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Rapid single nucleotide polymorphism detection for personalized medicine applications using planar waveguide fluorescence sensors

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ABSTRACT

Personalized medicine is an emerging field in which clinical diagnostics information about a patient's genotype or phenotype is used to optimize his/her pharmacotherapy. This article evaluates whether planar waveguide fluorescent sensors are suitable for determining such information from patient testing in point-of-care (POC) settings. The model system was Long QT Syndrome, a congenital disease associated with single nucleotide polymorphisms (SNPs) in genes encoding for cardiac ion channels. Three different SNP assay formats were examined: DNA/DNA hybridization, DNA/PNA hybridization (PNA: "peptide nucleic acid"), and single base extension (SBEX). Although DNA/DNA hybridization produced a strong intensity-time response for both wildtype and SNP analytes in a 5-min assay at 32°C, their hybridization rates differed by only 32.7%, which was insufficient for clinical decision-making. Much better differentiation of the two rates was observed at 53°C, where the wildtype's hybridization rate was two-thirds of its maximum value, while that of the SNP was essentially zero. Such all-or-nothing resolution would be adequate for clinical decision-making; however, the elevated temperature and precise temperature control would be hard to achieve in a POC setting. Results from DNA/PNA hybridization studies were more promising. Nearly 20-fold discrimination between wildtype and SNP hybridization rates was observed in a 5-min assay at 30°C, although the low ionic strength conditions required necessitated a de-salting step between sample preparation and SNP detection. SBEX was the most promising of the three, determining the absolute identity of the suspected polymorphism in a 5-min assay at 40°C.

Keywords: Biosensor, evanescent excitation, fluorescence, hybridization, long QT syndrome, molecular diagnostics, personalized medicine, peptide nucleic acid, pharmacogenetics, planar waveguide, point of care, single base extension, single nucleotide polymorphism

1. INTRODUCTION

With the completion of the Human Genome Project[1] in 2003 came the search for genetic polymorphisms within the genome. Such polymorphisms are responsible for diversity among individuals, but are also thought to be linked to predisposition to many common diseases[2-4]. Furthermore, genetic information about such polymorphisms can be used to assess the outcome of drug therapy (referred to as pharmacogenetics). For example, patient response to many drugs is highly individualized—plasma drug concentrations that are effective and well tolerated in one patient may cause adverse drug reactions in another. Often, such differences are linked to polymorphisms in the patient's genetic makeup. Personalized medicine is an emerging field that makes use of a patient's genetic information to optimize their therapy for both safety and efficacy.

Single nucleotide polymorphisms (SNPs) account for more than 90% of all polymorphisms[5]. They occur every 100-300 base pairs[5]; more than two-thirds are cytosine to thymine substitutions[5]. The human genome is thought to contain over 10 million SNPs[6]. To date, nearly 90% of these have been identified and deposited in public databases[7,8]. However, most of these have little if any impact on human disease. In fact, direct linkages between polymorphisms and human disease have only been established for about 1,950 genes, so far[9-11]. Interestingly, many of these genes contain multiple disease-linked polymorphisms—more than 49,000 have been reported in the 1,950 genes (average of about 25 polymorphisms per gene), 57% of these are SNPs[11]. Needless to say, establishing new linkages between

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polymorphisms and human disease (referred to as direct candidate association) is a very active area of research (see review by Suh and Vijg[10]). However, our work is more focused on personalized medicine—particularly developing rapid, inexpensive diagnostics assays for known SNPs, with the goal of selecting the most efficacious pharmacotherapy for a given individual. We believe that the development of POC diagnostics testing methods for both SNPs and phenotypic markers will be an important factor in the development of personalized medicine.

Presently, SNP testing is performed in reference or other clinical laboratories far from the point of care. Such testing is time-consuming (turn-around time of several days) and expensive ($500-$2,500 per assay). Several different SNP testing methodologies are employed including electrophoresis-based techniques[10,12,13], microarrays[14,15], real-time polymerase chain reaction[16-18] (real-time PCR), and sequencing[10]. Except for real-time PCR, all of these are too complex, slow and expensive for deployment in point-of-care (POC) environments. Real-time PCR is typically performed in low volume capillaries with fluorescence detection. Thus, it's both rapid and sensitive, and good progress has been made in reducing the size and cost of thermal cyclers. However, only a limited number of reactions (20-30) can be performed in parallel in a single machine. Still, we expect real-time PCR to migrate from clinical labs into POC environments over the next few years.

Over the past two decades, our laboratory has developed planar waveguide fluorescent biosensors for POC diagnostics applications. Our sensors are based on a patented injection-molded design that combines a 25 mm x 25 mm x 0.5 mm planar waveguide with an integral coupling lens[19-23]. The waveguide can be patterned with "capture" molecules such as antibodies or oligonucleotides. The integral lens enables precise optical alignment of the removable sensor unit with a fixed light source and detection system. Injection molding decreases piece cost to a few dollars (or less with multi-cavity injection) allowing waveguides to be used as one-shot, disposable sensors, which is important for clinical applications. Planar waveguide fluorescent biosensors offer the following attributes that make them ideal for rapid, POC diagnostics assays:

- Affinity assays (e.g., immunoassays, nucleic acid hybridization assays, drug-receptor assays, etc)
- Disposable sensor element (minimizes biosafety issues with human specimens)
- Point-of-care environments (e.g., physician's office, out-patient clinics, emergency department, etc)
- High sensitivity (low picomolar range)
- Fast response time (typically 5 minutes at room temperature)
- No wash or reagent addition steps after initial sample injection (resulting in low complexity assays)
- Multi-channel detection (enabling multi-analyte assays and on-board calibration)

Initially, we developed clinical POC immunoassays for analytes such as cardiac troponin subunit I, chorionic gonadotrophin, creatine kinase isoform MB, and myoglobin[24-27]. More recently, we've developed POC assays for nucleic acids[24,28,29], and biological warfare agents[30]. Our waveguide technology is potentially faster than all of the SNP detection methodologies discussed previously, and less complex than electrophoresis-based methods, microarrays, and sequencing. It also potentially rivals microarray technology in terms of assay multiplicity. In fact, a planar waveguide patterned with capture molecules can be viewed as a microarray with integrated detection system.

In this report we examine the feasibility of using planar waveguide fluorescent biosensors for rapid SNP detection in POC environments. We chose Long QT Syndrome (LQTS) as a model congenital genetic disease because it has been linked to a series of SNPs in genes encoding for cardiac ion channels[31-34]. Under physiological or emotional stress, affected individuals may develop a rare tachycardia referred to as *torsade des pointes* that results in syncope. Occasionally, this arrhythmia progresses into fatal ventricular fibrillation. These arrhythmias can be treated with an implantable defibrillator, or prophylactically with beta-blockers. However, affected individuals must first be diagnosed. Some (but not all) have a prolonged QT interval in their electrocardiograms. Thus, EKG is a useful preliminary screening procedure, but not necessarily conclusive because of false negatives[35]. SNP screening can potentially provide a more conclusive diagnosis, as well as information about the optimum beta-blocker to employ[36] (personalized medicine), but is not readily available because of high assay cost and the low frequency (1 in 5,000 to 1 in 7,000) of affected individuals. Thus, inexpensive SNP screening in LQTS is a significant unmet need.

We evaluated three different solid-phase molecular diagnostics assay formats that have been used for SNP detection—DNA-DNA hybridization, DNA-PNA hybridization (PNA is an abbreviation for "peptide nucleic acid"), and single base

extension (SBEX). The two hybridization assays involve the immobilization of a synthetic, single-stranded probe oligonucleotide (either DNA or PNA) that captures the analyte (also referred to as "target") nucleic acid from solution. The analyte is often a gene or exon fragment isolated from patient DNA and amplified using polymerase chain reaction (PCR). After 20-30 PCR cycles, the product of the PCR reaction (double-stranded DNA) is melted and snap cooled to give a single-stranded sample. For fluorescent assays, the PCR primers can be labeled with a fluorescent dye, conveniently giving fluorescently labeled analyte. Single base extension is a unique assay concept that involves both DNA hybridization and the enzyme-catalyzed incorporation of a dideoxynucleotide by DNA polymerase. Analyte DNA first hybridizes to the capture oligonucleotide, forming a DNA duplex that binds the polymerase. The sequence of the capture probe is designed so that the enzyme uses the suspected polymorphism in the analyte as its template for adding a complementary base to the 3' end of the capture probe. Fluorescently labeled dideoxynucleotide triphosphates are used as the substrate for the enzyme. The incorporated base can be identified by either wavelength or spatial resolution.

2. METHODS

A succinct description of methods is given below. Please see our recent review article[24] on planar waveguide fluorescent biosensors for a more complete description.

Biosensor. The biosensor assembly consisted of a planar waveguide sensor and a 3-channel flowcell. Planar waveguides were injection molded from polystyrene using a single cavity tool by Opkor, Inc. (Rochester, NY). Flowcells consisted of top and bottom plates machined from aluminum and anodized flat black. Three flow channels were milled into the top plate, each with small inlet and outlet ports and a 200-μL volume. A Peltier device (thermoelectric heat pump) and heat sink was also attached to the top plate enabling temperature control of the biosensor assembly over a range of 10-70°C. Three windows were milled into the bottom plate to allow fluorescence imaging of each channel. The waveguide was inserted between the two plates and sealed against the top plate with a composite gasket (Teflon/silicone rubber) using mechanical pressure. The gasket's low refractive index Teflon layer faced the waveguide and prevented the loss of guided modes into the gasket. The more compliant silicone rubber layer was used to ensure a watertight seal against the metal top plate. The three barrels of a computer-controlled syringe pump (Cavro Scientific Instruments, Sunnyvale, CA) were connected to the outlet ports of the flowcell. When actuated, the pump created negative pressure within each channel that pulled samples into the flowcell via "sipper" tubes attached to each inlet port.

Instrumentation. Biosensor instrumentation consisted of a 12 mW, 635 nm semiconductor laser, beam-forming optics, a mounting pedestal for the biosensor assembly, and a CCD camera (Santa Barbara Instruments Group, Model ST-6, Santa Barbara, CA). The laser was equipped with line-generating optics that produced a diverging beam with rectangular profile. The beam was expanded and collimated with beam-forming optics into a 20 mm x 1 mm sheet, which was then coupled with steering mirrors into the integral lens of the sensor. A computer-controlled, electromechanical shutter was placed between the laser and the beam-forming optics. It was closed between measurements to reduce photo bleaching. The CCD camera was equipped with a 55 mm f/2.8 macro lens (Nikon, Tokyo, Japan) that imaged the sensor's three channels. A 670 nm band-pass interference filter (Orion, Santa Cruz, CA) inserted in front of the macro lens rejected scattered laser light. A Macintosh 7600 computer (Apple Computer, Cupertino, CA) with a PowerForce 280 MHz G3 accelerator card (PowerLogix, Austin, TX) acquired images from the CCD camera at 6 sec intervals. Data reduction consisted of dark count subtraction and summation ("binning") of the individual pixels in each channel. All instrument control, data acquisition, and data reduction operations were programmed using LabView version 4.0.1 (National Instruments Corp., Austin, TX). Specific control macros were written for each assay protocol. Typically, these collected data over a given assay period (5 or 16 min) and computed the mean reaction rate (and its standard error) for each channel using ordinary least squares.

SNP detection assays. Because the present report is a methods evaluation study, highly purified synthetic analyte oligonucleotides were used instead of PCR-amplified human DNA. The PCR amplification process is not without error, so the use of synthetic DNA ensured that our analyte oligonucleotides actually contained the nominal sequences. A model LQTS SNP was chosen for the methods evaluation study. In particular, we chose a known polymorphism

occurring at position 760 in the KVLQT1 gene (encodes the alpha subunit of a cardiac muscle potassium channel), in which guanine is replaced by adenine (referred to as "G760A"). It's a fairly common polymorphism and de-identified patient samples are available.

For DNA-DNA hybridization, the following probe and analyte oligonucleotides were synthesized, HPLC-purified, and mass spectrometry validated by the DNA/Peptide core facility at the University of Utah: 1) capture probe: 5'-biotin-ATGAAGACCACGGAGCCCAGG; 2) wildtype analyte: 5'-Cy5-CCTGGGCTCCGTGGTCTTCAT; and 3) G760A analyte: 5'-Cy5-CCTGGGCTCCATGGTCTTCAT. The wildtype analyte sequence corresponded to positions 750-770 of the KVLQT1 gene. The same two analytes were used in DNA-PNA hybridization assays. The following PNA capture probe was purchased from Applied Biosystems (Foster City, CA): 5'-biotin-GAAGACCACGGAGCCCA. Different capture and analyte oligonucleotides were required for the SBEX assays: 1) capture probe: 5'-biotin-CCTGGCGGAGGATGAAGACCA; 2) wildtype analyte: 5'-TCCGTGGTCTTCATCCACCGCCAGGAGCT; and 3) G760A: 5'-TCCATGGTCTTCATCCACCGCCAGGAGCT. All three were synthesized, HPLC-purified and mass spectrometry validated at the DNA/Peptide core facility.

Capture oligonucleotides were immobilized to waveguides using a modular avidin/biotin approach. Clean waveguides were first coated with 150 nM neutravidin in PBS (phosphate buffered saline, pH 7.5) for 1 hr and washed twice in TE buffer (10 mM Tris, pH 7.4, with 1 mM ethylenediaminetetraacetic acid) to remove unadsorbed neutravidin. Capture oligonucleotide biotinylated at the 5' end (50 nM in TE buffer) was allowed to react with the immobilized neutravidin for an additional hour. Unreacted oligonucleotide was then removed by washing twice in TE buffer. Finally, waveguides were post-coated with trehalose (0.1% w/v in TE buffer) and vacuum dried for several hours at room temperature. Trehalose protected the immobilized oligonucleotides, giving a shelf life of several months when stored desiccated at 4°C. All coating and post-coating steps were performed in gasketed coating trays. Coating gaskets were cut to the same pattern and dimensions as the flowcell gasket, so only the portion of the upper waveguide surface in contact with the three flow channels was coated with capture oligonucleotide.

The same general assay protocol was used for both types of hybridization assay. Analyte oligonucleotide was injected into the three flowcell channels and hybridization rate was monitored over time. Usually the same analyte concentration was injected into each channel, giving triplicate measurements of the hybridization rate from a single biosensor assembly. The effects of several different parameters (monovalent cation concentration, divalent cation concentration, and temperature) on hybridization rate were examined. These parameters were refined using a "design of experiments"[37] approach to give optimal discrimination between wildtype and G760A sequences.

A version of spatial resolution was used in our SBEX assay in which a given analyte was mixed independently with each of the four Cy5-labeled dideoxynucleotide triphosphates. Each mixture was then injected into separate channels of a biosensor assembly, and the base incorporation rate monitored over a 5 min period. Analysis of variance (ANOVA) and Dunnett's post hoc test[38] were used to analyte the results. Thermo sequenase was chosen as the DNA polymerase because fluorescent labeling of the dideoxynucleotides has little impact on its incorporation rate. Kits containing thermo sequenase as the four Cy5-ddNTPs were purchased from GE Healthcare (product no. 27-2682-01).

3. RESULTS AND DISCUSSION

3.1. DNA/DNA Hybridization SNP Assays

Figure 1 shows the reaction kinetics at 32°C for both the wildtype (Wt) and G760A analytes (100 pM) hybridizing to a capture oligonucleotide complementary to the wildtype analyte. These data were reported in our previous *Analytical Biochemistry* article[28], and are included here as a reference point for the DNA/PNA hybridization and the single base extension assays. Both reactions took about 20 minutes to reach study-state, at which point the intensity of G760A was 36.2% lower than that of the Wt analyte. Thus, an end-point assay would take at least 20 minutes to run, with only marginal discrimination between perfectly matched and single-base mismatched species—clearly not adequate for POC SNP testing.

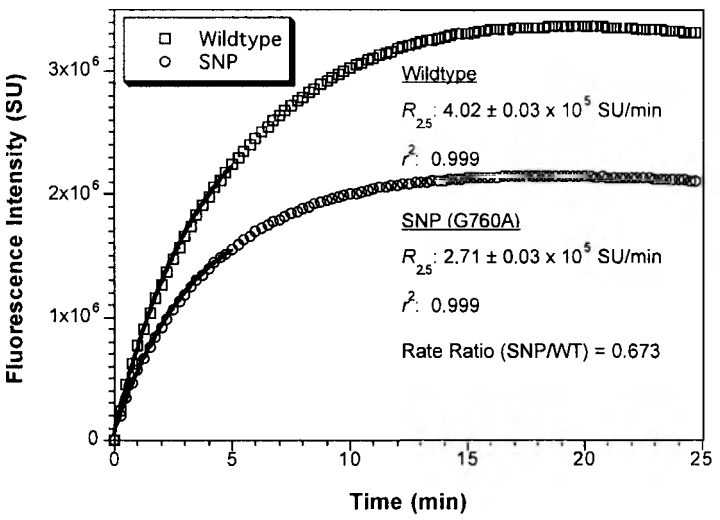

Figure 1 -- Reaction kinetics for the Wt (open squares) and G760A (open circles) analytes hybridizing to an immobilized capture oligonucleotide complementary to the Wt sequence (see *Methods*). The same reaction conditions were used for each analyte: 0.1 nM analyte; reaction temperature of 32°C; 10 mM Tris, pH 8.5, with 74 mM NaCl, 800 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. Fluorescence intensity measurements were taken every 20 seconds. Intensity measurements are given in arbitrary sensor units (SU). These data were previously reported by Tolley *et al.*[28], but are repeated here as a reference point for subsequent studies. Average hybridization rates (R_{25}) were determined by fitting Equation 1 to data obtained during the initial 5 minutes of the reaction (see text). Curve fits are shown as the solid line through the first 20 points of each data set.

Because our biosensor system has a 5-min assay time specification, we also compared hybridization rates obtained from the first 5 minutes of each data set. Rates were evaluated at the midpoint (2.5 min) of each 5-min data set using Equation 1:

$$I_t = I_0 + R_{t_i}\left[\frac{e^{k_{tech}t_i}}{k_{tech}}\right]\left[1 - e^{k_{tech}t}\right] \qquad (1)$$

where I_t is intensity at time t, I_0 is initial intensity, R_{t_i} is hybridization rate evaluated at time t_i, and k_{tech} is a technical (empirical) rate constant. Rate values of 4.02±0.03 x 10^5 SU/min and 2.71±0.02 x 10^5 SU/min were determined for the wildtype and G760A analytes, respectively by non-linear curve fitting by ordinary least squares. Curve-fits are shown as solid lines in Figure 1. Thus, the G760A analyte hybridizes more slowly (32.7%) than the Wt analyte. Although the rate-based method reduces assay time, discrimination is a bit worse that reported above for the end-point assay.

The situation would be more even complex in an actual clinical assay because the patients will have two alleles. Individuals with a Wt genotype will have two Wt alleles, both of which would hybridize at the maximum rate (which we'll define as 100%). Heterozygous individuals would have one Wt and one G760A allele. The observed rate would then be the weighted average of the Wt rate and the G760A rate, or 83.6% of the Wt rate. Exceedingly rare autosomal recessive individuals would have two G760A alleles, both of which would hybridize at the G760A rate, which is 67.3% of the Wt rate. The most common clinical decision would be between Wt and heterozygous individuals, but their observed hybridization rates would only differ by 16.4%. Such a decision point would require very good assay precision (coefficient of variation (CV) of ≤ 5%), and very tight control on assay conditions such as analyte concentration, temperature, and cation concentrations. Such tight control would be challenging to achieve in an inexpensive POC assay system. Thus, we investigated whether better discrimination between Wt and G760A could be achieved at higher temperatures.

Figure 2 shows the results of a temperature ramping experiment performed between 51°C and 55°C. Hybridization rates for both Wt and G760A analytes were measured at five different temperatures within the above range. Each data set was normalized by the maximum observed rate within the range. These experiments showed that the melting temperature (T_m) of the G760A analyte was 1.1°C lower than that of the Wt analyte. The data also showed that 53°C was the ideal temperature for distinguishing these two analytes. At that temperature, the hybridization rate of the Wt analyte was 66.1% of its maximum value, while that of the G760A analyte was essentially zero. Although this discrimination is adequate for a clinical decision point, maintaining a 53°C assay temperature with 0.1°C (or better) precision would be very challenging in a rapid, POC assay system. Furthermore, the ideal reaction temperature (i.e., temperature giving maximum discrimination between SNP and Wt) is expected to vary with different SNPs. This would be problem in screening for multiple SNPs with a single planar waveguide sensor (long term goal of this project)

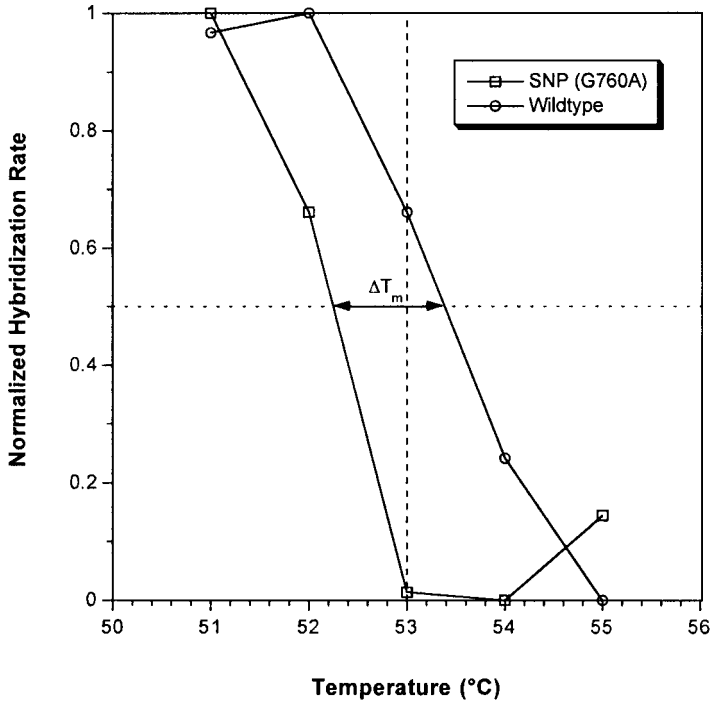

Figure 2 – Temperature ramping study using DNA/DNA hybridization assay. Hybridization assays were performed at 5 different reaction temperatures over a range of 51-55°C for both Wt and G760A analytes. A Peltier device attached to the flowcell top plate controlled temperature. Assay temperature was monitored with a thermister inserted in the top plate, immediately above the waveguide. Temperature was equilibrated for 5-10 minutes before performing assays. Assay conditions were identical to those given in Figure 1, except for the elevated temperatures and the 5-min data collection period. Hybridization rates were evaluated at the 2.5-min point using Equation 1. Data for a given analyte were normalized to its maximum hybridization rate observed over the temperature range. Melting temperature (T_m) was operatively defined as the temperature at which hybridization rate was reduced to 50% of its maximum value. Using this definition, the T_m of the Wt analyte was 1.1°C higher than that of the G760A analyte (denoted by the horizontal line with double arrows). The vertical dashed line shows the ideal assay temperature (see text).

because no single temperature would give ideal discrimination for all hybridization reactions. Temperature could be ramped (as was done in collecting the data shown in Figure 2), but assay times would be much longer because a 5-10 minute equilibration period is required after ramping to each new temperature, followed by the 5-min assay period, giving a total assay time of 50-75 minutes.

3.2. DNA/PNA Hybridization SNP Assays

Peptide nucleic acid (PNA) is a hybrid, synthetic polymer consisting of nucleoside bases covalently attached to a polypeptide backbone. It has some benefits over DNA as a capture strand in hybridization assays including: 1) reduced counter ion requirements; 2) faster hybridization rates; 3) greater duplex stability; and 4) better resistance to enzymatic digestion.[39,40] These benefits are potentially advantageous for planar waveguide biosensors as well, so we investigated PNA as a capture molecule in the hybridization assay described above. The analyte oligonucleotides (Wt & G760A) were the same, but the PNA capture probe was a 17-mer (sequence described in *Methods*) instead of the 21-mer DNA capture probe used above (17-mers were the longest PNA probes available commercially at the time these assays were performed). Hybridization assays were performed in triplicate for 5 min at 30°C in 10 mM Tris buffer, pH 8.4. Different concentrations of both monovalent (Na^+, K^+) and divalent cations (Ca^{2+}, Mg^{2+}) were added combinatorially to this buffer using a "design of experiments" approach[37]. Sixteen different combinations of cation concentrations were examined. The best discrimination between Wt and G760A analytes was observed at relatively low concentrations of both monovalent and diavalent cations (0.24 mM NaCl, 0.24 mM KCl, 0.2 mM $CaCl_2$, 0.2 mM $MgCl_2$), as shown in Figure 3. Under these conditions, the hybridization rate of G760A was 19-fold lower than that of the Wt analyte, which is a 94.7% decrease relative to Wt. The figure also shows a clear trend for better discrimination with decreasing NaCl and KCl concentration. Nevertheless, the hybridization rate of both analytes was markedly reduced in the absence of cations (data not shown). Although some additional gains in discrimination may be achieved by going to even lower cation concentrations, there is a trade off—namely, PCR is typically run in a buffer containing 10 mM Tris, pH 8.3, with 50 mM KCl and 1.5 mM $MgCl_2$. Thus, amplified analyte DNA from PCR would have to be desalted before being detected by DNA/PNA hybridization. Although not a problem in clinical laboratory settings, the desalting step would add complexity in a POC environment. Nevertheless, if this challenge can be overcome by microfluidic or other lab-on-a-chip approaches, the nearly 20-fold attenuation of the single-base mismatched species relative to the perfectly

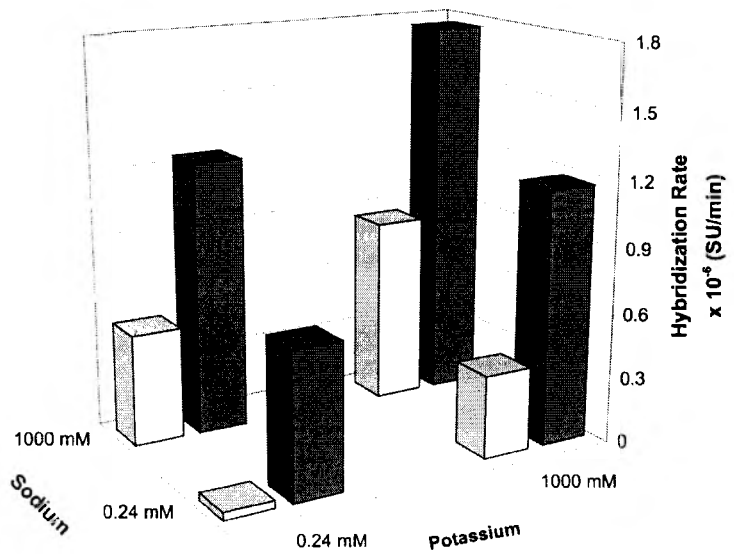

Figure 3 – Potassium and sodium cation effects on the DNA/PNA hybridization rates of Wt analyte (gray) and G760A analyte (white) at low divalent cation concentration. DNA/PNA hybridization assays were performed for 5 minutes at 30°C in 10 m*M* Tris buffer, pH 8.4 containing 0.2 m*M* $MgCl_2$ and 0.2 m*M* $CaCl_2$, and the sodium and potassium concentrations shown in the figure. Analyte concentration was 1 nM in all cases. Each assay was performed in triplicate. Average hybridization rates were determined by fitting Equation 1 to intensity versus time data as described above for the DNA/DNA hybridization assay.

matched species has the intrinsic resolution to detect SNPs in a hybridization-based method at near-ambient temperatures.

3.3. Single Base Extension SNP Assays

Because both types of hybridization assays had some shortcomings for POC testing, we investigated single base extension as a method for rapid SNP detection. SBEX is more accurate at detecting single base changes than hybridization assays because it exploits the intrinsic base incorporation fidelity of the DNA polymerase enzyme. Figure 4 shows single base extension reactions for all pair wise combinations of the Wt and G760A analytes with the four different dideoxynucleotide triphosphates (ddNTPs). A sham reaction containing buffer and DNA polymerase, but without analyte or ddNTP was run as a control blank. All reactions were run for 5 minutes at 40°C. For the Wt analyte, analysis of variance (ANOVA) followed by Dunnett's[38] post hoc test showed that only the incorporation rate of the complementary cytosine dideoxynucleotide triphosphate (ddCTP) was significantly higher ($P < 0.001$) than that of the blank. Similarly for the G760A analyte, only incorporation of its complemenary thymidine dideoxynucleotide triphosphate (ddTTP) was statistically significant ($P < 0.001$). Dunnett's test also showed that all reactions incorporating non-complementary bases were statistically indistinguishable ($P > 0.99$) from the control blank. Thus, SBEX gave nearly absolute discrimination between Wt and G760A analytes.

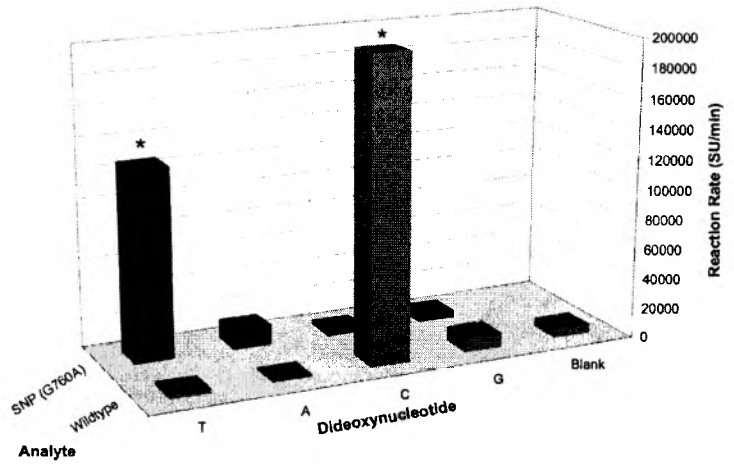

Figure 4 – Single base extension assays for the Wt and G760A analytes. All assays were performed with 0.1 nM analyte concentration for 5 min at 40°C in 10 mM Tris buffer (pH 8.5) with 10 mM $MgCl_2$ and 2 units of Thermo Sequenase. The following concentrations of Cy5-labeled dideoxynucleotide were used to compensate for reported[41] differences in reactivity: 4.0 nM of ddTTP, 4.9 nM of ddGTP, 2.0 nM ddCTP, and 2.5 nM ddATP. The intensity-time response of the sensor was linear over the assay period, so reaction rates were determined from the slopes of intensity vs. time curves using linear regression. Reaction rate values obtained for each analyte were compared to the blank rate using ANOVA and Dunnett's[38] post hoc test. Asterisks denote statistically significant ($P < 0.001$) rate values.

4. CONCLUSIONS

All three assays were able to detect SNPs at 1 nM analyte concentrations or lower, though the two hybridization assays required more stringent conditions than the single base extension assay. For example, the DNA/DNA hybridization assay exhibited best discrimination between matched and mis-matched sequences at temperatures above the T_m of the SNP, but below that of the Wt. This was a fairly strong condition for the sequences examined herein whose melting temperatures were both above 50°C and only differed by 1.1°C. More generally, it limits the practical application of DNA/DNA hybridization for SNP detection in rapid, POC assays. In contrast, the DNA/PNA hybridization assay exhibited excellent discrimination (19-fold) in a 5-min assay at only 30°C; however, such discrimination was only achievable with sub-millimolar concentrations of monvalent and divalent cations in the reaction mixture. This limitation has the practical consequence that samples prepared by polymerase chain reaction would have to be desalted before being assayed, which would complicate the fluidics of a POC SNP detection system. Single base extension gave the best results of the three assays—nearly absolute discrimination in a 5-min assay at 40°C. It is also the most readily adaptable of the three assay formats to multiple SNP assays on a single sensor chip. Shortcomings include a 40°C reaction temperature to achieve our 5 min assay specification and $5/assay reagent costs. The former should be obtainable in a POC instrument system, but may preclude a battery-powered handheld instrument.

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