



## Counterproliferation with Advanced Microarray Technology

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**I**n response to perceived terrorist biological threats within the United States, counterproliferation efforts have increased steadily in recent years. A variety of advanced technologies can be used to detect unknown organisms that may be potentially harmful to humans and/or the environment. However, first responders need more rapid, reliable, and accurate detection devices than are now available to detect harmful pathogens. The MAGICChip (Micro Arrays of Gel Immobilized Compounds on a Chip), a microarray-based assay that can detect and identify thousands of biological species in a matter of hours, has the capability of being one such technology.

### INTRODUCTION

In today's society there is an increasing, yet unfortunate, need for technologies that can accurately and rapidly detect the presence of bacterial organisms that might be used by terrorists. These bacteria have been identified as *select agents* and, according to the Centers for Disease Control, have "the potential to pose a severe threat to public health and safety." If there is a possible threat to human lives and safety, quick and accurate identification of these potentially harmful organisms is essential to determine possible postexposure prophylaxis methods. A determination would need to be made as to whether the incident is an actual biological event or a hoax. Classical bacterial identification methods that are routinely used in the laboratory are very accurate, but they often take several hours or days to complete.

Polymerase chain reaction (PCR) is a technique that is frequently used to identify bacteria. Although PCR

can be performed in a relatively short time, the chief limitation of the technique is that specific primers are needed to increase copies of the target organism so that enough material is available for detection. Without some foreknowledge of the target organism and associated specific primers needed, unknown organisms cannot be detected quickly or reliably.

Other factors contribute to the need for development of new devices that can quickly and easily identify unknown bacterial organisms. Success in using traditional identification methods is generally based on use by trained, experienced laboratory staff and technicians in a sterile laboratory setting. Most biological incidents are likely to take place in the field where untrained first responders will not have the requisite training to use complicated devices and may not be able to handle the biological material in such a manner as to avoid contamination. The net result would be unreliable results.

Multiple, large instruments are sometimes required for biological assays (e.g., incubators, thermal cyclers). In addition to the instruments being bulky and difficult to transport into the field, many of the reagents used with these instruments are perishable or are stable only at certain temperatures and under certain conditions.

Given the limitations of current biological detection devices, alternative identification methods need to be developed that provide accurate, timely results, can be easily transported by first responders to the site of a potential exposure, and are easy to use. The use of microarray analysis is a novel approach to bacterial identification that ameliorates many of these issues. Once a microarray has been designed for several biological strains, the cost to replicate the array is rather inexpensive. In addition, with such arrays, assay time is short and use is relatively uncomplicated, allowing first responders to employ the device with minimal training.

## THE CHALLENGES

Microarrays, which have been developed only within the last few decades, are small platforms that contain many different biological macromolecules logically arranged or “arrayed.”<sup>1</sup> The macromolecules used in arrays can be proteins, sugars, or nucleic acids (NAs).<sup>2-4</sup> Microarrays are analyzed by binding specific portions of target organisms to the array. Target molecules are labeled, for visualization, either directly or indirectly and then captured onto the platform surface through a process known as hybridization. Preferential binding (adherence of target sequences to capture probes) is realized by site-specific association of labeled molecules to the capture probes (sequences of an NA that exactly match the NA sequences that will be hybridized onto the microchip).

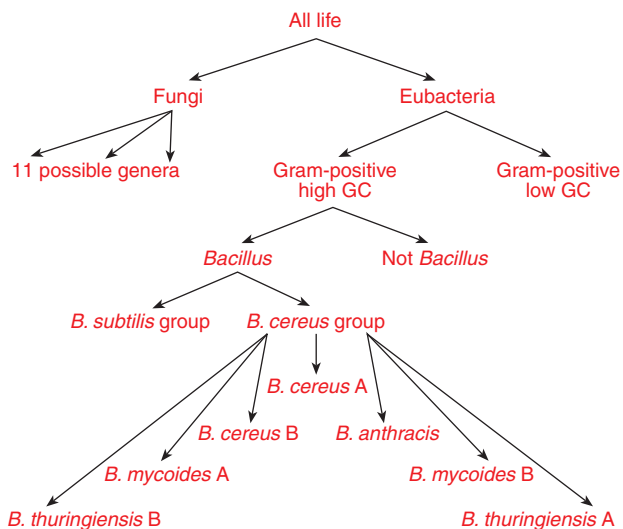
The MAGIChip (Micro Arrays of Gel Immobilized Compounds on a Chip) is a microarray-based technology developed to address the need to rapidly and accurately identify bacteria. It is able to detect and identify thousands of biological species in a matter of hours. The MAGIChip was initiated as a collaboration with Dr. David Stahl, now at the University of Washington, and Dr. Andrei Mirzabekov, formerly of Argonne National Laboratory.

The MAGIChip has been developed as an NA array using ribosomal RNA (rRNA) as the biological macromolecule target. rRNA molecules are extremely abundant, making up at least 80% of the RNA molecules found in a typical eukaryotic cell.<sup>5</sup> The rRNA is pre-amplified by bacteria and, therefore, is present in many thousands of copies per cell, making it a good target in a nonamplification-based assay. Single nucleotide variations present in canonical bacterial rRNA sequences are used to differentiate one bacterial species from another. This is a unique characteristic of the technology—that

no amplification step is required to create sufficient material to detect, in contrast to PCR. Identification using the MAGIChip can be completed in less than 2 h using only a handful of single nucleotide sequences on the chip, which can discriminate bacteria to the species level. Discrimination to the species level is important because the specific species of an organism is one of the determinants for assessing lethality. The other is the presence of toxins or virulence factors (discussed later). The potential hazard of many organisms cannot be determined unless species information is available. A sample phylogenetic tree<sup>6,7</sup> is shown in Fig. 1.

The process for detecting bacteria using the MAGIChip is relatively straightforward, minimizing the use of sophisticated laboratory techniques that may be more difficult for the naïve user. Bacteria are taken from a culture, washed, and pelleted. The pellet is then lysed using lysozyme, an enzyme that destroys the cell wall, allowing the release of NA. The lysed bacteria are then added to a preparation column where the NA from the cells remains immobilized while cell debris and soluble contaminating materials are removed. All the processes that take place after lysis (i.e., isolation, purification, fragmentation, and labeling of target rRNA) occur by chemical, rather than enzymatic, reactions. The use of stable chemicals reduces the logistics burden needed to transport other sorts of enzyme-based assays.

This purified NA then undergoes fragmentation and labeling simultaneously<sup>8</sup> through the use of chemical reactivity. Fragmentation into small, <500-base-pair, sequences allows the NA to be more easily hybridized onto the chip because smaller pieces of NA permeate more easily through the gel matrix. The gel matrix is the second unique feature of the MAGIChip. Unlike other



**Figure 1.** Phylogenetic tree: a schematic of hierarchical recognition on MAGIChip. Hierarchical-based identification strategy provides an internal consistency check. Probes are phylogenetically ordered by domain, kingdom, phylum, class, order, family, genus, and species (GC = guanine/cytosine base pair).

microarrays formed directly onto glass slides or into slides coated with a slab of gel, the MAGICChip platform is made of gel pads. The importance of this is discussed later. As the NA is cut into smaller pieces, free ends are created that are chemically labeled with the fluorescent probe. Once the NA is labeled and fragmented, it is eluted off the column and ready to be hybridized onto the chip. The total amount of all NA (DNA and RNA) eluted off of the chip is determined by ultraviolet spectrophotometry; then a determined amount is mixed with a hybridization buffer and put onto the chip for 1 h. The result of hybridization and data interpretation is visualized nearly simultaneously. Automated algorithms interpret the image and determine the identity of the microorganisms in less than 2 min. This, in turn, results in identification of an organism in less than 2 h (Fig. 2).

### UNIQUE MICROARRAY TECHNOLOGY

MAGICChips are three-dimensional (3-D) DNA microarrays.<sup>9</sup> Typical microarrays are printed on the flat surface of a glass slide that is usually treated with a specific compound upon which the capture probes adhere. MAGICChip microarrays are formed from oligonucleotide-impregnated gel pads, increasing the surface for hybridization by 50 times. The 3-D nature of this microarray allows more surface area of the capture probe to be exposed to the target sequence, with a probe density of more than 10<sup>12</sup> molecules per gel pad (Fig. 3).

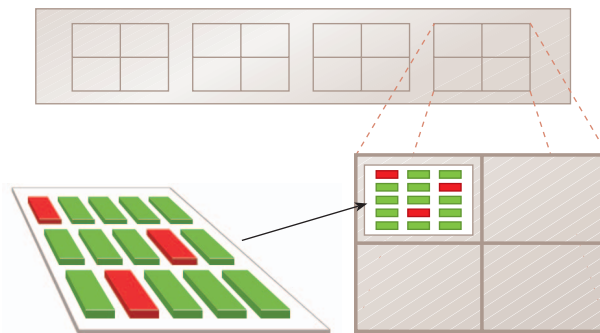


Figure 3. MAGICChip slide depicting the 3-D probes.

Specific probes are designed to recognize unique rRNA sequences and are placed into individual pads on the chip. The small size of the 3-D gel pad allows a high-density array to be deposited onto a chip. More than 2700 probes can be loaded onto the MAGICChip.

Considering the highly conserved nature of rRNA from species to species, using single base-pair mismatches in the design of the capture probes allows discriminating results requiring species-specific identification. Probes, which distinguish organisms using hierarchical recognition and are phylogenetically ordered by domain, kingdom, phylum, class, order, family, genus, and species, are loaded onto the chip. This hierarchical-based identification strategy provides an internal consistency check within the automated analysis program. Identification of *Bacillus* species is distinguished by hybridization to fewer than 100 probes; so, by virtue of available “real estate” on the chip, multiple probes for the same target species can be designed and analyzed simultaneously. Having multiple probes for the same target also increases the confidence of identification.

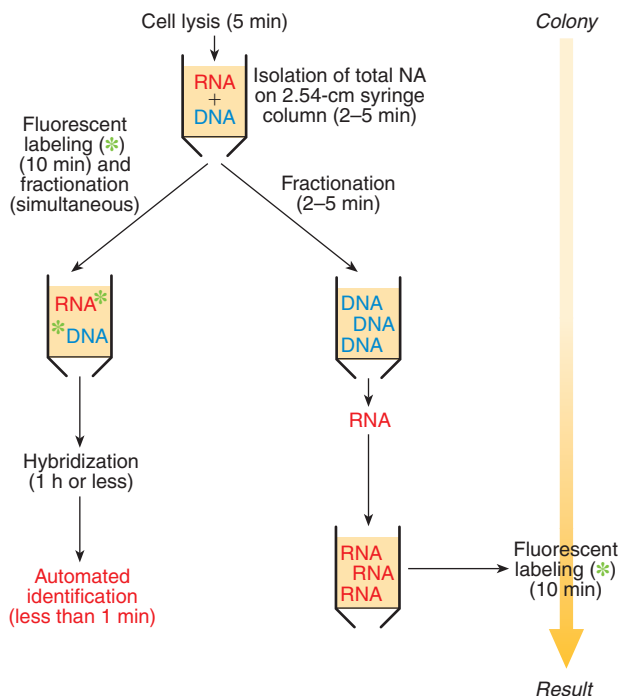


Figure 2. Isolation, fractionation, and labeling of nucleic acids on mini-columns.

### USE IN THE FIELD

MAGICChip technology is currently being tested by users to eliminate unnecessary processes that do not contribute to the sensitivity of the assay. Elimination of such processes would give the same result in less time. The reagents are also being tested for stability over time and temperatures. Optimized reagents for this technology to be used in the field would have a long shelf life and no required storage temperature. The microarray reader designed for these chips has been created to be portable. It is lightweight (under 2.7 kg) and is run by a standard laptop computer or minicomputer. User confidence controls are also being established to be incorporated into the protocol. As mentioned previously, the steps occurring in this procedure are cell lysis, fragmentation, and labeling of the NA. A variety of color metric control methods can be incorporated into the protocol to give the user the confidence that the assay has been performed correctly at each of these procedural steps.

## CURRENT APPLICATIONS

Currently the MAGICChip is being used to evaluate probe sequences designed for specific identification of various organisms. The sequences are being evaluated by methods designed specifically for MAGICChip as well as by harmonization with current PCR methods. The use of PCR in current applications aids in validating the probes used for detection on the chip.

Expanded applications include detection of virulence factors. Unlike rRNA, virulence factors, which include toxins or proteins that increase the invasive potential of an organism or its toxin, have a small number of copy transcripts. Also unlike rRNA, they are produced only under the specific conditions found in the host. Hence, identification strategies usually focus on the single-copy DNA sequence.

Current PCR technology provides a platform on which we can build to create amplicons specific to the probes on the chip. (Amplicons are segments of NA in large copy numbers that can be generated to complement a probe that is tested on a microarray.) Although bacterial genus and species can be identified without PCR, confirmation of lethal strains by microarrays requires the use of PCR probes. MAGICChip technology harmonizes with available PCR applications by directing users in the selection of PCR primers for specific amplification. Once generated, PCR amplicons would allow many probe validation experiments to be carried out without wasting reagents. Methods being tested are incorporation of fluorescent labels into PCR reactions and generation of PCR amplicons using prelabeled PCR primers, which also generate labeled PCR amplicons, for hybridization onto the chip. Various hybridization methods are also being evaluated for use with PCR amplicons. The use of PCR-generated labeled amplicons for hybridization allows generous amounts of probes to be produced that are specific to those being evaluated on the chip.

In addition to its role in detection of virulence, the MAGICChip can also be used to speed the selection of PCR primers and targets. Testing various specific probes using PCR can help determine the optimal size of probes used for hybridization. Probe specification is critical for determination of target-specific hybridizations. Cross reactivity of nonspecific probe binding can lead to the misidentification of an organism. Thus, using PCR for its specific nature is useful in validating probes while designing a chip. It is, however, not the intention to use PCR to detect organisms because of the limitations stated previously.

## TOOLS AND ANALYSIS

A large portion of the MAGICChip testing and evaluation is captured in a database and analysis application that helps provide performance evaluation, *in silico* experimentation, and hardware and software quality

control. The database application was developed to provide the traditional database collection, storage, and retrieval tasks for MAGICChip experimental data and to assist in probe design, assay evaluation, and component performance analysis.

At the lowest level, analysis is conducted by querying the database. Researchers and domain experts familiar with the types of data captured in the database application can formulate (or select) queries that provide insight into the performance of the assay. Analysis may also be conducted by using the statistical analysis tools and techniques currently available on the MAGICChip portable reader (developed by Argonne National Laboratory). Experienced users of the database application may apply the statistical analysis techniques to stored data or to the results from recently submitted queries. At the highest level, analysis is conducted by encapsulating domain expertise into more complex high-level queries. In some cases these high-level queries are constructed from a sequence of basic queries organized together by the domain expert to simulate the method by which a researcher might investigate the experimental data to draw some conclusions about the performance of the technology. In other cases the high-level queries may be constructed from data processing software that manipulates the stored data.

The MAGICChip database and analysis application consists of several components. To handle the traditional task of data collection, a secure Web-based data entry user interface was developed. To handle the traditional data storage and retrieval tasks, a secure relational database was also created. These two components give the system a Web-enabled, centrally located, secure infrastructure with distributed access to handle data from multiple research activities in different locations. The other components include a user-friendly analysis application that incorporates the analysis by query and a suite of software tools to accomplish some of the more complex querying of the system. The analysis application has been developed to be flexible and usable by researchers and domain experts and may prove to be quite useful in assessing the capabilities and potential of the MAGICChip.

## FUTURE USES

The potential for the use of MAGICChips encompasses a broad spectrum of applications in a variety of scientific settings. They have the potential to be developed to identify a broad spectrum of pathogenic organisms, target genes necessary for the development of drugs, and diseases in plants and animals. Some of these future endeavors may include protein MAGICChips, clinical diagnostics, bacterial forensics, viral identification, drug customization, pharmaco-genomics, and agricultural applications.

One valuable aspect of the MAGIChip is the single-nucleotide polymorphisms (SNPs) detection method it uses. Because bacterial DNA is highly conserved over time, SNPs are useful tools in bacterial identification methods, and since SNPs are the most abundant variations in the human genome, they have become the primary markers for genetic studies for mapping and identifying susceptible genes for complex diseases.<sup>10</sup>

Drug development can also be targeted by finding genes on this potentially high-capacity microarray platform. The expression patterns of many genes or gene products can be measured and analyzed in parallel on a single chip, helping to advance the discovery of potential gene targets for drug development.<sup>11</sup>

The structure and function of proteins are very different from those of DNA, but protein microarrays are exciting because a variety of proteins are contained in a single cell and they could all be researched in one array platform. Protein arrays are unique in that they have the potential to discover protein biomarkers that could indicate disease or different stages of a particular disease.<sup>12</sup> They can also aid in the study of the relationship between protein structure and function and could identify the amount or function of a protein or different proteins across the same or different cell types.<sup>12</sup> So, although the material being analyzed (proteins and DNA) is quite different, the applications and technique are quite standard.

Clinically, rapid detection time, high throughput, result confidence, hierarchical identification, and quantification are only some of the advantages of using this array as a diagnostic tool. The time required from sample collection to reporting of results in a clinical setting (the turnaround time) is crucial in the treatment of a variety of ailments. This technology, in its current state, can give results from sample to identification in less than 2 h. With collaborators, APL is working to reduce this time to less than 1 h. This rapid turnaround time is an attractive attribute of point-of-care testing that can be done as a patient awaits results.

High throughput is valuable because the real estate on the chip allows thousands of probes for microbial-, species-, or even strain-specific identification. One sample can test for an abundance of organisms at the same time, eliminating the need for multiple tests to identify a single organism (as is done in classical determination of microbiological organisms) and reducing the amount of sample needed to conduct multiple tests.

Every clinical assay requires a control, or set of controls, to determine the validity of the experiment and to give confidence in the results. "Within assay" controls are being developed and evaluated for use in the MAGIChip assay. The use of multiple probes for a single target is one control that is currently being implemented; it is a significant way to increase the reliability of the results.

Hierarchical determination will help identify organisms that do not have species-specific targets on the chip that is being tested but are included in the family-, order-, and perhaps genus-level identifications. This determination would allow a broad range of information to be captured on the organism in question, allowing specific research and analysis to be conducted based on the information gathered taxonomically.

Quantification could also be determined using the MAGIChip by identifying not only single organisms but a profusion of microbes that compose a variety of communities. This would be useful in background screening or baseline testing for newly acquired infections or even in screening for drug metabolism.

Because the potential to use a variety of bacteria, viruses, and fungi as biological weapons poses health risks to humans, agriculture, and the environment, future technologies must be developed with the capacity for accurate and sensitive detection. Bacterial forensics, viral identification, and agricultural applications are all prospective endeavors for development of this chip. MAGIChip arrays have been developed for discrimination of orthopox, and other important viruses and fungal probes have been recently introduced into rRNA MAGIChip arrays. Most agricultural pathogens are either viral or fungal.<sup>11</sup> A variety of agricultural aspects can be explored using the MAGIChip. Plant genetics, reproduction, and diseases, as well as crop protection, can be researched using the genetically diverse capabilities of the MAGIChip. Thousands of genes can be targeted simultaneously to look for genetic diversity or for microbial infestation by nature or by intentional release.

Budowle et al.<sup>13</sup> state, "Microbial forensics can be defined as a scientific discipline dedicated to analyzing evidence from a bioterrorism act, bio-crime, or inadvertent microorganism/toxin release for attribution purposes." As a response to bioterrorism, the Scientific Working Group on Microbial Genetics and Forensics (SWGMP) was established in July 2002 by the FBI to develop guidelines related to the operation of microbial forensics. Because the national government is implementing an infrastructure to lead the investigations of such a nature, the need for tools to assist in the detection is imminent.

The MAGIChip is a technology that has the possibility of being a major player in the counterproliferation efforts of this country. It has the capacity to detect a variety of unknown, potentially harmful organisms in a short period of time and is being transitioned as a portable detection device that can be used by first-response personnel.

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