.86 (± 0.0088)

Log signal-to-noise ratios were calculated for each spot on each array as $\log_2[(\text{intensity} - \text{background})/\text{background}]$. Standard errors for the array triplicates are shown in parentheses.

Table 2. Fold Change Estimates of 242 Significant Differentially Expressed Genes^a

Amount of Total RNA for Target (µg)	LSM of Average Fold Change ^b	Hybridization Time (Days)	LSM of Average Fold Change ^b
2.5	5.74	1	6.73
5.0	7.31	2	8.28
10.0	9.00	3	8.69
20.0	10.13	N.A.	N.A.

LSM, least-squares mean; N.A., not applicable.
^aAbsolute values were used without detriment to the analysis because up-regulated genes were consistently estimated to be up in all concentration/time combinations, and similarly, down-regulated genes were consistently estimated to be down for this set of genes.
^bResults from analysis of variance (ANOVA) testing using least squares means.

The number of genes with a *P* value less than 0.005 from testing each set of experimental conditions was seen to follow the general trend of increasing as the concentration of labeled target and/or incubation time also increased. For example, the number of genes with a *P* value less than 0.005 for 10 µg of starting RNA improved from 141 at day 1 to 352 at day 3. Similarly, the number for 3 days of incubation increased from 216 at 5 µg to 352 at 10 µg. To demonstrate that the increase in number of significant genes was not merely due to an increased false-positive rate, we performed an analysis using only genes that were detected on all arrays, showed a greater than 4-fold change in differential expression, and had a Bonferroni-adjusted *P* value of less than 0.05 from the overall test, in which there is 95% certainty that none of the genes are false positive (117 genes). The average *P* values at day 1, 2, and 3 from this analysis were 0.048, 0.041, and 0.035, respectively. The above results provide evidence that in general, higher concentrations of labeled target and longer hybridization times increase the number of significantly changed differentially expressed genes.

Our hypothesis and work from others (3,17) allow the prediction that increased hybridization times would also increase the fold change of gene expression. A set of 242 representative genes that had a Bonferroni-adjusted *P* value of less than 0.05 and an estimated change greater than 4-fold in either direction was selected from the full analysis. Thus, it was highly unlikely that any of the genes was a false positive. An ANOVA model that included categorical variables for gene, concentration, and time was used to test whether concentration and/or time had an effect on estimates of differential gene expression (Table 2). Fold change averages for the different experimental conditions showed significant differences. Absolute values of individual estimates (estimates from each concentration/time analysis carried out separately) were used for the dependent variables. Table 2 shows that as the concentration of labeled target increased, the estimated fold change also increased. Each increase in concentration resulted in a significant (*P* < 0.0001 with Tukey-Kramer adjustment) increase in estimated average fold change. Similarly, as the length of hybridization time in-

creased from 1 to 2 days, gene expression fold changes also increased with this difference, also having an adjusted P value of less than 0.0001. Three versus two days of incubation time was not significant at the 0.05 level after adjustment ($P = 0.075$).

A model in which target-to-probe hybridization nears thermodynamic equilibrium explains the mechanism by which an increase in hybridization time and labeled target concentration may increase the fold change of gene expression, as shown in Table 2. In regard to hybridization time, as the incubation time lengthens and equilibrium is approached, an increasing proportion of correct target-to-probe hybridizations occurs while concurrently removing unbound and potentially nonspecific targets from solution. The removal of the nonspecific targets decreases the nonspecific fluorescence signal equally for each dye in a given spot, which would proportionately increase the difference between the two fluorescence signals. This, in turn, would increase the average fold change, a model supported by the computer simulations and detailed physical chemical analysis generated by Bhanot et al. (4). In regard to labeled target concentration, a higher concentration of labeled target will approach equilibrium sooner than a

Figure 1. Effects of increased labeled target concentrations and hybridization times on spot detection and correlations. (A) Number of microarray spots identified for four different RNA amounts for target labeling (2.5, 5, 10, and 20 μ g) at three different hybridization times (1, 2, and 3 days). The average number of spots for each experimental condition was derived from three replicate microarray slides. The vertical bars show standard deviations. (B) Same data as shown in panel A, depicting the number of detected microarray spots versus the estimated proportion of target-probe complexes. The points in the graph are derived from a formula for calculating hybridization kinetics. The numbers in parentheses indicate total RNA amount in micrograms (left number) and hybridization incubation period in days (right number). The vertical bars show standard deviations. (C) A histogram showing the mean and standard deviations of spot intensity correlations among dye-flipped technical replicates for each total RNA amount used for target labeling and corresponding hybridization time. Pearson's correlation coefficient was used, and the horizontal line at 0.7 indicates the minimum acceptable level of correlation.

lower concentration of labeled target (4). Therefore, at a given time point in the hybridization period prior to equilibrium, the proportion of specific target relative to nonspecific target bound to a given probe will be greater at the higher concentration and produce a greater fold change.

Here we have reported a detailed and thorough study showing that for dual-channel long oligonucleotide microarrays carried out under laboratory conditions, an increase in the labeled target concentration and/or the hybridization time significantly improves results in multiple ways, including spot number, spot intensity, signal-to-noise ratio, number of differentially expressed genes, and fold change. Furthermore, no detrimental effects due to longer hybridization times were found under our laboratory conditions. The described work is applicable to all other nucleic acid microarray platforms, such as cDNA, short oligonucleotide, and genomic DNA microarrays. Although increasing the amount of labeled target is not always feasible, increasing the hybridization time 2–4 times that recommended in current published protocols

can always be applied free of the need and risk to change any other aspect of the microarray protocol and possibly with dramatically improved results.

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