

A novel, sensitive detection system for high-density microarrays using dendrimer technology

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Stears, Robin L., Robert C. Getts, and Steven R. Gullans. A novel, sensitive detection system for high-density microarrays using dendrimer technology. *Physiol Genomics* 3: 93–99, 2000.—To improve signal detection on cDNA microarrays, we adapted a fluorescent oligonucleotide dendrimeric signal amplification system to microarray technology. This signal detection method requires 16-fold less RNA for probe synthesis, does not depend on the incorporation of fluorescent dNTPs into a reverse transcription reaction, generates a high signal-to-background ratio, and can be used to allow for multichannel detection on a single chip. Furthermore, since the dendrimers can be detected individually, it may be possible, by employing dendrimer-binding standards, to calculate the numbers of bound cDNAs can be estimated. These features make the dendrimer signal detection reagent ideal for high-throughput functional genomics research.

high-density DNA microarrays; dendrimers; signal detection

HIGH-DENSITY DNA microarrays allow the rapid detection of thousands of expressed genes simultaneously (1, 2). Sensitive, accurate, and reliable detection methods are essential for genomic microarray analysis, as the RNA samples used to generate the probes can be rare or limiting. Unfortunately, the predominant method of synthesizing probes for glass slide microarrays, namely the direct incorporation of fluorescently modified nucleotides into the reverse transcription (RT) reaction, requires large amounts of starting material (up to 50 µg of total RNA or 1 µg of mRNA; Ref. 5). An important consideration for fluorescent nucleotide incorporation is that the amount of fluorescent dNTPs incorporated in each cDNA is sequence dependent; thus it is not possible to quantify cDNAs bound based on the fluorescence intensity of the signal. Also, high levels of dye incorporation into RT products can interfere with hybridization of the cDNA transcripts to the microarray (7). As an alternative, amplification methods involving *in vitro* transcription (6) have been used to enhance sensitivity in microarray analysis (3). However, this approach involves multiple steps, sequential enzymatic reactions, and can take up to 3 days for

probe preparation. Furthermore, these methods of probe synthesis all rely on the incorporation of modified nucleotides.

In this study, we developed a new detection system for DNA microarray analysis; it is a simple procedure that does not rely on modified nucleotide incorporation in the fluorescent labeling reaction and allows for high-quality signal detection for high-density DNA microarrays with a high signal-to-background ratio, even over an increasing number of scans of the microarray.

EXPERIMENTAL PROTOCOL

Dendrimer binding probes. Modified RT transcription (mRT) primer sequences were synthesized (Integrated DNA Technologies) as follows (5' to 3'): for cap3D01, g gCg ggA CAg AAg ACg CgC AgT gAg TCg gCC, oligo d(T); and for cap3D02, A CCg gAg Cgg CAC ggg AAA ATg AgC AAC Agg, oligo dT. One picomole of mRT primers were used with up to 40 µg of total RNA. Dendrimer contained the following capture sequences: cpl3D01 (complement to cap3D01), ggC CgA CTC ACT gCg CgT CTT CTg TCC CgC C; and cpl3D02 (complement to cap3D02), CCT gTT gCT CTA TTT CCC gTg CCg CTC Cgg T (Genisphere) were used for the RT reactions. The RT reaction was performed according to standard protocols (GIBCO, SuperScript RT II) after which 3.5 µl of 0.5 M NaOH/50 mM EDTA was added, and the reaction was heated to 65°C for 10 min to denature the DNA/RNA hybrids. This reaction was then neutralized with 5 µl of 1 M Tris, pH 7.5. For double-labeled detection, the Cy3 and Cy5 RT reactions were mixed and precipitated using 3 M ammonium acetate and 100% ethanol, then washed once with 70% ethanol. The pellets were resuspended in 3 µl sterile distilled H₂O and 15 µl of preheated (65°C) chip hybridization buffer (50% formamide, 10% dextran sulfate, 10 mM Tris pH. 7.5, 5 mM EDTA, 2× SSC, 1% SDS) was added. Probe was layered on a high-density DNA microarray and covered with a coverslip and incubated overnight in a water bath at 65°C in a hybridization cassette (Telechem). The next day the microarrays were washed for 5 min in 2× SSC/0.2% SDS at 65°C, then in 2× SSC and 0.2× SSC for 5 min each.

High-density DNA microarrays and direct incorporation probes were generated by standard methods (2). Briefly, 20–50 µg of total RNA was primed with 1 pmol of oligo dT primer, 1 µl of 10 mM dATP, dGTP, and dTTP was included in the reaction solution, along with 0.8 µl of 1 mM dCTP. Then, 1.2 µl of Cy3- or Cy5-labeled dCTP (Amersham) and 1.2 µl of SuperScript RT II enzyme were used per labeling reaction. Total RNA was prepared as follows: confluent HeLa cell cultures in 10-cm plates were transferred into 1% serum media for 18 h, then treated with either 100 nM phorbol myristic acid (GIBCO) or 15 ng/ml fibroblast growth factor

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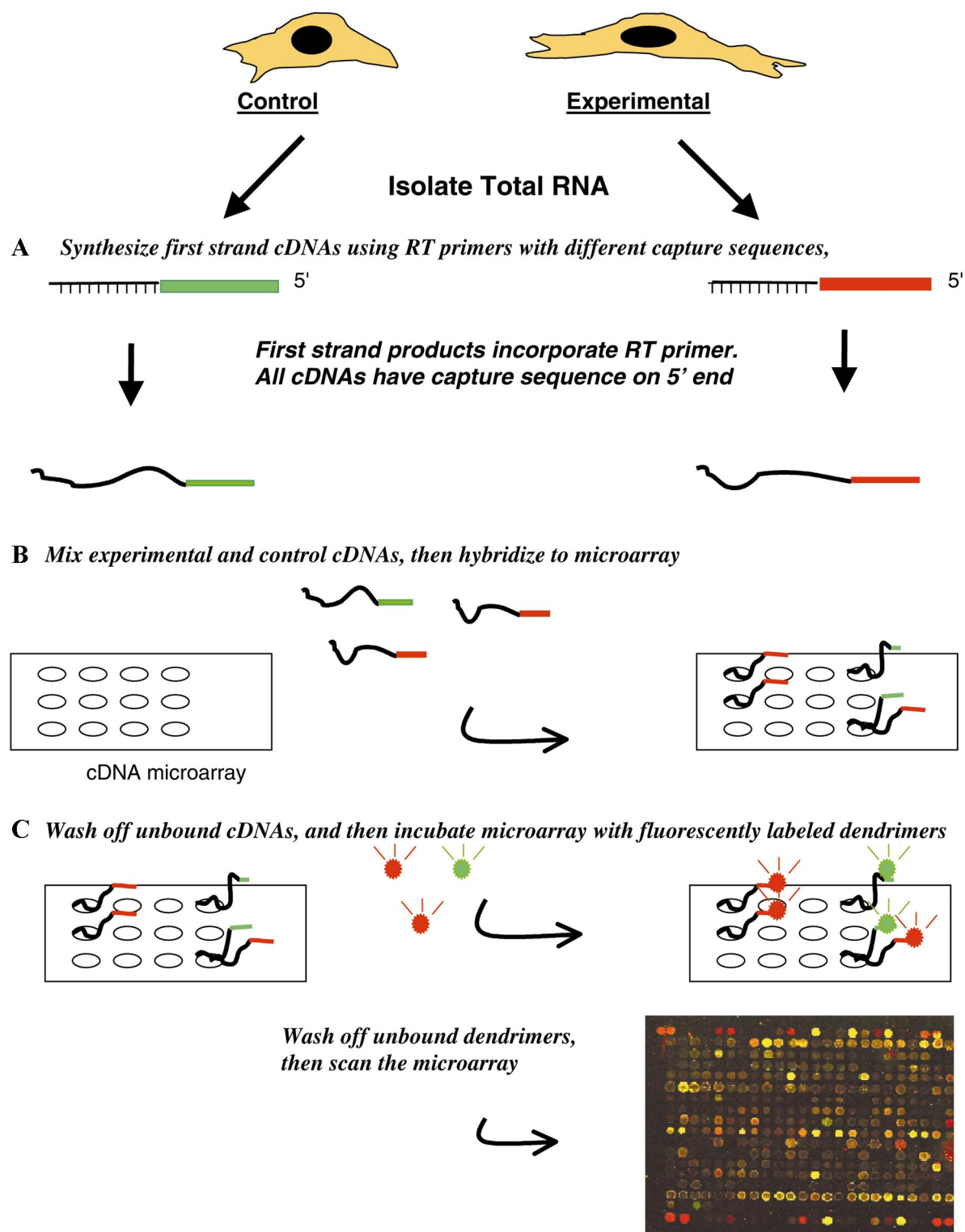


Fig. 1. Schematic diagram of the 3DNA dendrimer detection process. Microarray image at *bottom* is a false color overlay of a microarray detected with the dendrimer detection system and scanned in the Cy3 and Cy5 wavelengths.

(GIBCO) for 4 h. Total RNA was isolated from both stimulated sets by Trizol (GIBCO) extraction, then mixed to produce a stimulated large sample of stimulated control RNA. Microarrays were made by spotting 1,700 amplified cDNAs on a sialylated glass slide (Cell Associates) using a microar-

rayer (Cartesian Technologies), after which the cDNA was ultraviolet (UV) cross-linked onto the slide surface with a Stratalinker (Stratagene).

For dendrimer detection, 2.0 μ l of the Cy3- and Cy5-labeled dendrimers (Genisphere) were suspended in 15 μ l of

Table 1. *Dendrimers have a consistent number of fluorescent molecules*

Production Run	Fluorescent Molecules per Dendrimer	
	Cy3 labeled	Cy5 labeled
1	261	242
2	256	243
3	242	237
4	247	256
5	244	238
Average of 5 runs	250	243
SE	4	4

Quantitative analysis of 6 different lots of Cy3- and Cy5-labeled dendrimers. SE, standard error.

4× SSC-2% SDS and layered on the array. The arrays were covered with a coverslip and then incubated at 65°C for 6–8 h in a microarray hybridization cassette (Telechem International). The chips were then washed as above and scanned in a ScanArray 3000 confocal laser scanner (GSI Lumonics). The signal and background signals were then quantified with ImageQuant software. Signals were measured as the mean pixel intensity within each circumscribed spot, average background was measured using the mean pixel intensities of blank spots in the array (without cDNA) and as the mean pixel intensity outside the circumscribed spot diameter for calculating specific signals.

Preparation of dendrimer reagents. DNA dendrimers were prepared as previously described (4). Briefly, DNA dendrimers were assembled from seven oligonucleotides, *strands 1–7*. The seven strands were pairwise hybridized in $T_{50}N_{200}E_{10}8$ buffer ($T_{50}N_{200}E_{10}8$: 50 mM Tris·HCl, pH 8.0, 10 mM EDTA, and 200 mM NaCl) to form five building block “monomers”: A, B', B'', C', and C''. *Strand 1* and *strand 2* were hybridized to form the “A” monomer. Similarly, *strand 3* was hybridized to *strand 4* and *strand 4* was hybridized to *strand 5* to produce the B' and B'' monomers, respectively. The C' monomer was prepared by hybridizing *strand 2* and *strand 6*, and the C'' monomer was prepared by hybridizing *strand 2* and *strand 7*. Each monomer is composed of four 31-nucleotide single-stranded arms and one central 50-base waist. From these building blocks the core dendritic structure was assembled.

Dendrimer core assembly was accomplished by hybridizing one initiating A monomer with two B' and two B'' monomers in $T_{50}N_{200}E_{10}8$ buffer for 20 min at 42°C to form the 1-layer dendrimer. A second layer of C' and C'' monomers was added to the one-layer assembly to form a two-layer dendrimer regenerating the original sequences present on the A monomer. Three- and four-layer dendrimers were prepared by the sequential addition of B' and B'' monomers followed by C' and C'' monomers. At each layer of assembly 4,5',8-trimethylpsoralen (trioxsalen) was used to covalently cross-link the dendritic structure. This was accomplished by adding 1/15 of a volume of a trioxsalen-saturated ethanol solution to the hybridization mixture. After 20 min of incubation, the hybridization mixture was cross-linked in a UV photochemical reaction chamber (model HRI100, Simms Instruments) for 10 min at 42°C. After the fourth layer of assembly, the four-layer dendritic core was purified using ultracentrifugation on 10–50% sucrose gradients containing 50% deionized formamide run at 40°C. Gradient fractions containing four-layer dendrimer were pooled, ethanol precipitated, and resuspended in 50 mM Tris·HCl, pH 8.0, 10 mM EDTA.

To prepare fluorescent-labeled dendrimers, the cpl3D01 and cpl3D02 sequences were ligated, separately, to the puri-

fied dendritic core material. Thirty-nucleotide-long oligonucleotides complementary to the outer arms of the four-layer dendrimer having a 5' Cy3 or Cy5 were synthesized (Oligos Etc.). The Cy3 and Cy5 oligonucleotides were hybridized and covalently cross-linked to the outer surface of the 3DA2 and 3DB2 dendrimers, respectively. Excess capture and fluorescent-labeled oligonucleotides were removed by size exclusion chromatography. The concentration of dendrimer was determined by measuring the optical density of the purified material at 260 nm on a spectrophotometer. The fluorescence was measured at the optimal signal/noise wavelengths using a fluorometer (FluoroMax, SPEX Industries). Cy3 was excited at 542 nm, and the emission was measured at 570 nm. Cy5 was excited at 641 nm, and the emission was measured at 676 nm.

RESULTS

The dendrimer detection system. Essentially, the dendrimer detection method involves first hybridizing the unlabeled first-strand cDNA that contains 5' dendrimer binding sequence to the microarray, then washing off the excess RT primer and unbound cDNAs. The hybridized cDNAs are then detected by incubating the

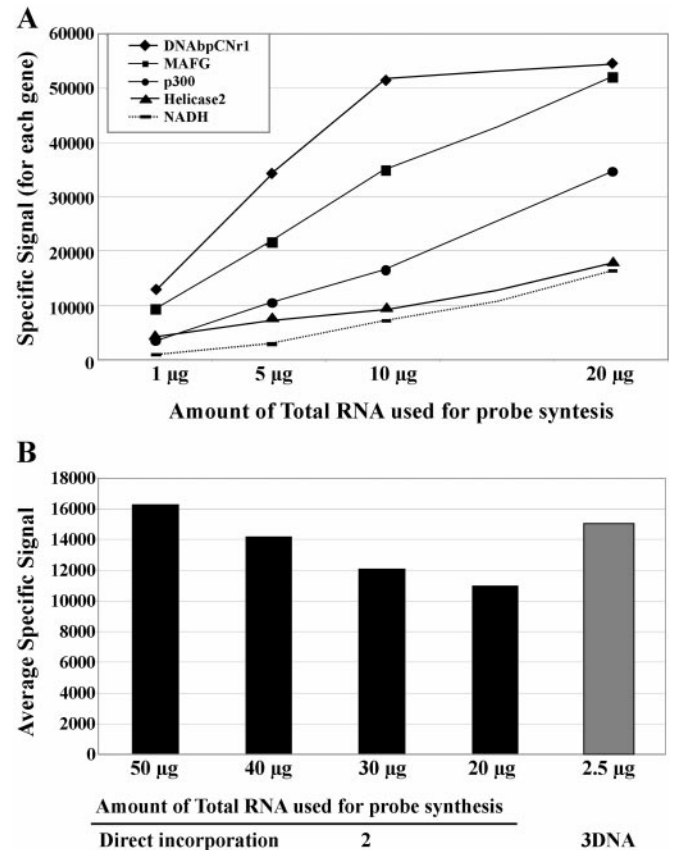


Fig. 2. The specific signal is proportional to the amount of starting RNA used for probe synthesis. A: specific signal (i.e., signal – background) for individual genes and increasing amounts of total RNA used for probe synthesis. B: comparison of the specific signals of direct incorporation probes with increasing amounts of total RNA used for probe synthesis to the specific signal from 2.5 µg of total RNA detected by dendrimer reagents (3DNA detection). MAFG, v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G.

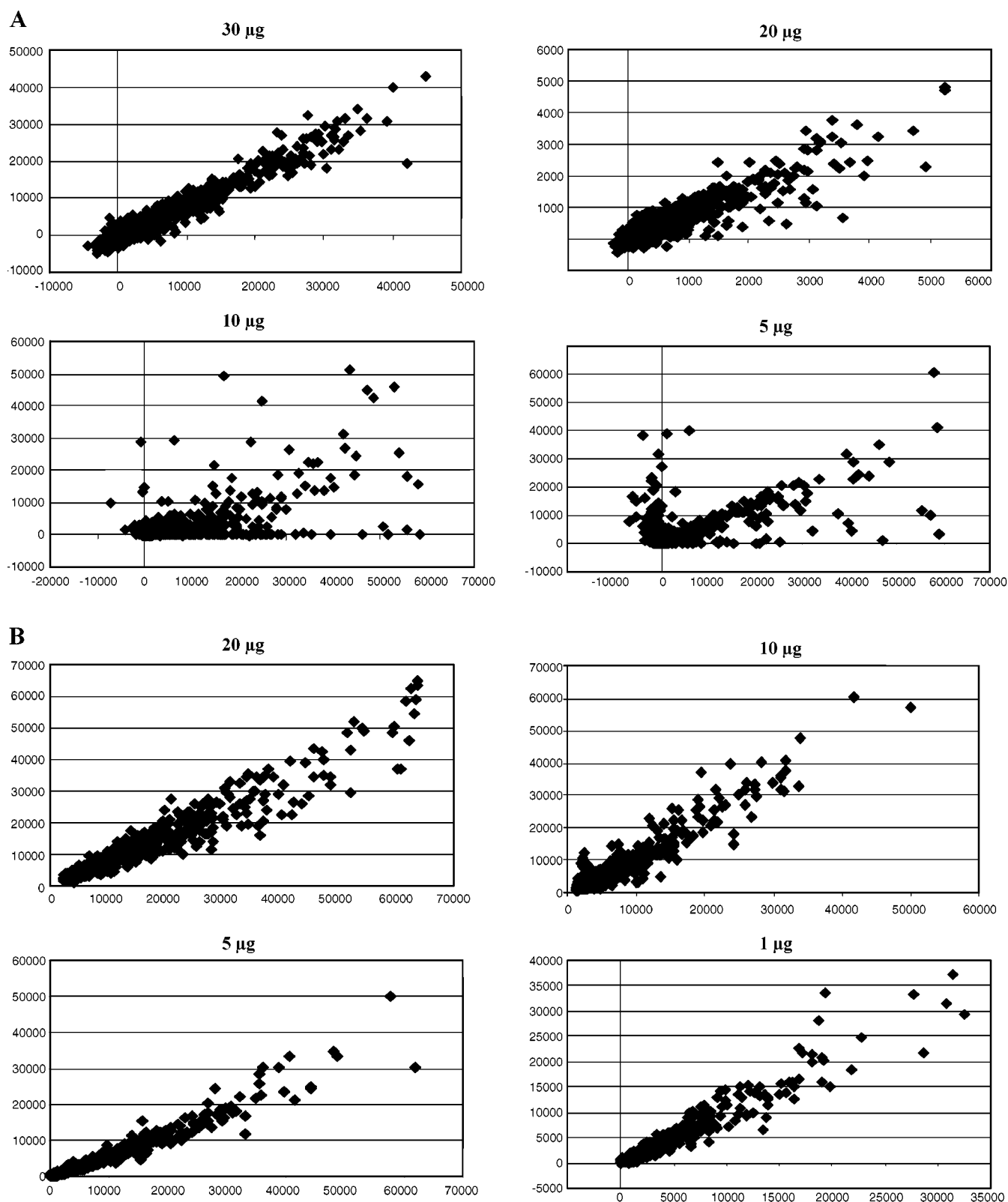


Fig. 3. Dendrimer detection provides sensitive detection to low amounts of input RNA. Total RNA was labeled separately with Cy3 and Cy5 for each input concentration tested. Microarrays were hybridized with both Cy3- and Cy5-labeled probes at each concentration, and scatter plot analysis of the Cy3-specific vs. Cy5-specific signals was plotted for the dendrimer detection method vs. the direct incorporation method over the concentration ranges tested. Probes were synthesized with 30, 20, 10, and 5 μg for the direct incorporation method (A) and 20, 10, 5, and 1 μg of total RNA for the dendrimer detection (B).

chips with dendrimers containing the capture sequence on the cDNAs (Fig. 1). Dendrimers are complexes of partially double-stranded oligonucleotides, which form stable, spherical structures with a determined number of free ends. Specificity of the dendrimer detection is accomplished through specific binding of capture oligonucleotide on a free arm of the dendrimer. By synthesizing an RT primer consisting of an oligo dT sequence coupled to a sequence complementary to the capture sequence on the dendrimer, we generated first-strand cDNA probes, without modified nucleotides, that are capable of binding the dendrimers via the complementary primers (Fig. 1A). These cDNAs are hybridized to the microarray, and then the unbound material is washed off (Fig. 1B). In a second short hybridization step, the dendrimers are incubated with the hybridized microarray and allowed to bind to the 5' end of the hybridized cDNAs via capture sequence (Fig. 1C). As the dendrimers are prelabeled with Cy3 or Cy5, a sensitive detection reagent for high-density DNA microarrays is generated (3DNA detection system).

Standardization of dendrimer detection reagents. An important feature of this detection method is the consistency of fluorescence intensity of the prelabeled dendrimers. As depicted in Table 1, dendrimers prepared from five independent synthesis reactions were quantified for fluorescence intensity per microgram of dendrimer DNA, and the average number of fluorescent molecules per dendrimer was calculated. The Cy3-labeled dendrimers had an average of 250 fluorescent molecules per dendrimer, whereas the Cy5-labeled dendrimers contained an average of 243 fluorophores per dendrimer, with a standard error (SE) of ± 4 molecules. These results show the tightly defined and quantified fluorescence intensity of the dendrimer detection reagent, a feature that should allow for the quantification of the numbers of cDNAs bound to the microarray.

Signal is proportional to amount of RNA used for probe synthesis. To determine whether the signal is proportional to the amount of input RNA used for probe synthesis, we synthesized first-strand cDNA probes using the mRT primer with increasing amounts of total RNA, from 1 to 20 μg . In Fig. 2A, we observe that with increasing amounts of total RNA used for the probe synthesis reaction, increasing total specific signals (i.e., signal – background) are obtained for individual expressed genes. This response is relatively linear within the concentration range tested, although signal saturation was observed with the most highly expressed gene. To compare the sensitivity of the 3DNA system to the standard direct incorporation method, we evaluated the specific signal obtained with a probe synthesized with 2.5 μg of total RNA, with the dendrimer detection system compared with the specific signal obtained with direct incorporated probes made from 10 to 50 μg of total RNA. In Fig. 2B, we found that the specific signal obtained with the dendrimer detection system using 2.5 μg of total RNA is comparable to the total specific signal of a standard direct incorporation probe generated with 40 μg of total RNA, indicat-

ing a 16-fold increase in sensitivity of detection with the 3DNA method.

Dendrimer detection generates high-quality signal with little background. Another important parameter that can compromise microarray analysis is the amount of background signal generated on the microarrays from the detection method used. To test this, we labeled equal amounts of RNA from the same source with Cy3 and Cy5 and probed a single microarray such that the microarray was double labeled. Scatter plot analysis was performed on the Cy3-specific signal vs. the Cy5-specific signal, over an increasing amount of input RNA for both the dendrimer-detected microarrays and microarrays probed with direct incorporation probes. The direct incorporation probes lose linearity in the scatter plot analysis when less than 20 μg of total RNA is used to generate the probes (Fig. 3A). However, the microarrays detected using the 3DNA system retained linearity in the scatter plot analysis down to 1 μg of total RNA used (Fig. 3B). To understand why the dendrimer detection system retained linearity, we examined the total background generated on the microarrays, looking at the 5–30 μg range for the direct incorporation probes and the 1–10 μg range for the dendrimer detection system (Fig. 4). We found that the background generated with the 3DNA detection system remained low and relatively constant over the concentration range tested, whereas the direct incorporation method generated background proportional to the amount of total RNA used for the probe synthesis and on average is much higher even at the low input RNA concentration. The low background for the 3DNA detection system allows for fluorescent signal to be detected at the relatively lower input RNA concentrations.

Increasing scan number does not degrade the specific signal. The use of increasing amounts of RNA is one way to increase the signal obtained from DNA microarrays. Another method is to increase the number of scans of the microarray for image generation prior to quantification. The increase in signal should be propor-

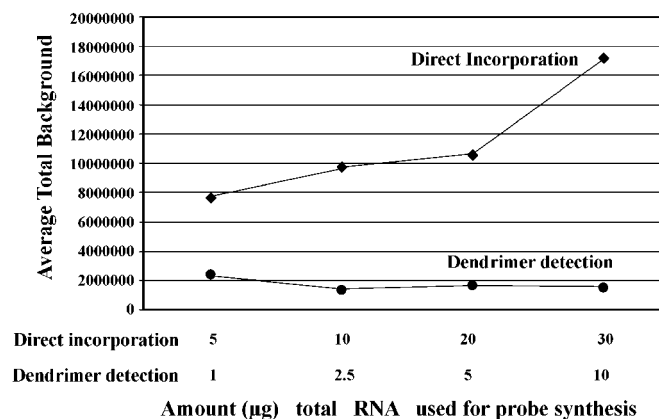


Fig. 4. 3DNA detection system does not generate high background. A comparison of the background generated with 3DNA detected probes and direct incorporation probes, with increasing amounts of RNA used for probe synthesis.

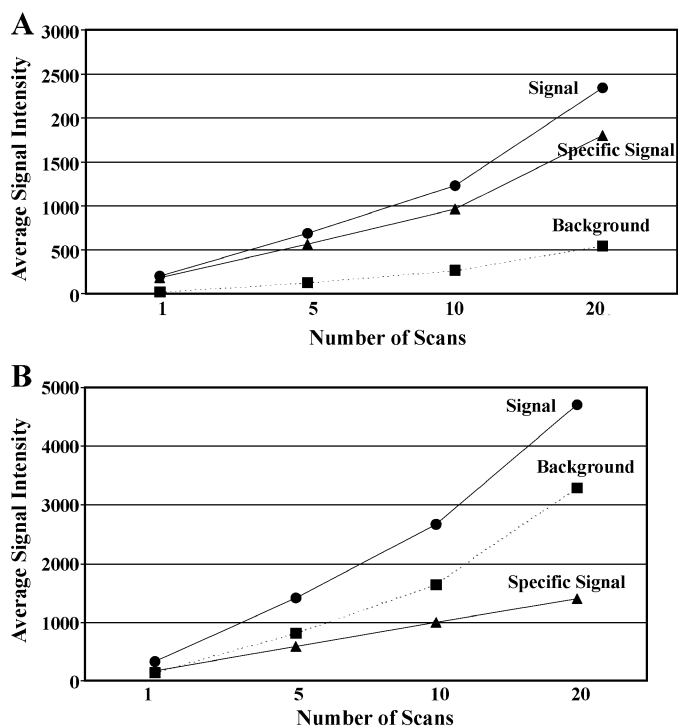


Fig. 5. Increase in specific signal (signal – background) on the arrays with increasing number of scans using the dendrimer detection method. Arrays were probed with 30 μg of total RNA for the direct incorporation and 20 μg of RNA for the dendrimer detection system. Of the 1,700 genes that were spotted on the microarrays, a set of 116 expressed genes were arbitrarily chosen for a more in-depth analysis of the signal, background, and specific signal generated. Each array was scanned 1, 5, 10, and 20 times. A: the 3DNA dendrimer detection maintained a high specific signal. B: the direct incorporation method exhibited a large increase in the background with increasing number of scans. Data show the average signal (●), background (■), and specific signals (▲) for the representative expressed genes.

tional to the number of scans of the microarray, and a high signal-to-background ratio should be maintained. To evaluate this parameter, we examined the total signal, total background, and total specific signal generated by 116 expressed genes over increasing number of scans of the microarray for both the dendrimer detection system and the direct incorporation method (Fig. 5), and equal amounts of input total RNA (20 μg) were used to make the probes for the direct incorporation probes and arrays detected using the 3DNA detec-

tion system. Indeed, a linear increase in signal is observed with an increasing number of scans for both the direct incorporation method and the 3DNA detection method. However, a comparison of the background generated with increasing scan number reveals an important difference between the two signal detection methods. First, with the direct incorporation method, the background is proportional to the number of scans, leading to a specific signal that is significantly less than background (Fig. 5B). With the 3DNA detection method, however, significantly less background is generated, so the specific signal is close to the actual total signal detected (Fig. 5A), and a closely correlated increase in the specific signal is obtained with an increase in scan number. Comparison of the retention of specific signal strength reveals the most significant differences in sensitivity. Although the specific signal vs. number of scans remains linear using both methods, the signal-to-background ratios of the expressed genes detected with the dendrimer detection system are significantly greater than those detected with the direct incorporation method over increasing number of scans. When the background is subtracted from the signal, the calculated specific signal strength from the dendrimer-detected arrays retained on average of 81% of the original signal, regardless of the number of scans. However, the calculated signal strength after background subtraction, for the genes probed with the direct incorporation probe, only retained an average of 42% of the signal strength.

DISCUSSION

Although the current method of signal detection with glass slide microarrays, direct incorporation of fluorescently modified dNTPs, generates good quality data, this new dendrimer based method has several advantages, the main points of which are summarized in Table 2. With the 3DNA system, high signal can be obtained while maintaining a low background, with up to 50-fold less total RNA for probe synthesis. This high signal-to-background ratio is also maintained over high scan numbers, facilitating the detection of low-abundance transcripts. Furthermore, as each dendrimer has predetermined, quantified fluorescent intensity (see Table 1) and as each cDNA transcript binds a single dendrimer, the amount of signal generated is directly proportional to the number of cDNA

Table 2. Attributes of dendrimer-based (3DNA) detection system

Attribute	Dendrimer Detection System	Direct Incorporation
Signal requires incorporation of modified nucleotides	No	Yes
Background	Low	Dependent on amount of RNA used and degree purification of labeled cDNA from unincorporated dNTPs
Signal-to-background ratio with optimal amounts of RNA used (50 μg for direct incorporation, 1 μg for dendrimer detection)	Signal 10-fold over background	Signal 2-fold over background
Amount of total RNA used for starting material	1–2 μg	Typically 40–50 μg
Quality of signal with increasing number of scans	High signal-to-background ratio maintained even up to 20 scans	Deteriorates significantly after 1 scan

molecules detected. This reagent thus has removed the barrier of variable incorporation of the modified dNTPs into an RT reaction that thus far has prevented direct calculations of cDNA molecules bound. We foresee that, by employing dendrimer-binding standards on the microarrays, it may be possible to estimate the numbers of bound cDNAs per gene. Third, as the fluorescently modified dNTPs are quite hydrophobic, probe aggregation may occur when these molecules are incorporated into large RT products with direct incorporation. However, with the dendrimer system, by generating first-strand cDNAs with unmodified nucleotides, the RT reaction is able to proceed under optimal conditions, and the solubility of the probe is more favorable. The incorporation of other fluorescent molecules will also facilitate deriving data from multiple probes in multiple detection wavelengths, from a single microarray.

In conclusion, the dendrimer detection (3DNA) reagents are an ideal signal detection reagent for high-density DNA microarrays, as they provide high-quality signal using low amounts of starting RNA material. In addition, they maintain a low background over increasing amounts of RNA used and an increasing number of scans. The dendrimeric reagents will also facilitate direct calculations of the number of transcripts bound due to their preordained fluorescent intensity and proportional relationship to the bound cDNAs on the microarray. Furthermore, since they are simple to use compared with direct incorporation probes and give

high-quality data, they are ideal for high-throughput functional genomic analysis.

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