Development of a Universal Probe for Electronic Microarray and Its Application in Characterization of the *Staphylococcus aureus* *polC* Gene

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Electronic microarray technology is an exceptionally accurate and effective technique for detecting and defining single nucleotide polymorphisms (SNPs) in DNA sequences. Target oligonucleotides are electronically addressed to a gel matrix containing streptavidin to which biotinylated polymerase chain reaction (PCR) amplicons are bound. Typically, a fluorescent-labeled reporter oligonucleotide specific for each locus of interest is hybridized and reported. We detail the development of a universal reporter system to replace the standard method that is used to detect many different sequences accurately. The universal reporter eliminates the need to synthesize specific labeled reporters for each SNP sequence thereby dramatically reducing the cost and time required for assay development. The feasibility of this approach was demonstrated by successfully analyzing eight SNPs distributed within a highly variable 1-kb region of the *polC* gene from six isolates of *Staphylococcus aureus*. (J Mol Diagn 2003, 5:28–33)

Molecular analysis of single nucleotide polymorphisms (SNPs) provides a convenient and cost-effective method for assessing individual genetic variation. For example, studies of the human genome have shown SNPs to be the most common form of human sequence variation, occurring on average every 1000 to 2000 bases.¹ Extensive genomic sequencing projects in bacteria are providing researchers with similar information on SNPs in chromosomal regions related to taxonomy, evolutionary genetics, epidemiology, pathogenicity, drug resistance, and biodegradation.² SNPs are well-suited to automated, high-throughput genotyping and are unlikely to undergo additional mutation, making them very useful tools for genetic analysis. While sequencing is currently the foundation for such analysis, these applications could all benefit from technology compatible with automation, decreased development time, and reduction of cost.

Microarrays are powerful tools for rapid and highly accurate analysis of large numbers of different DNA molecules. Microarray techniques provide simple graphic

interpretation of the data which is uncomplicated by extraneous sequences and can easily be databased and networked. Because DNA microarrays are still in the developmental stage, the full range of possible applications has yet to be realized. However, microarray technology can be generally divided into two categories: discovery or detection. For discovery, the most common approaches involve querying large numbers of data points using glass slides or GeneChips. Conversely, detection arrays relate more to individual SNP analysis that requires fewer data points. For this latter application, electronic microarray technology has been used recently in the study of human SNPs of clinical significance.^{3–5}

We used the electronic microarray technology for our study using the NanoChip Molecular Biology Workstation. The instrument is fully automated and uses a proprietary semiconductor microchip for the rapid concentration of negatively charged biomolecules through the application of a positive charge at selected test sites, followed by passive hybridization of SNP-specific Cy5- and Cy3-labeled oligonucleotide reporters. Thermal stringency is used to denature the mismatched reporter/target complex allowing for the discrimination between mutant, heterozygote, and wild-type samples. Although the technology provides an open platform for flexibility in the assay design, the expense involved in developing applications using the standard method of reporting is prohibitive for many laboratories since it requires specific fluorescent-labeled oligonucleotide probes for the wild-type and mutant sequences of interest. These probes are purchased at a cost of approximately \$150 to \$200 each and require about 1 week to obtain from a vendor. During assay development and optimization, many probes are often evaluated. Thus, to minimize cost, the assays are often performed in a step-wise fashion to avoid the expense of ordering several candidate probes simultaneously.

We developed a universal reporting system that eliminates the need to purchase large numbers of expensive-

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labeled reporters for SNP analysis. Sequence comparisons of *Staphylococcus aureus* (*S. aureus*) *polC*, the gene encoding DNA polymerase III, in GenBank (eg, accession numbers: D86727, AB053353, and Z48003) revealed a significant amount of heterogeneity, especially localized in a 927-bp region near the 3' end of the gene. With this region as a target, we demonstrate the ability of a universal reporter to detect any sequence of interest using an electronic microarray method with potentially widespread applicability for SNP analysis.

Materials and Methods

Initial procedures were performed as described in research application notes published by Nanogen, Inc., San Diego, CA.⁶

Materials

Expand Long Template PCR System was obtained from Roche Molecular Biochemicals, Indianapolis, IN. Bio-Spin chromatography columns used for desalting were obtained from BioRad Laboratories, Hercules, CA. NanoChip cartridges were obtained from Nanogen, Inc., San Diego, CA. Oligonucleotides were prepared by Invitrogen Life Technologies, Carlsbad, CA.

Genomic DNA was extracted using a phenol/chloroform extraction method⁷ and all PCR reactions were amplified in a 100- μ l reaction containing 0.5 mmol/L of each primer, 1.75 mmol/L MgCl₂, 200 μ mol/L of each deoxynucleoside triphosphate, 400 to 600 ng of template, and 1 unit of *Taq* polymerase. PCR cycling conditions included an initial at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute for 30 cycles.

The Pol2700F (5'-GTAGCGACAATGACTGAGA-3') and Pol3600R (5'-TATCATCAGACAACCAAT-3') primers were used for amplification and initial sequencing of a 927-bp internal region of the *polC* gene. Products for electronic microarray analysis were amplified with primer pairs Pol2700F, Pol3000R (5'-TGGAAATCAAATGTGTCGTC-3') and Pol3000F (5'-GACGACACATTTTGATTCCA-3'), Pol3600R generating 544- and 383-bp fragments, respectively. For array analysis, primers were biotinylated at the 5' end for PCR amplification of the desired target strand. The amplicons were desalted using Bio-Spin chromatography columns that had been previously re-equilibrated with water to assure subsequent optimal electronic manipulation.

Loading the NanoChip Cartridge

A heterozygous control sample was prepared by mixing equimolar amounts of amplicons derived from a known wild-type and mutant, as determined by optical density. Desalted amplicons, heterozygous control, and stabilizer oligonucleotides were transferred into a 96-well plate. The loader was programmed to electronically address each desalted amplicon to a specific pad on the cartridge, where they were rapidly concentrated and the biotinylated 5' end was covalently bound to streptavidin

in the permeation layer. The double-stranded amplicons were then denatured with a 0.1 N NaOH wash, followed by selective addressing of stabilizer oligonucleotides.

Sample Detection

The assay was initially optimized with the standard method of reporting. Subsequently, the assays were tested using the universal reporting system. Standard reporters were passively hybridized and detection was performed as an automated function on the reader of the NanoChip System using differences in hybridization energies of two fluorescent-labeled, allele-specific reporter oligonucleotides for each SNP (Table 1). The difference in hybridization energy between the matched and mismatched reporters was enhanced by a stabilizer oligonucleotide (Figure 1, Table 1) providing base-stacking interaction to improve discrimination. The cartridge was heated to a discriminatory temperature, washed, returned to 24°C, and scanned using a two-laser system. Data were analyzed and samples with a signal:noise ratio of >5:1 were classified as either wild-type or homozygous mutant for each SNP. Following reporting, the cartridge was regenerated by stripping the stabilizer/reporter complex by washing with 0.1 N NaOH as indicated in the reference method.⁶ The cartridge was then sequentially reported for analysis of additional SNPs. Sample detection with the universal reporter system followed a similar method once the universal reporter sequences were determined.

Generation of the Universal Reporter Sequence and Discriminator Oligonucleotides

To create the universal reporters, a random sequence was selected (consisting of 18 bases at roughly equal frequencies) and subjected to the BLAST database^{8,9} to assure that it did not contain known, naturally occurring bacterial sequences. This sequence was synthesized and labeled with Cy3 dye. Similarly, a second unique sequence was synthesized and labeled with Cy5 dye. Complementary sequences were determined and synthesized as "tails" on discriminator oligonucleotides (Figure 1), which also contained complementary sequences to the SNP and wild-type regions of the amplicons to be analyzed. The reporter and discriminator sequences (summarized in Table 1) were initially mixed together to allow hybridization of the reporter to the "tail" sequence of the discriminator. This complex was then placed over the array for passive hybridization to the amplicon, followed by analysis as described above. In several instances, degenerative stabilizers and discriminators were required for SNP analysis. These degenerative oligonucleotides were designed to be composed of an equimolar ratio of two possible nucleotides at one or more sites within the oligonucleotide.

Results

A 927-bp region of the *polC* gene from a group of six *S. aureus* isolates was amplified and sequenced using prim-

Table 1. Universal Reporter System Sequence Information*

SNP	Oligonucleotide	Sequence	Tm
114F	Discriminator C	ctgagtcggaacattgag AGGATCAGGATTC	36.6
	Discriminator T	gcagtatacgcttgaca AGGATCAGGATTT	35.7
	Stabilizer	GATTTACCTGATAAGACGTGTGAAACTTGTGGAGC	64
324R	Discriminator G	gcagtatacgcttgaca TTTTCAGCAACG	35.9
	Discriminator A	ctgagtcggaacattgag CTTTTCAGCAACA	37
	Stabilizer	GTACCAATTGTACCTGCACGGAATACT	60.5
393F	Discriminator C	gcagtatacgcttgaca TCCACAAAAGAGGC	44.1
	Discriminator T	ctgagtcggaacattgag ATCCACAAAAGAGGT	43.4
	Stabilizer	GCTGAAATAGATCGACTCGTTAAAGGATG	59.2
411F	Stabilizer	GCTGAAATAGATCGACTTGTAAAGGATG	57.9
	Discriminator T	gcagtatacgcttgaca TAGATCGACTT	26.3
	Discriminator C	ctgagtcggaacattgag TAGATCGACTC	27.6
648R	Stabilizer	GTAAAGGATGTACAGGTGTAAACGTACAACCTGG	62.5
	Discriminator C	ctgagtcggaacattgag TTTTGGATCAATC	32.2
	Discriminator G	gcagtatacgcttgaca TTTTGGATCAATG	32.6
759F	Stabilizer	CCCGATAAATCTTGAAGCATACGAATCATT	59.5
	Stabilizer	CCTGATAAATCTTGAAGCATACGAATCATT	57.8
	Discriminator G	gcagtatacgcttgaca TTATGTAAACAGGTACG	44.5
828F	Discriminator A	ctgagtcggaacattgag TTTATGTAAACAGGTACA	44.5
	Stabilizer	TTTGGGGTACCAGAATTCGGTACAG	60.3
	Stabilizer	TTTGGGGTTCAGAAATTCGGTACAG	60.7
861R	Discriminator T	gcagtatacgcttgaca ACAACATTTTCT	28.7
	Discriminator A	ctgagtcggaacattgag ACAACATTTTCA	29.3
	Stabilizer	GAATTAGTTCAAATCTCAGGATTATCTCATGG	57.7
861R	Discriminator A	gcagtatacgcttgaca CACACATCTGTA	32.1
	Discriminator C	ctgagtcggaacattgag CACACATCTGTC	30
	Stabilizer	CCATGAGATAATCCTGAGATTGAACTAATT	57.2
	TAIL-1	CTGAGTCCGAACATTGAG	
	TAIL-2	GCAGTATATCGCTTGACA	
	UNIV-Reporter-1	CTCAATGTTCCGACTCAG	
	UNIV-Reporter-2	TGTCAAGCGATATACTGC	

*Discriminator and stabilizer oligonucleotide sequences with their discrimination and melting temperatures are shown for the eight SNPs tested. Discriminators contained either tail sequence 1 or 2 (depicted in bold lower case and also shown separately) complementary to universal reporters 1 or 2, respectively.

ers Pol2700 and Pol3600. Within these sequences we identified eight individual SNPs to analyze using the NanoChip System. For array analysis, the 927-bp region of the *po/C* gene was amplified into two separate PCR amplicons (544 and 383 bp) to avoid assay complications typical with very large amplicons. The 544-bp fragment contained SNPs at positions 114, 324, 393, and 411 (Figure 2). The 383-bp fragment contained SNPs at positions 648, 759, 828, and 861 (Figure 2). To demonstrate

that these sequences were suitable for array analysis, several SNP sites were successfully detected by the standard method of reporting as illustrated in Figure 2.

The universal reporter approach was then used to analyze eight SNPs in six different MRSA strains. For each sample a signal:noise ratio of greater than 5:1 was obtained allowing for proper designation of the nucleotide present at each SNP. Figure 3 shows typical data output using the NanoChip System for *po/C* SNP 411,

Figure 1. Standard SNP and universal reporter probe design. The standard method of SNP reporting using the NanoChip System (A) is comprised of a reporter and stabilizer oligonucleotide both complementary to the gene sequence. The universal reporter system (B) consists of the discriminator sequence (a binding sequence complementary to the gene, and a tail sequence unrelated to the gene) and stabilizer oligonucleotide. The universal reporter oligonucleotide is complementary to the tail sequence.

5' **ttttagcgacaatgactgagattactgaagtaaaccggtaccgccacactatattgtccgaactgtaaacgagtgaattttcaatgat**
ggttcagtaggatcaggattT/Cgatttacctgataagacgtgtgaaactgtggagcgcacttattaaagaaggacaagatattccgtttg
 SNP 114
aaaaatttttaggatttaaG/Aggagataaagttcctgatatcgacttaaactttagtggtgaatatcaaccgaatgcccataactacacaaaa
gtattatttggtgaggataaagtattccgtgcaggtacaattggtacT/CgttgctgaaaagactgcttttggttatgttaaaggT/Ctatttga
 SNP 324
 SNP 393 binding sequence T
 ATCCACAAAAGAGGT
 SNP 411
 atgatcaaggT/CaTCCACAAAAGAGGC**gctgaaatagatcgactC/Tgttaaaggatgtacaggtgttaaagctacaactgg**
 SNP 393 binding sequence C
 SNP 393 stabilizer
acagcatccaggG/Aggtattattgtagtacctgattacatggatatttatgattttacgccgatacaataatcctgccgatgatcaaaattcagc
atggatgacgacacattttgatttccattctattcatgataatgtattaaaacttgataacttggacacgatgatccaacaatgattcgtatgcttc
aagatttataA/GggA/C/GattgatccaaaaA/CcA/GatactgtagatgaT/CaaagaagtT/CatgcaG/AatA/Ttttagt
 SNP 648
acacctgaaagtttG/AggtgttactgaagatgaaattttatgtaaacagggtacA/GtttggggtA/TccgaattcggacaggG/Attcg
 SNP 759
tcgtcaaatgtagaagatacaaaagccaacaacattttT/AgaattagttcaaatctcaggattatctcatggT/Gacagatgtgtggttag
 SNP 828
 SNP 861
gC/Taatgtcaagaattaattaaaaccggtatatgtgatttatcaagtgttaattggtgtcgtgatgatcat 3'

Gray: PCR Primers
 Bold: Stabilizer Sequence
 SMALL CAPS: Binding Sequence
 Bold Caps: SNP Locations

Figure 2. The *polC* gene sequence. The *polC* sequence illustrating the relationship of PCR primers, binding sequence, and stabilizer oligonucleotides (binding and stabilizer sequences for SNP 393 are specifically detailed, while the remaining seven SNP locations are shown in bold). A tail sequence, unrelated to the bacterial genome, is conjugated to the 5' end of each binding sequence. The universal reporter sequence is complementary to the tail sequence.

which is representative of data for all of the SNPs analyzed. For a variety of reasons (eg, initial sample concentration, differences in loading efficiency, etc) the concentration of the DNA addressed varied somewhat from sample to sample resulting in differences in the fluorescence intensity. Nevertheless, the results obtained through the universal reporter approach were totally concordant with those from direct DNA sequencing. Table 2 summarizes these data for the eight SNPs which were analyzed in the six *S. aureus* strains.

To determine the optimal melting temperature (T_m) for the binding piece of the universal reporters, we evaluated

discriminator pairs with values ranging from 26.3°C to 48.5°C. While all discriminator pairs were suitable for analysis, those with T_m values above 30°C yielded more consistent results since they were less subject to changes in ambient temperature. For example, discriminators with T_m values below 30°C often resulted in denaturation before application of any stringency. We also found it important to ensure that the first nucleotide in the tail sequence had no complementary to the corresponding nucleotide on the amplicon. When such situations existed in early experiments, we found that the mismatch discriminator remained bound to the sample at the dis-

Figure 3. The *poC* SNP 411 genotyping results. NanoChip System genotyping results for SNP 411 are listed. Results are representative of data obtained for all SNPs tested using the NanoChip System. The x-axis lists strain numbers and a constructed heterozygote (Het389) used to standardize fluorescence.

crimination temperature, resulting in improper calls (data not shown). The initial sequencing of *poC* revealed that the nucleotide at position 648 could be G, C, or A. The ability of specific discriminators to accurately detect this SNP variation was evidenced by the loss of both G and C fluorescence signals at the discrimination temperature with appropriate amplicons.

Analysis of the sequences around specific SNPs of interest (eg, 393, 648, and 759) revealed additional SNPs nearby. Our results indicated that even a single mismatch within the stabilizer-binding site could result in an inability to obtain proper calls. However, when these additional SNPs were incorporated into the stabilizer (ie, an equimolar mixture of stabilizers with both possible sequences) all isolates were properly analyzed. Finally, multiplexing for multiple SNP reporting was performed through alkaline denaturation to remove the initial discriminator/reporter complex from the PCR amplicon followed by hybridization of the second reporting complex. We observed that secondary reporting resulted in a sufficient amount of signal to obtain correct calls. This process was repeated until all SNPs on an amplicon had been analyzed.

Discussion

The rapid progress currently being made in the sequencing of microbial genomes portends an increasing number of potential sequence-based microbiological applications in the years ahead.^{10–13} Such analysis may well require

the detection of multiple SNPs which could benefit from an electronic microarray approach. The universal reporter system described here has the potential to facilitate an economically feasible approach to the use of electronic microarrays in such analysis. Once important SNPs are identified, appropriate universal reporters can be quickly synthesized and arranged in a variety of configurations resulting in rapid throughput screening of the region of interest. The individual discriminator oligonucleotides used in the universal reporting method were also readily synthesized and were much less expensive than their conventional counterparts since no extra conjugations were necessary. With the universal reporter method, only one labeled oligonucleotide each for wild-type and mutant sequence must be synthesized along with several inexpensive, unlabeled discriminator oligonucleotides, thereby speeding up assay development and optimization while greatly reducing the cost. We anticipate that use of this system will result in an approximate 10-fold decrease in method development costs and an approximate fivefold decrease in assay development time.

Several important aspects of reporter, discriminator, and stabilizer design became apparent with our initial design and analysis of the assay components. In order for the concept to be useful, the universal reporter and the discriminator “tail” must have a unique sequence not found in nature, to eliminate the possibility of non-specific binding. The sequences that we describe contained no significant homology to bacterial genomes as determined by BLAST^{8,9} analysis. In considering the design of specific discriminators, it was clear that the universal reporter and tail sequences must have a greater affinity for each other (ie, higher *T_m* value) than that for binding sequence and amplicon interaction. These parameters were achieved in this study, since reporters remained bound to discriminator tails at the temperatures required for stringent probing of the array (ie, greater than 30°C). In addition, no interference related to amplicon binding or secondary structure within the reporter was observed. The ability to perform analysis on larger amplicons such as those examined here helps to eliminate the need to generate multiple PCR products. While the NanoChip System has been demonstrated to detect SNPs on a 1-kb PCR product, the assay is optimized for analysis of products 100 to 200 bp in size. The obvious concern in analyzing large amplicons is the elimination and inhibition

Table 2. Sequencing/NanoChip System Genotyping Summary*

Strain	SNP															
	114F		324R		393F		411F		648F [†]		759F		828F		861R	
	Seq	Arr	Seq	Arr	Seq	Arr	Seq	Arr	Seq	Arr	Seq	Arr	Seq	Arr	Seq	Arr
SA1	T	T	T	T	T	T	C	C	A		A	A	A	A	T	T
SA6	C	C	T	T	C	C	T	T	G	G	G	G	A	A	G	G
SA10	C	C	T	T	C	C	T	T	G	G	G	G	A	A	G	G
SA13	T	T	T	T	T	T	C	C	A		A	A	A	A	T	T
SA19	C	C	T	T	T	T	C	C	G	G	A	A	T	T	G	G
SA20	C	C	C	C	C	C	C	C	C	C	G	G	A	A	T	T

*For each SNP, results are shown for sequencing and the array analysis.

[†]No entry represents no fluorescence signal indicative of the presence of an alternative nucleotide. SNP 648 contains three possible nucleotides (A, C, or G) the first of which was not being assessed with universal reporters.

of secondary structure. Thus, the system utilizes a low salt environment to decrease the formation of secondary structures following alkaline denaturation. However, we found that analysis could be performed on a 383- and 544-bp PCR product of the *po/C* gene using both traditional and universal reporting approaches. In addition, following optimization, several SNPs were successfully analyzed on the entire 927-bp fragment with either reporting method (data not shown).

Unfortunately, in heterogeneous sequences not all SNPs occur at a convenient distance to avoid interfering with the binding of the various assay components. In the *po/C* gene, we observed several instances of closely spaced SNPs. Three of the SNPs investigated had an adjacent SNP that we designed to fall within the stabilizer binding sites. We found that proper analysis of such heterogeneous sequences in all isolates could be obtained if the stabilizers were synthesized with degeneracy at the "secondary" SNP site, using an equal concentration of both possible stabilizer sequences.

In some instances genotyping may involve situations where more than a single mutant type must be detected. In our study, sequencing of SNP648 detected a G, C, or A in the isolates examined. However, we found that such situations could be analyzed by the detection of one fluorescence signal or by the loss of both fluorescence signals following thermal stringency.

Overall, these results demonstrate the ability of the universal reporting system to facilitate the development of electronic microarray analysis for the detection of large numbers of SNPs. In addition, we determined that this assay is capable of dealing with issues typical of heterogeneous sequences, such as closely spaced SNPs. Thus, the decrease in development cost and the flexibility derived from the use of universal reporters make the NanoChip electronic microarray technology an attractive platform for potential use in a variety of future genotyping applications.

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