DNA microarrays – their mode of action and possible applications in molecular diagnostics

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Introduction

DNA microarrays offer the latest technological advancement for multi-gene detection and diagnostics. They were conceived originally to examine gene expression for large numbers of genes [8, 18, 24], but have also been applied to DNA sequence analysis [22], immunology [14], genotyping and diagnostics [6, 10, 27]. In the latter context, they can be used to distinguish between DNA sequences that differ by as little as a single nucleotide polymorphism (SNP) [26]. In addition, their flexibility and high throughput capabilities hold tremendous potential for pathogen detection, identification, and genotyping in molecular diagnostic laboratories. Nevertheless, an important factor in the employment of any technique is cost. High-density arrays may be very valuable in disease diagnosis [1], but at US $500 to US $1,000 per commercial array they are unlikely to see routine use in veterinary medicine. Fortunately, there are a number of low-cost alternatives now available both for array fabrication and detection chemistries.

The purpose of this review is to outline the basic principles of microarray construction and use. At the same time emphasis will be placed on low-cost methods for producing and using low-density arrays suitable for eukaryotic, prokaryotic, and viral diagnostics. Most examples will highlight genotyping arrays used to detect the presence of specific genes derived from genomic DNA. Many of these methods are also applicable to low-density expression arrays.

Microarray principles

DNA microarrays are typically composed of DNA "probes" that are bound to a solid substrate such as glass (Figure 1). Each spot (50 to 150 μm) in the array lattice is composed of many identical probes that are complementary to the gene of interest. During hybridisation DNA "targets" diffuse passively across the glass surface, when sequences complementary to a probe will anneal and form a DNA duplex. Hybridised targets can then be detected using one of many reporter molecule systems. In essence, a microarray is a reverse dot-blot that employs the same principles of hybridisation and detection used for many years with membrane-bound nucleic acids (e.g. Southern and Northern blots).

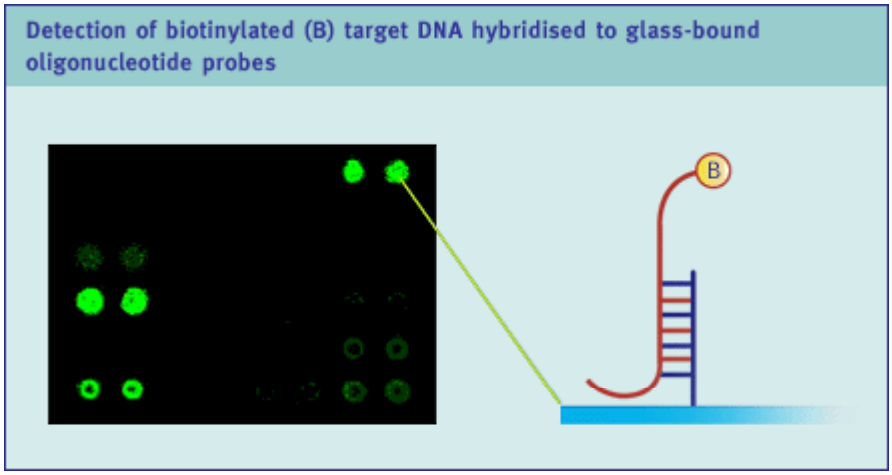

Figure 1. Detection of biotinylated (B) target DNA hybridised to fluorescent-labelled oligonucleotide probes bound to a glass slide. Individual spots are just discernible within the fluorescent circles and represent many identical oligonucleotide probes hybridised to the target DNA, which was labelled first with a single biotin molecule (B).

Probes and array fabrication

Probes used for genotyping microarrays are either polymerase chain reaction (PCR) products from cloned genes or oligonucleotide DNA. PCR products made from cDNA clones are useful for detecting the presence or absence of genes, but their lengths (300 to 1000 base pairs) make it difficult to discriminate between minor sequence mismatches, which are important for molecular diagnostics. Oligonucleotide probes, on the other hand, are typically much shorter (9- to 50-mer) and are usually modified to incorporate an amine or thiol linker that permits covalent attachment of the oligonucleotide to a coated glass surface [2, 3, 7, 9]. The modification of probes adds considerable expense to array construction, but in practice is not necessary for probe attachment [5]. For instance, unmodified oligonucleotide probes can be suspended in alkaline buffer (pH 12) and deposited mechanically onto acid-washed slides; they adhere to the slide surface via hydrogen bonds and electrostatic attraction and are then available to form duplexes with complementary strands of target DNA. This attachment scheme is robust across a wide range of pH (1 to 10), temperature (4°C to 95°C) and ionic buffers (e.g. 0 to 4 M NaCl). The sensitivity of detection can be enhanced if acid-washed slides are coated with epoxy-silane before probe deposition [5].

The quality of the glass slides used to make arrays can also affect the success of microarray application [23]. Poor quality slides have uneven surfaces and may auto-fluoresce, thereby producing background signal that interferes with spot detection and quantification. Auto-fluorescence can be particularly problematic when signal intensity is low, as is the case with expression arrays. To avoid these problems, high quality, commercially prepared slides are available – albeit at a premium. As an alternative, standard Teflon-masked slides (Erie Scientific Co., Portsmouth, NH, USA) have been used successfully for low-density genotyping arrays, and a unique array can be printed in each well. This provides a convenient format for the simultaneous screening of multiple samples or replicates on a single slide (Figure 2). There are several techniques available for the mechanical deposition of oligonucleotides and other probes onto solid substrates [23], although the associated hardware can be expensive. Exceptions do exist, however, and include manually operated array spotters and low volume robotic spotters (TeleChem International Inc., Sunnyvale, CA, USA).

Figure 2. A Teflon-coated slide is used to process multiple samples during hybridisation studies. Each of the slide's 12 wells contains an independent, low-density microarray. Some hybridisations may be carried out at low temperatures (4°C to 42°C) in a simple, humidified chamber, as shown.

Target labelling and detection systems

For genotyping arrays, targets may be either PCR products, into which reporter molecules (e.g. biotin and Cy-3) can be incorporated directly via 5-prime primer modifications, or genomic DNA. When targets are present in high molar concentrations (e.g. PCR reaction) the strategy of using a single reporter per target works well. Less abundant targets, however, may require incorporation of multiple reporter molecules or signal augmentation using enzymatic amplification. Multiple reporter molecules can be incorporated into genomic DNA using nick translation, random priming, or chemical incorporation. Regardless of the system being used, detection of hybridised targets is usually by fluorescent scanner, or by phosphoimager for isotopic reporters and membrane-based arrays. Many combinations of fluorescent dyes and conjugates are suitable for detecting hybridised targets on microarrays (Table 1).

Examples of reporter systems used to detect hybridised targets

Target label	Secondary conjugate	Detection
Haptens Biotin Digoxigenin (DIG) Dinitrophenyl (DNP)	Streptavidin or antibiotides conjugated to alkaline phosphatase, horseradish peroxidase or fluorescent dyes	Enzyme-labelled fluorescence, tyramide signal amplification or direct fluorescence
Fluorescent dyes* AlexaFluor 546 Cy-3 and Cy-5 Phoerythrin	None	Direct fluorescence

*Specifications of alternative fluorescent dyes are provided in references
NB. Choice of reporter system will depend in part on the type of scanner available for imaging

Table 1. Reporter systems used for detecting hybridised targets on microarrays.

ELF-97 signal generation. A fluorescent signal can be generated for hybridised targets using several enzyme-based systems. One approach is to use "Enzyme-Labelled Fluorescence" (ELF-97; Molecular Probes, Eugene, OR, USA) [5, 20], for which a biotinylated target is hybridised to its complementary probe on the array. Non-specific targets are then removed by washing, and streptavidin conjugated to alkaline phosphatase is added followed by soluble ELF-97 substrate. Alkaline phosphatase cleaves ELF-97 producing a fluorescent, insoluble crystal that adheres to the slide surface (Figure 3). The crystals are excited using UV light (350 nm) and finally emissions are collected with a wide band pass filter (>520 nm). The ELF-97 system is very inexpensive (pennies per array) and is especially ideal for low resolution imaging systems, such as the gel documentation imagers commonly found in molecular laboratories. Using this type of equipment, ELF-97 is suitable for detecting 5-prime biotinylated PCR products when more than 10^9 target copies are present [5, 6]. Unless the system is optimised, however, ELF-97 can produce significant background fluorescence that is easily detected with high-resolution scanners. This probably occurs when 3-dimensional 'piles' of crystals accumulate on the glass surface as substrate cleavage proceeds. Subsequent slide processing can easily scatter the crystals across the slide surface. Low-resolution scanners are generally insensitive to this type of background, but scanners designed specifically for microarrays can easily detect these wayward crystals.

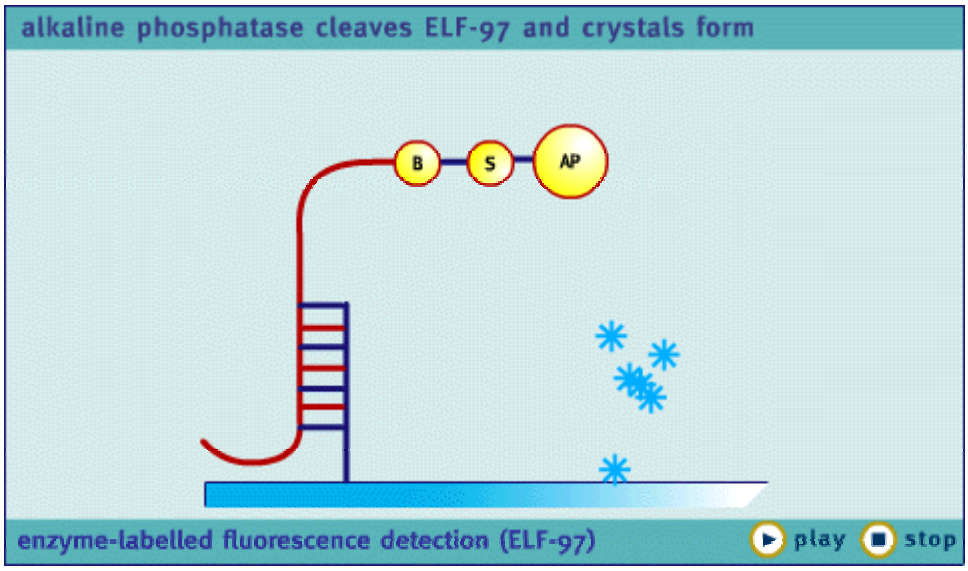

Figure 3. Animation demonstrating the technique of enzyme-labelled fluorescence detection (ELF-97). First, a biotinylated target (B) anneals to the oligonucleotide probe bound to the surface of a glass slide. Non-specific targets are removed from the reaction using several washing steps before streptavidin conjugated to alkaline phosphatase (S-AP) is added, followed by soluble ELF substrate. The alkaline phosphatase cleaves the ELF-97 to produce insoluble, fluorescent crystals that are deposited on the surface of the slide. ELF-97 crystals are detected using a BioRad Fluor-S MultiImager. The photograph below the animation shows array spots detected using this method. The dark spots represent hybridisations of a biotinylated oligonucleotide (bottom row) and a PCR product (top row).

Fluorescent dyes. There are many fluorescent dyes available for direct incorporation into target DNA via enzymatic modification or chemical incorporation [23]. Cy-3 and Cy-5 (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) are two dyes widely used for expression array experiments, even though they are relatively expensive compared with many equally suitable alternatives. The Alexa dye series (Molecular Probes) is particularly useful for diagnostic work. For example, AlexaFluor 546 has nearly identical excitation and emission spectra as Cy-3, but has a higher signal intensity and is less prone to photo-bleaching [21]. At a very low cost per reaction, streptavidin conjugated to AlexaFluor 546 can be used for the detection of biotinyated PCR products without the background problems of ELF-97, but with a similar degree of sensitivity (Figure 4).

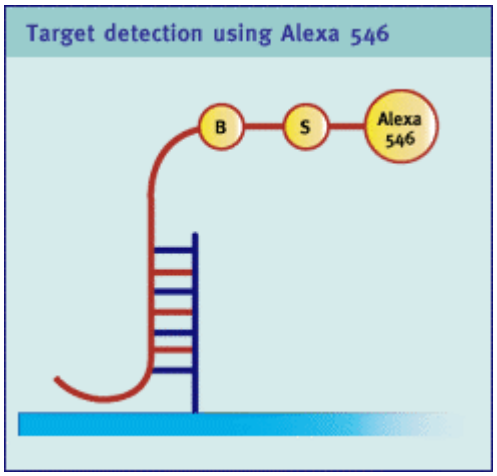

Figure 4. Illustration of target detection using AlexaFluor 546. A biotinylated target (B) anneals to its complementary array probe. After non-specific targets are removed, streptavidin conjugated to AlexaFluor 546 is added for direct detection using a fluorescent scanner.

Tyramide signal amplification. A particularly exciting development for target detection is the adoption of immunohistochemistry systems that use tyramide signal amplification (TSA) [13]. The process of hybridisation occurs as in other examples, except that streptavidin is conjugated to horseradish peroxidase (HRP) (Figure 5). HRP catalyses proximal deposition of soluble tyramide conjugates onto the slide surface. When the slide surface has been pre-coated with a protein (e.g. equine serum albumin), the tyramide is covalently bound via dimers with tyrosine residues at the protein surface. The soluble form of tyramide can be conjugated to many different fluorescent dyes or haptens, such as biotin or dinitrophenol (DNP) [15]. AlexaFluor 546 can be coupled with biotin-tyramide conjugates to enhance signal strength from scarce target hybridisations (Figure 5). TSA has been commercially adapted for microarrays (PerkinElmer, Boston, MA, USA), but it is relatively easy to adapt TSA immunohistochemistry kits for diagnostic applications. The adaptation is more expensive than other alternatives described above, but still costs below US $2.00 per slide (using eight arrays per Teflon-masked slide) and the detection sensitivity is dramatically improved compared to direct labelling methods.

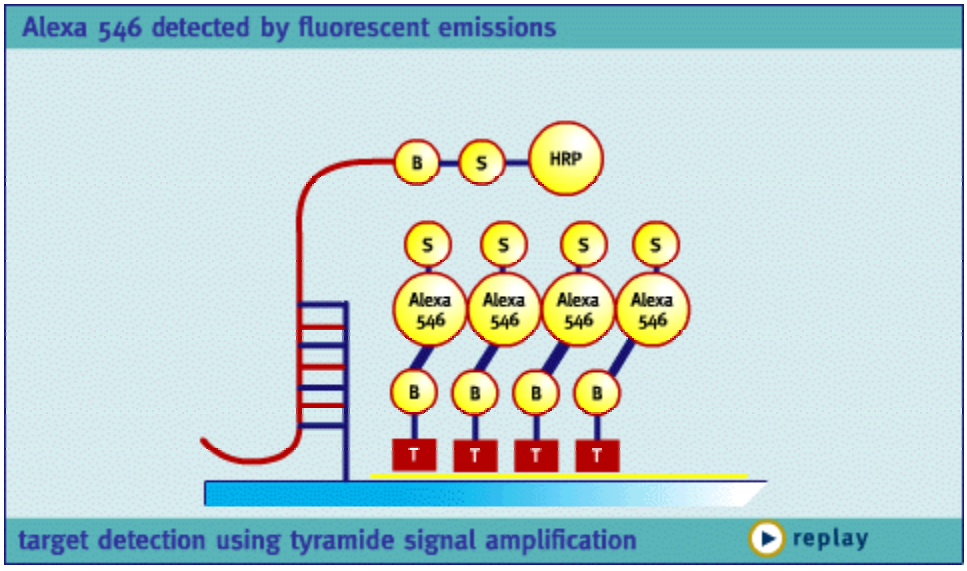

Figure 5. Animation demonstrating target detection using tyramide signal amplification. After targets hybridise to the oligonucleotide probe, non-specific targets are removed and a layer of protein (albumin) is deposited onto the slide surface. Streptavidin (S) conjugated to horseradish peroxidase (HRP) is added to the reaction, followed by soluble tyramide (T) conjugated to biotin (B). HRP causes proximal deposition of tyramide-biotin conjugate at the slide surface, which is then detected using streptavidin conjugated to AlexaFluor 546 (A).

When TSA is used in the manner depicted in Figure 5, there is nearly a three-fold increase in signal intensity and more than a 100-fold increase in signal sensitivity ($< 10^7$ copies for a biotinylated PCR product) when compared to a direct detection system using AlexaFluor 546 (Figure 6a). Importantly, this degree of sensitivity permits detection of single copy genes without the need for PCR. For example, genomic DNA from *E. coli* O157:H7 and *E. coli* O91:H2 was extracted and nick-translated in the presence of biotin-dATP. After hybridisation to a simple array, the positive control probes were visible for both samples (Figure 6b, lower row of spots), but the gene-specific probe for the *eae*A locus (intimin) was only visible for the O157:H7 DNA. Although this result has been demonstrated before, it had required PCR to generate sufficient copies for detection using ELF-97 and a low-resolution scanner [6]. TSA makes it possible to probe quickly for many genes without relying on multiplex PCR. There are other amplification strategies emerging that may prove more sensitive while remaining cost effective for molecular diagnostic laboratories [25].

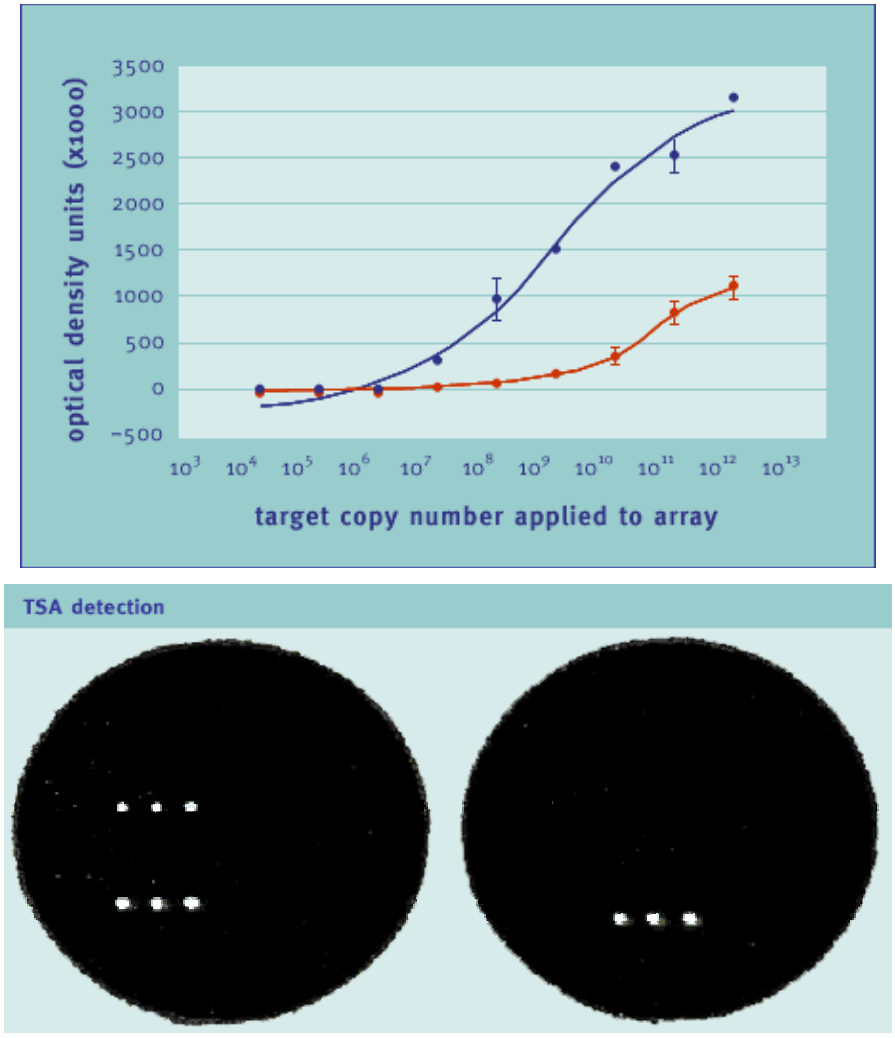

Figure 6. A graph reveals that detection of biotinylated PCR products using tyramide signal amplification (TSA; solid circles) produces a 3-fold greater signal intensity and more than 100-fold improvement in detection sensitivity compared with a direct detection system (open circles). The photograph in the lower portion of the figure illustrates TSA detection of a chromosomally encoded *eae*A gene from *E. coli* O157:H7 genomic DNA (top row of three spots in left hand well). The lower row of spots represents a positive control oligonucleotide. The right hand well is a negative control for *E. coli* O91:H2.

Hybridisation and detection

Once targets are labelled with reporter molecules (e.g. biotin or Cy-3) they are hybridised to the array in a salt buffer. Experimental conditions can be varied (temperature and salt concentration) to achieve the desired degree of specificity for a given set of probes. For PCR targets, array hybridisation plus detection can be completed in less than two hours and, depending on the assay characteristics, may be carried out at room temperature [5]. Imaging arrays can be accomplished using relatively low-resolution gel documentation systems when ELF-97 substrate is used to detect hybridised targets. More sensitive laser or charge coupled devise (CCD)-based systems are probably required for the majority of fluorescent dyes. This equipment probably represents the biggest financial obstacle for the incorporation of microarrays into routine veterinary diagnostics.

Potential applications in veterinary diagnostics

DNA microarrays can be used to detect multiple pathogens based on differences in 16S rDNA sequences. For example, nucleic acids can be extracted from a sample and 16S rDNA sequences amplified by PCR using universal 16S primers [11]. The resulting PCR products can be hybridised to an array consisting of many oligonucleotide probes,

which can be designed to detect and characterize pathogens by taxonomy (e.g. Gram type) [17], by genus, or by species if sufficient discriminatory sequences are available. McCabe *et al.* [19] have constructed a similar pathogen detection array in a membrane-based, macroarray format that could be adapted to microarrays. This type of PCR genotyping is ideal where multiple sequence differences are known to exist between conserved primer regions. For example, the author's laboratory is collaborating in an effort to construct an array suitable for genotyping multiple haplotypes of the bovine MHC genes. Detection of pathogens can include other specific markers such as intimin, Shiga-like toxins and haemolysin A from *E. coli* O157:H7 [6]. Indeed, most multiplex PCR assays can be adapted for rapid detection on microarrays, although it is advantageous to limit total product length to less than 150 bp in order to enhance PCR and hybridisation efficiency. Under ideal conditions, it may be possible to multiplex ten or more PCR primer pairs for detection on a microarray. Clearly, probe sequences must be identified a priori before these types of arrays can be constructed.

PCR-based target preparation is necessary when testing heterogenous samples or cultures, or when characterizing fastidious organisms. With the introduction of TSA, it is now feasible to detect genes when target DNA is not a limiting factor. For example, the author's laboratory is collaborating in an effort to construct an array for detecting and characterising tetracycline resistance genes without PCR. For this system, one microgram of extracted genomic DNA is labelled with biotin using nick translation. The labelled target is then hybridised to an oligonucleotide array with probes specific for sixteen different tetracycline resistance genes. Using TSA it is possible to detect both chromosomal and plasmid-borne resistance genes (Call, unpublished data). In theory, it should be possible to construct a much larger array suitable for detecting large numbers of resistance genes while simultaneously providing information about gene identity – a feature that is particularly useful for studies of the molecular epidemiology of antibiotic resistance. It may be possible to streamline this assay so that the entire process (genomic extraction to imaged array) can be completed within eight hours, rather than the typical 24 to 48 hours required for phenotypic resistance assays.

Other potential applications of microarrays are the fingerprinting of bacterial isolates using very short, random hexamers [16] or by adapting expression arrays, and the identification of microbes by direct detection of 16S rRNA [12]. These methods are also suitable for expression arrays. If a number of diagnostic RNA markers are identified for specific disease conditions [1], then more cost effective, low-density arrays could be designed for use in diagnostic laboratories. The methods described here could also be adapted for use with protein arrays [4]. These could be useful for detecting diagnostic proteins in diseased tissue, or could potentially be used to develop an array suitable for serotyping bacteria. *Salmonella enterica*, for example, has over 2,400 recognized serotypes; a serotyping array could in a single reaction simultaneously screen for all known serotypes. In short, imagination is currently the only factor limiting the number of array applications that could be developed and employed in a molecular diagnostic laboratory.

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