

Development of a microarray assay that measures hybridization stoichiometry in moles

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Microarray data is most useful when it can be compared with other genetic detection technologies. In this report, we designed a microarray assay format that transforms raw data into a defined scientific unit (i.e., moles) by measuring the amount of array feature present and the cDNA sequence hybridized. This study profiles a mouse reference universal RNA sample on a microarray consisting of PCR products. In measuring array features, a labeled DNA sequence was designed that hybridizes to a conserved sequence that is present in every array feature. To measure the amount of cDNA sample hybridized, the RNA sample was processed to ensure consistent dye to DNA ratio for every labeled target cDNA molecule, using labeled branched dendrimers rather than by incorporation. A dye printing assay was then performed in order to correlate molecules of cyanine dye to signal intensity. We demonstrate that by using this microarray assay design, raw data can be transformed into defined scientific units, which will facilitate interpretation of other experiments, such as data deposited at the Gene Expression Omnibus and ArrayExpress.

INTRODUCTION

After DNA sequencing, microarray data is the most widely accessed form of genetic information. The multidimensional nature of microarray data enables its use in different applications. Researchers are currently encouraged to deposit their microarray data into public microarray data repositories such as the National Center for Biotechnology Information (NCBI), Gene Expression Omnibus (GEO), and the European Bioinformatics Institute's (EBI) ArrayExpress.

In order to assist in organizing microarray experiments, the Microarray Gene Expression Database group (MGED; <http://www.mged.org>) was created to define specific guidelines for the submission of microarray data referred to as minimal information about a microarray experiment (MIAME) (1–4). Ideally, a microarray system that measures gene expression levels in terms of natural units, such as mRNA copies per cell with an estimated error model, would be highly useful in interpreting microarray data. There have been many reports that improve the accuracy of microarray

experiments by describing error model schemes (5–7). The objective of this report is to describe a microarray assay design that measures sequence abundance using a defined scientific unit by measuring hybridization stoichiometry in molar units. This described method of microarray analysis advances the process of measuring gene expression in terms of mRNA copies per cell, since it quantifies gene expression abundance in molar terms and facilitates comparison of microarray data across different platforms.

MATERIALS AND METHODS

Mouse 9K Array Amplification

The mouse UniGEM Clone List sequence-verified clone collection was purchased from InCyte Pharmaceuticals (Palo Alto, CA, USA). Bacterial clones containing cDNA inserts for growth and replication were inoculated using a pin replicator (VP Scientific, San Diego, CA, USA) into 96-well Falcon™ U-bottom plates (BD Biosciences, Bedford, MA, USA) containing LB/ampicillin

(50 µg/mL) and incubated overnight at 37°C with agitation. PCRs were assembled in 96-well microplate format using the BioRobot® 3000 (Qiagen, Hilden, Germany) to dispense the various components of the reaction mixture. The clones comprising the library have conserved sequences to enable amplification using a single primer set. Each reaction mixture consisted of 10 µM dNTP, 20 µM primer mixture (forward primer 5'-CTGCAAGGCGATTAAGTTGGGTAAC-3' and reverse primer 5'-GTGAGCGGATAACAATTTCA-CACAGGAAACAGC-3'), 3 µL of overnight culture, and 3.75 U Eppendorf MasterTaq® (Brinkmann Instruments, Westbury, NY, USA). Amplifications were carried in 150-µL volumes in 96-well plate format to maximize PCR product yield using a Tetrad® Thermal Cycler (MJ Research, Waltham, MA, USA). Cycling conditions were as follows: an initial denaturation (5 min) at 95°C, 35 cycles (94°C for 1 min, 58°C for 1 min, 72°C for 4 min) followed by 10 min at 72°C. An aliquot of each reaction (10 µL) was analyzed on a 0.8% agarose

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gel containing ethidium bromide and visualized with an AlphaImager™ 2200 Documentation and Analysis system (Alpha Innotech, San Leandro, CA, USA). An aliquot of representative PCR products were quantified using PicoGreen® dye (Molecular Probes, Eugene OR, USA).

Arraying and Postprocessing

PCR products corresponding to the 8734 mouse cDNAs and the *Bacillus subtilis* control sequences were printed on Amersham Type 7* slides using the Molecular Dynamics GenIII spotter (Amersham Biosciences, Piscataway, NJ, USA), as described previously (8). The GenIII spotter has 12 capillary printing pins. The printing pins deposit 0.8 nL per contact with the slide. After printing, DNA feature adhesion to the slides was achieved by cross-linking in a Stratalinker® Model 2400 UV Illuminator (Stratagene, La Jolla, CA, USA) with UV light at 254 nm and energy setting at 500 mJ.

Construction of a Cy™3 Reference Sequence

The cDNAs for fabrication of the mouse array were amplified with the following primers (forward primer 5'-CTGCAAGGCGATTAAGTTGGGTAAC-3' and reverse primer 5'-GTGAGCGGATAACAATTTACACAGGAAACAGC-3'). This ensured that in addition to the complete cDNA insert being amplified, some vector sequence was also amplified (8). The sequences from the plasmids, which comprise the Mouse UniGene collection, were aligned. All of the plasmids had homologous sequences 5' and 3' of the polylinker cloning sites, 79 and 29 bp in length, respectively. This conserved sequence was used to validate the amount of bound feature on a microarray. The first step in creation of this construct was an extension reaction with oligonucleotides representing the homologous sequences. The oligonucleotides were as follows: the 56-mer sense primer, 5'-GTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTG-3' and the 71-mer

antisense primer with a complementary sequence overhang, 5'-GGAATTGTG-GAGCGGATAACAATTTACACAGGAAACAGCTATGACCATGATTAC-GCAACGTCGTGACTGGG-3'.

The extension reaction was carried out using PCR conditions described above on the Tetrad thermal cyclers. The extension cycle was as follows: 94°C for 5 min, 60°C for 2 min, and 68°C for 5 min. A double-stranded DNA product was created, and 1 µL was used as a template for PCR amplification using the forward and reverse primers utilized for amplification of the array features. The PCR program consisted of a 94°C hot start for 5 min, 25 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 30 s, and a final extension for 5 min at 68°C. One microliter of the resultant 101-bp product was used as a template for a final round of PCR to attach a T7 promoter sequence.

PCR was performed with the following sense primer 5'-TAATACGACTCACTATAGGGAGGGTTTTCCCAGTCACGACGTTG-3' and the reverse primer utilized for amplification of the UniGene set (see above). The resultant

127-bp product was transcribed using the MegaShortScript™ in vitro transcription kit (Ambion, Austin, TX, USA).

Sample Labeling

Genisphere labeling method. The Array 350RP™ Kit (Genisphere, Hatfield, PA, USA) was used to label 2 µg of total mouse universal RNA (Stratagene). This Genisphere protocol was followed for the labeling reactions with the modification where the QIAquick® PCR purification columns (Qiagen) was used for every purification step. The cDNA samples were quantified by spectrophotometry at A₂₆₀ and A₂₈₀ nm and by using an RNA nanochip on the Bioanalyzer microfluidic system (Agilent, Santa Clara, CA, USA). Although both measurement parameters gave consistent readings, the values determined by spectrophotometry were used in the hybridization experiment.

Incorporation labeling method. To label the array feature reference RNA sequence, 5 µg of RNA were reverse transcribed in a reaction that incorporates amino allyl-labeled dUTP

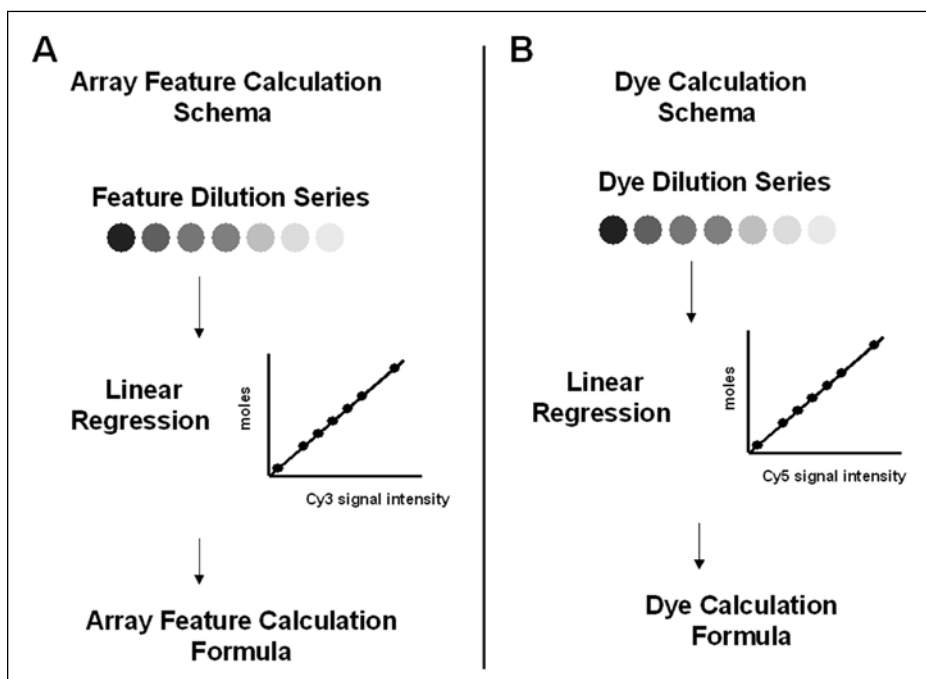


Figure 1. The process of quantifying hybridization stoichiometry in moles. (A) Illustration of how array features are measured in moles. It involves printing a dilution series of a known amount of DNA in a microarray and hybridizing the Cy3-labeled reference to these standards. The signal intensities derived from this hybridization are plotted against the molar amount of DNA printed. (B) Illustration of how the assay is used to determine the amount of dye present in a hybridized cDNA sample. The graphic correlates signal intensity to moles of cyanine dye printed.

(Sigma, St. Louis, MO, USA). Then, the cyanine monoreactive dyes (Amersham Biosciences) were conjugated via an amino allyl group. After labeling, the cyanine-labeled DNA products were analyzed in a spectrophotometer at A_{260} nm to measure DNA, at A_{550} nm to measure Cy3, and at A_{650} nm to measure Cy5. In order to determine how much cyanine dye was incorporated into the fluorescent target, the following equations were used:

$$\text{Cy3 pmol dye/target} = (A_{550} \times \text{dilution} \times \text{volume}) / 0.15$$

$$\text{Cy5 pmol dye/target} = (A_{650} \times \text{dilution} \times \text{volume}) / 0.25$$

Hybridization and Scanning

Four hundred nanograms processed reference cDNA sample, spikes (see concentration represented in Table 2), and 10 pmol (250 ng) of Cy3-labeled sequence were mixed in 50% formamide, 8× standard saline citrate (SSC), 1% sodium dodecyl sulfate (SDS), and 4× Denhardt's solution. To avoid nonspecific hybridization, 2 μ L of Array 350RP dT Blocker (from the Genisphere kit) were added in the hybridization mixture. Forty microliters of hybridization mixture were manually pipeted onto a microarray slide under a coverslip. The hybridization was incubated at 42°C for 12 h. The slides were then washed using the following conditions, 1× SSC and

Table 1. Measurement of Stoichiometry of cDNA Sequences Bound to Array Features

GenBank® Accession No.	Cy5	Cy3	Cy5 pmol DNA	Cy3 pmol DNA	Probe Saturation (%)
AA166336	50430	654	2.58×10^{-7}	8.54×10^{-5}	3.02×10^{-1}
W18585	42809	1580	2.16×10^{-7}	1.78×10^{-4}	1.21×10^{-1}
AA030527	40085	4315	2.01×10^{-7}	4.52×10^{-4}	4.45×10^{-2}
AA276835	35617	896	1.77×10^{-7}	1.10×10^{-4}	1.61×10^{-1}
AA271223	26201	1717	1.27×10^{-7}	1.92×10^{-4}	6.60×10^{-2}
AA271588	24782	991	1.19×10^{-7}	1.19×10^{-4}	1.00×10^{-1}
W14332	22172	1503	1.05×10^{-7}	1.70×10^{-4}	6.19×10^{-2}
AA277159	18676	55	8.75×10^{-8}	2.55×10^{-5}	3.43×10^{-1}
AA000655	18454	3827	8.64×10^{-8}	4.03×10^{-4}	2.14×10^{-2}
AA221886	17232	19938	8.01×10^{-8}	2.01×10^{-3}	3.98×10^{-3}
AA058055	16334	2043	7.56×10^{-8}	2.24×10^{-4}	3.37×10^{-2}

Utilizing the equations that were described in Figures 2 and 3, the picomoles of labeled targets and array features were calculated. This table discloses the most abundant sequences derived from the Stratagene mouse universal reference total RNA sample that hybridized to the array as represented in the Cy5 pmol DNA column. The picomole amount of array probe is represented by the Cy3 pmol DNA column. By transforming hybridization data into picomoles, the percentage of array probe saturation was then calculated (% probe saturation).

0.2% SDS for 10 min, 0.1× SSC and 0.2% SDS for 10 min, 0.1× SSC for 10 min, and 0.1× SSC for 1 min. Slides were then dipped in water and dried by centrifugation. Finally, the slides were scanned with an Axon Scanner (Axon Instruments, Union City, CA, USA) at 600 photomultiplier tube (PMT) settings for both the Cy3 and Cy5 settings.

Dye Printing Experiment

A dye printing experiment was designed to measure the correlation

between cyanine dye molar amount and signal intensity. Cy5-labeled deoxycytosinetriphosphate (dCTP) was purchased from Amersham Biosciences. A titration of dye was printed in replicates of 24 with the concentration in the source of printing plate ranging from 1×10^{-6} to 10 pmol/ μ L. As the printing pins deposit approximately 0.8 nL per contact, it was possible to calculate the amount of dye deposited per slide. After printing, the slides were scanned on an Axon Scanner at the Cy5 setting at 600 PMT. The average intensity values for each concentra-

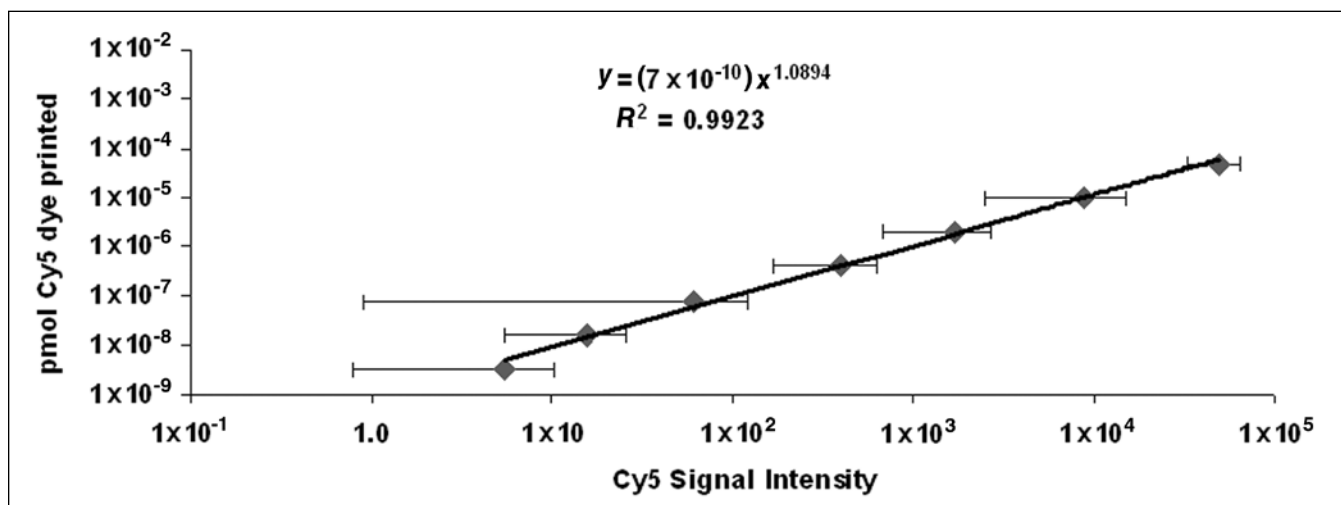


Figure 2. Correlation of Cy5 dye molecules to signal intensity. A dye printing experiment was performed where a titrated dilution series of Cy5 dye was spotted on slides. The plot represents the average signal intensity of 48 printed replicates. The Cy5 signal intensity is plotted on the x-axis, while Cy5 dye in picomoles is plotted on the y-axis. After plotting, a curve fit was calculated, and the formula was used to transform signal intensity derived from microarray hybridization experiments to amount of dye molecules present for each hybridized spot.

tion range were calculated. A plot was derived of signal intensity versus molar dye amount.

Calculation of Array Features

The *B. subtilis* *ycxA* sequences were positioned in a printing plate so that each clone was printed with each of the twelve printing pins. The clones were diluted at the following concentrations (ng/ μ L) in the source printing plate: 200, 50, 20, 5, 2, 0.5, and 0.05. The plate was used twice during the printing run, resulting in 24 replicates for every clone at each dilution point. Since the printing pins deposit 0.8 nL of material per contact, the pmols of DNA deposited on the slide were plotted against the derived Cy3 signal intensities after hybridization of the reference Cy3-labeled product.

RESULTS

Description of Assay Design

The molar relationship between a given array probe and its corresponding target is very useful for a variety of applications. Profiling reference standards facilitate the transformation of data sets that have been generated using different assay techniques. Furthermore, it can be used to evaluate the efficiency of specific DNA probe sequences in detecting and measuring gene expression as well as determining the sensitivity of a particular assay.

Our system employs a method of measuring the amount of DNA in every array feature after printing. In order to do this, a Cy3-labeled DNA sequence is hybridized to every printed feature. Since the array features consist of PCR products that are amplified with a single conserved primer set, the Cy3-labeled sequence hybridizes with the corresponding conserved sequence. This feature enables a means to measure the amount of DNA printed on the array. Figure 1A illustrates the schema of how array features are measured in moles. It involves printing a dilution series of a known amount of DNA in a microarray and hybridizing the Cy3-labeled reference to these standards. The signal intensities derived from this

hybridization are plotted against the molar amount of DNA printed. A standard curve was then generated in which the formula that characterizes the slope of the line was used to transform signal intensities in moles of array features.

A separate series of dye printing experiments were performed to determine the amount of cyanine dye present in the hybridized cDNA sample. Figure 1B illustrates how this measurement was performed. A dilution series of a known amount of cyanine dye was printed on a blank microarray slide. Then the slide was scanned in the same scanner at the identical settings as the array hybridization experiments. A standard curve was then generated that correlated signal intensity to moles of cyanine dye printed. The formula that characterizes the slope of this line was used to transform the signal intensities derived by the hybridized cDNAs to the amount of Cy5 dye detected in moles.

We implemented a labeling system that incorporates a constant defined amount of dye molecules per DNA molecule. The Genisphere labeling technique was used to accomplish this. This system generates approximately 360 ± 20 molecules of dye per labeled DNA by hybridizing DNA dendrimers containing cyanine dyes to the modified cDNA sequences (9). By using this labeling system, the amount of DNA hybridized can then be measured on a molar basis. Based on this experimental approach, a stoichiometric unit that represents the ratio between a known amount of sample cDNA hybridized to a known amount of corresponding homologous array probe can be calculated.

Measuring Hybridization Stoichiometry of a Reference RNA Sample

The hybridization mixture consisted of three different labeled targets: (i) Cy5-labeled cDNA derived from universal reference RNA; (ii) Cy5 individually labeled spike sequences; and (iii) a Cy3 reference DNA sequence that recognizes every array probe. In transforming the derived data set from this hybridization in units whereby stoichiometry can be measured, we compared the signal intensities of the hybridized array probes to signal inten-

Table 2. Array Signal Intensities and Transformed Data Sets of the *Bacillus subtilis* Dynamic Range Control Spikes

Clone	Hyb (ng)	Hyb (pmol)	Cy5 (\bar{x})	Cy5 (sd)	Cy3 (\bar{x})	Cy3 (sd)	Target DNA (\bar{x} in pmol)	Probe DNA (\bar{x} in pmol)	Probe Saturation (%)
ybhR	2500	1×10^{-2}	62316.96	5116.92	775.63	208.17	3.25×10^{-7}	9.76×10^{-5}	3.33×10^{-1}
ybaQ	500	3×10^{-3}	22491.08	4987.38	960.75	346.77	1.07×10^{-7}	1.16×10^{-4}	9.25×10^{-2}
ycxA	100	5×10^{-4}	9044.63	1138.82	612.42	217.36	3.97×10^{-8}	8.12×10^{-5}	4.89×10^{-2}
ybaS	40	2×10^{-4}	5290.42	1242.05	1217.71	449.24	2.22×10^{-8}	1.42×10^{-4}	1.57×10^{-2}
ybaF	20	1×10^{-4}	4683.33	847.79	1048.42	382.71	1.94×10^{-8}	1.25×10^{-4}	1.56×10^{-2}
ybdO	4	2×10^{-5}	828.04	318.84	465.33	231.24	2.96×10^{-9}	6.65×10^{-5}	4.44×10^{-3}
ybaC	0.2	1×10^{-6}	1132.92	501.26	1280.46	541.00	4.16×10^{-9}	1.48×10^{-4}	2.81×10^{-3}
yacK	0	0	878.67	590.53	2328.71	939.20	3.19×10^{-9}	2.53×10^{-4}	1.26×10^{-3}

The data displayed is derived from the dynamic range control spike hybridizations. The clone, hyb (ng), and hyb (pmol) columns represent the *B. subtilis* clone names and nanograms and picomoles of spike DNA in the hybridization mixture, respectively. This table contains mean background-subtracted Cy5 and Cy3 signal intensities and corresponding standard deviations of 24 replicates. The target DNA column lists the mean (in picomoles) of target DNA hybridized, and the probe DNA column lists the mean (in picomoles) of printed corresponding microarray probe that was measured. The probe saturation column lists the percentage of probe saturation that was observed for every amount of spike sequence that was hybridized in the hybridization mixture.

sities that were generated upon printing Cy5 dye. This dye printing experiment was performed on the same type of microarray slide and was scanned at the same scanner settings. Figure 2 plots the amount of dye printed to signal intensity of purified Cy5 dye. The curve fit regression derived from this assay generated the following formula: $y = (7 \times 10^{-10}) x^{1.0894}$. This formula was used to calculate the picomole amount of Cy5 dye that was hybridized to a specific array probe.

Our Cy3-labeled reference sequence is a DNA sequence that hybridizes to conserved regions on every array probe. Therefore, hybridization of this sequence to a dilution series of array probes printed on the slide can be used as a reference to calculate the amount of DNA molecules printed. Figure 3 plots the amount of picomoles of probe DNA printed against signal intensity of the Cy3 homologous reference DNA sequence hybridized. A regression formula can be derived from this data set and used to calculate the amount of DNA printed for every array probe. For this experiment the regression formula is: $y = (1 \times 10^{-7}) x + 2 \times 10^{-5}$.

Based on measurements of sample cDNA sequence and microarray features, the stoichiometric ratio between labeled target samples and array probes can be measured. Table 1 represents the data of the highest expressed sequences measured for the Genisphere-labeled universal reference cDNA sample. The data shows that the probe was in excess

even for the highest molar amount of Cy5-labeled sequence bound. Table 2 displays data generated using the dynamic range control spikes. By using known concentrations in the hybridization mixture, this assay serves as a guide in determining what are the upper and lower limits of sensitivity. The table contains data for the percentage of array probe saturation as well as raw signal intensity values. As the concentration of the target DNA in the hybridization increased, so did the percentage of array probe saturation for every input spike sequence. However, this

situation was not observed with the raw data since the Cy5 signal intensities did proportionately increase for the 0 , 1×10^{-6} , and 2×10^{-5} concentrations.

DISCUSSION

Microarray data is most useful when it can be compared with other genetic detection technologies. Microarray data has been compared to serial analysis of gene expression (SAGE), real-time PCR, Northern blot analyses, and other array formats (10–12). A recent study

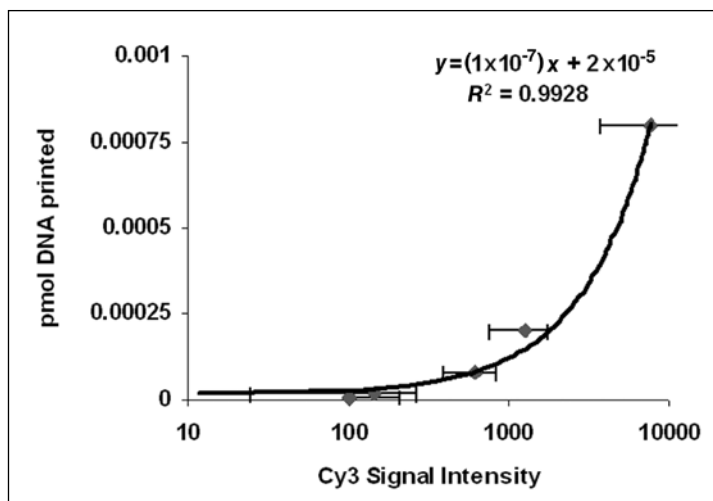


Figure 3. Calculation of printed array probe utilizing a Cy3-labeled homologous reference labeled target. This plot represents the correlation of known amounts of DNA probe printed to signal intensity after hybridization with the Cy3 reference array probe. This is the average signal intensities of 24 replicates. The Cy3 signal intensity is plotted on the x-axis, and the picomole amount of feature DNA is plotted on the y-axis. The curve fit was calculated and was used to determine the picomole amount of every microarray feature.

utilizing identical mRNA preparations on independent microarray platforms revealed considerable divergence in the data obtained, suggesting the need for the establishment of appropriate standards for data analysis (13). Expressing microarray data by accounting for the stoichiometric interaction of target and array feature will significantly improve the accuracy of such comparisons.

Many groups have used a common reference to make accurate comparisons of multiple microarray data sets (14,15). We elected to use the Stratagene universal reference RNA sample because it was designed for this application. It has also been used in real-time PCR assay systems (16). There are many problems associated with using biological references: (i) determining what is a good biological reference; (ii) labeling the biological reference consistently; (iii) and quantifying the labeled sequences in the reference accurately (17,18). Further dye bias needs to be compensated for when comparing two samples in an array experiment (19,20,21). Therefore, it is important to be able to accurately measure and characterize the reference before using it in extensive comparative studies like microarray projects.

Ideally, gene expression profiles should be expressed in natural units with each intensity value articulated uniformly. The assay design described here is an attempt to express microarray data in molar terms. This microarray analysis quantifies the hybridization stoichiometry between array feature and target. The design uses the Cy3-labeled DNA sequence as the common reference for quantifying the molar amounts of array features present, and it uses a dye printing experiment to correlate signal intensity to molecules of cyanine dye. By using a labeling method that generates a consistent and defined ratio of dye molecules to sample DNA molecules, it is possible to measure the stoichiometry of hybridized sample DNA sequences to array features.

Array features also need to be present in molar excess compared to the targets for the signal intensities to remain within linear range (11). Our system demonstrates that array features are considerably in excess since signals reach the highest limit of quantifiable detection at 0.5% feature saturation

when scanned at 600 PMT. This shows a limitation of using cyanine dyes in array experiments and demonstrates how this experimental model can be useful in improving dyes for this purpose. Furthermore, our system can generate usable data from defective arrays where some of the printing pins are clogged or where features have not evenly attached to the slide.

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