

Detection of CFTR mutations using ARMS and low-density microarrays

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Abstract

The amplification refractory mutation system (ARMS) is routinely used for the identification of specific mutations within genomes. This PCR-based assay, although simple, is performed at a low-throughput scale, usually requiring gel-electrophoresis for the identification of specific mutations. We have applied the ARMS technology to a low-density microarray system to facilitate the needs of the medical clinic; high-throughput capabilities and ease-of-use. Mutations within the cystic fibrosis transmembrane regulator (CFTR) gene ($\Delta F508$, 1717–1G>A, G542X, 621+1G>T, and N1303K) were detected by multiplex-ARMS-PCR, and fragments were post-PCR labeled with Cy5. Amine-modified probes specific for both the wild-type and mutant forms of each mutation site were attached to glass substrates. Following hybridization of the PCR fragments to the attached probes (in a low-density microarray format), confirmation of the presence of specific sequences was achieved using a commercial scanner, as well as a fabricated low-cost fluorescent detector and applicable software. The novel combination of the ARMS and low-density microarray technologies allows for a high-throughput, simple means to rapidly identify multiple known mutations for many genetic diseases including cystic fibrosis.

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1. Introduction

The capacity to detect mutations in genes involved in genetic disorders is critical for an accurate diagnosis to be achieved. Once the exact mutations are known, a variety of methods can be used to identify their presence in patients, i.e. restriction fragment length polymorphisms (RFLPs), allele specific oligonucleotides (ASOs), oligo-ligation assays (OLAs), quenched-fluorophore systems, and the amplification refractory mutation system (ARMS). ARMS can detect the presence of point mutations or small deletions in nucleic acids, and has been well established for detecting mutations in the cystic fibrosis gene cystic fibrosis trans-

membrane regulator (CFTR) (Newton et al., 1989; Ferrie et al., 1992). ARMS primers are designed so that the 3' end of one of the primers contains the exact complement with the mismatch, and will only extend if the mismatch is present. If the mutation is not present, no fragment is made. The same can be done with a wild-type primer, allowing only amplification if the wild-type form is present. By specifically altering the orientation of each primer set (e.g. wild-type primers amplifying a sense fragment of a certain length, mutant primers amplifying an antisense fragment of a different length), the genotype of a patient can be determined by gel-electrophoresis. However, when analyzing large sets of mutations in a clinical setting, gel-based assays are not desirable due to their low-throughput and lack of automation. Attempts have been made to make ARMS detection more automated, such as modifications for colorimetric assays, ELISA-based

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detection systems, fluorescence polarization, real-time PCR using fluorescent probes or dyes, and computerized gel imaging software (Haque et al., 1998; Gibson et al., 1997a, 1997b; Whitcombe et al., 1998; Thorpe and Porteous, 1999; O'Dell et al., 2000; Bartlett et al., 2001). However, none are routinely used in the clinical diagnostic setting, primarily due to the cost of required equipment and availability of trained personnel.

Microarrays have been used for a wide variety of applications, including gene expression studies and single nucleotide polymorphism (SNP) detection for drug discovery applications. Oligonucleotides or cDNA sequences are attached to substrates (such as glass or nylon), and through the hybridization of the amplified target to the substrate, the identification of the presence of specific sequences within an unknown sample can be determined. This represents a form of the reverse dot-blot assay, and the hybridization event can be detected through chemiluminescence, fluorescence, radioactivity or electrical conductivity. Although the original force behind microarrays was in screening for gene regulation variations between two separate samples, the true power of this technology is in the identification of gene subgroups which can be used in a panel format for grouping individuals into categories. Gene expression studies within childhood leukemia's will allow for specific drug regimens to be selected based on the patients own genetic markers (Yagi et al., 2003; Ross et al., 2003; Cheok et al., 2003). For example, patients in group A (grouped by the expression levels of specific genes) may respond well to drug X while patients in group B (a separate level of gene expression) will not respond well. Therefore, possible clinical outcomes can be assessed before the drug is even given. Microarrays can be produced at a large scale, and multiple glass slides can be hybridized and processed in a single day.

Cystic fibrosis (CF) is an autosomal recessive disease that affects 1 in 2500 Caucasians in the United States. Over 1200 mutations within the CFTR gene have been discovered to either cause or represent polymorphic markers for CF in humans, and mutational heterogeneity exists with respect to certain ethnicities (see the Cystic Fibrosis Mutation Database, <http://www.genet.sickkids.on.ca/cftr/>). The CFTR protein is a member of the ABC (ATP-binding cassette) family of transporters, functioning as a chloride channel in the plasma membrane of epithelial cells (for review of diagnosis and mutation testing, see (Kant et al., 1995; Richards et al., 2002). The current average survival age of CF patients is 25, most succumbing to respiratory infections or heart failure. An accurate diagnosis of CF is critical for early detection so that treatment can be initiated. CF diagnosis also plays a role in genetic counseling for partners considering parenthood.

We have combined (1) the capability of ARMS to amplify PCR fragments only in the presence of specific cystic fibrosis mutations, with (2) the hybridization efficiency of low-density microarrays. This combination provides a high-throughput and simple system that requires minimal hands-

on intervention and the removal of size-specific discrimination using gel electrophoresis. The convergence from a high-density microarray (spot sizes ranging from 50–250 μm diameter) to a low-density microarray system (multiple spots providing an area of 1 mm in diameter), along with a cost-effective fluorescent detector, provides more efficient fluorescence detection and a minimization of spotting variability and noise from background fluorescence.

2. Materials and methods

2.1. Low-density microarray production, probes and DNA samples

Amine-labeled oligos (purchased from IDT, Coralville, IA, and resuspended in water) were spotted at 10^{-11} mol/ μl onto epoxysilane-treated slides (Erie Scientific, Portsmouth, NH) and incubated at 80 °C for 1 h for attachment. Eighteen carbon spacers were placed between each sequence (increasing hybridization efficiency) and the amine group, leaving the amine-labeled end of the probe to be tethered to the surface (Southern et al., 1999). Spotting was performed on an ArrayIt SpotBot (TeleChem, Sunnyvale, CA). The low-density microarray format was designed so that each "spot" contained 21 individual spots (0.2 mm in diameter) within a 1.0 mm area. This increased area allows for the optimal signal detection range for each diode on the RapidReaderTM detector (a 5 × 5 array of 1 mm photodiodes). Control and mutant genomic DNA was obtained from Coriell Cell Repositories (Camden, NJ).

2.2. ARMS

ARMS primers in this work were previously described (Ferrie et al., 1992). Each reaction contained the following: 0.5 μl of AmpliTaq Gold (2.5 U, Applied Biosystems, Foster City, CA), 2.5 μl of 10X PCR buffer (containing 1.5 mM MgCl_2), 1 μl dNTP's (final concentration of each nucleotide was 200 μM), 1 μl of each primer (100 ng per 25 μl reaction volume), and 200 ng of genomic template. Multiplexed PCR conditions were as follows: 10 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C; 10 min at 72 °C. Each reaction was verified on a 2% agarose gel.

2.3. Fluorescent labeling and hybridization

PCR fragments were fluorescently labeled using the LabelIt Cy5 kit as per the manufacturer's protocol (Mirus Corp., Madison, WI). Fragments were purified using Microspin S-400 columns (Amersham Biosciences, Piscataway, NJ). Slides were pre-hybridized/blocked in 5X SSC (Fisher Scientific), 0.01% Tween-20 (Fisher Scientific), 1% bovine serum albumin (Sigma, St. Louis, MO) and 0.115 mg/ml herring sperm DNA (Promega, Madison, WI) for 30 min at room temperature. Samples were then placed on the

slide in the above solution and hybridized for 15 min at 70 °C. Amine-labeled oligos containing Cy5 on the 3'-end were used for positive control hybridization detection. Following hybridization, slides were washed in: (1) 5X SSC, 0.01% Tween-20, and (2) 0.1X SSC for 4 min each at room temperature. The slides were then spin-dried, and placed in a commercial scanner (GenTAC, Ann Arbor, MI) and the HealthSpex RapidReader™ for fluorescence detection.

2.4. Fluorescence detection device

The Healthspex RapidReader™ contains a 635 nm laser source (Stocker Yale), filters (Omega Optical) and the Healthspex Integrated Optical Sensor (IOS). The IOS (Fig. 3A) contains a 5 × 5 array of photodiodes and processing channels. It was created in a 0.6 μm CMOS technology and fabricated by X-Fab, a semiconductor foundry in Erfurt Germany (Vo-Dinh et al., 1999). The IOS chip is cooled to 5 °C to reduce photodiode leakage current. Future versions of the system may be cooled even further. The cooling is achieved by using a Peltier module, with its hot surface tied to a water-cooled heat exchanger. Also, the diodes are run with near-zero bias, as speed of response is not an issue for this application. This also reduces leakage current.

There are 25 diode channels on the HealthSpex IOS, implemented as a 5 × 5 array of 1-mm diodes on a 1.5-mm grid, allowing for 25 simultaneous spot readings. Each diode channel includes an ultra-low-leakage (on the order of one femto-ampere) integrator, a window comparator, and a reset switch. The integration capacitor has a value of 10 pF, giving an output slope of 0.1 mV/s per fA of photo current. The present IOS has two sets of outputs per channel to the data system: pulsed outputs from the CTF (current to frequency) system, and analog ramp outputs from the integrators. At high light levels the CTF has a fairly linear output up to about 50 kHz, equivalent to about 10⁵ V/s, or about 1 μA of photo current. For normal fluorescence measurements, slopes of a few mV/s are measured over several time periods of 15–30 s each. All 25 channels are acquired in parallel by the data system.

The fluorescence sensitivity of the RapidReader™ is based on pure Cy5 dye studies and shows about 3.5 decades of response (Supplemental Fig. 1). The saturation at the high concentrations is due to self-absorption in the dye, as tests with a LED source have shown about an 8-decade dynamic range. The lower limit is defined by the background fluorescence and light bleed through of the system. Future improvements in the background level will require better filtering, and the identification of other sources of fluorescence, some of which may not be reducible. We have limited flexibility with the epoxysilane slide treatment used for probe attachment, though we are working to reduce other sources of scattered light. We also hope to increase the top end by improving our illumination techniques. In general the top end is above clinical concern since one can readily reduce

sample dye incorporation, at a cost of reducing the low-level response.

3. Results and discussion

3.1. Performance of ARMS

To evaluate the efficiency of the ARMS-PCR assay, genomic DNA containing known mutations was used. Using each primer set for each mutation along with mutant and wild-type DNA, the generation of bands of the correct length were determined by gel electrophoresis. Each mutation on the panel (ΔF508, 1717–1G>A, G542X, 621+1G>T, and N1303K) was tested individually (Fig. 1), and in a multiplex reaction containing all five primer pairs (Fig. 1B, lanes 8 and 9). Once the ARMS assay was tested for its accuracy, each reaction was post-PCR labeled with Cy5 so that fluorescence detection could be determined on the arrays.

3.2. Low-density microarray

Amine-labeled oligonucleotides were designed for each mutation in the panel (Table 1). The low-density microarray format was designed so that each “spot” contains 21 individual spots (0.2 mm in diameter) within a 1.0 mm area. This increased area allows for the optimal signal detection range for each diode on the RapidReader™ detector.

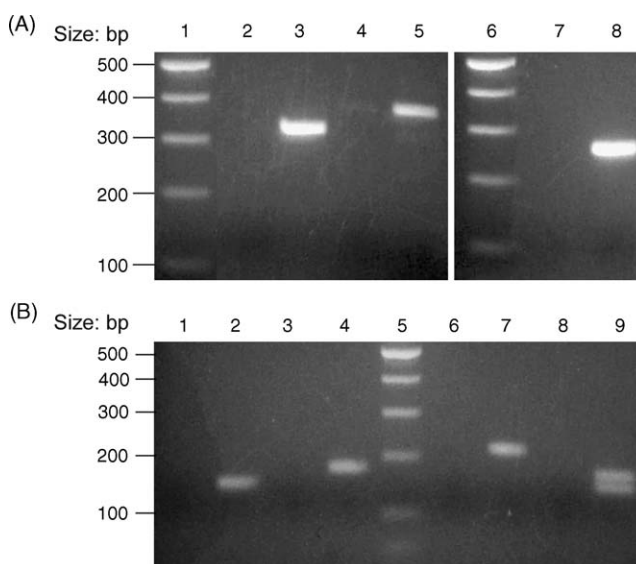


Fig. 1. Gel electrophoresis of ARMS reactions. Lanes A1, A6 and B5 contain a DNA ladder. PCR reactions containing various DNA templates to evaluate the performance of the ARMS reaction to detect the specific mutations are listed as follows: (A2) wild-type (wt); (A3) N1303K; (A4) wt; (A5) 621+1G>T; (A7) wt; (A8) G542X; (B1) wt; (B2) F508; (B3) wt; (B4) W1282X; (B6) wt; (B7) 1717–1G>A; (B8) wt; (B9) multiplex F508 and W1282X (using F508/W1282X compound heterozygous DNA template).

Table 1

Probe sequences specific for each mutation, 5'-amine-modified with C6 spacers

Mutant probe	Sequence
621+1G>T	TTT GAT TTA TAA GAA G7T AAT ACT TCC TTG CAC AGG
F508	GGC ACC ATT AAA GAA AAT ATC ATT GGT GTT TCC TA
1717–1G>A	CTA TTT TTG GTA ATA AGA CAT CTC CAA GTT TGC AG
G542X	ATA GTT CTT 7GA GAA GGT GGA ATC ACA CTG
N1303K	TAG AAA AAA GTT GGA TCC CTA TGA ACA GTG G
W1282X	TTT GCA ACA GTG AAG GAA AGC CTT T

To each array/glass slide, Cy5-labeled PCR products were hybridized to each array/glass slide, and the fluorescence measured by both a commercial scanner and an inexpensive detection device designed in-house (Fig. 2). A refer-

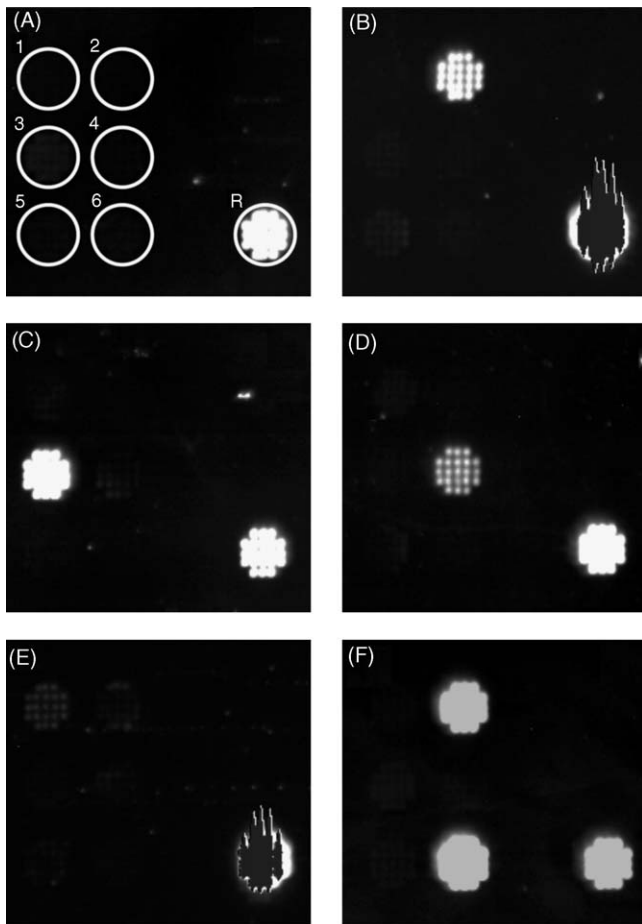


Fig. 2. Diagram of spotting orientation on the low-density microarrays. Each spot is 1 mm in diameter and contains: (1) F508; (2) 1717–1G>A; (3) N1303K; (4) 621+1G>T; (5) W1282X; (6) G542X; and (R) reference spot containing amino-linked 15-mer with a terminal Cy5 label (seen as a smear in B and E due to bleaching of the scanner). Each box represents a separate hybridization with the PCR products from ARMS reactions containing known DNA: (A) wt; (B) 1717–1G>A; (C) N1303K; (D) 621+1G>T; (E) F508; (F) G542X.

ence spot was placed on each array so that a correct orientation can be established on each slide. The reference spot contains an amino-linked 15-mer with a terminal Cy5 label. After establishing a baseline fluorescence for each slide, ARMS-products from Δ F508, 1717–1G>A, G542X, 621+1G>T, and N1303K DNA templates were hybridized, and detection on each corresponding spot was achieved, leaving the other mutant spots unbound and unlabeled. This was seen for the 1717–1G>A, N1303K, 621+1G>T, and weakly for F508. Fig. 2F shows that the ARMS product from G542X DNA bound both to the G542X probe as well as the 1717–1G>A probe. This arose due to the fact that the G542X ARMS primers overlap the 1717–1G>A region. However, the 1717–1G>A ARMS primers do not overlap the G542X sequence, and does not produce a product which binds to the G542X probe (Fig. 2B). This is important to remember when designing and multiplexing ARMS primers for PCR and in combination with microarrays.

3.3. Fluorescence detection

A commercial fluorescent scanner was used to take the images for Fig. 2. However, the cost of these detectors are not well suited for the clinical diagnostic field. We have designed a low-cost fluorescence detector (RapidReader™)

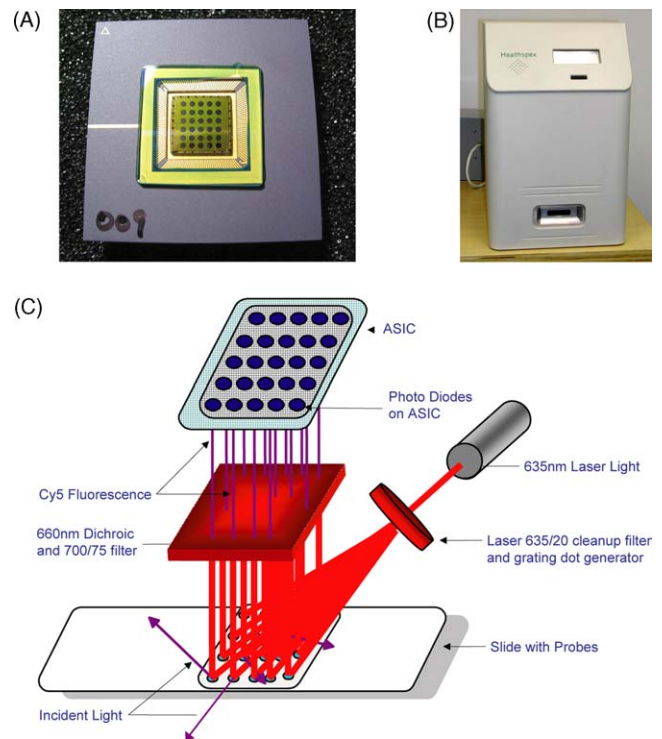


Fig. 3. Detection device for low-density microarrays. (A) The HealthSpex biosensor chip containing a 5×5 array of photodiodes. (B) The housing for the HealthSpex chip, RapidReader™, a closed device containing excitation lasers and filters. (C) A schematic of the reader including optics and laser positioning.

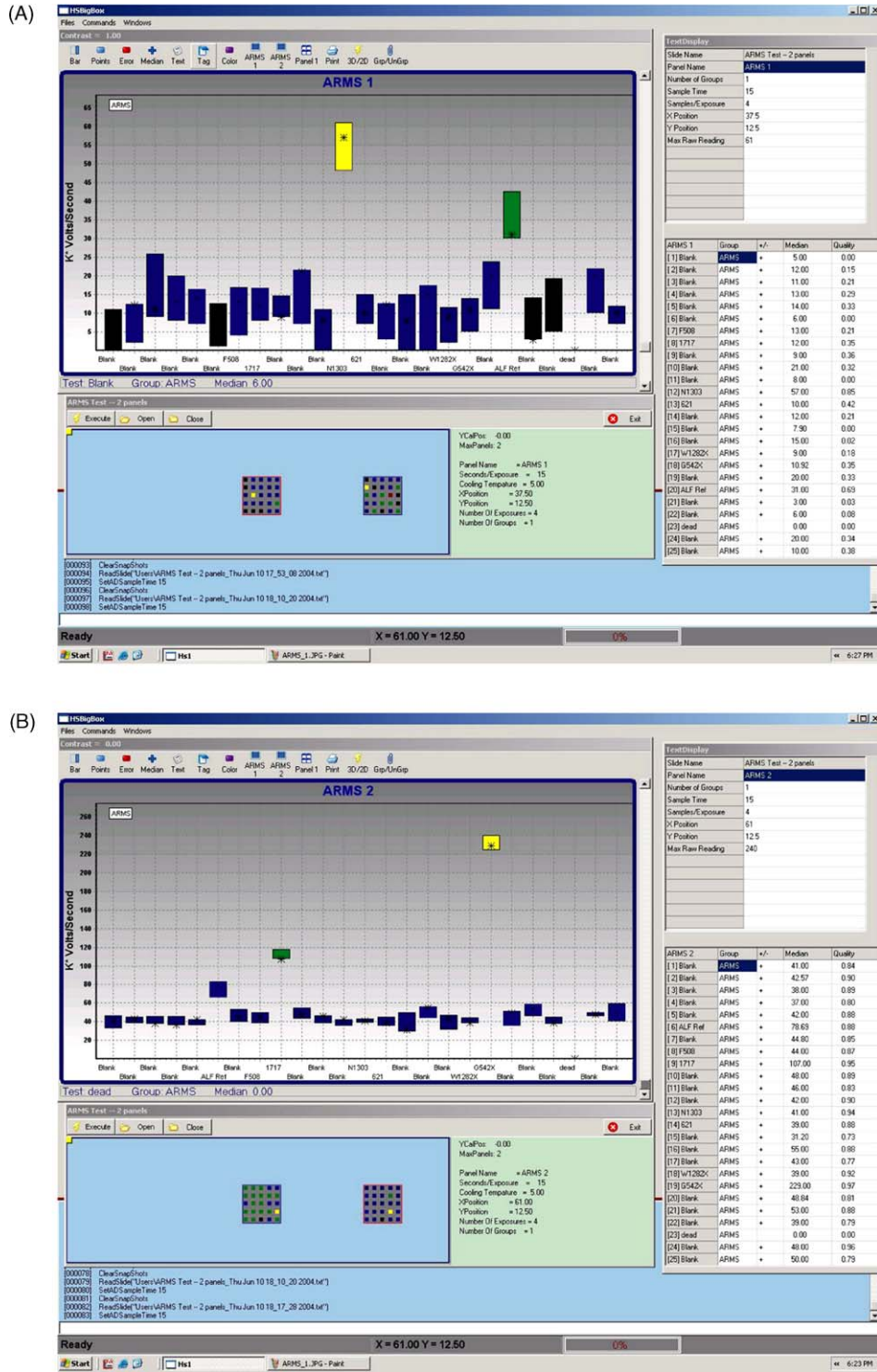


Fig. 4. Each slide is placed in the reader individually, and a readout of fluorescence intensity displayed using the HealthSpex software. (A) and (B) show the readings (screen shots) using slides from Fig. 2(A) N1303K and (B), G542X. Within each screen shot, a bar graph of each diode/mutation (x axis) and fluorescence intensity readings (y axis) can be seen. When a signal is detected, the bars (each bar averaging multiple readings) increase accordingly to the amount of signal. In (A), the green bar represents a positive control (a spotted oligo with an attached Cy5). The yellow bar represents a positive fluorescence reading for the N1303K spot (detecting hybridization). All other diodes are considered at or below background levels. Background is determined by a separate diode detecting a region containing spotting buffer alone. A readout of signals from each diode is given on the right-hand side of the screen, and a lay-out of the slide itself below the bar graph.

built around a 5×5 array of photodiodes (Fig. 3A). Each diode can be read independently of one another. The IOS is built into a fluorescence macro-scope system that controls the illumination of the sample slide by selected laser light, filters the fluorescent emission, and focuses it on the detector array. Fig. 3C shows a block diagram of the central optical system, while a picture of the present system is shown in Fig. 3B. In addition to the IOS subsystem the instrument contains a motor-controlled X–Y stage. Thus multiple 5×5 arrays can be placed in the view of the sensor, one at a time. Up to 12 array positions can be placed on a standard “1 by 3” slide, and even more with reduced margins. Thus up to 300 probes can be included, though this usually includes blanks, references, controls, and duplicates, reducing the true number of analytical measurements possible on a single slide. Data for each array are typically acquired in about one minute, or up to ten minutes for a full slide, depending on the number of arrays printed.

Software which correctly positions each slide and takes background readings was used to retrieve the generated data, and compile each slide or area of a slide into a bar graph. By correctly aligning the slide under the biosensor chip (the image of the slide is focused on the biosensor chip), a fluorescent signal for each 1 mm spot can be determined. Raw data, as well as the comparison of each diode can be displayed in real time. Fig. 4A shows a screen-shot using the N1303K slide from Fig. 2C, displaying the reference spot (yellow bar) and the single N1303K spot (green bar). Everything else resides within the background fluorescence levels. Multiple spots can be displayed as well, seen in Fig. 4B using the G542X slide from Fig. 2F (reference, blue bar; 1717–1G>A, green bar; G542X, yellow bar). These findings show that when adapted to a low-density microarray containing large areas of probes, fluorescence can be easily detected, minimizing printing variability and slide impurities.

4. Conclusions

The ARMS technology relies on the ability to amplify only specific fragments which contain the exact mismatch complementary to the one of the primers, such as in a SNP. Each primer to be used for ARMS must be rigorously tested so that false priming is not seen. Fig. 1 shows that ARMS primers can be efficiently used to detect specific mutations with the CFTR gene. The ability of these primers to be multiplexed into one reaction is also important for the clinical diagnostic setting. It is also important to note that the direction of the PCR fragments should be considered when designing probes and primers for the detection of multiple mutations which are close to each other in the genome, as overlapping ARMS products may be picked up by multiple probes (Fig. 2F). This hurdle may be achieved though the optimization of mutant probes to be attached to the arrays, binding only the mutant and not wild-type fragments.

Although PCR products were post-labeled with Cy5 in this work, the incorporation of Cy5-labeled nucleotides (or other fluorophores) into the PCR reaction can easily be achieved. When combined with ARMS, allele-specific oligonucleotides bound to solid substrates (arrays) have been shown to efficiently bind to their complementary sequences (Fig. 2), and that their fluorescence can be measured by an inexpensive detection device (Fig. 3). This allows for not only the accurate detection of fluorescence, but also the combination of specific hybridization events and allele specific PCR for a highly accurate molecular detection assay. It is important to note that most commercially available scanners can be used in this assay. We constructed our fluorescence detector so that high through-put assays could be easily designed.

ARMS detection methods can be classified into three levels: (1) One that detects that PCR has taken place (e.g. real-time PCR intercalation methods, colorimetric assays, and methods that rely on detection of a primer tail sequence either via solid capture methods or in solution), (2) one that detects that PCR has taken place and the product is the correct size (e.g. agarose gel electrophoresis and other size discrimination methods such as fluorescence polarization), and (3) one that detects that PCR has taken place and that the product contains the correct sequence (e.g. ARMS with allele specific probes in solution or in situ). For the development of a diagnostic assay, a level 2 or 3 (the method described in this work) is desired so that non-specific priming and/or primer dimers can be ruled out. It will be important to expand the number of mutations tested, add positive controls to test for PCR efficiency, and test the incorporation of labeled nucleotides to future assays (manuscript in preparation). The work above represents CF mutation screening only. For carrier screening of CF, or any other genetic disease, a separate reaction/slide would have to be performed containing wild-type ARMS primers. The combination of ARMS and low-density microarrays is a reliable means for the detection of specific mutations present within the CFTR gene, and could be easily modified for existing genetic diagnostic assays.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bios.2004.10.033](https://doi.org/10.1016/j.bios.2004.10.033).

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