study. We observed no evidence of such an increase (see Fig. 4 in the online Data Supplement).

We observed significant variation in tear glucose concentrations between the 2 eyes of individual study participants, as well as over time within a single eye. During this study, we were cognizant of potential confounding events such as yawning and eye rubbing. Although the 2 individuals who rubbed their eyes during the study had the highest glucose concentrations, events like these do not explain the large within-individual differences observed.

Variations in tear glucose concentrations within a single individual must derive from the sum of the biological variance and any variances associated with sampling and measurement. The error in our tear fluid collection volumes was negligible (9); the SD observed for an individual was ~3 times the SD of the 3 replicate mass spectral measurements of a single tear fluid sample. The relative SDs of these 3 replicates varied somewhat with glucose concentration but had a median value of 14%. Thus, the observed SD in tear glucose measurements derive mainly from actual variations of the glucose concentration in the different tear fluid samples.

We did not observe a systematic increase over time in glucose concentration variations that could result from the effect of tear depletion during repeated measurements. Tear glucose concentration appeared to vary randomly over the repeated sampling events.

We observed variations in tear glucose concentrations among fasting individuals and a significant correlation between ln(tear glucose concentration) and blood glucose concentration. Mean fasting tear glucose concentrations did not differ significantly in relation to contact lens use. Further studies are needed to investigate the apparent difference in the correlation between tear glucose and blood in these subpopulations.

The extremely low glucose concentrations in tear fluid, more than 100 times lower than in blood, raise questions about the physiologic role of tear glucose. Future studies are needed to address the correlation between tear and blood glucose in hypoglycemic and hyperglycemic states and in the presence of diabetes.

Grant funding/support: This research was supported by the National Institutes of Health Grant DK-55348 (to S.A.A.). Financial Disclosures: S.A.A. is the scientific founder of Glucose Sensing Technologies LLC, a company developing glucose-sensing contact lenses. Acknowledgments: We thank Drs. Gary Foulks and Chaloppadi Sundar-Raj for helpful discussions and critical reviews of the manuscript.

References

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Microtransponder-Based Multiplex Assay for Genotyping Cystic Fibrosis, Xin Lin,1 James A. Flint,1 Marco Azaro,1 Thomas Coradetti,1 Wesley M. Kopacka,1 Deanna L. Sreek,2 Zhiying Wang,1 James Dermody,2 and Wlodek Mandecki*1 (1PharmaSeq, Inc., Monmouth Junction, NJ, 2Department of Microbiology & Molecular Genetics, University of Medicine and Dentistry, New Jersey-New Jersey Medical School, Newark, NJ; *address correspondence to this author at: PharmaSeq, Inc., 11 Deer Park Drive, Suite 104, Monmouth Junction, NJ 08852; fax 732-355-0102, e-mail mandecki@pharmaseq.com)

Background: We developed and evaluated a genotyping assay for detection of 50 cystic fibrosis (CF) mutations. The assay is based on small (500 μm) electronic chips, radio frequency (RF) microtransponders (MTPs). The chips are analyzed on a unique fluorescence and RF readout instrument.

Methods: We divided the CF assay into 4 panels: core, Hispanic, African-American, and Caucasian. We amplified 18 CF transmembrane regulator (CFTR) DNA frag-
Cystic fibrosis (CF) is caused by one or more mutations in the gene encoding for the CF transmembrane conductance regulator (CFTR) protein. CF occurs when both copies of the CFTR gene function abnormally, and one functional copy is sufficient to prevent the disease. In the Caucasian population, CF is inherited with a frequency of 1:3300, making it the most lethal inherited disease of childhood, but carrier frequency and incidence of CF vary with race and ethnic group (1). A single mutation causing loss of the phenylalanine residue at position 508 (ΔF508) accounts for nearly 70% of all mutations observed in Caucasians with CF, but more than 1000 other mutations of the CFTR gene have been reported in all races and ethnic groups. In May 2001, the American College of Medical Genetics published a recommended panel of 25 mutations and 6 polymorphisms for population-based CF screening (2).

We describe a new platform for performing a multiplexed genotyping assay based on radio frequency (RF) microtransponders (MTPs) and provide a working example, the CF assay. CF mutations tested and the rationale are provided in Table 1 and Supplemental Data (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue7). We designed this assay to be ethnic-group specific, thus simplifying the design and potentially reducing costs. The mutations are grouped into 4 panels: core, Caucasian, Hispanic, and African-American. Because the assay is ethnic specific, efficiency is increased, with fewer DNA probes and reagents needed. A patient would be tested with 2 panels, the core panel and one ethnic panel; more than one ethnic panel might be used for patients with complex ethnic backgrounds.

The key element of the assay method is the MTP (Fig. 1A), a monolithic 500 μm × 500 μm integrated circuit chip that can transmit its identification code by RF. Each chip consists of photocells, read-only memory (ROM), transmit logic circuitry, and an antenna loop. Visible light, typically red or green, is pulsed at 1.5 MHz to provide power and a stable clocking signal for the logic circuitry. The circuitry accesses the contents of ROM (the ID value) and modulates the current through the antenna in correspondence with the ID value. The resulting variable magnetic field in the vicinity of the MTP can then be measured with a nearby receiving coil and decoded to provide the ID value, which identifies the oligonucleotide immobilized on the MTP. The current MTP design uses 10 bits to encode the ID value, allowing 1024 unique values; however, the ROM contains an additional 40 unused bits, so the MTPs could be manufactured to have as many as 2^{50} (~10^{15}) unique ID values. Before use, the MTPs are coated with a polymer that places both hydroxy and amino groups on the surface of the chip. Such derivatized MTPs are subjected to oligonucleotide synthesis.

In preparation for the flow reader analysis, the MTPs are suspended in a liquid medium that prevents sedimentation of MTPs but allows flow characteristics comparable to water when sheared. The suspension is repeatedly passed through a narrow channel of the instrument, where the ID values are read and fluorescence measurements are made. The flow system is designed to support a transfer rate of up to 1000 MTPs/s. The time needed to read the ID can be as short as 300 μs and the time to read fluorescence 1–2 ms. Multiple forward and reverse passes, typically 50 total passes, of the MTPs through the flow channel are required to obtain enough data for analysis. The instrument in the present study, Tsunami IV, uses a 532-nm, 300-mW laser for both RF identification and fluorescence at a single location on one side of the flow channel. We used custom software named Retro for data analysis. Both the flow reader and MTPs are described in more detail in a recent report (3).

The principle for detecting mutations is allele-specific primer extension (ASPE) (4, 5) on the PCR-amplified CFTR DNA; the schematic of the assay is shown in Fig. 1B. For each mutation, 2 primers were prepared, one specific to the wild-type allele and another specific to the mutant allele. The sequence differences between the 2 primers are the tag sequence at the 5′ end and a single nucleotide at the 3′ end (an “anchor” sequence). Thus, for any particular allele, only 1 primer was extended in a reaction with DNA polymerase in the presence of 4 dNTPs. After the ASPE reaction, the target DNA was hybridized to a capture probe, the sequences of which are complementary to tag sequences commonly referred to as universal tags (4, 6–8). The capture probe is a synthetic oligonucleotide (24 nucleotides) covalently bound to the MTP surface. Because the tag sequence was present at the 5′ end of allele-specific primers, the capture reaction was very specific. In the ASPE reaction, we used biotin-labeled dCTP in place of dCTP. Thus, the ASPE target typically contained several biotin moieties, which were subse-
The treated multiplex PCR products (10–20 ng of each DNA fragment) were added to the ASPE reaction mixture, final volume 40 μL, containing 20 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 25 mmol/L allele-specific primers, 5 μmol/L biotin-CTP, 0.1% Triton-100, 28 μmol/L dCTP, 100 μmol/L dNTP (dCTP–), and 3 units Tsp DNA polymerase. The reactions were incubated at 96 °C for 2 min to denature DNA, followed by 30 PCR cycles (94 °C for 30 s, 60 °C for 1 min, and 74 °C for 2 min) and 72 °C for 7 min.

Hybridization of MTPs was performed in the 1× prehybridization buffer [50 mmol/L Tris-HCl, PH 8.0, 150 mmol/L sodium chloride, 0.1% (wt/vol) SDS, 0.5% (wt/vol) Ficoll (type 400), 50 mmol/L EDTA, pH 8.0, 200 μg/mL sheared, denatured salmon sperm DNA, 1 μg/mL BSA] at 48 °C for 10 min. After removing the prehybridization buffer, the MTPs were hybridized in 1× hybridization solution (80 μL ASPE products and 80 μL 2× hybridization buffer) at 48 °C for 2 h and rinsed 3 times.

Streptavidin-phycoerythrin conjugate was diluted 1:10 in PBS (1.06 mmol/L potassium phosphate monobasic, 155.17 mmol/L sodium chloride, 2.97 mmol/L sodium phosphate dibasic, pH 7.4), and 10 μL was added to 120 μL 1× washing buffer at a final concentration of 8 μg/mL. The MTPs bearing the hybridized DNA were incubated in the above solution for 30 min at room temperature in the dark, rinsed, and analyzed.

Allele-specific primers containing 24-nt tag sequences were designed using 3 custom programs written in Python: ExtractProbes.py, FindOptProbe.py, and Tags2Probes.py. The tag sequences were at the 5′ end of the primers, and each allelic-specific sequence was at the 3′ end. The allele-specific sequences varied in length, but always possessed a Tm of 50 °C. For each biallelic single nucleotide polymorphism analyzed by ASPE, 2 allele-specific primers (ASP) were synthesized, with each ASP differing in the tag sequence and in the polymorphic nucleotide contained at its 3′ terminus.

We conducted 2 separate series of experiments to validate the performance of the assay. In the internal

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**Table 1. CF genotyping results for the core panel from internal validation.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Allelic Variant</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<td>1</td>
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<td>A455E</td>
<td>G551D</td>
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<td>R334W</td>
<td>1078delT</td>
<td>1717-1G&gt; T</td>
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* Mutation calls for 2 alleles are abbreviated as follows: dot, wild-type/wild-type; m, wild-type/mutant; M, mutant/mutant; *, incorrect call; blank, not assayed for the particular mutation in accordance with the work plan. Mutation panels are defined as follows: core, 1–24 (using the numbers on the top of table); Caucasian, Ficoll (type 400), 5 mmol/L EDTA, pH 8.0, 200 mmol/L KCl, 25 mmol/L sodium chloride, 0.1% (wt/vol) SDS, 0.5% (wt/vol) Ficoll (type 400), 5 mmol/L EDTA, pH 8.0, 200 μg/mL sheared, denatured salmon sperm DNA, 1 μg/mL BSA at 48 °C for 10 min. After removing the prehybridization buffer, the MTPs were hybridized in 1× hybridization solution (80 μL ASPE products and 80 μL 2× hybridization buffer) at 48 °C for 2 h and rinsed 3 times.

ExtractProbes.py, FindOptProbe.py, and Tags2Probes.py. The tag sequences were at the 5′ end of the primers, and each allele-specific sequence was at the 3′ end. The allele-specific sequences varied in length, but always possessed a Tm of 50 °C. For each biallelic single nucleotide polymorphism analyzed by ASPE, 2 allele-specific primers (ASP) were synthesized, with each ASP differing in the tag sequence and in the polymorphic nucleotide contained at its 3′ terminus.

We conducted 2 separate series of experiments to validate the performance of the assay. In the internal
study, we used 23 standard (Coriell) genomic DNA samples. In addition, we used 32 coded genomic DNA samples in an external study completed in Dr. Dermody’s laboratory at the University of Medicine and Dentistry of New Jersey. During the course of this project, >100 CF reagent sets were prepared. Each reagent set consisted of a vial containing derivatized MTPs that compose the mutation panel and the assay file on electronic media. Typically, 3 MTPs were used for each probe to achieve multiple readouts for statistical accuracy.

The results from the internal study (Table 1) indicate that correct calls were 98.8% of all determinations (807 total calls), and false-positive and false-negative calls were 1.1% and 0.12%, respectively. The results from the external study are shown in the Supplemental Data. Correct calls were 95.7% of all determinations (1086 total calls), and false-positive and false-negative calls were 3.9% and 0.36%, respectively. In addition, 27 synthetic 60-nt oligonucleotides were designed to simulate DNA mutations not present in the Coriell DNA samples. Assays performed on the synthetic samples resulted in 100% correct calls of homozygous mutation.

We are generally pleased with the results obtained in both the internal and external testing. The overall percentage of correct calls was high: 98.8% and 95.6%, respectively. Especially encouraging were high fluorescence ratios (wild type-to-mutant oligo probe), approaching 100 in many cases, indicating a high potential of the assay for DNA testing, and in particular CF testing. The wrong calls seem to be clustered for specific mutations, suggesting difficulties with certain oligonucleotides or PCR products.

In summary, MTPs were used as solid phase in a CF assay. Although the reported rate of false positives (1%–4%) is higher than in commercially available CF assays [Luminex (12), Roche, and Applera (13)], we are confident that it can be improved, because the biochemical basis of the assay is well understood and the biochemical principle is similar to that implemented in the Luminex CF assay (12). The main advantage of the MTP platform is the large number of ID codes available, currently 1024 but readily expandable. The expansion might be justified if the number of mutations being tested for increases, or if other types of assays require a higher multiplex level.

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Financial disclosures: The authors associated with PharmaSeq, Inc., have equity interest in the company. J.D. is a consultant to the company.

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4. Ye F, Li MS, Taylor JD, Nguyen Q, Colton HM, Casey WM, et al. Fluorescent microsphere-based readout technology for multiplexed human single nucle-


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Fig. 1. (A), microtransponders used in the assay, the integrated circuit side and the back of the microtransponder (dark gray squares).

To coat MTPs, the chips were treated with coating solution containing 3-aminopropyltriethoxysilane. Capture probes were synthesized on the coated MTPs at PrimeSyn. The capture probe sequences were complementary to the tag sequences in each allele-specific primer; in addition, they had a spacer of 9 T residues at the 3′ end. (B), schematic of the assay. (C), multiplex PCR results. Eighteen DNA fragments were PCR amplified from the CFTR gene. Lane 1, 100 bp DNA ladder; lane 2, 18-plex standard; lane 3, 18-plex-PCR-1; lane 4, 18-plex-PCR-2; lane 5, 6-plex-PCR-1; lane 6, 6-plex-PCR-2; lane 7, 6-plex-PCR-3. The presence of proper PCR products was confirmed on a 10% polyacrylamide Tris-boric acid-EDTA gel. All 18 DNA fragments can be readily identified by PAGE (lanes 5–7) when the PCR is done in 3 sets of 6 amplicons each.

To construct the multiplex primer set, all the primer pairs were combined at a final concentration of 74 nmol/L each, although in some cases an adjustment was made to achieve more efficient amplification. PCR reactions were performed on the GenAmp PCR System 9700 (Applied Biosystems). The 18- and 6-plex amplifications were performed in 30-μL reactions containing 3 units AmpliTaq Gold DNA polymerase (Applied Biosystems), 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 250 μmol/L deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, and dTTP), 6 μL primer mix, and 0.65 μL DNA template at a concentration of ~184.4 ng/μL. Forty thermal cycles were implemented, and the extension temperature was between 55 and 64 °C.

Equal volumes of all PCR products to be simultaneously assayed were pooled and enzymatically treated to degrade the excess PCR primers and dNTPs using 1 unit shrimp alkaline phosphatase and 2 units Exo I exonuclease per 10 μL of the PCR products in a reaction performed at 37 °C for 30 min, followed by a 15-min incubation at 80 °C to inactivate the enzymes.

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