

# DNA analysis with multiplex microarray-enhanced PCR

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## ABSTRACT

We have developed a highly sensitive method for DNA analysis on 3D gel element microarrays, a technique we call multiplex microarray-enhanced PCR (MME-PCR). Two amplification strategies are carried out simultaneously in the reaction chamber: on or within gel elements, and in bulk solution over the gel element array. MME-PCR is initiated by multiple complex primers containing gene-specific, forward and reverse, sequences appended to the 3' end of a universal amplification primer. The complex primer pair is covalently tethered through its 5' end to the polyacrylamide backbone. In the bulk solution above the gel element array, a single pair of unattached universal primers simultaneously directs pseudo-monoplex PCR of all targets according to normal solution-phase PCR. The presence of a single universal PCR primer pair in solution accelerates amplification within gel elements and eliminates the problem of primer interference that is common to conventional multiplex PCR. We show  $10^6$ -fold amplification of targeted DNA after 50 cycles with average amplification efficiency 1.34 per cycle, and demonstrate specific on-chip amplification of six genes in *Bacillus subtilis*. All six genes were detected at 4.5 pg of bacterial genomic DNA (equivalent to  $10^3$  genomes) in 60 independent amplification reactions performed simultaneously in single reaction chamber.

## INTRODUCTION

Methods for the simultaneous analysis of multiple genes are in ever growing demand. Microarrays are an ideal platform for such analysis in scientific, clinical and environmental contexts, since their miniature size allows one to arrange up to hundreds or thousands of biological probes in a relatively small space and reaction volume (1,2). However, the overall

system-level sensitivity of microarray detection technology is relatively low (3). PCR is one of the most powerful methods for nucleic acid amplification, but using PCR for multi-target analysis is constrained by two main limitations. First, identifying solution-phase multiplex PCR amplicons typically requires a secondary method for size separation or sequence verification prior to analysis and data interpretation. Second, multiplex PCR is restricted in the number of targets that can be faithfully amplified simultaneously, because of uncontrollable primer-primer interactions (4,5).

An attractive solution for increasing the number of different targets that are amplified in a single PCR reaction is the spatial separation of different primer pairs. Microarrays appear to be ideally suited for this task: tethering each pair of primers to a discrete spot on a surface directs the amplification of different targets in a number of non-overlapping micro-surroundings. Given the miniature dimensions of microarrays, highly multiplexed amplification would likewise occur in a homogenous, minimal volume and avoid the split assay. There have been several attempts to perform PCR on a solid support where the amplification primers were cross-linked to a surface (6–9) (D. H. Bing, C. Boles, F. N. Rehman, M. Audeh, M. Belmarsh, B. Kelley and C. P. Adams, <http://www.promega.com/geneticidproc/ussymp7proc/0726.html>). Invariably, these studies revealed that solid-phase PCR (SP-PCR) is significantly less efficient than conventional solution-phase reactions.

Supplementing the reaction mixture with unbound primers identical to those immobilized to the surface, and allowing the reaction proceed in the liquid phase and on the surface of the solid phase simultaneously seems to dramatically increase the product yield on solid supports (10–21). However, adding a multitude of gene-specific, unbound primers to the solution-phase reintroduces the potential for primer interference, a major problem for soluble multiplex PCR. The dilemma is, therefore, that a researcher must sacrifice either the highly multiplexing capability of microarray, or the sensitivity of the PCR. The aim of this study is therefore to develop the base technology platform that provides both the sensitivity of PCR and the multiplexed capacity of microarrays in a homogenous assay.

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## MATERIALS AND METHODS

### DNA targets

*Bacillus mycoides* genomic DNA was obtained from *B. mycoides* ATCC10206 cells using a silica minicolumn purification method as described previously (22), with a VectaSpin Micro Anapore™ 0.2 µm centrifuge filter unit (Whatman International Ltd, England) operated at 14000 *g*. *Bacillus subtilis* ATCC23857 genomic DNA was purchased from ATCC (Manassas, VA). Genomic DNA was fragmented to an average size of 850 bp for 3 min at 37°C in a 100 µl reaction mixture containing 5 µg of genomic DNA; 10 µl of 10× reaction buffer (Ambion, Austin, TX), containing 100 mM Tris-HCl, pH 7.5, 25 mM MgCl<sub>2</sub> and 5 mM CaCl<sub>2</sub>; and 0.1 U of DNase I (Ambion). The reaction was stopped by adding EDTA to a final concentration of 10 mM and incubating the samples at 75°C for 7.5 min. Fragmented DNA was then purified by a standard phenol-chloroform extraction procedure.

Positive control DNA amplicons were prepared by conventional PCR by using 1–5 ng of *B. mycoides* or *B. subtilis* genomic DNA and specific primers listed in Table 1. PCR amplification was performed in 25 µl containing 1× reaction buffer (10 mM Tris-HCl, pH 8.3, 10 mM KCl), 2.5 mM MgCl<sub>2</sub>, 250 µM each dNTP, 0.08 U/µl of AmpliTaq DNA Pol, Stoffel fragment, 400 nM each of the forward and reverse primers, and 10<sup>3</sup>–10<sup>6</sup> copies of target DNA. All PCR reagents were from Applied Biosystems (Foster City, CA). Thermal cycling parameters included an initial denaturation at 94°C for 3 min; 30 cycles of 45 s at 94°C, 1 min at 55°C, 45 s to 1 min at 72°C; and a final extension at 72°C for 3 min. Mastercycler gradient (Eppendorf Scientific, Inc., Westbury, NY) and DNA Engine Dyad PTC-220 (MJ Research, Inc., Waltham, MA) thermal cyclers were used in the study. The PCR fragments were separated on 1% low-melting temperature agarose gel (Sigma, St. Louis, MO) and isolated by organic extraction as described previously (23).

### Gel element arrays

Polyacrylamide 676-gel elements arrays were prepared as aldehyde matrices (24) using photopolymerization techniques as described previously (25). All PCR primers were synthesized in-house with a 394 DNA/RNA synthesizer (Perkin-Elmer/Applied Biosystems) and high-performance liquid chromatography purified according to the standard protocols. Gel-immobilized primers were synthesized with a 5' C<sub>5</sub>, and C<sub>18</sub> (Glen Research, Sterling, VA) or PEG-900 amino-modified spacer (Table 1). PEG-900 coupled primers were synthesized as described previously (26). After polyacrylamide matrix activation (24), the amino-modified complex primers were applied to the gel elements with a custom robotic arrayer (27). Each gel element (100 × 100 × 20 µm) contained both the forward and reverse complex primer pair, and each pair of primers was loaded on 10 replicate gel elements per array. Fluorescently labeled, internal reporting probes were synthesized with a Texas Red sulfonyl chloride fluorescent compound (Molecular Probes, Eugene, MA) as described previously (28).

### Multiplex microarray-enhanced PCR (MME-PCR)

Single-target, on-chip amplification protocols were performed as follows. The on-chip reaction vessel was created with Small

Frame-Seal Chamber (25 µl vol) (MJ Research) affixed around an individual 676-gel element array. The PCR amplification mixture was assembled off-chip as described above, except that we included 10<sup>5</sup>–10<sup>6</sup> copies of target DNA, 40 ng/µl yeast tRNA (Ambion), 0.5% BSA (Sigma), 400 nM each of the universal amplification primers (Table 1) and 0.2 U/µl of AmpliTaq DNA Pol, Stoffel fragment. In the experiments where universal primers were omitted from solution, an equal volume of deionized water (dH<sub>2</sub>O) was added instead. Prior to MME-PCR, the reaction mixture was degassed in a Centrivap Concentrator (Labconco, Kansas City, MO) at 0.01–0.02 bars for 90 s at ambient temperature. To compensate for evaporative loss, 5 µl of dH<sub>2</sub>O was then added back into the reaction mixture for each 30 µl of the reaction cocktail. An aliquot of 25 µl of degassed amplification mixture was then applied within the framed gel element array, and the chamber was sealed with a plastic lid. The PCR chips were placed either on top of the In Situ Adapter (Eppendorf Scientific, Inc.) attached to a Mastercycler gradient machine, or into the Twin Tower Block of the DNA Engine Dyad PTC-200. On-chip thermal cycling included an initial heating at 93°C for 3 min, followed by 25 cycles of (92°C for 45 s, 57°C for 150 s and 72°C for 180 s), 25 cycles of (92°C for 45 s, 57°C for 75 s and 72°C for 75 s) and a final extension for 180 s at 72°C. At least two replicate arrays (manufactured on separate glass substrates) were processed and analyzed for each test condition.

Multiplexed PCR chips were manufactured with primer pairs designed for six functional genes of *B. subtilis* (Table 1). Each primer pair was immobilized within 10 replicate gel elements per array, such that there were 60 loaded gel elements on each PCR chip. MME-PCR was performed with a dual-phase amplification strategy. In the first phase, universal primers were omitted from the amplification cocktail. Thus, 10 µl of MME-PCR amplification mixture (described above) containing 10<sup>1</sup>–10<sup>6</sup> copies of target DNA and 0.8× Self-Seal Reagent (MJ Research) was degassed as described above and loaded onto the center of a microarray. A No.1 premium glass cover slip (18 × 18 mm, Fisher Scientific, Bohemia, NY) was placed directly over each array. The first round of the MME-PCR included an initial denaturation at 93°C for 3 min, 40 cycles of (92°C for 45 s, 59°C for 90 s and 72°C for 60 s) and a final extension at 72°C for 3 min. After the first round of PCR, the chips were soaked in dH<sub>2</sub>O for 5 min to remove the cover slips, rinsed briefly in 6× SSPE (900 mM NaCl, 60 mM sodium phosphate, pH 7.4, 6 mM EDTA), 1% Tween-20 and washed for 15 min in dH<sub>2</sub>O at ambient temperature. Chips were air-dried and then used for a second round of PCR, wherein universal primers were now added to the reaction cocktail at a concentration of 40 nM each. Amplification proceeded as described above, except that the number of amplification cycles was reduced to 30. After second-stage amplification, cover slips were removed and gel element arrays were treated for 1.5 h at 37°C in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.4% SDS and 100 µg/ml of Proteinase K (Amresco Inc., Solon, OH) to remove non-specific protein deposits.

### Detection

The detection of amplified target by hybridization was performed in Small Frame-Seal chamber in a buffer containing

**Table 1.** Primers, probes and targets

Oligo ID <sup>a</sup>	Sequence <sup>b</sup>
<b>Complex primers</b>	
U1-bm16S-1 <sup>c</sup>	<b>gctaaatcggactagctacc</b> acactgggactgagacac
U2-bm16S-2 <sup>c</sup>	<b>taatccagctacgctgcate</b> gccagcttattcaactagcac
U5-bm16S-5 <sup>c,d</sup>	<b>ttttctctctcccaatctc</b> gtacaagagctgcaagacc
U6-bm16S-4 <sup>c,d</sup>	<b>atcagcaatgccttctaagtc</b> gccgattactagcgattcc
U5-bsDnaK-R <sup>c</sup>	<b>ttttctctctcccaatctc</b> gctccagcttactgatatcc
U6-bsDnaK-F <sup>c</sup>	<b>atcagcaatgccttctaagtc</b> gttttctttctctgtgcc
U5-bsEbrA-R <sup>c</sup>	<b>ttttctctctcccaatctc</b> aaccatattcccctgagcc
U6-bsEbrA-F <sup>c</sup>	<b>atcagcaatgccttctaagtc</b> agtaaacacgaccctgatag
U5-bsFruR-F <sup>c</sup>	<b>ttttctctctcccaatctc</b> gatcaaacaggcaaaacac
U6-bsFruR-R <sup>c</sup>	<b>atcagcaatgccttctaagtc</b> aatgtaagctctcagcgtc
U5-bsGrpE-R <sup>c</sup>	<b>ttttctctctcccaatctc</b> gaagccgacaatgaacagac
U6-bsGrpE-F <sup>c</sup>	<b>atcagcaatgccttctaagtc</b> aggatcaaatctgccctac
U5-bsSpo0A-R <sup>c</sup>	<b>ttttctctctcccaatctc</b> aggacaggaagacatggaag
U6-bsSpo0A-F <sup>c</sup>	<b>atcagcaatgccttctaagtc</b> ccctcagcctctctaaaac
U5-bsYisY-F <sup>c</sup>	<b>ttttctctctcccaatctc</b> ccgatgatgtgaaagcag
U6-bsYisY-R <sup>c</sup>	<b>atcagcaatgccttctaagtc</b> cccgcagacagtaaaatcag
<b>Universal primers</b>	
U1	<b>gctaaatcggactagctacc</b>
U2	<b>taatccagctacgctgcate</b>
U5/AP000522/12788-12808	<b>ttttctctctcccaatctc</b>
U6/AP000522/14015-13995	<b>atcagcaatgccttctaagtc</b>
<b>Specific primers</b>	
bm16S-1/AF155957/310-329	cacactgggactgagacac
bm16S-2/AF155957/474-453	gccagcttattcaactagcac
bm16S-3/AF155957/1198-1218	tcacatgcccttatgacc
bm16S-4/AF155957/1354-1335	gccgattactagcgattcc
bm16S-5/AF155957/1245-1264	gtacaagagagctgcaagacc
bm16S-7/AF155957/36-56	gcctaatacatgcaagtcgag
bm16S-8/AF155957/758-738	ctcagtgctcagttacagaccag
bm16S-9/AF155957/770-790	gtggggagcaaacaggattag
bm16S-10/AF155957/1495-1475	acttcacccaatcctctgtc
bsDnaK-R/Z99117/12237-12217	gctccagcttactgatatcc
bsDnaK-F/Z99117/12110-12130	gttttctttctctgtgcc
bsEbrA-R/Z99113/57310-57292	aaccatattcccctgagcc
bsEbrA-F/Z99113/57162-57181	agtaaacacgaccctgatag
bsFruR-F/Z99111/96526-96545	cgatcaaacaggcaaaacac
bsFruR-R/Z99111/96652-96633	aatgtaagctctcagcgtc
bsGrpE-R/Z99117/13794-13775	gaagccgacaatgaacagac
bsGrpE-F/Z99117/13669-13698	aggatcaaatctcctcctac
bsSpo0A-R/Z99116/108839-108820	aggacaggaagacatggaag
bsSpo0A-F/Z99116/108693-108712	ccctcagcctctctaaaac
bsYisY-F/Z99109/157532-157550	gccgatgatgtgaaagcag
bsYisY-R/Z99109/157677-157658	cccgcagacagtaaaatcag
<b>Probes</b>	
P-bm16S/AF155957/1301-1320	tcggattgtaggctgcaact
P-bsDnaK/Z99117/12141-12161	gctcttagctttacgataaccg
P-bsEbrA/Z99113/57190-57209	gaatcccgataagccctttg
P-bsFruR/Z99111/96549-96568	tacgtcttagcagacccttc
P-bsGrpE/Z99117/13718-13737	ttcaagccttctcagcagctg
P-bsSpo0A/Z99116/108795-108814	gtgtccgttataagcaagcc
P-bsYisY/Z99109/157602-157583	cccattgaaaaccggcaag
<b>Targets</b>	
T-bm16S-723/AF155957/36-758	723 bp; bm16S-7/bm16S-8
T-bm16S-726/AF155957/770-1495	726 bp; bm16S-9/bm16S-10
T-bm16S-157/AF155957/1198-1354	157 bp; bm16S-3/bm16S-4
T-bsDnaK/Z99117/12110-12237	128 bp; bsDnaK-F/bsDnaK-R
T-bsEbrA/Z99113/57162-57310	149 bp; bsEbrA-F/bsEbrA-R
T-bsFruR/Z99111/96526-96652	127 bp; bsFruR-F/bsFruR-R
T-bsGrpE/Z99117/13669-13794	126 bp; bsGrpE-F/bsGrpE-R
T-bsSpo0A/Z99116/108693-108839	147 bp; bsSpo0A-F/bsSpo0A-R
T-bsYisY/Z99109/157532-157677	146 bp; bsYisY-F/bsYisY-R

<sup>a</sup>Oligonucleotide ID includes the oligonucleotide's name/GenBank accession number/numbers of first and last nucleotides in the deposited DNA sequence.

<sup>b</sup>Universal primers and universal segments of complex primers are shown in bold font.

<sup>c</sup>These primers contain amino-linker PEG-900 on their 5' ends.

<sup>d</sup>Both of these primers were synthesized in two variants, with PEG-900 and C<sub>18</sub> amino-linkers on their 5' ends. We did not find a significant difference in performance of these two types of primers (data not shown).

The name of each complex primer includes the name of a universal primer and the name of a sequence specific primer. For instance, the name *U1-bm16S-1* means that the complex primer contains the *U1* universal primer in its 5'-half and *bm16S-1* specific primer in its 3'-half, and so on. Universal primers are denoted as U1, U2, U5 and U6.

Sequence specific primers, whose names start with *bm16S*, were designed for 16S rRNA gene of *B. mycoides*. Odd numbers in the end of the names denote forward primers and even numbers denote reverse primers. The names of specific primers designed for six functional genes of *B. subtilis* start with *bs*, with the name of a gene immediately following. *F* in the names stands for forward and *R* stands for reverse.

The titles of reporting probes and specific DNA targets start with P and T, respectively. The name of biological species *B. mycoides* (*bm*) or *B. subtilis* (*bs*) and genes (*16S* or *DnaK*, for instance) follow them. Names of targets generated for the *B. mycoides* 16S rRNA gene contain the length of the target in bp in the end of each name. All targets listed in the table were synthesized by PCR. The length of each target and the specific primers used for the synthesis are shown in the right column.

1 M guanidine thiocyanate (Fisher Scientific, Fair Lawn, NJ), 50 mM HEPES, pH 7.5, 5 mM EDTA, 40 ng/ $\mu$ l of yeast tRNA, 0.1% BSA and 2.5 nM of internal reporting probe labeled at the 3' end with Texas Red. Amplified PCR chips were incubated at 92°C for 2 min, 70°C for 10 min and 30°C overnight. Amplified PCR chips were then washed in 6 $\times$  SSPE, pH 7.4, 1% Tween-20 at ambient temperature for 5 min and analyzed on a custom fluorescent microscope as described in detail previously (29). The local background was deducted and confidence intervals were computed for the significance level of 95%, or  $\alpha = 0.05$  as described previously (29).

## RESULTS

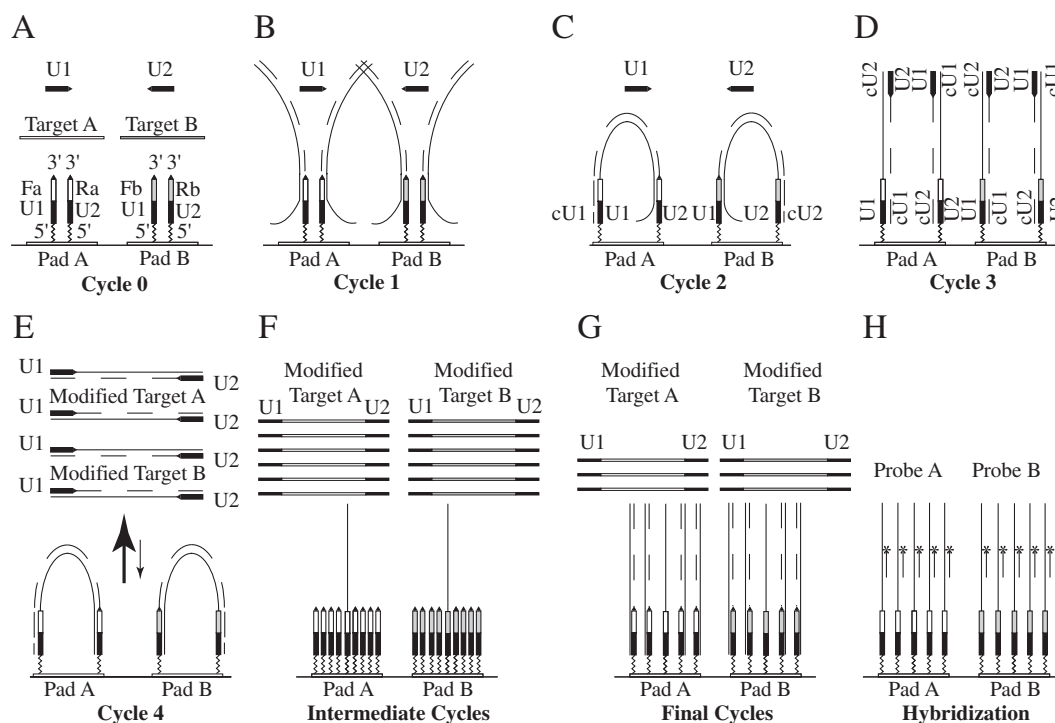
### MME-PCR design

The working model for MME-PCR is illustrated in Figure 1. The salient and distinguishing feature of the PCR chip is the combined use of multiple gene-specific gel-immobilize primers, and a single universal primer set in solution. A gene-specific amplification is initiated from the individual gel elements, and the accumulation of modified targets (containing the target gene and universal primer sequences) in solution serves to accelerate the within-gel amplification. By immobilizing

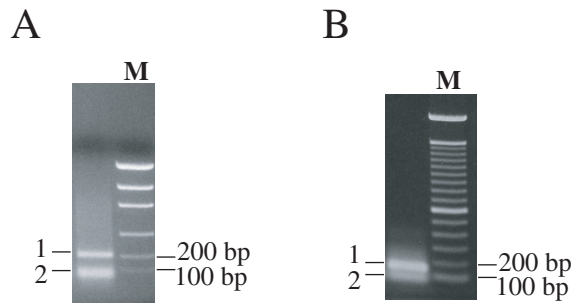
multiple gene-specific primers within the gel elements and using a single pair of universal primers in solution, we minimize primer interference effects common to conventional solution-phase, multiplexed PCR. In this study, the nucleic acid targets were applied to the PCR chip in the presence of amplification mixture for immediate thermal cycling.

### MME-PCR proceeds through the formation of modified targets

The working on-chip amplification model in Figure 1 was verified by demonstrating the synthesis of modified target in solution. Primers U1-bm16S-1 and U2-bm16S-2 were immobilized in replicate gel elements, and are designed to amplify a 165 bp amplicon from *B. mycooides* DNA. The universal primer linkers are each 20 nt; thus, we expect an amplicon of 205 bp to accumulate in solution according to Figure 1. Using  $10^6$  copies of target T-bm16S-723 (Table 1), the expected 205 bp fragment is detectable in the amplification mixture after 50 cycles of on-chip amplification (Figure 2A). The two visible fragments in Figure 2A were excised from the gel, purified and sequenced. Sequence analysis confirmed that 205 bp product contained the expected *B. mycooides* 165 bp target, whereas the shorter amplicon appeared to be a mixture



**Figure 1.** Working model for MME-PCR. (A) Cycle 0 shows two gel elements (Pad A and Pad B) containing unique complex PCR amplification primers. Every complex primer consists of a universal primer (U1 or U2) and gene-specific forward ( $F_a$ ,  $F_b$ , etc.) or reverse ( $R_a$ ,  $R_b$ , etc.) primers. Free-floating U1 and U2 primers are supplied as part of the amplification cocktail in solution over the chip. (B) In Cycle 1, the DNA targets in solution anneal to their respective gene-specific PCR primer sequences within the gel elements, and the DNA polymerase extends from the gel-immobilized primers. (C) In Cycle 2, a nucleic acid 'bridge' is formed between one extended primer on each pad is shown in (C). Once the 'bridge' is formed, the polymerase can extend the second strand and synthesize a modified target containing sequences (cU1 and cU2) that are complementary to universal primers U1 and U2. (D) By Cycle 3, free-floating universal amplification primers U1 and U2 can then serve as amplification primers, not only on the gel elements themselves (D) but also in solution (E). (E) Beginning in Cycle 4, then, a pseudo-monoplex PCR amplification becomes established in the bulk solution over the gel element array. (F) Pseudo-monoplex PCR eventually dominates the reaction due to higher amplification efficiency in solution. (G) In parallel, hybridization kinetics will force more of the amplified product into the gel element arrays and therefore 'accelerate' the within-gel PCR amplification reaction. (H) After PCR, the microarrays were washed and the immobilized PCR products detected and confirmed by hybridizing with internal fluorescently labeled reporting probes. Thus, only the products of within-gel amplification are specifically detected. Parental and newly synthesized nascent DNA strands are shown as solid and dashed lines, respectively.



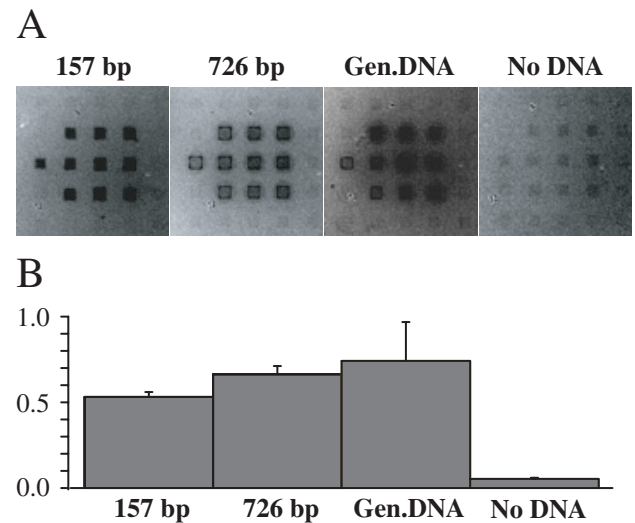
**Figure 2.** Modified targets containing universal sequences are produced and released into solution during MME-PCR. (A) Microarrays were manufactured by immobilizing complex primers U1-bm16S-1 and U2-bm16S-2 into 10 replicate gel elements. Universal primers U1, U2 and  $10^6$  copies of target T-bm16S-723 were included in the amplification cocktail. (B) Amplification with different combinations of gene-specific and universal primers. Primers U5-bm16S-5 and U6-bm16S-4 were immobilized within gel elements, and universal primers U5, U6 and  $10^6$  copies of target T-bm16S-726 were supplied in the amplification cocktail. In both experiments, MME-PCR proceeded for 50 cycles. The solution-phase reaction mixture was analyzed on 1.2% agarose gels. M is low weight marker (A) and a 100 bp DNA marker (B). Numbers 1 and 2 shown to the left of figures indicate amplified fragment and primer dimers amplification artifacts, respectively.

of primer dimers and amplification artifacts with no relation to the *B.mycoides* target. Changing the sequence of both the universal and gene specific primers (Table 1; universal primers U5 and U6; gene-specific complex primers U5-bm16S-5 and U6-bm16S-4) also resulted in the expected (152 bp) amplification product in solution using  $10^6$  copies of target T-bm16S-726 (Figure 2B). In all the cases, excluding the universal primers from the amplification mixture resulted in no amplified material in the solution (data not shown). These data verify the amplification model of Figure 1, and indicate that the on-chip PCR scheme is extensible to different gene targets and/or universal primer sequences.

The end-point yield of the 205 bp target (Figure 2A) was  $\sim 300$  ng. The PCR process may be described by the equation  $N = N_0 \cdot K^n$  (30–33), where  $K$  denotes the amplification efficiency,  $N_0$  and  $N$  are the initial and final numbers of targeted molecules, respectively, and  $n$  is the number of cycles. Using this equation, the average on-chip amplification efficiency ( $K$ ) in MME-PCR was  $\sim 1.335$  for this particular modified target over 50 cycles.

### Template size and complexity

MME-PCR amplification may be initiated from on or within the gel elements, yet polyacrylamide gel has a restricted pore size. Here, we compared how DNA samples of different size and complexity are amplified by the method. Gene-specific complex primers U5-bm16S-5 and U6-bm16S-4 (Table 1) designed to amplify a 152 bp amplicon from *B.mycoides* 16S rRNA gene were immobilized in replicate gel elements. The amplification reactions were supplied with either  $10^6$  copies of 157 bp PCR fragment, 726 bp PCR fragment (Table 1; targets T-bm16S-157 and T-bm16S-726, respectively), *B.mycoides* genomic DNA with average size of the fragments of 850 bp, or no DNA. After 50 cycles of thermal cycling, the microarrays were hybridized with reporting probe P-bm16S. As shown in Figure 3, the amplification was supported by all three types of templates, whereas the negative control generated little background signal (A). Quantitative analysis of the



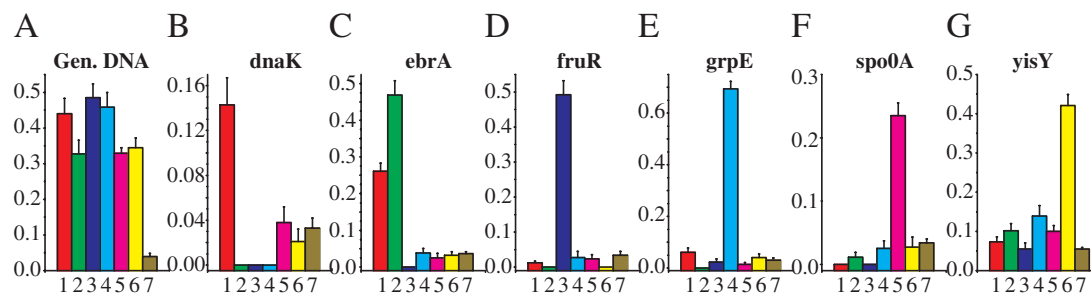
**Figure 3.** MME-PCR with template DNA of different complexity and sizes. (A) Microarrays were manufactured by immobilizing complex primers U5-bm16S-5 and U6-bm16S-4 into 10 gel elements on replicate arrays. The amplification of the 152 bp fragment was supplied with universal primers U5, U6 and either  $10^6$  copies of target T-bm16S-157 (157 bp DNA fragment), T-bm16S-726 (726 bp DNA fragment), *B.mycoides* genomic DNA or no DNA. After 50 cycles of MME-PCR, the microarrays were hybridized with internal probe P-bm16S. (B) The images were quantified, and the data plotted in arbitrary fluorescent units as the mean  $\pm$  confidence interval, as described in Materials and Methods.

images showed that each of the templates was amplified to approximately the same end-point signal intensity (B). Approximately 100–300 ng of 152 bp modified target was generated for each target size, corresponding to an average amplification efficiency of 1.306–1.335 over all 50 cycles (data not shown).

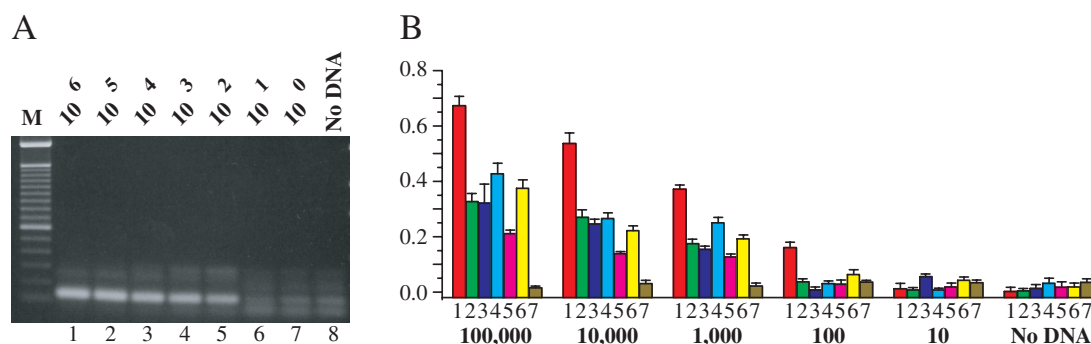
It has been previously shown in our laboratory that DNA fragments  $>500$  bp do not diffuse in the gel elements of the 3D microarrays readily (S. Bavykin, unpublished data). The observation that 157 and 726 bp PCR fragments and genomic DNA with average fragment size of 850 bp support the amplification to approximately the same degree further confirms our model that the amplification in MME-PCR starts on the surface of the gel elements and then proceeds in solution with the synthesis of modified targets (see Figure 1). Thus, neither the size of the DNA template nor the complexity of the DNA sample has a considerable effect on the end-point yield or amplification efficiency of the described MME-PCR method.

### Specificity of the MME-PCR

Two important procedural modifications were introduced for MME-PCR based on our observations and experiments with single target protocol. First, because we were no longer interested in identifying modified targets in solution above the gel element arrays, we replaced the 25  $\mu$ l Frame-Seal chamber with glass coverslips and Self-Seal Reagent to significantly reduce the formation of air bubbles in the chamber during thermal cycling. Second, we performed a two-phase amplification protocol wherein the universal (solution-phase) primers were only added after several cycles of amplification from the gene-specific gel elements. In doing so, we minimized the formation of universal primer dimers early in the



**Figure 4.** MME-PCR is specific. (A) Microarrays were manufactured by immobilizing six different pairs of complex primers, designed for the amplification of six different functional genes of *B. subtilis*: dnaK, ebrA, fruR, grpE, spo0A and yisY (see Table 1 for the primer list). Each pair of primers was immobilized into the 10 replicate gel elements on each array. The reaction mixture was supplied with universal primers U5, U6 and 4.5 ng of *B. subtilis* genomic DNA ( $10^6$  copies). After PCR, the microarrays were hybridized with a mixture of six reporting probes, P-bsDnaK, P-bsEbrA, P-bsFruR, P-bsGrpE, P-bsSpo0A and P-bsYisY (Table 1), to verify the identity of the resulting amplicons. Intensities of hybridization signals are shown in arbitrary units. (B–G) The experiment described in (A) was repeated, except that the genomic DNA was replaced with  $10^6$  copies of one of the six amplicons, T-bsDnaK, T-bsEbrA, T-bsFruR, T-bsGrpE, T-bsSpo0A or T-bsYisY (Table 1). Labels above each panel (A–G) identify a target supplied with amplification cocktail. Numbers 1–6 below each bar in the bar graphs represent complex immobilized primers for *B. subtilis* genes dnaK, ebrA, fruR, grpE, spo0A and yisY, respectively. Bar number 7 represents average background signal from empty gel elements on the chip.



**Figure 5.** Detection limit of the MME-PCR. (A) Serial dilutions of total genomic DNA of *B. subtilis* were prepared. Eight separate conventional PCR reactions with bsGrpE-F/bsGrpE-R primers (Table 1) were run for 50 cycles. The products of the reactions were separated on 1.2% agarose gel. The labels above the lanes denote the amount of *B. subtilis* genomic DNA used in the reaction, expressed in equivalents of the bacterial genomes. ‘No DNA’ lane contains the product of mock PCR reaction, performed without the addition of DNA. M, 100 bp DNA ladder. (B) Microarrays, identical to those described in Figure 4 were used for MME-PCR with different amounts of input *B. subtilis* genomic DNA ( $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ ,  $10^1$  or zero copies). After PCR, the microarrays were hybridized with the set of six *B. subtilis* reporting probes used in the experiment shown on Figure 4 to verify the identity of the amplicons. The numbers below each bar in the bar graph panels denote the name of targeted genes, or the background as shown in Figure 4. Labels below each panel denote the amount of the bacterial genomic DNA, expressed in the number of copies of the genome.

PCR, and their subsequent competition with the target DNA for enzyme and nucleotides in later cycles.

MME-PCR chip was designed and fabricated for six functional gene targets in *B. subtilis* (dnaK, ebrA, fruR, grpE, spo0A and yisY) by immobilizing six pairs of complex primers: U5-bsDnaK-F/U6-bsDnaK-R, U5-bsEbrA-F/U6-bsEbrA-R, U5-bsFruR-F/U6-bsFruR-R, U5-bsGrpE-F/U6-bsGrpE-R, U5-bsSpo0A-F/U6-bsSpo0A-R and U5-bsYisY-F/U6-bsYisY-R (Table 1). To demonstrate multiplexed amplification, on-chip PCR was conducted in the presence of universal primers U5 and U6, and 4.5 ng ( $10^6$  copies) genomic DNA. The six *B. subtilis* functional genes were amplified and detected with similar efficacy, with end-point product accumulation ranging from 7- to 12-fold above background (Figure 4A).

To demonstrate amplification specificity, MME-PCR was conducted in the presence of  $10^6$  copies of the individual, PCR-generated amplicons from dnaK, ebrA, fruR, grpE, spo0A and yisY genes (see Targets in Table 1). During primer design for the MME-PCR, we have noticed that the 3' ends of U6-bsDnaK-R and U5-bsEbrA-F differ by only a single base in 8 nt (Table 1). We utilized these primers as a mean for the evaluation of chances for possible false-positive outcomes. As

shown in Figure 4B–G, amplification of the six individual targets in the MME-PCR resulted in robust signals on gel elements loaded with corresponding primers. We observed only one exception from this pattern, when, expectedly, T-bsEbrA target also supported amplification on gel pads loaded with U5-bsDnaK-F/U6-bsDnaK-R primers to a level significantly higher than the background (Figure 4C). This was the only false-positive out of the 36 possible outcomes, and there were no false negatives. Therefore, we demonstrated high specificity of MME-PCR. Importantly, the efficacy of the 6-plex PCR was approximately the same as the efficacy of the individual monoplex reactions, as assessed by end-point product accumulation (i.e. signal intensity; Figure 4A versus 4B–G). Thus, using MME-PCR allows amplification of multiple genomic targets in an independent and specific fashion, in a single reaction, on a single biochip.

#### Detection limit of the MME-PCR

Conventional PCR for a 126 bp target (T-bsGrpE) showed a lower detection limit of 100 copies per reaction (Figure 5A; compare lanes 1–5 and 6–8). In order to determine minimum

detection limits for MME-PCR, we manufactured microarrays as described for Figure 4 and performed six separate MME-PCR reactions with decreasing amounts of the *B.subtilis* genomic DNA. The resulting data are shown in Figure 5B. Remarkably, we found that the lower detection limit of MME-PCR was nominally 1000 copies of input genomic DNA, with two of the six targets (*dnaK* and *yisY*) amplified at 100 copies of input DNA. Thus, we have successfully demonstrated the specific, simultaneous amplification of six targets in 60 gel elements in a homogenous assay, at 1000 copies of input genomic DNA.

## DISCUSSION

Currently, SP-PCR represents an analog of nested PCR (34), where the first stage amplifies DNA of interest in solution using free-floating primers and the second step results in attachment of amplified fragments through immobilized PCR primers (10–21). During SP-PCR DNA, amplification occurs mostly in solution, but the increased concentration of pre-amplified target facilitates PCR on the solid support. This combination of PCR and microarray technologies were suggested as a potentially useful technique for SNP identification (12,13,15,18), allele detection (11,18), detecting infectious organisms or drug-resistant mutations (10,17,19), *in vitro* transcription/translation studies (21) and the quantification of SP-PCR products (14). Regardless of amplification efficiency, SP-PCR suffers from the common problem of any multiplex PCR, namely primer interference and dimer formation. The net result of primer interference is a reduction in PCR amplification efficiency and multiplexing capacity, such that previous SP-PCR platforms have not been as effective or widely applied in the aforementioned fields of use. Trying to resolve problem of primer interference, Tillib *et al.* (19) created a microarray bearing hundreds of monoplex PCR chambers separated from each other by mineral oil. However, this protocol is time consuming and complicated.

'Bridge' amplification on solid supports [Bridge-SP-PCR, (6–9) (D. H. Bing, C. Boles, F. N. Rehman, M. Audeh, M. Belmarsh, B. Kelley and C. P. Adams, <http://www.promega.com/geneticidproc/ussymp7proc/0726.html>)] does have the potential to resolve primer interference for multiplex PCR on a microarray. In this method, a unique pair of PCR primers is immobilized on the solid support (i.e. there are no primers in solution) in individual compartments or locations (spot, microbead, etc.). However, the obvious benefit of spatial primer immobilization that excludes interference with one another is offset by the very low amplification efficiency of PCR on the solid support. Low amplification efficiency introduces a need for signal amplification, either with isotopes, antibodies or enzyme-linked reporting strategies in order to detect amplified fragments. For example, Adessi *et al.* (6) reported a lower detection limit of  $1.5 \times 10^6$  copies of a 866 bp fragment in a 25  $\mu$ l Bridge-SP-PCR amplification chamber, using  $^{32}$ P-labeled probes or anti-digoxigenin mouse antibodies for detection and identification of amplified sequences. Using the data from Figure 4A in (6), the calculated amplification efficiency for bridge PCR on a glass support was 1.0223 per cycle over 45 cycles. Shapero *et al.* (7) used polyacrylamide microspheres as a solid support and demonstrated a detection sensitivity of  $3 \times 10^5$ – $3 \times 10^6$  copies of human

genomic DNA in conjunction with  $^{32}$ P-labeled dCTP or missequencing for amplified DNA fragment identification. D. H. Bing, C. Boles, F. N. Rehman, M. Audeh, M. Belmarsh, B. Kelley and C. P. Adams (<http://www.promega.com/geneticidproc/ussymp7proc/0726.html>) achieved a detection sensitivity of  $2.8 \times 10^4$  copies of human genomic DNA, but carried out identification of amplified fragment with  $^{32}$ P-labeled precursors. Adams and Kron (8) and Onodera *et al.* (9) do not provide data on detection sensitivity or amplification efficiency.

To circumvent the limitations caused by low amplification efficiency on a solid support and a low degree of multiplexing in solution-phase PCR, we conceived of and implemented a MME-PCR strategy where multiplex PCR occurs on and within gel element arrays and is enhanced by pseudo-monoplex PCR in solution. The key features of the method are that two types of primers (solution-phase and immobilized) work in concert, and that 3D gel elements support enzymatic activity throughout the solution-phase volume of the microarray element. Multiple specific complex primers are immobilized to the microarray gel elements and initiate PCR amplification of multiple targets directly from the gel element; and a single pair of universal primers in solution provides amplification of multiple complex targets (flanked by sequences that are complementary to universal primers) that result from the initial rounds of amplification from the gel element (Figures 1 and 2). The extent to which increased product yield translates into a reduced number of cycles or absolute detection sensitivity is the subject of ongoing experiments. We demonstrated (Figure 2B) that MME-PCR can produce up to 300 ng of amplicon (a  $2 \times 10^6$ -fold increase in target abundance) with an average amplification efficiency of 1.34 per cycle over 50 cycles, estimates that begin to approach amplification efficiencies reported for conventional PCR [i.e. 1.46–1.48 (30); 1.58–1.62 (31); and 1.79–1.99 (32,33)].

The low average amplification efficiency on planar microarrays (6) may have several explanations. For example, Adessi *et al.* (6) concluded that at optimal primer density ( $10^{10}$  primers/mm<sup>2</sup>) only 1 in 300 primers was converted into target DNA. This result may be explained by a screening effect, wherein the high negative charge density of immobilized primers in close proximity to a solid support disturbs polymerase functioning on the 2D surface, and/or 'repels' of target DNA in solution, reducing hybridization and extension efficiency. Alternatively, abnormal target–probe and probe–probe interactions on planar microarrays (35) may serve to decrease the effective concentration of target in solution near the surface and amount of accessible probes on the chip and therefore reduce initial priming or later-stage amplification efficiency. In distinct contrast, we randomly immobilize primers to the polyacrylamide backbone throughout the 3D volume of a  $100 \times 100 \times 20 \mu$ m gel element. Therefore, most of the immobilized primers are located far from a solid (2D) surface. Molecular interactions in 3D polyacrylamide elements are close to those in solution (36,37), which facilitates protein–protein and DNA–protein interactions (38,39) and may improve DNA polymerase function with immobilized primers relative to a 2D surface. Moreover, MME-PCR is constantly accelerated by an ever increasing concentration of modified target in solution that improves hybridization and extension efficiency. In a similar manner, the modified targets also

contain a 40 bp recognition sequence on each terminus, which we believe increases target hybridization with immobilized complex primers and the resulting amplification efficiency on and within the gel elements themselves (Figure 1G).

Testing the MME-PCR technology with template DNAs of different size suggested that DNA length and complexity have only a marginal effect on end-point signal intensity (Figure 3). This was an unexpected result, because large DNA fragments (ca. 500 bp) normally diffuse into gel elements ~10 times slower than 200 bp fragments (S. Bavykin, unpublished data). These data suggest that MME-PCR amplification is initiated (Figure 1B–D) from the surface of the gel element, proceeds in solution with the synthesis of modified targets (Figure 1E and F), which in turn diffuse into the gel elements and accelerate solid-phase amplification (Figure 1G).

The possibility of highly multiplexed MME-PCR was demonstrated on six single-copy functional genes in *B.subtilis*. In actuality, data in Figure 5 demonstrate 60 independent amplifications on each microarray, because each of six different functional genes was amplified and detected in 10 replicate gel elements in a single reaction at  $10^3$  copies (4.5 pg) of input genomic DNA. Moreover, the amplification was highly specific, with no false-negatives out of 36 possible outcomes. Consequently, the reported format of MME-PCR, detection limits and estimated amplification efficiency are consistent with most solution-phase, split-sample PCR assays and microarray platforms, which are now in common use. These features of the gel-element PCR chip therefore make the technology suitable for highly sensitive and accurate parallel genetic analyses, using primer sequences already developed and validated elsewhere.

Future efforts of our study will focus on simplifying the analytical procedure, increasing the complexity of the chip, simplifying the labeling and reporting chemistry and extending the technology for the analysis of nucleic acids extracted from clinical and environmental sample matrices. For example, preliminary experiments (D. Lewicki, personal communication) indicate that degassing the amplification cocktail is not necessary for experiments performed in 10  $\mu$ l volumes under the glass cover slip and in the presence of Self-Seal Reagent. Similarly, primary amplification from the gel element surface and subsequent amplification of targets up to 152 bp in length within the gel element itself (Figure 3) suggest that DNA fragmentation prior to MME-PCR may be unnecessary. Finally, one-stage amplification was successfully employed to demonstrate that MME-PCR proceeds through modified target formation and to estimate the influence of DNA size and its complexity on the yield of the amplification reaction (Figures 2 and 3). Therefore, a more thorough analysis and careful selection of universal primer sequences may help to resolve primer dimerization during the first cycles of the dual-phase MME-PCR scheme (Figures 4 and 5), significantly simplifying the analytical process and reducing overall assay times.

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