Microsphere Bead Arrays and Sequence Validation of 5/7/9T Genotypes for Multiplex Screening of Cystic Fibrosis Polymorphisms

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The development of simple and rapid methods for the detection of the common genetic mutations associated with cystic fibrosis (CF) requires access to positive-control samples including the 5/7/9T variants of intron 8. We used PCR and a simple multiplex bead-array assay to identify 5/7/9T control samples from 29 commercially available DNA samples. Unpurified PCR products were directly hybridized to color-coded beads containing allele-specific capture probes for 5/7/9T detection. The performance of the assay was investigated using reverse-complement oligonucleotides, individual PCR products, and multiplex PCR products for 5/7/9T detection within a complex CFTR screening assay. Samples were genotyped by grouping the relative signal intensities from each capture probe. Of 29 commercially available DNA samples analyzed, 2 5T/7T, 2 5T/9T, 9 7T/9T, 11 7T/7T, and 5 9T/9T genotypes were identified. The genotype within each sample group was confirmed by DNA sequencing. The assay was compatible with the analysis of 10 to 1000 ng of genomic DNA isolated from whole blood and allowed for the separate identification of primary CFTR mutations from reflex variants. The correct identification of positive controls demonstrated the utility of a simple bead-array assay and provided accessible samples for assay optimization and for routine quality control in the clinical laboratory. (*J Mol Diagn* 2004, 6:348–355)

Molecular testing for inherited single-gene disorders such as cystic fibrosis (CF) has grown significantly in scope and volume fostering the need for simple and rapid detection technologies. The mutation carrier frequency for CF is approximately 1 in 29 among Caucasian North Americans, making CF the most common genetic disorder in this population.¹ CF is a life-shortening, autosomal recessive condition characterized by abnormally thick and viscous mucus, excess sweat electrolytes, poor nutritional uptake, pancreatic endocrine insufficiency, and declining pulmonary function secondary to chronic

lung infection.^{1,2,3,4} The cloning of the cystic fibrosis transmembrane regulator (CFTR) gene in 1989, provided the capability of direct DNA analysis for heterozygote carriers of cystic fibrosis.⁵ In consideration of the benefits associated with neonatal screening for cystic fibrosis,⁶ the American College of Medical Genetics (ACMG) and the American College of Obstetrics and Gynecology (ACOG) recommended carrier screening in the general population for the 25 most common mutations in the CFTR gene along with reflex testing for six additional polymorphisms.¹ The ACOG/ACMG included recommendations to test for reflex variants only in the presence of a common CF mutation, placing special considerations on the technologies used for CF screening.

The detection of primary CF mutations separately from reflex polymorphisms is necessary to distinguish classical CF and non-CF-associated disease. For example, a polythymidine tract in intron 8, known as 5/7/9T, modifies the phenotype of the disease associated with the R117H polymorphism. The R117H polymorphism is associated with either classical CF or with congenital bilateral absence of the vas deferens (CBAVD) depending on the presence and chromosome location of 5T or 7T. The presence of 5T on the same allele (5T in *cis*-) with R117H is associated with classical CF; however, 7T in *cis*-with R117H is associated with infertility due to CBAVD in otherwise healthy men.⁷ The ambiguity arises when 5T is paired in a trans configuration with R117H and no other CF mutation is present, or if 5T is detected alone, because the phenotype is associated with CBAVD only.⁸ Testing for the R117H mutation and the intron 8 variant in a large population was recognized as a potential complication in CF screening program because it would expand the risk ascertainment beyond that for classical CF.² Since the 5T variant alone occurs in about 5% of the US population,¹ the combination of primary mutations and reflex variants in a single test can result in the detection of a 5T genotype without the R117H polymorphism. The discovery of the 5T mutation alone has led to an increase in fetal sample testing following amniocentesis; a risky procedure for a non-disease causing variant.⁹ Thus, to minimize the ambiguity of reporting non-CF mutations,

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the guidelines for CF screening specify reflex testing for 5/7/9T only with the positive detection of R117H.¹

However, the number of mutations and increased throughput requirements for CF screening resulted in many labs and vendors combining 5/7/9T and primary mutation panel testing in the same assay. For example, reverse dot-blot hybridization (RDB), currently the most common method for CF screening,⁹ uses probe pairs complementary to mutant and normal DNA sequences printed as line-probes on nylon membranes. Due to the relatively complex workflow for RDB assays, commercial versions of RDB include probes for 5/7/9T alongside primary CF mutations on a single strip.^{2,10} Another commercially available CF assay based on oligo ligation and capillary electrophoresis separates the detection of primary mutations from reflex alleles, but requires a second enzymatic reaction for detection of PolyT variants.¹¹ Therefore, there is a need to separate the analysis of 5T from the primary CF mutations without increasing complexity or reducing throughput of the assay.

Microsphere bead arrays provide simple and high-throughput analysis of DNA polymorphisms with discrete detection of wild-type and mutant alleles in a complex genetic assay. The bead-array platform uses multiplex PCR and allele-specific capture probes covalently immobilized on spectrally distinct polystyrene microspheres. The microspheres, or beads, are dyed internally with two fluorophores, the ratio of which can be combined to make 100 bead sets. For a specified bead set, a standard probe-hybridization surface for allele detection is generated using covalently attached amine-modified capture probes. Bead arrays offer significant advantages over other array technologies in that hybridization occurs rapidly in a single tube, the testing volume scales to a microtiter plate, and unlike glass or membrane microarrays, bead solutions can be quality tested as individual components or easily reformulated depending on the target analytes. Biotin-modified targets such as PCR products are hybridized to allele-specific capture probes on different beads. Following hybridization, the solution is mixed with a reporter fluorophore, typically streptavidin-phycoerythrin and analyzed for bead identity and associated hybridization signal intensity using a Luminex IS 100 system. Since up to 100 beads can be theoretically detected in a single assay, the bead array can be used for detection of all wild-type and mutant alleles within a multiplex genotyping assay.

The general approach of using photo-addressable beads for multiplexed nucleic acid assays has been demonstrated for single-nucleotide polymorphism screening,^{12,13,14} identification of bacteria,^{15,16} gene expression arrays,¹⁷ and detection of CF mutations.¹⁸ A commercially available assay for cystic fibrosis mutations has been adapted for the Luminex system using allele specific primer extension (ASPE) and universal tags for bead hybridization. ASPE, however, requires multiple enzymes and cleanup steps before detection.¹⁶ We adapted the bead-array approach for direct detection of unpurified PCR products without additional enzymatic reactions or post-hybridization cleanup steps. Two bead arrays were developed: one for detection of primary mutations, the other for separate de-

tection of reflex alleles. This approach resolved issues associated with the detection of 5T without R117H and complied with ACOG guidelines for CF reflex testing.

To validate and optimize the performance of this bead-array assay for 5/7/9T detection within a multiplexed assay for cystic fibrosis mutations, positive-control DNA samples were required.¹⁹ The Coriell Institute for Medical Research, funded by the National Institute of General Medical Sciences (NIGMS), maintains an excellent bank of cell lines and purified DNA for most of the mutations in the CFTR gene recommended for population screening.²⁰ Assuming that representative genotypes for 5T, 7T, and 9T would be available in the existing Coriell DNA panel of CF mutations,²¹ we used a combination of DNA sequencing and bead-array assays to identify 5/7/9T genotypes in a group of 29 samples. These data provided a set of characterized genotypes for improving our bead-array assay and are of general use to other investigators requiring positive-control samples for CF test development.

Materials and Methods

Reagents

Oligonucleotides, including amine-modified probes, biotin-modified reverse complement probes, and PCR primers were obtained from Integrated DNA Technologies (Coralville, IA). PCR primers were HPLC purified. All oligonucleotides were resuspended in deionized water as 1 mmol/L stock solutions and stored at -20°C. Solutions of 1 mol/L Tris-HCl, pH 8.0, 1X TE (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0), 10% SDS, and RNase-free water were obtained from Ambion (Austin, TX). AmpliTaq Gold and 10X Gold Buffer were obtained from Applied Biosystems (Foster City, CA). Deoxynucleotides were obtained from Bioline (Randolf, MA); dUTP and uracil-N-glycosylase (HK-UNG) were obtained from EpiCentre (Madison, WI). Streptavidin- β -phycoerythrin (SA-PE) was obtained from ProZyme (Alameda, CA). Tetramethyl ammonium chloride (TMAC), sodium sarkosyl, and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma (St. Louis, MO). The MES was prepared at pH 4.5 with the addition of 5 N NaOH and stored at 4°C. EDC, (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride), was obtained from Pierce (Rockford, IL). Human genomic DNA samples were obtained from the Coriell Institute for Medical Research (Camden, NJ) as DNA isolated from cell lines. These DNA solutions were stored at -80°C or at 50 ng/ μ L in deionized water at -20°C.

General Workflow for Bead-Array Hybridization and Detection

The general workflow for detecting and analyzing 5/7/9T alleles included multiplex PCR for generating primary and reflex allele amplicons and bead-array hybridization included the following steps. 1) single-tube multiplex PCR with biotin-modified primers incorporating dUTP and UNG digestion for amplicon carryover control; 2) transfer of an aliquot of unpurified PCR products to a bead solu-

tion for 5/7/9T detection; 3) hybridization of the sample for 20 minutes, followed by addition of SA-PE without additional purification; 4) detection using a Luminex IS100 system; 5) data analysis of median fluorescence intensity per bead.

Capture Oligonucleotide Design and Coupling

Oligonucleotide capture probes were modified at the 5' terminus with a C₁₂-amine for coupling to the carboxylated microspheres. The following probes were used: IVS8–5T, 5'-TGTGTGTTTTTAACAGGG-3'; IVS8–7T, 5'-GTGTGTTTTTTTAACAGG-3'; and IVS8–9T, 5'-GTGTGTTTTTTTAACAG-3'. Immobilizing the amine modified probe to the carboxylated surface of the photo-addressable microspheres were completed using a standard carbodiimide-coupling procedure.¹⁸ Briefly, a set of 5 × 10⁶ microspheres were pelleted, resuspended in 50 μ L of 100 mmol/L MES, pH 4.5, and mixed with 200 pmol of amine modified oligonucleotide. A 2.5- μ L aliquot of freshly prepared 10 mg/ml EDC was added, the solution briefly vortexed and incubated for 30 minutes in the dark. A fresh 10 mg/ml solution of EDC was prepared for a second addition of EDC and 30-minute incubation. The microspheres were washed with 1 ml of 0.02% Tween-20 followed by pelleting the beads, removal of supernatant, and addition of 1.0 ml of 0.1% SDS. After pelleting the beads and removing the supernatant, the coupled microspheres were resuspended in 100 μ L of 1X TE buffer, pH 8.0 and stored at 4°C in the dark. A typical coupling reaction of 5 million beads was sufficient for 900 to 1000 assays such that repeating the coupling reaction was minimized. After probe selection, the coupling reaction scale could be increased to 10 or 100 million beads.

Oligonucleotide coupling efficiency was characterized using a reverse-complement oligonucleotide modified with biotin at the 5' terminus.¹⁴ Different quantities of an individual complimentary oligo were aliquoted to each well and brought up to 17 μ L with 1X TE buffer. Briefly, a three-plex bead array corresponding to the probes for 5T, 7T, and 9T was diluted in 1.5X TMAC hybridization buffer (4.5 mol/L tetramethylammonium chloride, 75 mmol/L Tris-HCl, 6 mmol/L EDTA, and 0.15% Sarkosyl, pH 8.0) so that each bead was at an approximate concentration of 150 beads/ μ L. The oligonucleotide samples were gently mixed with the addition of a 33- μ L aliquot of a bead mix, containing the beads for 5/7/9T detection in a single well. The components were hybridized at 95°C for 5 minutes followed by 52°C for 15 minutes. After hybridization, the samples were transferred to the Luminex instrument (Luminex Corporation, Austin, TX), the hot-plate of which had been previously warmed to 52°C. The samples were gently mixed with a 25- μ L aliquot of a 40 ng/ μ L SA-PE solution and allowed to incubate for an additional 10 minutes before analysis. The output file of median fluorescence intensity per bead was analyzed using MS Excel (Microsoft Corporation, Redmond, WA).

PCR Amplification

PCR primers, specific for intron 8 of the CFTR gene, GenBank Accession No. M55114, were selected to generate a 120-bp product. The forward primer was 5'-AA-CAAGCATCTATTGAAAATATCTGA-3', and the reverse primer, biotin modified at the 5'-terminus was 5'-biotin-TTGTGTTGTTTGCTTTCTCAA-3'. PCR reactions were prepared in 50- μ L volumes using 5 mmol/L Mg²⁺, 5 U of AmpliTaq Gold, 0.1 U of HK-UNG, and 1 pmol of each primer. The final nucleotide mixture was 0.2 mmol/L dATP, dCTP, and dGTP, 0.05 mmol/L dTTP, and 0.4 mmol/L dUTP. Reactions were completed with an initial incubation at 37°C for 15 minutes, and 10 minutes at 95°C followed by 35 cycles of 94°C for 30 seconds, 58°C for 90 seconds, and 72°C for 90 seconds followed by 72°C for 2 minutes. Single-tube multiplex reactions were performed with the intron 8-specific primer pair and additional primers for primary CFTR amplicons. PCR products were stored at –20°C until analyzed.

Bead-Array Hybridization Assays

A three-plex bead array for 5T, 7T, and 9T detection was prepared in 1.0 X hybridization buffer (3.0 mol/L TMAC, 50 mmol/L Tris-HCl, 4 mmol/L EDTA, and 0.1% Sarkosyl, pH 8.0) such that each of the three beads was at a final concentration of ca. 100 beads/ μ L or 5000 beads/capture probe/assay. The bead solution, comprised of the probes for 5/7/9T detection in a single well, was dispensed in 48- μ L aliquots to the wells of a microtiter plate. Samples were analyzed by mixing the bead solution with a 2- μ L aliquot of PCR products. The samples were hybridized at 95°C for 5 minutes and 52°C for 15 minutes. Following hybridization, the samples were immediately transferred to the XYP stage of a Luminex xMAP system maintained at 52°C. The samples were gently mixed with the addition of 25 μ L of a 40 ng/ μ L SA-PE solution that had been freshly diluted in 1X TMAC hybridization buffer. Median fluorescence intensity (MFI) values were obtained from a minimum of 100 beads and background subtracted using an average MFI obtained in the absence of PCR product. Allele fractions were derived by dividing the background-corrected signal of the test allele by the sum of the background-corrected signals of the other alleles.

Analysis of DNA Purified from Whole Blood

Genomic DNA was purified from whole blood obtained from 10 healthy, consenting volunteers using a QIAmp-Blood DNA kit (Qiagen, Valencia, CA). After purification, the DNA from individual donors was quantified and stored at –20°C until analyzed. Before PCR, a dilution series of DNA from 3 to 1000 ng from each donor was prepared in a microtiter plate, for a total of 94 samples. These samples were amplified as described and analyzed as 2- μ L aliquots using the 5/7/9T bead array. The median fluorescence intensity and allele fraction was compared by source and by quantity of DNA.

DNA Sequencing

The 5/7/9T region of the CFTR gene was amplified using bipartite PCR primers containing an intron 8 CFTR-specific oligo and universal M13-forward and M13-reverse binding sites. The forward primer was 5'-AGGAAACAGCTATGAC-CAT-GGCCATGTGCTTTTCAAAC-3' and the reverse primer was 5'-GTTTTCCAGTCACG-ACGCGCCATGTG-CAAGATACAGT-3'. PCR was completed using 1 U Super-TAQ (Ambion), 100 ng template DNA, and 35 cycles of 94°C for 30 seconds, 58 for 30 seconds, and 72°C for 60 seconds followed by 7 minutes at 72°C. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). Purified PCR products were checked on 1% agarose gel for purity and quantity and were sent to Lark Technologies (Houston, TX) for dye-terminator DNA sequencing using M13-28 reverse and M13-40 forward primers.

Results and Discussion

The development of new technologies for the diagnosis of genetic diseases requires sufficient attention to quality assurance and the routine testing of positive-control samples.²² Regardless of the methodology, the detection of the actual mutation from a DNA source is a requisite for demonstrating the clinical utility of an assay. However, for diseases with infrequently encountered or rare mutations, like CF, positive-control samples may be difficult to obtain or even completely unavailable.¹⁹ To acquire positive-control samples for 5/7/9T testing, we hypothesized that 5/7/9T genotypes would be represented in DNA samples commercially available in the Coriell panel of characterized CF mutations. We screened these samples using bead-array hybridization and verified the results by DNA sequencing. The assay was developed to comply with ACMG/ACOG guidelines for separate testing of primary mutations and reflex alleles using a single PCR and two separate bead arrays. We report on the development of the bead-array assay for detection of 5/7/9T variants, sequence confirmation of observed genotypes and application to clinical samples.

Bead-Array Characterization

Optimal signal intensity and specificity for bead-array assays depends on the sequence of the capture probe oligonucleotide and to a lesser extent, the hybridization protocol and quantity of target amplicon. We investigated the use of three capture probes for the detection of 5/7/9T variants in intron 8 of the CFTR gene using a single-hybridization protocol and varying concentrations of target molecules. The capture probes were covalently attached to color-specific microspheres and were designed such that the first base mismatch in the 5' direction occurred between bases 8 and 11 of an 18-mer probe (19-mer for the 9T probe). Following allele-specific capture probe hybridization to the target amplicon, the genotype of a DNA sample would be determined using the relative signal intensity between the three beads. Dominant signal intensity on a single bead would indicate a homozygous sample whereas equiv-

Figure 1. Probe hybridization signal intensity for (●) 5T, (◆) 7T, and (▲) 9T capture probes versus quantity of 5T complementary oligonucleotide. Conditions: Biotin-modified 5T complementary oligo added to hybridization reaction containing 5000 beads/capture probe/assay for 5T, 7T, and 9T probes; 1000 ng of SA-PE added post-hybridization. Lines were used for visual reference and data points corresponded to average of three values.

alent signal intensity between two beads would indicate a heterozygous sample. Optimizing the assay depended on generating good signal intensity while maintaining high-specificity between closely related alleles. The coupling and hybridization efficiency was characterized using single-stranded oligonucleotides. The specificity of the capture probes was evaluated using PCR targets.

Characterization with Single-Stranded Oligonucleotides

Complementary single-stranded oligonucleotides are routinely used in final testing of various types of capture probes including molecular beacons²³ probes for bead arrays.¹⁵ For bead arrays, biotin-modified complementary oligonucleotides have been used to check the amount of coupled oligo on the bead surface and its hybridization characteristics.¹⁵ The use of biotin-modified complementary oligonucleotides was a rapid method for characterizing the coupling efficiency of the various capture probes. In these experiments, a single complementary oligonucleotide was added in increasing quantity to a bead solution containing the probes for 5T, 7T, and 9T. By titrating the quantity of oligonucleotide, estimates on surface saturation and specificity were made.

Plots of signal intensity were obtained using a single complementary oligo in the presence of all three capture probes. The results for the 5T capture probe and complement oligo are shown in Figure 1. As the quantity of oligonucleotide increased, the signal increased on the matched probe to a greater extent than observed for the mismatch probes. The signal intensity increased to a maximum value of approximately 20,000 MFI for the 5T probe. Similar results were observed when the same bead solution was analyzed using the oligonucleotides complementary to the 7T and 9T probes. The signal intensity on the respective mismatched probes reached a maximum above 20 fmol, similar to results observed using the 5T complementary oligo (Figure 1).

Table 1. Hybridization Assays with PCR Targets Listing Signal Intensity for 7T, 5T, and 9T Capture Probes

Single-PCR				Multiplex PCR			
Quantity [fmol]	7T	5T	9T	Volume [μ L]	7T	5T	9T
16	1620	22	0	0.1	800	33	0
32	1860	15	0	0.25	890	9	0
65	2030	17	0	0.5	1070	0	0
130	2270	55	0	1	1200	7	0
260	2500	21	3	2.5	1200	0	0
520	2400	82	0	5	1040	16	2
1200	2240	79	0	10	560	9	0

*Results are divided by quantity of DNA for analysis of a single-plex PCR and by volume hybridized for analysis of a multiplex PCR. Single PCR amplicon generated with one primer pair for intron 8 amplicon. Within the multiplex PCR, the same primer pair was mixed with other primers used to generate amplicons for detecting primary CF mutations.

The change in signal intensity with increasing complementary oligonucleotide was consistent with saturation of the available capture probes on the bead surface. As the available capture probes on the bead surface were hybridized to a complementary oligonucleotide, the signal intensity did not increase. However, the increase in signal on the mismatched probes corresponded to a decrease in specificity for the target oligonucleotide. This decrease in specificity suggested that the actual specificity of the capture probes was better characterized using PCR targets.

Characterization with PCR Targets

In contrast to hybridization assays using oligonucleotide targets, the signal intensity and specificity for PCR targets would be influenced by the quantity of PCR product, competition between probe capture and strand re-annealing, and excess biotin from unincorporated primers or unbound PCR targets. The effect of PCR product quantity on signal intensity and specificity was evaluated using intron 8 PCR products. The resulting signal intensity on the 5T, 7T, and 9T beads listed by quantity of individual PCR product is summarized in Table 1. The targets from an individual primer-pair PCR were titrated by quantity in fmol. PCR targets from a multiplex PCR, in which the intron 8 amplicon was generated with other CFTR amplicons, were analyzed by varying the volume of the reaction added to the bead array containing probes for 5/7/9T hybridization. The amplicon size was 120 bp, which overlapped with the size of several other amplicons generated in the multiplex PCR (data not shown) preventing simple quantitation. The signal intensity by volume of multiplex PCR is summarized in Table 1.

Bead-array hybridization resulted in surface saturation without a corresponding decrease in specificity at higher quantities of PCR product. Using the single PCR target, the signal intensity increased to a maximum of 2400 MFI at 260 fmol. Increasing the quantity of PCR target above 260 fmol to 1200 fmol resulted in a 10% decrease in signal intensity. Over this range of PCR product, the signal intensity on the 5T and 9T beads was less than 100 MFI. A similar trend but lower signal intensities were observed using the PCR target generated in the multiplex reaction. A maximum signal of 1200 MFI was observed with the addition of 1.25- μ L PCR products to the hybridization solution. Hybridizing volumes greater than 2.5 μ L

resulted in a decrease in signal. The decrease in signal was 50% between 5 and 10 μ L.

The results from the hybridization assays of PCR products indicated a greater degree of specificity than indicated using only complementary oligonucleotides. Over a 100-fold range in either quantity (individual PCR) or volume (multiplex PCR), the signal intensity on the 7T bead was similar, although decreasing at high volume. The signals on the mismatch beads, 5T and 9T, were consistently less than 100 MFI. The specificity over a broad range of quantity was much greater than observed using single-stranded oligonucleotides, in which the signal intensity on the mismatch-capture beads increased with increasing target quantity.

The specificity of the three capture probes, at least for a 7T/7T sample were similar for detection of amplicons generated in a single- or multiplex PCR. The decrease in signal with multiplex PCR was associated with the 20-fold increase in biotin-modified primers used in the multiplex PCR. As the volume of the reaction increased, the quantity of unbound biotin increased in the reaction consuming the available SA-PE. In the absence of DNA or in the absence of the intron 8 primers, background signal intensities were observed on the 5T, 7T, or 9T beads in the multiplex reaction. Therefore, the hybridization assay was specific to IVS8 targets. These results confirmed the requirement for characterizing probe specificity with PCR targets and the subsequent need to determine capture-probe specificity with a 5T and 9T allele.

Detection of 5/7/9T Genotypes

A panel of commercially available DNA samples, obtained from cell lines and containing characterized CF mutations, were evaluated using the bead-array hybridization assay on the expectation that positive-control samples for 5T, 7T, and 9T would be identified. The PCR and bead-array hybridization of 29 genomic DNA samples resulted in distinct groups of signal intensity between the three beads, represented as a 3-D scatter plot in Figure 2. Five groups of samples based on 5T, 7T, and 9T signal intensities were observed. These groups corresponded to 7T homozygous, 9T homozygous, and three heterozygote sample groups: 5T/7T, 5T/9T, and 7T/9T. The sample groupings were substantially distinct indicating good specificity for different 5/7/9T target alleles. A

Figure 2. Scatter plot of signal intensity for 5T, 7T, and 9T capture probes for PolyT assays of 29 genomic DNA samples. As labeled, each axis corresponded to median fluorescence signal intensity for 5T, 7T, and 9T probes. Conditions: 100 ng of genomic DNA amplified in a multiplex PCR with a 2 μ l aliquot hybridized to beads containing probes for 5/7/9T detection; 1000 ng SA-PE added post-hybridization. Data points corresponded to single sample assays and plotted by MFI for all three probes. The indicated genotype was labeled for each group of samples.

single sample within each group was analyzed by DNA sequencing to confirm the genotype.

Genotype Verification by DNA Sequencing

DNA sequencing has the advantage of resolving different cystic fibrosis genotypes through direct base identification²⁴ and is recognized as a means of validating positive controls for CF testing.² DNA sequencing results were obtained from five samples representing each of the various genotypes indicated in Figure 2. DNA sequencing traces yield a distinct pattern of peaks corresponding to heterozygote samples and the type of mutation. In the 5/7/9T region of intron 8, a single or double heterozygote peak in the sequencing electropherogram would be observed allowing for the identification of 5T, 7T, and 9T heterozygote samples. The polyT tract is 3' of a TG repeat, usually 12 repeats, but the variable length of which has also been associated the severity of a 5T allele.²⁵ Polymorphisms would be distinguished by the number of thymidines preceding a two-peak signal near the TG repeat region. Using the forward sequencing reaction, in which the strand detected is the reverse complement, a 5T/7T heterozygote would be detected by five A peaks followed by a single A/C peak as part of the TG repeat region. A similar pattern would be observed for a 7T/9T genotype differing only by the number of preceding adenonine peaks. A 5T/9T heterozygote-sequencing pattern in this direction would result in five adenonine peaks followed by two double A/C peaks. Corresponding differences in the heterozygote samples would be observed using the reverse reaction with the number of TG repeats not affecting the sequencing reaction or the interpretation of the genotype. Sequencing results were obtained from five samples corresponding to the group-

ings shown in Figure 2. DNA sequencing in the forward and reverse reaction confirmed the homozygous and heterozygote genotypes grouped in Figure 2.²⁶

Identifying 5/7/9T Positive-Control Samples

From the DNA sequencing data and the signal intensity grouping between the three beads (Figure 2), the 5/7/9T genotype for 29 commercially available DNA samples was assigned. The signal intensity between the three beads was converted to an allele fraction by dividing the fluorescence intensity of the test allele by the total fluorescence signal for the alleles in that group (eg, $MFI_{5T} + MFI_{7T} + MFI_{9T}$). The sample identity, characterized mutation and allele fractions are listed by genotype in Table 2. Because the allele fraction normalized differences in signal intensity, genotypes were easily assigned into different groups. Within this group of DNA samples, two 5T/7T and two 5T/9T samples were identified. With regard to the other genotypes, 11 were 7T homozygous, five were 9T homozygous, and nine were heterozygous 7T/9T. Within this sample set, the nine $\Delta F508$ heterozygote samples and one $\Delta F508$ homozygous sample were associated with a 9T allele.

The PolyT genotype for these samples was available for 23 of the 29 samples listed in Table 2; within these 23 samples, there was 100% concordance.²¹ The average allele fraction for 7T/7T was 0.91 ± 0.03 in the 7T allele, whereas for 9T/9T, the allele fraction was 0.83 ± 0.03 with a higher degree of cross-hybridization on the 5T allele. A similar amount of cross-hybridization on the 5T allele was observed for 7T/9T samples. Within each genotype, however, the relative SD was less than 4%, such that a target range of allele fraction values for each genotype could be assigned. To maximize the allele fraction for each possible genotype, positive-control DNA samples were required. These results confirmed the use of a simple hybridization protocol and direct analysis of PCR products for the distinct detection of closely related alleles. Having identified positive-control samples for validating the specificity of the capture probes, the clinical utility of the bead-array assay was demonstrated using genomic DNA isolated from whole blood.

Bead-Array Hybridization with DNA Isolated from Whole Blood

The source of genomic DNA is often whole blood, from which purified DNA may vary significantly in quantity, quality, and the presence of PCR inhibitors. These variations may detrimentally affect the PCR amplification and detection of alleles using any "downstream" assay. Therefore, we tested a wide range of DNA quantity obtained from 10 donors using the multiplex PCR and bead-array hybridization assay for 5/7/9T detection. Whole blood samples were obtained from 10 healthy volunteers. The samples were PCR amplified using 3–1000 ng DNA per donor. Eight of the 10 samples were identified as 7T homozygous with the other two as 7T/9T heterozygous. The resultant signal intensity by quantity of DNA for the

Table 2. Intron 8 Genotype by Coriell Number, Characterized CF Mutation and Allele Fraction for 5/7/9T

Intron 8 genotype	Coriell sample	Characterized mutation	Allele fraction by probe		
			5T	7T	9T
7T/7T	NA09947	Normal	0.04	0.93	0.03
	NA11277	Δ 1507/normal	0.06	0.90	0.04
	NA11761	G551D/R553X	0.06	0.92	0.02
	NA11859	2789+5G>A/2789+5G>A	0.02	0.96	0.02
	NA11860	3849+10kbC>T/3849+10kbC>T	0.03	0.94	0.03
	NA12444	1717-1G>T/normal	0.06	0.87	0.07
	NA12585	R1162X/normal	0.07	0.86	0.08
	NA12785	R347P/G551D	0.04	0.92	0.05
	NA12960	R334W/normal	0.06	0.92	0.02
	NA12961	V520F/normal	0.06	0.89	0.05
	NA13033	F508C/normal	0.03	0.93	0.04
	NA01531	Δ F508/ Δ F508	0.14	0.04	0.82
	NA11281	621+1G>T/ Δ F508	0.14	0.04	0.82
	NA11283	A455E/ Δ F508	0.13	0.05	0.82
9T/9T	NA11290	A455E/621+1G>T	0.12	0.01	0.87
	NA11496	G542X/G542X	0.14	0.05	0.81
	NA11723	W1282X/normal	0.53	0.44	0.03
	NA13032	I506V/normal	0.58	0.39	0.03
5T/7T	NA11279	129G>C/ Δ F508	0.51	0.00	0.49
	NA13591	R117H/ Δ F508	0.52	0.00	0.48
7T/9T	NA07441	3120+1G>A/621+1G>A	0.08	0.41	0.51
	NA07552	R553X/ Δ F508	0.09	0.36	0.55
	NA07830	556dA/ Δ F508	0.11	0.37	0.52
	NA11275	3659dC/ Δ F508	0.10	0.37	0.53
	NA11278	Q493X/ Δ F508	0.09	0.38	0.53
	NA11280	711+1G>T/621+1G>A	0.09	0.37	0.54
	NA11282	G85E/621+1G>A	0.07	0.39	0.53
	NA11284	R560T/ Δ F508	0.08	0.39	0.52
	NA11472	N1303K/G1349D	0.08	0.39	0.54

eight homozygous samples is represented as a scatter plot in Figure 3. The signal intensity increased from between 3 and 10 ng and was similar within error between 10 and 1000 ng.

Similar to the profiles observed with titrating the PCR product, the signal intensity on the 5T or 7T beads was low and independent of DNA quantity above 10 ng. At 3 and 5 ng, the variance by source of DNA increased and one of the DNA samples had signal below a 200 MFI low signal threshold. The increase in variance in signal intensity below 10 ng of input DNA affected the allele fraction,

Figure 3. Effect of DNA quantity on probe hybridization for (●) 7T, (◇) 5T, and (△) 9T capture probes. Conditions. Single-tube multiplex PCR for CFTR mutation detection performed with varying quantity of DNA isolated from whole blood. Aliquots of PCR hybridized to beads containing probes for 5/7/9T detection; 1000 ng of SA-PE added post-hybridization. Data points corresponded to single-assay of eight individual samples.

and subsequent allele call, for two 7T/9T individuals within this sample set (data not shown). The increase in variance and change in allele fraction at 3 and 5 ng suggested that 10 ng was the minimum quantity required for PolyT reflex testing using this bead-array approach. These results demonstrated the compatibility of the bead-array assay with genomic DNA isolated from whole blood over a wide range of input DNA from 10 to 1000 ng from multiple donors.

Summary

The use of multiplexed bead-arrays was demonstrated for the detection of reflex polymorphisms within a complex genetic assay for CF mutations. By separating the detection of reflex variants from primary mutations using different bead arrays, a simple and high-throughput method compliant with ACOG/ACMG recommendations was achieved. Multiplex PCR products were generated with the incorporation of dUTP allowing for prevention of carryover contamination following enzymatic digestion with uracil-N-glycosylase.²⁷ As part of a simple workflow, PCR products were analyzed without pre- or post-hybridization treatment. As required for assay optimization, positive-control samples for 5/7/9T alleles were identified and confirmed by DNA sequencing. The use of positive-control samples from genomic DNA was a much better indicator of capture-probe specificity and assay performance than synthetic controls. Having a number of commercially available positive controls addresses the

substantial issues for third-party developers of genetic assays and the quality control and validation demands for clinical laboratories.

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