Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology

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ABSTRACT

The use of DNA-based molecular detection tools for bacterial diagnostics is hampered by the inability to distinguish signals originating from live and dead cells. The detection of live cells is typically most relevant in molecular diagnostics. DNA-intercalating dyes like ethidium monoazide and propidium monoazide (PMA) offer a possibility to selectively remove cells with compromised cell membranes from the analysis. Once these dyes enter a cell, they bind to DNA and can be covalently crosslinked to it by light exposure. PCR amplification of such modified DNA is strongly inhibited. In this study we evaluated the suitability of propidium monoazide treatment to exclude isopropanol-killed cells from detection in defined mixtures using diagnostic microarray technology. The organisms comprised *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Serratia marcescens*, and *Escherichia coli* O157:H7. PCR products obtained from amplification of chaperonin 60 genes (*cpn60*; coding for *GroEL*) were hybridized to a custom-designed microarray containing strain-specific *cpn60*-based 35-mer oligonucleotide probes. Results were compared with data from quantitative PCR, which confirmed that PMA could successfully inhibit amplification of DNA from killed cells in the mixtures. Although microarray data based on analysis of end-point PCR amplicons is not quantitative, results showed a significant signal reduction when targeting killed cells and consistently agreed with qPCR results. Treatment of samples with PMA in combination with diagnostic microarray detection can therefore be considered beneficial when analyzing mixtures of intact and membrane-compromised cells. Minimization of detection signals deriving from dead cells will render data more relevant in studies including pathogen risk assessment.

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1. Introduction

The selective detection of live microorganisms poses a great challenge for molecular DNA-based diagnostic technology due to the persistence of DNA in the environment after cell death. Often, meaningful data can only be obtained if dead cells are excluded from the analysis. For bacteria, a relatively recent approach is the treatment of samples with the DNA-intercalating dyes ethidium monoazide (EMA) or propidium monoazide (PMA) (Nogva et al., 2003; Rudi et al., 2005a,b; Nocker et al., 2006). This fast and simple treatment was proposed to lead to the exclusion of membrane-compromised bacterial cells from the analysis. Live cells are traditionally considered to have the potential for an active metabolism and to be intact, whereas lack of membrane-integrity has been accepted as an indication of cell death. Although loss of membrane integrity is a very conservative definition of death and dead cells, after

having lost viability, can still have intact membranes for some time, the exclusion of damaged cells would pose an important step in limiting DNA-based detection to a more relevant faction of a bacterial population. In addition to bacterial diagnostics, the PMA method has also been successfully employed for assessing fungal viability by discriminating between live and heat-inactivated cells of different infectious fungi (Vesper et al., 2008).

The principle is based on the assumption that PMA (or EMA), when added to a mixture of intact and membrane-compromised cells, selectively enters only the compromised cells. Once inside the cell, it intercalates into nucleic acids. The presence of an azide group allows crosslinking the dye to the DNA by exposure to strong visible light. The light leads to the formation of a highly reactive nitrene radical, which can react with any organic molecule in its proximity including the bound DNA. This modification strongly inhibits the PCR amplification of the extracted DNA. At the same time when the crosslinking occurs, the light reacts unbound excess dye with water molecules. The resulting hydroxylamine is no longer reactive, so the DNA from cells with intact membranes is not modified in the DNA extraction

procedure. Although the mechanism of action of these chemicals has not been elucidated yet and could be result of a combination of factors, the overall result of treatment of membrane-compromised cells is a reduction in PCR amplification. Comparing the two dyes, PMA has the advantage over EMA in being more selective for dead cells as it is more membrane-impermeant (Nocker et al., 2006; Flekna et al., 2007; Cawthorn and Witthuhn, 2008) and it has not been observed to enter intact cells of many bacterial species after 5 min of exposure. The use of PMA for live-dead distinction has so far been validated in combination with quantitative PCR (qPCR) and end-point PCR-based denaturing gradient gel electrophoresis (DGGE) (Nocker et al., 2007a, b). The purpose of this study was to evaluate whether treatment of samples with PMA is also beneficial in combination with microarray analysis of end-point PCR products. Diagnostic microarrays are one of the major downstream analysis tools of PCR amplicons.

In this investigation we chose defined mixtures of different live and isopropanol-killed bacterial species. In a first experiment, PMA-treated and untreated samples were analyzed by qPCR and by microarray hybridization of end-point PCR products. Results allowed for comparing the effect of PMA treatment on detection signals from the two detection methods and for validating of microarray probes. In a second experiment, the effect of PMA-treatment on signal intensities was studied using different live-dead ratios of one selected organism (*Salmonella typhimurium*) while the total cell number was held constant. Finally, an environmental sample was seeded with different amounts of killed *Salmonella* cells to evaluate the efficiency of PMA treatment.

2. Materials and methods

2.1. Cultivation

The strains used in this study were: *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella enterica* serovar *Typhimurium* (environmental isolate), *Serratia marcescens*, and *Escherichia coli* O157:H7 (strain 932); all strains were derived from the Department of Microbiology, Montana State University. Cultures were grown in Brain Heart Infusion (BHI) medium for 10 h (30 °C, 180 rpm) before adjusting the OD_{600} to 1.0 by dilution with BHI broth. Mixtures of culture aliquots were made in 1.5 ml microcentrifuge tubes.

2.2. Isopropanol killing

Cells were killed by adding 1 ml isopropanol to 500 µl of cell suspension (OD_{600}=1.0) and exposing cells for 10 min. Isopropanol was removed by harvesting cells using centrifugation at 5000 ×*g* for 5 min and removing the supernatant. Pellets of killed cells were resuspended in 500 µl of BHI broth.

2.3. Environmental sample

Two liters of turbid water (43 NTU) from a spring-fed creek were centrifuged in aliquots for 15 min at 7000 rpm to harvest biosolids and biomass. All supernatant was discarded except 8 ml. Pelleted material was resuspended in this remaining volume. Aliquots of 400 µl were distributed in 1.5 ml microcentrifuge tubes and spiked with 100 µl of isopropanol-killed *Salmonella* suspensions (in BHI, different dilutions) or BHI broth (control). The dilutions of the *Salmonella* suspensions were calculated to obtain cell numbers ranging from 10^1 to 10^8 cells per sample.

2.4. Direct cell counts

One milliliter of the 25-fold diluted creek water sample was filtered through 0.22 µm polycarbonate filters (25 mm, Millipore) and stained with 100 µg ml^{-1} DAPI (4',6-diamidino-2-phenylindole;

Sigma, St. Louis, MO) for 15 min. Enumeration of cell counts was performed with a Nikon Eclipse E 800 epifluorescence microscope using a 1.4-numerical-aperture 100× oil objective. Twenty randomly chosen microscopic fields (93×69 µm) were enumerated.

2.5. PMA crosslinking

PMA (Biotium, Hayward, CA) was dissolved in 20% dimethyl sulfoxide to create a stock solution of 20 mM and stored at −20 °C in the dark. Typically, 2.5 µl of the PMA stock solution was added to 500-µl aliquots of mixed species to a final concentrations of 100 µM. Light-transparent 1.5-ml microcentrifuge tubes (T5015G; Marsh, Rochester, NY; available through: www.abgene.com) were used. Following an incubation period of 5 min in the dark with occasional thorough mixing (by inverting tubes multiple times in different directions), samples were laid horizontally on ice with the more transparent side facing upwards and were light exposed using a 650-W halogen light source (sealed beam lamp, FCW 120 V, 3,200 K; GE Lighting, General Electric Co., Cleveland, OH; available from: www.replacementlight-bulbs.com). Pure culture samples were light-exposed for 2 min and spiked environmental samples for 4 min. The sample tubes were placed about 20 cm from the light source. Placing the samples horizontally on ice should avoid excessive heating and might optimize light exposure by reflection. Shaking (by tilting the ice box) was performed to make sure that every single droplet received good light exposure. After photo-induced crosslinking, cells were pelleted at 5000 ×*g* for 5 min prior to DNA isolation.

2.6. DNA isolation

Genomic DNA from pure culture experiments was extracted using the QIAGEN DNeasy® blood and tissue kit. Cell pellets were resuspended in 180 µl of enzymatic lysis buffer (20 mM TRIS pH 8.0, 2 mM EDTA, 1.2% Triton® X-100, 20 mg/ml of freshly added lysozyme), incubated at 37 °C for 30 min with occasional mixing, before adding 25 µl of proteinase K and 200 µl of buffer AL (included in the kit). Samples were incubated for 10 min at 70 °C and cooled down to room temperature before addition of 200 µl of 98% ethanol. Longer incubation with proteinase K was observed to result in DNA fragmentation and an increase in C_T values in qPCR independent of PMA treatment. DNA was purified over spin columns following the kit's instructions. Elution was achieved by applying 200 µl of buffer AE (supplied by the kit), incubating samples for at least 1 min and centrifugation at 6000 ×*g* for 1 min. As the QIAGEN kit resulted in complete inhibition of amplification of DNA from the creek water sample, the genomic DNA from the environmental sample was extracted using the Qbiogene soil kit (Qbiogene, Carlsbad, CA). Cell lysis was achieved by bead beating using a FastPrep machine (Qbiogene) for 30 s at a speed setting of 4.5 m/s. Cell debris was removed by centrifugation at 18,000 ×*g* for 5 min, both directly after bead beating and after the addition of 250 µl of protein precipitation solution (included in the kit) to the supernatant after bead beating. DNA was eluted with a volume of 120 µl DNA elution solution buffer (included in the kit).

2.7. Quantitative PCR

One microliter aliquots of extracted DNA were used as templates for relative quantitative PCR. Reactions and data analysis were performed using a SmartCycler II (Cepheid, Sunnyvale, CA). Cycle threshold (C_t) values were automatically calculated by the SmartCycler software using a threshold of 30 fluorescence units. For all experiments, 1 µl of extracted genomic DNA was added to 24 µl of PCR mixture containing Power Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 10 pmol of each primer. Primers used for quantification are given in Table 1. The cycling parameters for

Table 1
Gene targets and qPCR primers used for relative quantification of genomic DNA from the bacterial strains

Strain	Gene target	Primer name and sequence (5'-3')	Product length	References
P. aeruginosa	*oprI*	OPR-1 GCTCTGGCTCTGGCTGCT OPR-2 AGGGCACGCTCGTTAGCC	197 bp	(Qin et al., 2003)
Listeria sp.	hly	hly-F GGGAAATCTGTCTCAGGTGATGT hly-R CGATGATTTGAACTTCATCTTTTGC	106 bp	(Guilbaud et al., 2005)
Salmonella sp.	*invA*	invA2-F ATTCTGGTACTAATGGTGATGATC invA2-R GCCAGGCTATCGCCAATAAC	288 bp	(Fey et al., 2004)
S. marcescens	16S rRNA gene	SMSF GGTGAGCTTAATACGTTCATCAATTG SMSR GCAGTTCCCAGGTTGAGCC	178 bp	(Iwaya et al., 2005)
E. coli 0157:H7	*stx1*	stx1-F GACTGCAAAGACGTATGTAGATTCG stx1-R ATCTATCCCTCTGACATCAACTGC	150 bp	(Sharma and Dean-Nystrom, 2003)

quantification of *S. typhimurium* were: 9 min at 95 °C (initial polymerase activation and denaturation) followed by 45 cycles of 20 s at 95 °C, 30 s at 60 °C and 25 s at 72 °C; for *L. monocytogenes*: 9 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 62 °C and 15 s at 72 °C; for *E. coli* 0157:H7: 9 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 25 s at 57 °C and 25 s at 72 °C; for *S. marcescens*: 9 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 30 s at 62 °C and 20 s at 72 °C; for *P. aeruginosa*: 9 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 30 s at 62 °C and 25 s at 72 °C. For melt curve analysis the temperature was increased in 0.2 °C increments from 60 to 94 °C.

2.8. Microarray design

The microarray used probes selected from the conserved regions of *cpn60* genes (Goh et al., 1998, 2000; Maynard et al., 2005). The corresponding sequences were retrieved from the sequenced genomes of the organisms of interest as follows: *S. enterica* serovar Typhimurium strain LT2, GenBank Accession Number NC_003197.1, *L. monocytogenes* strain EGD, Accession Number AL591824.1, *E. coli* O157:H7 strain EDL933 Accession Number NC_002655, *P. aeruginosa* PAO1, Accession Number NC_002516.2, and *S. marcescens* strain Db11, (Sanger Institute, http://www.sanger.ac.uk/Projects/S_marcescens/). Specific oligonucleotides of 35 bases long for each organism were selected from the *cpn60* conserved regions using the OligoPicker software (4). Negative controls consisted of buffer spots and of spots carrying a 70-mer DNA probe for *Arabidopsis thaliana* DNA. Specificity was tested by hybridization to DNA from each of the five species individually.

2.9. Construction of the microarray

The selected 35-base oligonucleotides were purchased from a commercial supplier (IDT, Coralville, IA, USA) and were printed onto Corning Ultra GAPS™ slides (Corning Canada, Whitby, Ontario) using a commercially available Bio-Rad Versarray Pro™ robotics array printer at the Microarray laboratory of the Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, Canada. The sequences of the universal primers used for amplification and of the probes for each species are shown in Table 2.

2.10. Amplification of cpn60 genes from genomic DNA mixtures and Cy5 labeling of PCR products

The universal primers wdF and wdR were used to amplify the cpn60 conserved regions using amplification and purification meth-

ods described by Maynard et al. (2005). PCR amplicons were labeled using a random primer linear amplification method (BioPrime DNA labeling system—Invitrogen) substituting dCTP-Biotin indigenous to the kit with Cy5 fluorophore-tagged dCTP (Perkin Elmer, Boston, MA). Observed FOI (frequency of incorporation) was in the range of 10–12 units/10^3 bases.

2.11. Hybridization of labeled cpn60 PCR products

Experiments were carried out under the following conditions: 25 ng of labeled *cpn60* amplicon were used per hybridization in DIG Easy Hyb™ buffer (Roche, Indianapolis, IN) supplemented with salmon sperm ssDNA (1.0 μg/μL final conc.). Hybridizations were performed at 50 °C using a Slidebooster™ hybridization station (Advalvtixs SB800, The GelCompany, San Fransisco, CA, USA). Microarray slide scans were generated using a Canberra-Packard ScanArray Lite™ fluorescence scanner. Laser power output and photomultiplier gain (PMT) of the microarray scanner were set at fixed levels for the entire dataset.

2.12. Analysis of digitized scan results

Hybridization data (scanned images) were numerically extracted using Imagene 8.0 (BioDiscovery). Data was normalized to control for hyb to hyb variation. Invariant positive spots were averaged over the dataset to generate normalization factors used to adjust spot intensities. All results used to generate linear graphs were based on average of three technical replicates for each of two biological replicates. Microarray probe validation results represent averages of three technical replicates from a single biological replicate.

3. Results

3.1. Validation of principle (experiment 1)

The first experiment studied the efficiency of PMA treatment to minimize detection signals from dead cells. Seven mixtures of five model species were analyzed comprising *P. aeruginosa*, *L. monocytogenes*, *S. typhimurium*, *S. marcescens*, and *E. coli* O157:H7. Mixture I contained only live cells, mixture VII only isopropanol-killed cells (isopropanol treatment induces membrane damage). All remaining

Table 2
Primers and probes used for microarray work

Universal *cpn60* primers (5′–3′) (Maynard et al., 2005)	
wdF	GAIIIIGCIGGIGAYGGNACNACNAC
wdR	KIYKITCICCRAANCCNGGNGCYTT
Microarray probes (5′–3′)	
Escherichia coli	
Ec35Cs1	CTGGCTCAGGCTATCATCACTGAAGGTCTGAAAGC
Ec35Ca1	GCTTTCAGACCTTCAGTGATGATAGCCTGAGCCAG
Listeria monocytogenes	
Lm35Cs1	AATGTAACAGCTGGAGCAAACCCAGTAGGCGTTCG
Lm35Ca1	CGAACGCCTACTGGGTTTGCTCCAGCTGTTACATT
Pseudomonas aeruginosa	
Pa35Cs1	GACGAGTCCATCGGCCAGATCATTGCCGAAGCCAT
Pa35Ca1	ATGGCTTCGGCAATGATCTGGCCGATGGACTCGTC
Serratia marcescens	
Sm35Cs1	TCCGTACCTTGCTCCGATTCCAAAGCTATCGCTCA
Sm35Ca1	TGAGCGATAGCTTTGGAATCGGAGCAAGGTACGGA
Salmonella typhimurium	
St35Cs1	GGCGCAGTCCATCATTACCGAAGGCTTGAAAGCCG
St35Ca1	CGGCTTTCAAGCCTTCGGTAATGATGGACTGCGCC
Negative control	
Arab72c[a]	TCTTGGTATTGCTGGAATATTAGATGTGTGGGCAGGG (contin.) CATACCACTCCCACTGTCTTCTATCTTGCTTTG

All microarray probes except the negative control are based on *cpn60* gene sequences.
[a] ATG4/CHLG/G4 (*Arabidopsis thaliana*): ATG4/CHLG/G4 (chlorophyll synthase); chlorophyll synthetase.

mixtures contained 4 live species and 1 killed species each; the pipetting scheme is shown in Fig. 1A. Extracted genomic DNA from non-PMA treated and PMA-treated mixtures was analyzed for the presence of amplifiable sequences using qPCR. Gene targets and primers for quantification of the individual species are given in Table 1. Mean threshold cycles (C_T) values and standard deviations were obtained from three independent experiments. Whereas the amplification of DNA without PMA treatment did not depend on the viability status of the cells, PMA treatment resulted in a substantial increase in C_T values when targeting killed cells (Fig. 1B). Mean signal reductions obtained from subtracting C_T values of non PMA-treated mixtures from C_T values of PMA-treated mixtures are shown in Fig. 1C and are in the range between 10.39 cycles (for *L. monocytogenes* in mixture III) and 15.86 cycles (for *S. typhimurium* in mixture VII). Signal reductions tended to be greater for mixture VII (only dead cells) compared to mixtures which only contained one dead species.

For the validation of microarray probes, the experiment was complemented by a set of mixtures where the killed species symbolized by empty circles in Fig. 1A were absent (instead of adding killed cells). PCR amplicons from *cpn60* genes were subjected to

microarray hybridization with mixture I (containing only live cells) as baseline. Relative microarray signal intensities are shown in Fig. 2. No signal was obtained when the corresponding species was absent in the mixture. Killed cells yielded signals in the absence of PMA treatment. These signals from killed cells were greatly reduced upon PMA treatment. This result correlated with the signal reduction seen in qPCR.

3.2. Determination of signal detection sensitivity (experiment 2)

As qPCR and microarray signals could be efficiently suppressed by PMA if mixtures contained exclusively dead cells of the detected species, a second experiment was designed to address the sensitivities of the detection strategies. The intent was to see how well different ratios of live and dead cells would be reflected by the signal intensities. All bacterial species with the exception of *Salmonella* were kept constant and represented 20% of the total volume in eight mixtures. The relative proportion (vol/vol) of live *Salmonella* cells was decreased in steps from 20% (mixture I) to 0% (mixture VIII), relative to mixture I (Fig. 3A). The difference in volume was either compensated

Fig. 1. Effect of PMA sample treatment on qPCR and microarray signals. (A) Pipetting scheme for seven mixtures of five bacterial species. Filled circles indicate live cells and empty circles indicate isopropanol-killed cells added to the mixture. (B) Average threshold cycle (C_T) values from qPCR without and with PMA treatment. Standard deviations from three independent experiments are given in brackets. Grey shading indicates presence of killed cells of the corresponding species in the mixture. (C) Average signal reductions obtained from subtracting C_T values of non PMA-treated mixtures from C_T values of PMA-treated mixtures. Standard deviations from three independent experiments are given in brackets. Grey shading indicates presence of killed cells of the corresponding species in the mixture.
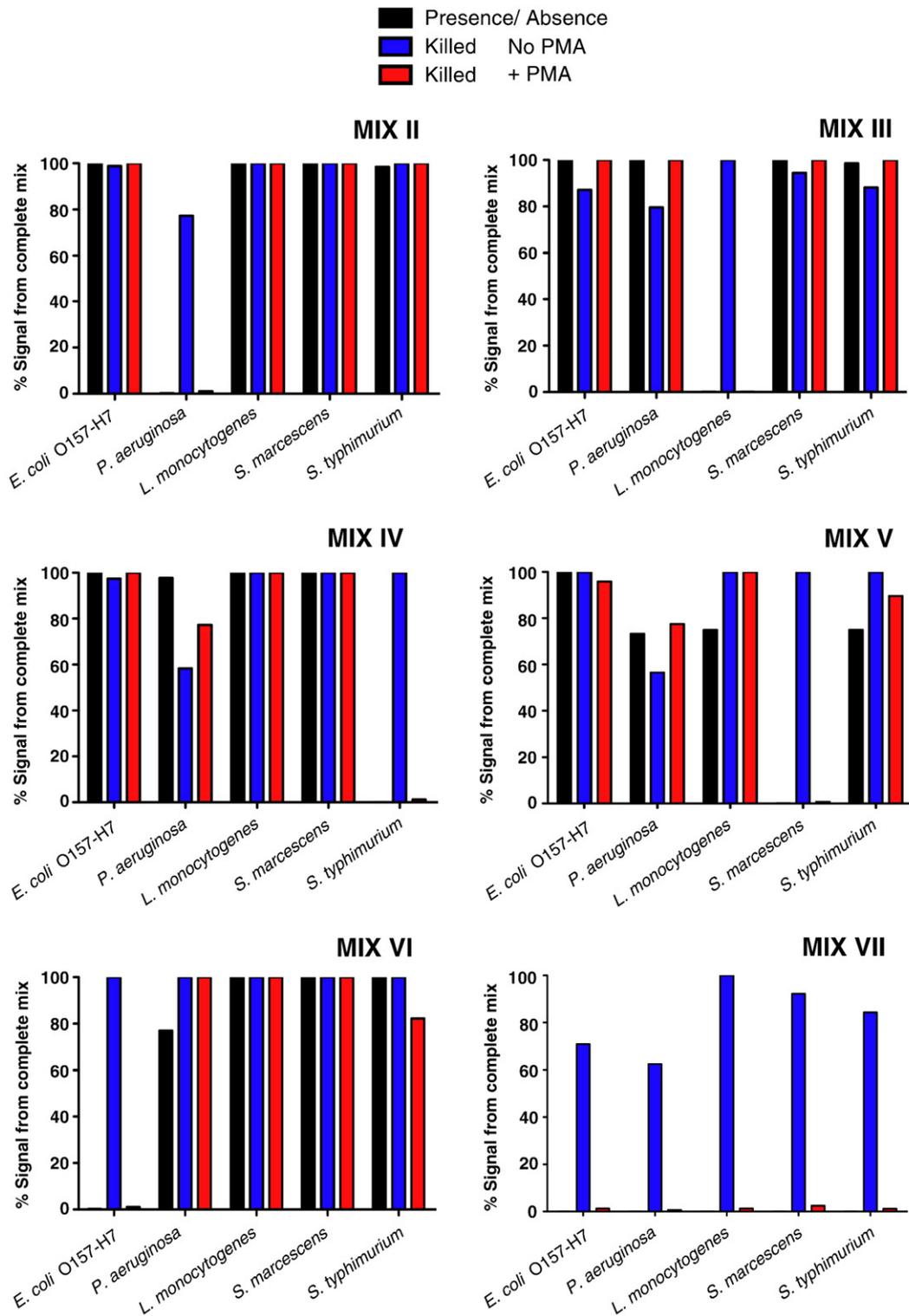
Fig. 2. Validation of microarray performance outlining the appropriate sensitivities and specificities of the selected probes. The same pipetting scheme for mixtures I to VII applies as represented in Fig. 1A. The mixtures were additionally complemented by a set where the killed species symbolized by empty circles in Fig. 1A were absent (instead of adding killed cells). PCR products obtained from all mixtures were subjected to microarray hybridization. Mixture I, *i.e.* the one in which all 5 species were alive, served as a reference. The histogram bar heights correspond to the intensities of hybridization signals in a particular mixture expressed as percentages of the signal obtained with mixture I, to a maximum of 100%.

by addition of culture broth or by addition of isopropanol-killed cells. *Salmonella* genomes in the extracted genomic DNA from these mixtures were quantified by qPCR (Fig. 3B). Without PMA treatment, C_T values increased when the proportion of live *Salmonella* dropped

(while the difference in volume was compensated by broth). This result was expected as the total number of *Salmonella* cells decreased. In the case where the difference in volume was compensated by the addition of dead cells, the C_T values remained constant without PMA
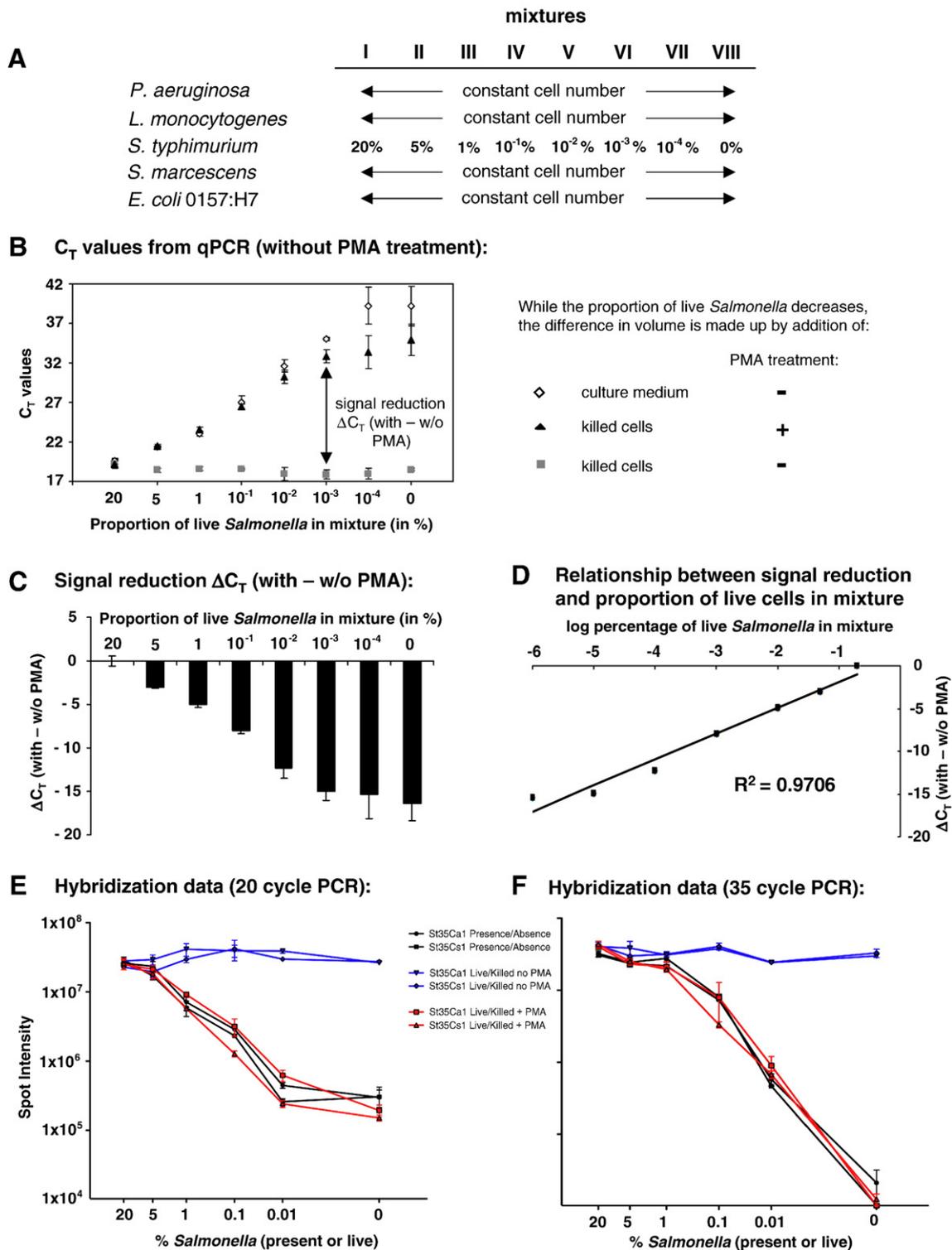
Fig. 3. Detection sensitivity of live cells in PMA-treated samples with a background of killed cells. (A) Pipetting scheme for eight mixtures of five bacterial species. With the exception of *Salmonella*, the cell number of every species is held constant. Dots indicate that the corresponding species comprises 20% of the total volume by addition of pure culture with an OD600 of 1.0 to the mixture. The proportion of live *Salmonella* in the mixtures was reduced in steps from an initial 20% to 0% relative to mixture I. The difference in volume was made up by addition of culture medium or killed cells. The percent values refer to the proportions in mixture 1. (B) Average qPCR threshold cycle (C_T) values for the different mixtures without and with PMA treatment. Error bars indicate standard deviations from three independent experiments. The difference in C_T values between PMA-treated and non PMA-treated mixtures containing killed *Salmonella* cells is referred to as 'signal reduction'. (C) Average signal reduction obtained from subtracting C_T values of non PMA-treated mixtures (with killed cells) from C_T values of PMA-treated mixtures (with killed cells). Error bars indicate standard deviations from three independent experiments. (D) Relationship between signal reduction and the log proportion of live *Salmonella* in mixtures (in the presence of killed *Salmonella*). (E–F) Microarray hybridization results showing *cpn60* signal intensities (arbitrary units) versus amount of live *Salmonella* cells present in the mixture for 20 and 35 cycle PCR amplification products. Blue lines represent values obtained when killed *Salmonella* cells are added to replace live cells in the absence of PMA treatment. Black lines indicate results when live *Salmonella* cells are replaced by culture medium. Red lines indicate results obtained when killed *Salmonella* cells are added to replace live cells followed by PMA treatment. Error bars correspond to the standard deviations of three technical replicates.

treatment as the genomic DNA from live and killed cells amplified equally well. After PMA-treating the mixtures with both live and killed *Salmonella* cells, the C_T values rose with decreasing percentages of live cells. PMA obviously suppressed the amplification of genomic DNA from killed cells. The obtained C_T values from PMA-treated samples with live and killed cells were comparable to C_T values from non-PMA treated samples that only contained the same amount of live *Salmonella*, but no killed cells (compare white diamonds and black triangles in Fig. 3B). Only with very low numbers of live *Salmonella* or in the complete absence of live *Salmonella* was a difference in C_T values seen. Fig. 3C shows the signal reductions resulting from PMA treatment of samples containing both live and killed cells: With decreasing proportions of live cells (and simultaneously increasing proportions of killed cells) the signal reductions caused by PMA treatment increased. The range where a linear relationship between the log percentage of live *Salmonella* in the mixtures and signal reduction ΔC_T was observed spanned over app. 5 $\log_{10}$ units (Fig. 3D).

The microarray results support the qPCR results. In the absence of PMA-treatment and when the decreasing number of live *Salmonella* cells was compensated by the addition of killed *Salmonella* cells (blue lines), the fluorescence signal for the *Salmonella* probes remained at a high level, indicating that *cpn60* amplification was equally effective on live or killed cells. Replacement of live *Salmonella* by culture broth (black lines, Fig. 3E and F) led as expected to a decrease of signal with decreasing proportions of live *Salmonella*. A similar decrease was observed when replacing live *Salmonella* cells with killed ones and treating the mixtures with PMA. This again suggested that PMA-modification of DNA from killed cells suppressed amplification leading to reduced spot intensities on the microarray. Comparative results between 20, 25, and 35 cycles of amplification were not significantly different (Fig. 3E and F; data with 25 cycles not shown).

3.3. Validation with environmental sample (experiment 3)

After evaluating the effect of PMA treatment on detection signals with defined mixtures, we addressed the question how efficiently signals would be suppressed in the presence of an environmental sample background. For this purpose, solids and biomass from 2 l of turbid creek water (turbidity of 43 NTU) were harvested by centrifugation and resuspended in a small volume of the same water. The concentrated environmental sample was split in nine different aliquots. DAPI direct counts and qPCR (using 16S rRNA gene primers and a standard curve, data not shown) suggested app. 10^7 or more bacterial cells per concentrated aliquot (corresponding to 10^5 cells per ml original sample). Eight aliquots were seeded with isopropanol-killed *Salmonella* cells. *Salmonella* numbers ranged from 10^1 to 10^8 cells per aliquot. Samples were either treated with PMA or not prior to genomic DNA extraction. The resulting DNA was 10-fold diluted to avoid inhibition of amplification. Lower dilution factors were found to result in significant inhibition of PCR amplification in spiking tests (data not shown). No amplification was obtained in qPCR with a non-seeded sample (serving as a control) and samples containing 10^3 *Salmonella* cells or less per sample (Fig. 4). This suggested a detection limit of the qPCR assay of 10^4 cells per test sample under the conditions chosen. Above the detection threshold and in the absence of PMA treatment, the C_T values became as expected increasingly smaller for increasing numbers of *Salmonella* cell numbers. After PMA treatment, no amplification within 45 PCR cycles was obtained for all samples suggesting that all dead *Salmonella* were efficiently removed from analysis up to 10^8 cells per sample.

The microarray results paralleled the qPCR results except for the observation of a poorer detection threshold for the *cpn60* amplification (Fig. 4). No signal was obtained at or below 10^5 added cells, whereas qPCR gave results both at 10^5 and 10^4 cell additions in the absence of PMA treatment. Treatment with PMA completely abolished the *cpn60* signal even at 10^8 cells added, further reinforcing the

A **C_T values from qPCR:**

Number of killed *Salm.* cells in sample	C_T values with standard deviation	
	w/o PMA	with PMA
no *Salm.*	no amplif.	no amplif.
10^1	no amplif.	no amplif.
10^2	no amplif.	no amplif.
10^3	no amplif.	no amplif.
10^4	40.20 (± 2.23)	no amplif.
10^5	34.53 (± 0.95)	no amplif.
10^6	31.53 (± 0.11)	no amplif.
10^7	26.48 (± 0.01)	no amplif.
10^8	22.93 (± 0.17)	no amplif.

no amplif. = no amplification within 45 cycles
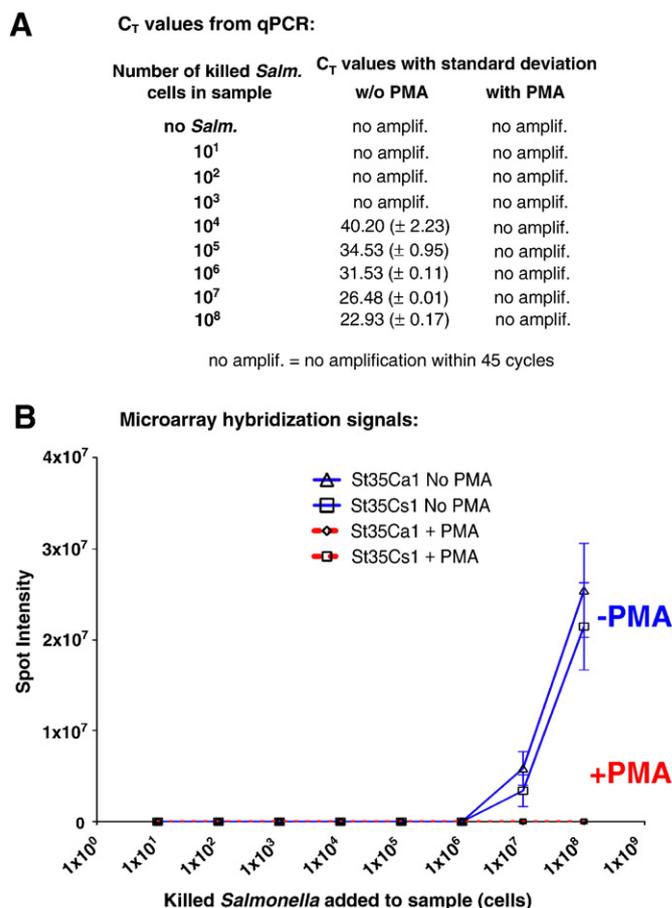
B **Microarray hybridization signals:**

Fig. 4. Efficiency of PMA treatment to eliminate detection signals from isopropanol-killed *Salmonella* spiked into concentrated creek water samples. (A) Average qPCR threshold cycle (C_T) values for PMA-treated and non-treated samples spiked with different numbers of dead *Salmonella* cells. Standard deviations from three technical replicates are given in brackets. The genomic DNA template was 10-fold diluted to reduce inhibition of amplification. If no amplification was observed within 45 cycles, no C_T value is reported. (B) Microarray results showing *cpn60* signal intensities (arbitrary units) versus amount of dead *Salmonella* cells added to the concentrated creek water samples. Signals for the sense probe (St35Cs1) and the antisense probe (St35Ca1) are shown separately. Error bars correspond to the standard deviations obtained from three technical replicates.

evidence for efficacy of PMA in crosslinking DNA under the experimental conditions used.

4. Discussion

Sample treatment with PMA prior to PCR amplification is increasingly gaining acceptance as a means to exclude membrane-compromised cells from analysis (Cawthorn and Witthuhn, 2008; Pan and Breidt, 2007; Vesper et al., 2008). The method reduces qPCR and end point PCR-DGGE signals (Nocker et al., 2007a,b). This study extended the potential use of PMA when using a diagnostic microarray as a downstream analysis tool of PCR-amplified target sequences. Microarray results were complemented with qPCR data as a reference. Both methods belong to the most promising molecular techniques for detection of bacterial pathogens, but do not differentiate between live and dead cells if detection is based on DNA.

4.1. Experiment 1

When analyzing defined mixtures of five bacterial species with qPCR, PMA treatment resulted in efficient removal of the signals from dead cells (Fig. 1). The differences in C_T values between PMA-treated and non-treated samples were generally more pronounced in mixture

VII with all five species being dead compared to the mixtures where only one species was dead. The reason for this tendency is unclear and needs more information about the mechanism of how PMA leads to decreased PCR signals. The observation might be helpful in clarifying this aspect in future work.

PMA treatment efficiently suppressed both qPCR and microarray signals of dead cells. In addition to showing a nice correlation between the two downstream detection methods, the first experiment also validated the performance of the *cpn60* microarray. For this purpose a set of mixtures with one of the five species missing was included. No false positive signals caused by cross-reactivity were observed. The conserved sequences within the coding region of the chaperonin 60 gene show more divergence than 16S rRNA genes. This fact is very useful in differentiating closely related species such as *E. coli* versus *Salmonella* spp., while remaining capable of using universal primers. The extra variability due to codon degeneracy also permits the use of longer probes, which provide more uniform probe-to-probe signal relative to the sequences needed on 16S arrays. As shown in Fig. 2, the probes selected showed very good discrimination, even between closely related species such as *E. coli* and *Salmonella.*

4.2. Experiment 2

The second experiment was performed to test how well different ratios of live and dead cells would be reflected by the signal intensities with the two downstream detection methods. PMA treatment of mixtures with decreasing numbers of live *Salmonella* cells and simultaneously increasing numbers of killed cells resulted in signals that were close to the ones obtained by substituting the killed cells with culture broth (Fig. 3). This applied to both downstream detection methods. In other words, PMA treatment efficiently excluded the killed cells from analysis and gave nearly the same results as if they were completely absent. Only at very low ratios of live/killed cells and when the C_T values were higher than around 30 cycles, could signals from killed cells not be completely suppressed by PMA (Fig. 3B). The reason might lie in the sensitivity of exponential amplification. A high number of cycles can lead to amplification if only a few copies of amplifiable template DNA escape inactivation by PMA.

The qPCR data correlated nicely with the microarray hybridization results. No great difference in microarray signals was observed when analyzing PCR products using 20, 25, and 35 amplification cycles. This suggests that increased cycle numbers did not result in PCR bias in this experiment. Bias could potentially occur, as end-point PCR tends to favor the amplification of low-abundance templates with higher cycle numbers resulting in increasing loss of the quantitative aspect and in a mitigation or elimination of differences in initial template abundances in the final PCR product pool. An initial very low quantity of amplifiable DNA would ultimately reach the same signal intensity as a larger number of template molecules. Although not seen here, it would be prudent with other more complex samples to hold cycle numbers as low as possible when amplifying PMA-treated samples, while balancing the need to have sufficient PCR product for analysis. The approach was already outlined in a previous publication employing DGGE analysis of end-point PCR products (Nocker et al., 2007b). Considering this potential bias of end-point PCR, the PMA-caused microarray signal suppression of dead cells seen in this study appears particularly promising.

4.3. Experiment 3

The last experiment showed that PMA treatment can efficiently suppress the detection of dead cells in an environmental sample background. With both downstream detection methods, no signals were obtained with up to 10^8 dead *Salmonella* cells. Based on the experience from previous experiments with environmental samples, the efficiency of PMA treatment naturally depends on the sample type

(Wagner et al., 2008). Light transparency might be the most important factor. If light cannot penetrate the sample, the crosslinking of PMA to DNA and the simultaneous inactivation of unbound excess PMA will not be efficient. Insufficient light transparency caused by the nature of the sample might have been the major reason for the insufficient removal of signals from dead cells in a recent publication applying PMA and EMA treatment to anaerobic fermentor sludge (Wagner et al., 2008). The dark black appearance of the sample described by the authors might not have allowed sufficient light penetration. Although the concentrated sample processed in this study was substantially turbid, good light penetration was achieved by choosing only 500 µl volumes in a 1.5 ml microcentrifuge tube, horizontal positioning of the tubes on ice during light exposure and mixing of the samples as described in Materials and methods.

5. Conclusion

This study has for the first time combined PMA treatment of mixtures of live and dead bacterial cells with microarray analysis of PCR products. The effect of sample treatment on microarray signals correlated very well with the results from qPCR analysis. Although microarray detection has a lower intrinsic sensitivity, its benefits lie in its high-throughput and its versatility. The highly multiplexed format of the technology allows for rapid detection of specific gene targets of a great number of different microorganisms in a single assay. The combination of the obtained data with viability information would represent a signficant improvement of the technology. The successful suppression of *cpn60* hybridization signals from dead bacteria in defined five-species mixtures as presented in this study appears a promising step in this direction.

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