

Impact of prehybridization PCR amplification on microarray detection of nitrifying bacteria in wastewater treatment plant samples

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Summary

A gel-based microarray that included a set of 26 oligonucleotide probes targeting all nitrifying bacteria at varying levels of specificity suggested the presence of targeted microorganisms when hybridized to RNA isolated from a wastewater treatment plant, but could not discriminate between perfectly matched and mismatched sequences due in part to low signal intensity. To enhance sensitivity and improve discrimination, polymerase chain reaction was used to selectively amplify the 16S rRNA genes of specific nitrifier groups. RNA transcribed from these DNA templates was hybridized to the microarray and thermal dissociation analysis was used to characterize the specificity of hybridization. Amplification with *Nitrospira*-specific primers resulted in the selective amplification of this target group, confirmed by both a significant increase in signal intensity and a melting profile identical to the reference RNA. In contrast, *Nitrobacter* was not detected in the environmental samples with probe Nbac1000 despite pre-amplification with *Nitrobacter*-specific primers, indicating the absence of strains containing this *Nitrobacter*-specific sequence. Pre-amplification using primers specific for β -Proteobacterial ammonia-oxidizing bacteria resulted in a significant increase in signal intensity for probe

Nso190, but melting profiles for probe Nso190 showed a slight deviation between amplified RNA and the reference microorganism, suggesting that the amplification products contained some sequences that varied by a single nucleotide difference in the probe target region.

Introduction

DNA microarrays are becoming more widely used for the detection of bacterial genes in environmental samples (Koizumi *et al.*, 2002; El Fantroussi *et al.*, 2003; Wu *et al.*, 2004; Kelly *et al.*, 2005; Loy *et al.*, 2005). Two of the main challenges associated with the application of DNA microarrays to environmental samples are specificity and sensitivity (Zhou and Thompson, 2002; Kelly, 2003). Non-target organisms or contaminants, such as remnant RNA or DNA, can cause cross-hybridization with nucleic acids immobilized on DNA microarrays. Therefore, achieving a high level of specificity is critical to ensuring accurate detection. Many strategies have been proposed to solve discrimination problems, including the use of DNA polymerase and DNA ligase to help discriminate mismatches from perfect-match duplexes (Mikhailovich *et al.*, 2001; Busti *et al.*, 2002; Gharizadeh *et al.*, 2003; Rudi *et al.*, 2003). Previous work with oligonucleotide probes in a gel-based microarray format has demonstrated that melting profiles can also be used to achieve single-base-pair mismatch discrimination of target and non-target hybridizations (Liu *et al.*, 2001; Urakawa *et al.*, 2002; 2003). Recent work has suggested that the data-analysis tools used to assess melting profiles on gel-based microarrays can result in poor-quality melting profiles (Pozhitkov *et al.*, 2005), but work by our group (e.g. Urakawa *et al.*, 2002; 2003; El Fantroussi *et al.*, 2003; Kelly *et al.*, 2005) has demonstrated good melting-profile replication and has not shown any of the problematic data types described by Pozhitkov and colleagues (2005).

Sensitivity is another limiting factor for the application of DNA microarrays to environmental samples. Previous work has demonstrated that DNA microarrays can be used to detect bacterial rRNA directly from environmental samples (Koizumi *et al.*, 2002; El Fantroussi *et al.*, 2003; Peplies *et al.*, 2004; Kelly *et al.*, 2005). The advan-

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tages of direct rRNA detection include avoiding polymerase chain reaction (PCR) bias and avoiding the time required for PCR. The main disadvantage of direct rRNA detection is that it may not be sensitive enough to detect low-abundance targets. The sensitivity of DNA microarrays can be improved by amplification of targets by PCR prior to microarray hybridization, and several groups have used this approach (Bodrossy *et al.*, 2003; Kim *et al.*, 2004; Loy *et al.*, 2005). Polymerase chain reaction amplification prior to hybridization should result in stronger signals and should allow the detection of specific target groups of interest, even if they are present in low abundance. However, direct hybridization of PCR amplicons is not optimal for gel-based DNA microarrays because re-annealing of double-stranded PCR amplicons can compete with hybridization of the target strand to the immobilized probe, reducing signal intensity, and because the optimal target size for gel-pad microarray hybridization is approximately 100–200 base pairs (Kelly *et al.*, 2002).

The goal of the current study was to develop a prehybridization PCR amplification approach to improve the sensitivity and specificity of gel-based microarray detection of low-abundance nitrifying bacteria in environmental samples. The microarrays used in this study included

probes targeting all known nitrifying bacteria at various phylogenetic levels. In order to avoid the difficulties associated with hybridization of double-stranded PCR amplicons, we included an *in vitro* transcription step to produce single-stranded RNA targets from the PCR amplicon. In order to simplify the validation of probe specificity, we incorporated into the array two single-mismatch variants of each of the probes. We collected the environmental samples used to test this approach from a municipal wastewater treatment plant (WWTP).

Results and discussion

Hybridization to RNA extracted from WWTP samples

We hybridized the DNA microarray with 17.5 µg of RNA extracted from 2 ml of mixed-liquor suspended solids (MLSS) obtained from an activated sludge WWTP. Figure 1 clearly demonstrates successful hybridization of rRNA extracted from WWTP samples. In particular, the high signal intensities from general probes Univ1390, Univ907 and Eub338 confirm successful hybridization of native rRNA without PCR amplification. Note that Fig. 1 shows signal intensities of each probe normalized to the signal intensity of probe Eub338.

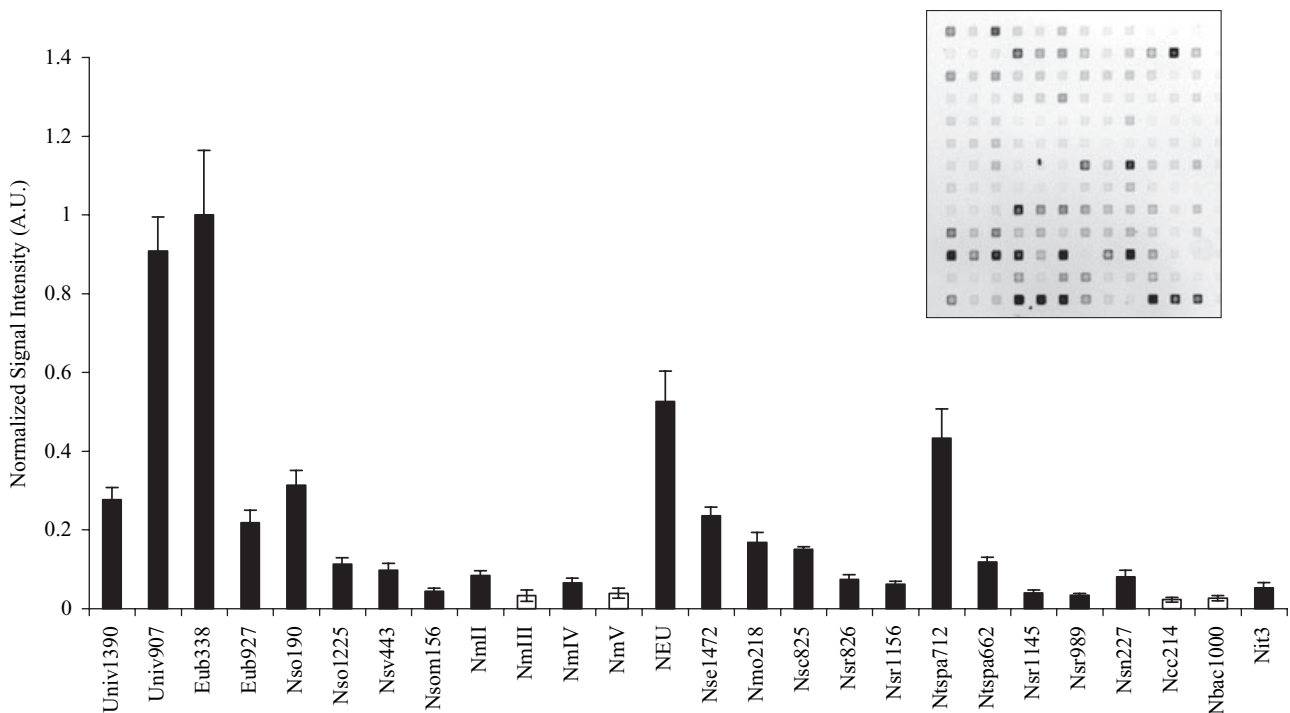


Fig. 1. DNA microarray image hybridized with rRNA extracted from mixed-liquor samples collected from the WWTP (top) and the normalized signal intensities of each probe on the microarray (bottom). The microarray was hybridized and washed at 20°C. Signal intensities were normalized to signal intensity of probe Eub338. A dark column represents a significant signal, while a white column indicates a non-significant signal. Data points represent mean values ($n = 2-10$, depending on the number of replicate probes on the microarray), and error bars reflect the standard deviations for each mean.

Probes targeting the same group of bacteria at different sites on the 16S rRNA gene, e.g. Eub338 and Eub927, are redundant probes (El Fantroussi *et al.*, 2003). The signal intensities for some redundant probes varied significantly when hybridized with environmental native rRNA. For example, probe Univ907 had significantly higher signal intensities than probe Univ1390 ($P < 0.001$; $n = 6$), and probe Eub338 had significantly higher signal intensities than probe Eub927 ($P < 0.001$; $n = 6$). Differences in signal intensities between redundant probes may result from minor differences in specificity (e.g. Nso190 and Nso1225 differ slightly in specificity). However, we also observed different signal intensities from redundant probes when we hybridized the DNA microarray with rRNA obtained from a single reference organism, *Nitrosomonas eutropha* (Fig. 2). Probes Univ907, Eub338 and Nso190 showed signal intensities 1.6, 9.5 and 2.7 times higher than their corresponding redundant probes Univ1390, Eub927 and Nso1225 when hybridized with *N. eutropha* rRNA. Site-specific differences in signal intensities originating from redundant probes could be a result of specific site preferences during fragmentation and fluorescent labelling of the target sequences (Bavykin *et al.*, 2001; El Fantroussi *et al.*, 2003), different binding capabilities of the different probes, or a combination of these factors. Because Univ907, Eub338 and Nso190 gave higher signal intensities than Univ1390, Eub927 and Nso1225, respectively, Univ907 and Eub338 appear to be more sensitive general probes, while Nso190 appears to be a more sensitive probe for β -Proteobacterial ammonia-oxidizing bacteria (AOB) when using the microarray format.

When hybridized at 20°C with RNA extracted directly from WWTP samples, nitrifier probes Nso190, Nso1225, Nsv443, Nsom156, NmII, NmIV, NEU, Nse1472, Nmo218, Nsc825, Nsr826, Nsr1156, Ntspa712, Ntspa662, Nsr1145, Nsr989, Nsn227 and NIT3 gave significant positive signals (Fig. 1). Thus, native rRNA potentially from

low-abundance nitrifiers was detected directly without PCR. We used the structured mathematical model of Rittmann and colleagues (1999) and the operating condition of the Egan Water Reclamation Plant (EWRP) (Table 2) to estimate that all nitrifiers should have constituted approximately 7.7% of the total community. Being able to detect organisms present at a few per cent of the biomass is crucial. A previous study (Denef *et al.*, 2003) was able to use glass-slide DNA microarrays with PCR amplification to detect bacteria present at 1–5% of the analysed community. Our results with environmental RNA hybridized directly to a gel-based microarray are comparable. However, the signal intensities of probes targeting less abundant nitrifiers, especially *Nitrobacter* (probes NIT3 and Nbac1000), were near background in direct hybridization of environmental RNA (Fig. 1). These low signal intensities complicate the analysis of microarray data because it is very difficult to achieve accurate and reproducible melting profiles for hybridizations with such low signal intensities.

Pre-amplification of environmental 16S rRNA genes

To increase sensitivity, we selectively amplified 16S rRNA genes for *Nitrospira*, *Nitrobacter* and β -Proteobacterial AOB using the universal forward primer 11f (Kane *et al.*, 1993) and specific reverse primers S-G-Ntspa-0685-a-A-16 (Hovanec *et al.*, 1998), S-G-Nbac-1035-a-A-18 (NIT3) (Wagner *et al.*, 1996) and S-*Nso-1225-a-A-20 (Mobarry *et al.*, 1996) respectively. *In vitro* transcription was then used to produce single-stranded rRNAs from these PCR amplicons.

Pre-amplification using the Ntspa0685 primer resulted in an approximate 100-fold increase in hybridization to probe Ntspa662 relative to native RNA (Fig. 3A). The specificity of Ntspa662 hybridization was confirmed by melting-profile analysis: pre-amplified RNA from the WWTP samples showed a melting profile identical to the

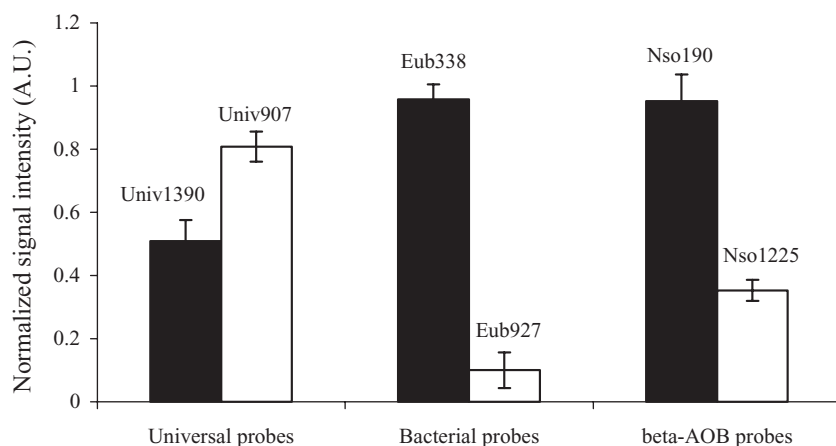


Fig. 2. Normalized signal intensity of redundant probes from hybridization of *N. eutropha* pure culture rRNA at 20°C. Signal intensities were normalized to signal intensity of probe Eub338. Data points represent mean values ($n = 4$), and error bars reflect the standard deviations for each mean.

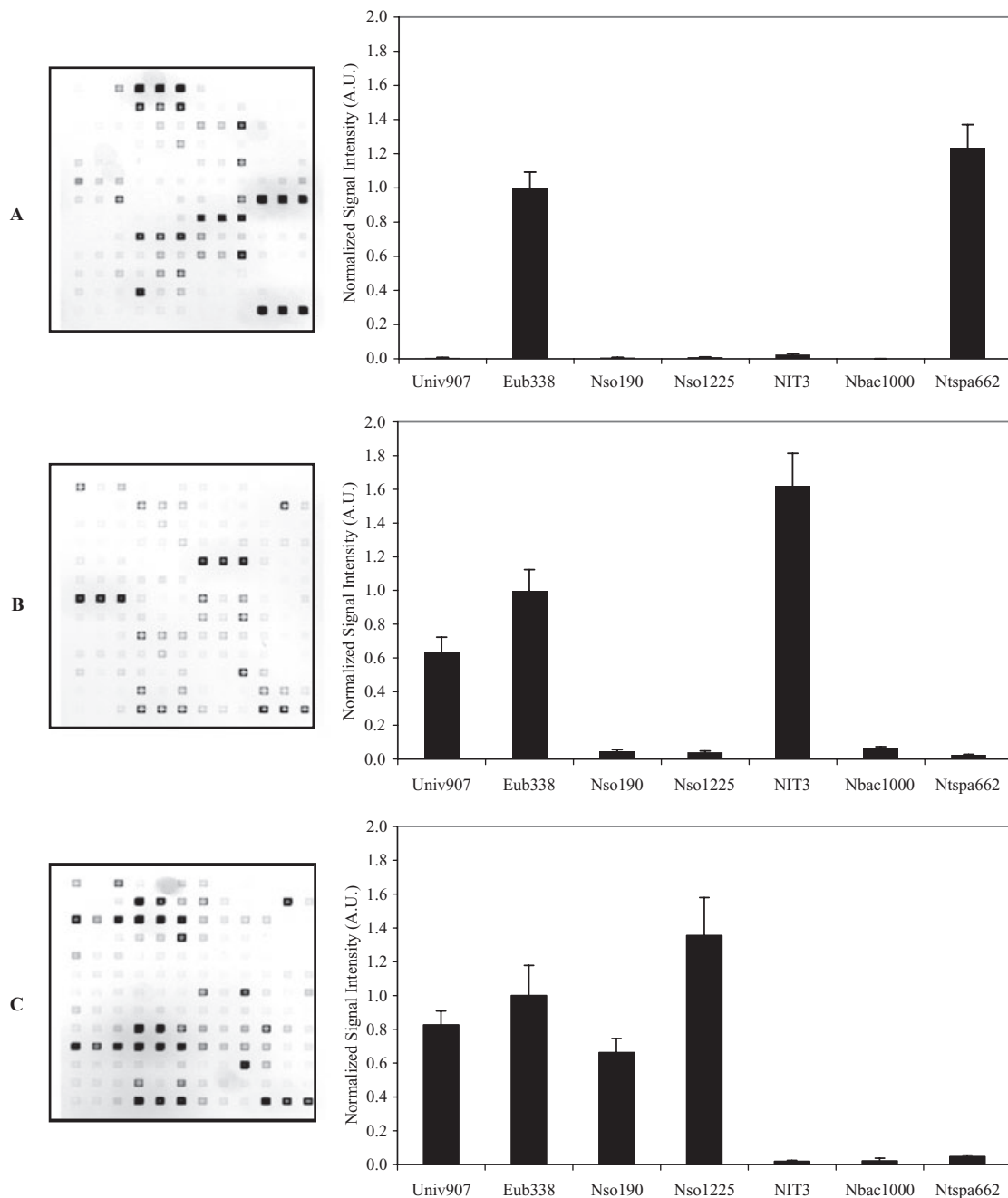


Fig. 3. DNA microarray images of specifically pre-amplified rRNA when hybridized at 20°C: (A) *Nitrospira*-specific rRNA; (B) *Nitrobacter*-specific rRNA; and (C) β -Proteobacterial AOB-specific rRNA. Signal intensities were normalized to the signal intensity of probe Eub338. Data points represent mean values ($n = 4$), and error bars reflect the standard deviations for each mean.

corresponding reference strain (Fig. 4) and the T_d of environmental pre-amplified RNA (47.39 ± 0.35 ; $n = 4$) was not significantly different from the T_d for the reference culture (48.10 ± 0.44 ; $n = 4$). In contrast, the T_d s for two single-mismatch probes (Ntspa662-c7g and Ntspa662-g9c) were significantly lower ($T_d = 33.20 \pm 0.34$; $n = 4$) and the melting profiles were clearly distinct (Fig. 4). Together, these results demonstrated that single-base

mismatch discrimination was achieved for probe Ntspa662 using melting-profile analysis, and they confirmed the specific detection of *Nitrospira* in the WWTP samples after pre-amplification.

Hybridization of RNA directly recovered from the WWTP to probe Nbac1000 did not produce a significant hybridization signal (Fig. 1) and no signal increase was observed for this probe following amplification with the

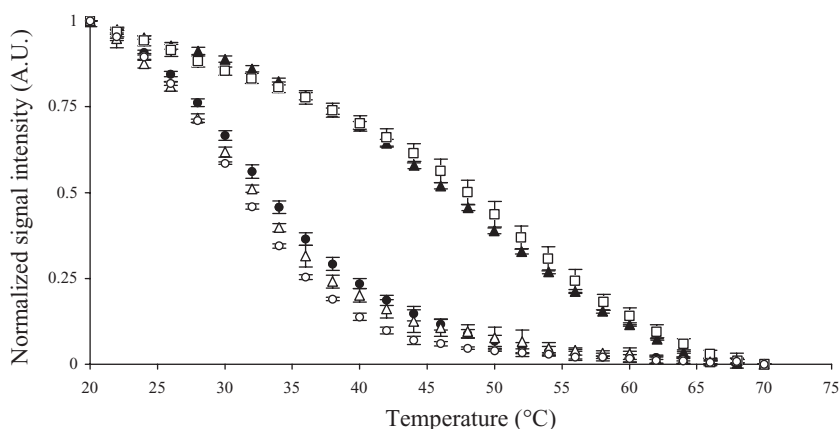


Fig. 4. Normalized melting profiles of probe Ntspa662, targeting *Nitrospira* group of NOB, to perfect-match *in vitro* transcribed RNA of *Nitrospira* spp. (□), and to environmental native RNA (●) and *in vitro* transcribed RNA of environmental *Nitrospira*-specific PCR products (▲). One-mismatch melting profiles were derived from the hybridization of the *in vitro* transcribed RNA of *Nitrospira* spp. to probes Ntspa662-c7g (△) and Ntspa662-g9c (○). Error bars indicate the standard deviations from the means of replicate probes ($n = 4$).

Nitrobacter-selective primer set (Fig. 3B). This indicated the absence of *Nitrobacter* strains containing the Nbac1000 target sequence, a result supported by our previous detection of *Nitrobacter* with probe NIT3, but not with Nbac1000, using a membrane hybridization format for analysis of WWTP samples from a different reactor system (Kelly *et al.*, 2005).

Hybridization of RNA directly recovered from the WWTP to probe NIT3 was near background (Fig. 1), but the signal intensity of NIT3 was significantly increased after pre-amplification (Fig. 3B). The specificity of NIT3 hybridization was confirmed by melting-profile analysis: the melting profile of the reference strain, *Nitrobacter agilis*, was distinct from the melting profiles of the corresponding single-mismatch probes (Nit3-c10g and Nit3-g13c) at temperatures above the T_d of probe NIT3 ($47.95 \pm 0.14^\circ\text{C}$) (Fig. 5), and the melting profile (Fig. 4) and T_d of environmental pre-amplified RNA (47.53 ± 0.31 ; $n = 4$) were not significantly different from the melting profile and T_d of *N. agilis* (47.95 ± 0.14 ; $n = 4$). The correspondence between dissociation profiles for reference rRNA and RNA derived from the pre-amplified product confirms the utility of dissociation analysis to distinguish between matched and mismatched hybrids. However,

selective amplification of *Nitrobacter* could not be confirmed as hybridization to NIT3 confirms only the incorporation of this primer sequence in the amplification product, which may or may not have been derived from fully complementary target sequences.

Probes Nso190 and Nso1225 target most β -Proteobacterial AOB. Hybridization of Nso190 with a perfect-match reference organism, *Nitrosospira briensis*, showed good discrimination in melting profiles between the perfect-match probe and two single-mismatch probes (Nso190-c11g and Nso190-g10c) (Fig. 6), and the melting profiles of these single-mismatch probes generated T_d s [40.73 ± 0.36 ($n = 4$) for Nso190-c11g and 39.21 ± 1.10 ($n = 4$) for Nso190-g10c] that were significantly lower than the T_d for the perfect-match probe (43.49 ± 0.66 ; $n = 4$). These results demonstrated that single-base mismatch discrimination was achieved for probe Nso190 using melting-profile analysis. The melting profile (Fig. 6) and T_d of environmental native RNA (34.30 ± 0.28 ; $n = 4$) were markedly lower than those of *N. briensis* (43.49 ± 0.66 ; $n = 4$), suggesting a low abundance, or absence, of the Nso190 target population in the reactor system. Pre-amplification with the 1225 primer increased the normalized signal intensity of probe Nso190 (Fig. 3C) and shifted

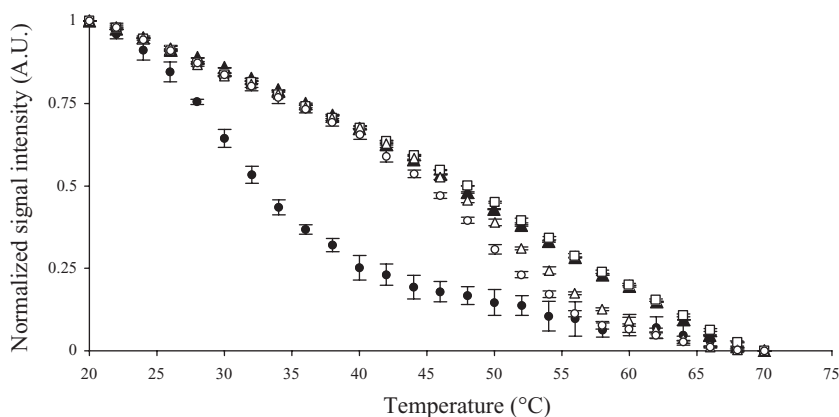


Fig. 5. Normalized melting profiles of probe NIT3, targeting *Nitrobacter* group of NOB, to perfect-match *in vitro* transcribed RNA of *Nitrobacter agilis* (□), and to environmental native RNA (●) and *in vitro* transcribed RNA of environmental *Nitrobacter*-specific PCR products (▲). One-mismatch melting profiles were derived from the hybridization of the *in vitro* transcribed RNA of *N. agilis* to probes Nit3-c10g (△) and Nit3-g13c (○). Error bars indicate the standard deviations from the means of replicate probes ($n = 4$).

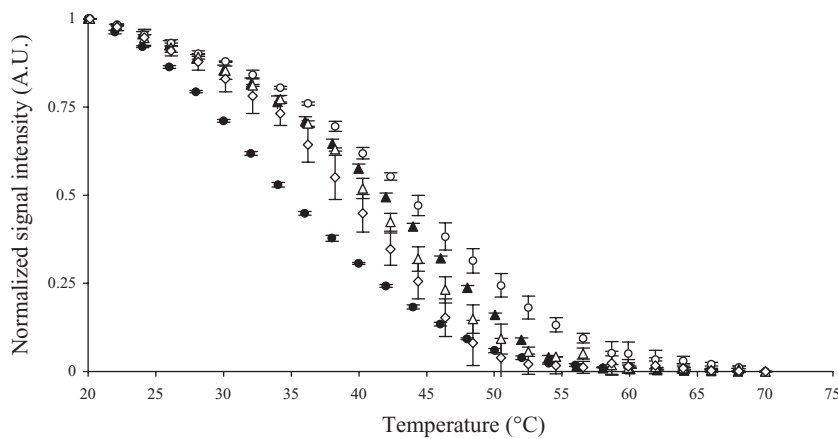


Fig. 6. Normalized melting profiles of probe Nso190, targeting β -Proteobacteria ammonia-oxidizing bacteria, to perfect-match *in vitro* transcribed RNA of *Nitrosospira briensis* (\circ), and to environmental native RNA (\bullet) and *in vitro* transcribed RNA of environmental AOB-specific PCR products (\blacktriangle). One-mismatch melting profiles were derived from the hybridization of the *in vitro* transcribed RNA of *N. briensis* to probes Nso190-c11g (\triangle) and Nso190-g10c (\diamond). Error bars indicate the standard deviations from the means of replicate probes ($n = 4$).

the melting profile of environmental RNA closer to the reference strain (Fig. 6). However, the T_d of the pre-amplified rRNA (41.79 ± 0.29 ; $n = 4$) was still significantly lower than the T_d of *N. briensis* (43.49 ± 0.66 ; $n = 4$), suggesting that the amplification products contained some sequences that varied by a single nucleotide difference in the probe target region. As the Nso1225 primer has only one mismatch to *Desulfovibrio* and other unidentified soil bacteria in the β -Proteobacteria, these or other closely related populations may have contributed to hybridization.

Melting profiles for Nso1225 demonstrated that single-base mismatch discrimination was also achieved for probe Nso1225: melting profiles for *N. eutropha* and *N. briensis* (both perfect matches for Nso1225) were identical (Fig. 7) and the T_d s for these two reference organisms were not significantly different from each other [41.35 ± 0.08 ($n = 4$) for *N. eutropha* and 41.11 ± 0.60 ($n = 4$) for *N. briensis*], whereas the T_d s of the two single-mismatch probes were significantly lower [39.14 ± 0.16 ($n = 4$) for Nso1225-c14g and 37.79 ± 0.06 ($n = 4$) for Nso1225-g8c]. Hybridization of Nso1225 with native and pre-amplified RNA could not confirm specific detection of this target: the melting profiles and T_d s of native RNA (34.30 ± 0.28 ; $n = 4$) and pre-amplified RNA (38.92 ± 0.07 ; $n = 4$) were significantly lower than those of *in vitro* transcribed rRNA of the refer-

ence strains (Fig. 7). In addition, as the Nso1225 probe sequence was used for amplification (as with the NIT3 probe above), the presence of the Nso1225 target could not be confirmed by hybridization to this probe.

Conclusions

Non-target organisms or contaminants, such as remnant RNA or DNA, are expected to be part of a positive signal from DNA microarray hybridization at 20°C. Therefore, we performed melting-profile experiments to evaluate our ability to distinguish perfect-match probe-target hybridizations from single-base mismatch hybridizations, and to evaluate the specificity of microarray detection of nitrite oxidizing bacteria (NOB) and AOB in WWTP samples. We compared the melting profiles of probe-target duplexes using *in vitro* transcribed rRNA of pure cultures with those obtained from environmental samples with and without PCR amplification.

In the current study using municipal activated sludge, we detected native RNA of moderately low-abundance nitrifiers (less than 7.7% of total population) with a polyacrylamide-gel-based DNA microarray, but could not resolve the presence of less abundant microorganisms. In order to enhance the sensitivity of microarray detection,

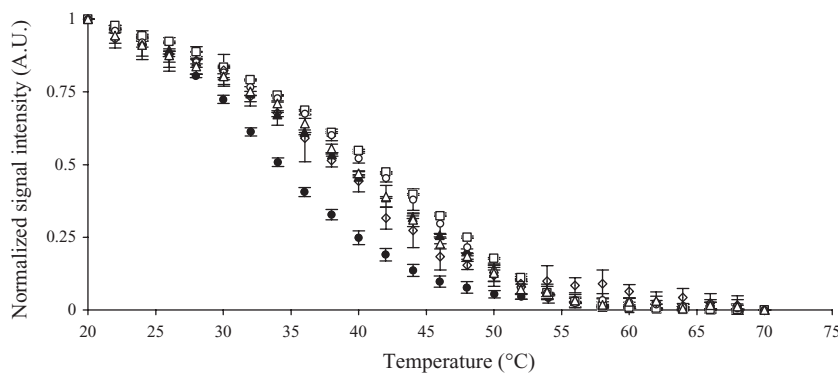


Fig. 7. Normalized melting profiles of probe Nso1225, targeting β -Proteobacteria ammonia-oxidizing bacteria, to perfect-match *in vitro* transcribed RNA of *N. eutropha* (\square) and *N. briensis* (\circ) and to environmental native RNA (\bullet) and *in vitro* transcribed RNA of environmental AOB-specific PCR products (\blacktriangle). One-mismatch melting profiles were derived from the hybridization of the *in vitro* transcribed RNA of *N. briensis* to probes Nso1225-c14g (\triangle) and Nso1225-g8c (\diamond). Error bars indicate the standard deviations from the means of replicate probes ($n = 4$).

we hybridized the microarrays with rRNA transcribed from PCR products that were produced using primers specific to certain groups of nitrifiers. This pre-amplification strategy helped increase the concentration of targeted sequences and allowed for preliminary detection of *Nitrospira* NOB and β -Proteobacteria AOB in WWTP activated sludge.

Hybridization at a single stringency (20°C) suggested the presence of NOB and AOB, but did not discriminate between targeted and mismatched sequences. Therefore, we used melting profiles to discriminate perfectly matched organisms from non-targeted ones, and we demonstrated that single-base mismatch discrimination was achieved on the microarrays for probes Ntspa662, NIT3, Nso190 and Nso1225. Combined amplification and melting-profile analysis confirmed the presence of bacteria containing the *Nitrospira* target sequence in WWTP and a low abundance or absence of *Nitrobacter* species containing a sequence complementary to probe Nbac1000. The presence of AOB containing a sequence complementary to probe Nso190 could not be confirmed, although related population(s) differing by a single nucleotide polymorphism were suggested to be present.

These results indicate that a microarray protocol including pre-amplification with specific primers, *in vitro* transcription and melting-profile analysis can be a useful tool for highly sensitive, as well as highly specific, detection of microbial nucleic acids in environmental samples. Additional studies will be needed to determine if detection of AOB and *Nitrobacter* could be improved by modifications of PCR conditions or by selection of alternative probes and/or primers.

Experimental procedures

Microarray design and fabrication

We designed and built a microarray that included a set of 26 oligonucleotide probes targeting all nitrifying bacteria at varying levels of specificity, along with general probes for all life, the *Bacteria* domain and some subclasses (α , β , γ) of the Proteobacteria. We used the Probe Match program provided by the Ribosomal Database Project II (<http://rdp.cme.msu.edu>) and BLAST (<http://www.ncbi.nih.gov/BLAST>) searches to check the probes for internal complementarities and to evaluate probe specificity. We also designed two kinds of single-mismatch (MM) variants of each probe by substituting G for C (c-MM probes) or C for G (g-MM probes). In order to avoid selecting MM probes that were matches for other organisms, we checked the complementarity of MM probes against the ARB (<http://www.arb-home.de>) and GenBank (<http://www.ncbi.nih.gov/Genbank>) databases. Table 1 lists the probe names, oligonucleotide sequences, target organisms and the names of single-mismatch probes. Probes were synthesized with an amino-linker at the 3' end of each probe, as described previously

(Vasilikov *et al.*, 1999). Gel-pad microarrays were fabricated as described previously (Urakawa *et al.*, 2002).

Reference RNAs

We used *N. eutropha* strain C91, *N. briensis* strain C128, *N. agilis* (ATCC 14123) and *Nitrospira* spp. as reference organisms. For each reference organism, we extracted DNA by bead-beating (Kuske *et al.*, 1998) using the FP120 Cell Disrupter (Qbiogene, Carlsbad, CA). We amplified 16S rRNA genes of reference nitrifiers by PCR using primers 11F (5'-GTTTGATCCTGGCTCAG-3') and 1512AR (5'-ACGGYTACCTTGTTACGACTT-3') and cloned the amplified DNA using TOPO Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). We then isolated plasmids containing the 16S rRNA genes using Ultra Clean Mini Plasmid Prep Kit (MoBio Laboratories, Carlsbad, CA), linearized them by restriction digestion, and used them as templates for *in vitro* transcription using a commercial RNA transcription kit (New England Bio Laboratories, Beverly, MA). We determined the number of mismatches between the probes and the reference RNA sequences, using the *in silico* Prediction Calculator software package (available at <http://stahl.ce.washington.edu>).

Mixed-liquor samples

On 25 February 2003, we collected activated sludge grab samples from the EWRP (Chicago, Illinois) from the end of the four aeration basins, where the mixed liquor combines before going to the settling tanks. The EWRP mainly receives residential wastewater. The average total flow was 19 MGD (71 900 m³ per day) during the sampling period, and the maximum design capacity of this plant is 30 MGD (114 000 m³ per day). The EWRP achieved stable nitrification of the ammonium- and organic-N (collectively measured as Total Kjeldahl Nitrogen, or TKN) to nitrate, as shown by operating data in Table 2. Clear evidence of nitrification is the very low effluent NH₄⁺ - N (0.13 mgN l⁻¹). The effluent NO₂⁻ + NO₃⁻, 13.5 mgN l⁻¹, is further good evidence of good nitrification. Also, the solids retention times (SRT) was sufficient for stable nitrification. For 14°C, a safety factor of 5 requires that the SRT for ammonia-oxidizing bacteria be 10.5 days (Rittmann and McCarty, 2001), and the SRT of EWRP was 13.9 days.

For RNA extraction, we dispensed duplicate 1 ml samples of MLSS samples into 2 ml cryovials containing 0.5 g of baked zirconium beads (0.1 mm diameter; BioSpec Products, Bartlesville, OK). We also collected 500 μ l of MLSS for DNA extraction. We stored sample tubes on dry ice until transferred to a -80°C freezer in the laboratory.

RNA extraction

We extracted RNA from duplicate activated sludge samples as previously described in the study by Stahl and colleagues (1988) with modification for RNA purification. After we extracted RNA from the cells by the bead-beating method, we isolated and purified RNA in the supernatant using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's manual. Finally, we eluted the RNA from the kit

Table 1. Oligonucleotide probes included on the DNA microarray.

No.	Probe name	Probe short name	Sequence (5'→3')	Target	g-Mismatch probe ^a	c-Mismatch probe ^a	Source
1A	S*-Univ-1390-a-A-18	Univ1390	GACGGGGGTGTGTACAA	All life	Univ1390-t10g	Univ1390-g13c	Amann <i>et al.</i> (1990)
1B	S*-Univ-0907-a-A-18	Univ907	CCCCGTCAATTCCTTTGAGTTT	All life	Univ907-a 18g	Univ907-a9c	Amann <i>et al.</i> (1992)
2A	S-D-Eub-0927-a-A-17	Eub927	ACCCGTTGTGGCGGCCOC	Most <i>Bacteria</i>	Eub927-19g	Eub927-g8c	Giovannoni <i>et al.</i> (1988)
2B	S-D-Eub-0338-a-A-18	Eub338	GCTGCCTCCCGTAGGAGT	Most <i>Bacteria</i>	Eub338-17g	Eub338-g11c	Amann <i>et al.</i> (1990)
3	S*-Nso-0190-a-A-19	Nso190	CGATCCCTCGCTTTCTCC	Most β-Proteobacterial AOB	Nso190-c11g	Nso190-g10c	Mobarry <i>et al.</i> (1996)
4	S*-Nso-1225-a-A-20	Nso1225	CGCCATTGTATTACGTGTGA	Most β-Proteobacterial AOB	Nso1225-c14g	Nso1225-g8c	Mobarry <i>et al.</i> (1996)
5	S-G-Nsv-0443-a-A-19	Nsv443	CCGTGACCGTTTCGTTCCG	<i>Nitrosospira</i> lineage	Nsv443-c7g	Nsv443-g9c	Mobarry <i>et al.</i> (1996)
6	S-G-Nsom-0156-a-A-19	Nsom156	TATTAGACATCTTTTCGAT	<i>Nitrosomonas</i> spp.	Nsom156-c12g	Nsom156-g17c	Mobarry <i>et al.</i> (1996)
7	S-S-Nsom-0120-a-A-20	Nmill	TTAAGACACGTTCCGATGTA	<i>Nitrosomonas communis</i> lineage	Nmill-c9g	Nmill-g10c	Pommerening-Röser <i>et al.</i> (1996)
8	S-S-Nsom-0998-a-A-21	Nmilli	TAGCGAATTTCTAGAGATAG	<i>Nitrosomonas marina</i> lineage	Nmill-c12g	Nmill-g15c	Pommerening-Röser <i>et al.</i> (1996)
9	S-S-Nsom-1004-a-A-19	Nmiv	ACTCACCTCTCAGCGAGCT	<i>Nitrosomonas cryotolerans</i> lineage	Nmiv-c9g	Nmiv-g13c	Pommerening-Röser <i>et al.</i> (1996)
10	S-G-Nsom-0174-a-A-18	Nmv	TCTCAGAGACTACGCGG	<i>Nitrosomonas mobilis</i> lineage	Nmv-c11g	Nmv-g9c	Pommerening-Röser <i>et al.</i> (1996)
11	S-G-Nsom-0653-a-A-18	NEU	CCCCTCTGCTGCACCTA	<i>Nitrosomonas</i> spp.	NEU-c12g	NEU-g11c	Wagner <i>et al.</i> (1995)
12	S-S-Nse-1472-a-A-18	Nse1472	ACCCAGTCTACGCCCC	<i>Nitrosomonas europaea</i>	Nse1472-c15g	Nse1472-g7c	Juretschko <i>et al.</i> (1998)
13	S-S-Nmo-0218-a-A-18	Nmo218	CGGCCGCTCCAAAAGCAT	<i>Nitrosomonas oligotropha</i> lineage	Nmo218-c10g	Nmo218-g6c	Gieseke <i>et al.</i> (2001)
14	S*-Nsc-0825-a-A-18	Nsc825	CCCTCCCAACGCTAGTT	γ-Proteobacterial AOB	Nsc825-c10g	Nsc825-g11c	This study
15	S-G-Nsr-0826-a-A-18	Nsr826	GTAACCCGCGACACTTA	<i>Nitrospira</i> spp.	Nsr826-c9g	Nsr826-g11c	Schramm <i>et al.</i> (1998)
16	S-G-Nsr-1156-a-A-18	Nsr1156	CCCGTTCTCCTGGGCGAGT	<i>Nitrospira</i> spp.	Nsr1156-c9g	Nsr1156-g12c	Schramm <i>et al.</i> (1998)
17	S-P-Nispa-0712-a-A-21	Nispa712	CGCCTTCGCCACCGGCTTCC	<i>Nitrospira</i> spp.	Nispa712-c10g	Nispa712-g8c	Daims <i>et al.</i> (2000)
18	S-G-Nispa-0662-a-A-18	Nispa662	GGAATTCGCGCTCCTCT	<i>Nitrospira</i> spp.	Nispa662-c7g	Nispa662-g9c	Daims <i>et al.</i> (2000)
19	S*-Nsr-1145-a-A-18	Nsr1145	GGCAGTCTTTTCAGAGTG	<i>Nitrospira</i> spp.	Nsr1145-c8g	Nsr1145-g5c	Harms <i>et al.</i> (2003)
20	S-S-Nsr-0989-a-A-18	Nsr989	ACACGAGCATGTCCAAC	<i>Nitrospira marina</i>	Nsr989-c9g	Nsr989-g8c	This study
21	S*-Nsn-0227-a-A-18	Nsn227	ATGGTCCGCGAACTCATC	<i>Nitrospina gracilis</i>	Nsn227-c9g	Nsn227-g8c	This study
22	S-S-Ncc-0214-a-A-18	Ncc214	CATCCATCGGTGCGAGCT	<i>Nitrococcus mobilis</i>	Ncc214-c8g	Ncc214-g10c	This study
23	S-G-Nbac-1000-a-A-15	Nbac1000	TGCGACCGGTCATGG	<i>Nitrobacter</i> spp.	Nbac1000-c7g	Nbac1000-g8c	Mobarry <i>et al.</i> (1996)
24	S-G-Nbac-1035-a-A-18	NIT3	CCTGTGCTCCATGCTCCG	<i>Nitrobacter</i> spp.	Nit3-c10g	Nit3-g13c	Wagner <i>et al.</i> (1996)

a. Descriptive names of MM probes are given. Nomenclature following the hyphen indicates the nucleotide change and the position of nucleotide change.

Table 2. Operating data for the activated-sludge stages of the Egan Water Reclamation Plant.

Parameter (units)	Value
Total flow (m ³ /day)	71 900
Solids retention time (days)	13.9
Mixed-liquor suspended solids (mg l ⁻¹)	1 720
Mixed-liquor pH	7.3
Mixed-liquor temperature (°C)	14
Sludge recycle ratio (%)	76
Influent BOD ₅ (mg l ⁻¹)	145
Effluent BOD ₅ (mg l ⁻¹)	2.0
Influent TKN (mgN l ⁻¹)	32.0
Effluent NH ₄ ⁺ - N (mgN l ⁻¹)	0.13
Effluent NO ₂ ⁻ + NO ₃ ⁻ (mgN l ⁻¹)	13.5

Each datum represents the average of daily measurements taken over the 25 days prior to sample collection. BOD, biochemical oxygen demand.

membrane in 30 µl of DEPC-treated water and treated RNA samples with RNase-free DNase (Amersham Pharmacia Biotech, Piscataway, NJ) to degrade unwanted DNA. We confirmed the presence of extracted RNA and the absence of DNA by electrophoresis on 1% agarose gels, and we determined the concentration of nucleic acids by spectrophotometric analysis at 260 nm.

DNA extraction and in vitro transcription of nitrifier-specific PCR products

We used 500 µl of the activated sludge sample to isolate DNA directly using UltraClean Soil DNA kits (MoBio Laboratories) following the manufacturer's protocol. We confirmed the presence of DNA by electrophoresis on 1% agarose gels.

To increase the amount of nitrifier-specific nucleic acids, we amplified the DNA using T7 promoter-conjugated 11f (5'-AATACGACTCACTATAGGTTTGATCCTGGCTCAT) (Kane *et al.*, 1993; Koizumi *et al.*, 2002) and nitrifier-specific (Nso1225r, NIT3r, Ntspa685r) primers on a DNA thermocycler (MJ Research, Watertown, MA). Nso1225r (CGCCATTG TATTACGTGTGA) targets 16S rRNA gene of AOB in the β-Proteobacteria, NIT3r (CCTGTGCTCCATGCTCCG) targets *Nitroacter* spp., and Ntspa685r (CGGGAATTCCGCGCTC) targets *Nitrospira* spp. The reaction mixture (50 µl) contained 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.5% Tween 20, 0.1% Triton X-100, 5.0 µg of bovine serum albumin, 0.02 mM of each deoxynucleoside triphosphate, 0.4 µM of each primer and 1 µl of DNA template. After a 10 min hot start at 80°C, we added 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) to each reaction tube. The thermal profile used for the nitrifier-specific amplification was: initial denaturation for 5 min at 95°C; 35 cycles of 90 s at 95°C, 30 s at 60°C and 90 s at 72°C; and a final extension at 72°C for 10 min. We purified PCR products using the QIAquick® PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

We *in vitro* transcribed RNA from PCR-generated templates in a reaction mixture containing 20 µl of 5× T3/T7 RNA buffer, 20 µl of 10 mM NTP, 20 U Ribonuclease Inhibitor (Invitrogen), 5 µl of 0.1 M DTT, 31.5 µl of DEPC-treated water and 20 µl of PCR product. We equilibrated the mixture at

37°C in the DNA thermal cycler for 15 min and then added approximately 120 U of T7 RNA polymerase enzyme. We incubated the reaction at this temperature for 2 h, added another 60 U of T7 RNA polymerase, and let it incubate for another hour. To dissolve any precipitate after transcription was completed, we added 100 µl of 0.1 M Na₂EDTA. We purified RNA using phenol/chloroform followed by chloroform extraction. After RNA was precipitated out by ethanol, we re-suspended the pellet in DEPC-treated water.

RNA fragmentation, labelling and hybridization

The fragmentation and labelling step was performed as previously described (Kelly *et al.*, 2002). Hybridizations were conducted as described previously (Urakawa *et al.*, 2003) with slight modifications. We hybridized the microarray in the dark at 20°C for 22–26 h in a hybridization chamber CoverWell (Grace Bio-Laboratories, Bend, OR). Following hybridization, we washed the microarray five times at room temperature with 100 µl of washing buffer. After the final wash, we added 100 µl of washing buffer to the viewing chamber (Grace BioLabs), which was affixed to the microarray for fluorescence monitoring.

Microarray imaging and melting-profile experiment, and melting-profile analysis

The melting-profile system was described previously (Urakawa *et al.*, 2003). We used special software called Microchip Imager (Argonne National Laboratory, Argonne, IL) to capture the image and analyse the signal intensity for each gel element. We ran and recorded melting profiles for all gel elements (27 × 27 matrix) simultaneously. We gradually increased the temperature of the thermal table from 20°C to 70°C at a rate of 1°C per minute with image acquisition every 2 min, or at 2°C intervals. We converted signal intensity data to a text format by special software called Melting Experiment Converter (Argonne National Laboratory).

We analysed melting profiles using the *T_d*, Melt Quality and Shape Calculator software package (available at <http://stahl.ce.washington.edu>) that was previously described (Urakawa *et al.*, 2002). We used this software to calculate normalized signal intensities and dissociation temperatures (*T_d*s) for the melting profiles. *T_d*s are reported in the text as mean values ± standard error. Differences in *T_d*s were analysed for statistical significance by Student's *t*-test (Larson, 1975).

Signal intensity analysis

Hybridization signals were analysed using Student's *t*-test (Larson, 1975). Hybridization signals were considered positive if the fluorescent signal intensity was significantly higher than background signal intensity, 6.62 ± 0.51 A.U. (*n* = 30) for gel pads that did not contain any probe. For each probe, we calculated the *t*-value ($t = \frac{\bar{x} - 6.62}{\sigma}$), where \bar{x} is an average signal from that probe, and σ is the square root of the pooled variance or $\sqrt{\frac{\sigma_p^2}{n} + \frac{0.51^2}{30}}$, where σ_p^2 is variance of the

particular probe and n is the number of replicate probes. A t -value higher than 2.04 indicated positive detection with 95% confidence for degrees of freedom between 30 and 34, where the degree of freedom for any probe is $n + 28$, and n ranges from 2 to 6 for probes on DNA microarray used here.

Acknowledgements

The authors wish to thank the following: G. Yershov, A. Kukhtin, A. Gemmell and D. Chandler from Argonne National Laboratory for their efforts in manufacturing the oligonucleotide microarrays, and S. Surzhikov from Argonne National Laboratory for synthesis of the oligonucleotide probes. Support from DARPA and NASA is acknowledged.

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