Electronic Detection of Single-Base Mismatches in DNA with Ferrocene-Modified Probes

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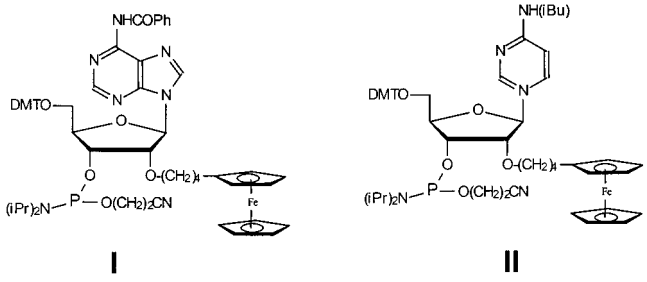
Abstract: Genotyping and gene-expression monitoring is critical to the study of the association between genetics and drug response (pharmacogenomics) and the association of sequence variation with heritable phenotypes. Recently, we developed an entirely electronic method for the detection of DNA hybridization events by the site-specific incorporation of ferrocenyl derivatives into DNA oligonucleotides. To perform rapid and accurate point mutation detection employing this methodology, two types of metal-containing signaling probes with varying redox potentials are required. In this report we describe a new ferrocene-containing phosphoramidite **9** that provides a range of detectable redox potentials. Using automated DNA/RNA synthesis techniques the two ferrocenyl complexes were inserted at various positions along oligonucleotide probes. Thermal stability analysis of these metal-containing DNA oligonucleotides indicates that incorporation of **9** resulted in no destabilization of the duplex. A mixture of oligonucleotides containing compounds **9** and **I** was analyzed by alternating current voltammetry (ACV) monitored at the 1st harmonic. The data demonstrate that the two ferrocenyl oligonucleotide derivatives can be distinguished electrochemically. A CMS-DNA array was prepared on an array of gold electrodes on a printed circuit board substrate with a self-assembled mixed monolayer, coupled to an electronic detection system. Experiments for the detection of a single-base match utilizing two signaling probes were carried out. The results demonstrate that rapid and accurate detection of a single-base mismatch can be achieved by using these dual-signaling probes on CMS-DNA chips.

Introduction

A key element in genetics research is the study of the association between genetics and drug response (pharmacogenomics) and the association of sequence variation with heritable phenotypes. Variations in sequence are known as single nucleotide polymorphisms (SNPs) and therefore SNP detection is an area of intense research.[1] A primary goal of this work is to construct a map of the molecular "circuitry" of cells and cell signaling and ultimately the genotyping of large numbers of patients so that treatment can be tailored to individuals. Chip-based DNA diagnostics are revolutionizing the way the genome is being sequenced and examined.[2] For example, DNA arrays have been developed to profile gene expression in cells that are exposed to test compounds in an approach known as toxicogenomics.[3]

As part of our research efforts to develop DNA arrays for electronically detecting nucleic acids we have focused on the site-specific incorporation of ferrocenyl derivatives into DNA oligonucleotides that function as electrochemical probes.[1a,4] We have reported the preparation and characterization of deoxyuridine nucleosides and nucleotides where ferrocene was conjugated to the nucleobase through unsaturated bonds[5] and the preparation of adenosine and cytidine modified with ferrocene at the 2′-positon through butoxy linkers.[6] The ferrocene-modified DNA oligonucleotides prepared from phosphoramidites **I** and **II** ($E_{1/2}$ of 0.120 V vs Ag/AgCl) have proven to be excellent signaling probes for the electronic detection of nucleic acids employing CMS-DNA chips.[1a,7,8]



I **II**

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(1) (a) Umek, R. M.; Lin, S. W.; Vielmetter, J.; Terbrueggen, R. H.; Irvine, B.; Yu, C. J.; Kayyem, J. F.; Yowanto, H.; Blackburn, G. F.; Farkas, D. H.; Chen, Y. P. *J. Mol. Diag.* **2001**, *3*, 74−84. (b) Schork N. J.; Fallin D.; Lanchbury J. S. *Clini. Genet.* **2000**, *58*, 250−264.

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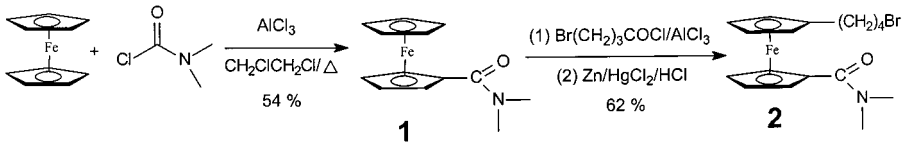
Scheme 1



However, to achieve rapid and accurate detection of single-base mismatches for genotyping and gene-expression analysis, two different metal-containing signaling probes with different redox potentials must be developed. To this end, we report the design and synthesis of a new phosphoramidite **9** with a dimthylcarbamyl group attached to the ferrocenyl moiety (Scheme 2). Employing phosphoramidite **9** and automated DNA/RNA synthesis techniques, the ferrocenyl derivatives can be incorporated into any position of a DNA sequence. In addition, the thermal stability and electrochemical properties of the resulting metal-containing DNA oligonucleotides were investigated. Finally, we describe the detection of a point mutation using dual-signaling probes on CMS-DNA arrays coupled to an electrochemical reader.

Results and Discussion

Preparation of Ferrocenyl Moiety. The redox potentials of the ferrocenyl derivatives can be tuned by substituting functional groups into the cylopentadienyl rings.[9] Electron-donating groups, such as alkyls, shift the $E_{1/2}$ value of ferrocenyl derivatives to lower potential relative to ferrocene,[6,10] while electron-withdrawing groups such as carbamyls shift the redox potential to higher values.[11−13] To alter the redox potential of the ferrocenyl complex originating from phosphoramidite **I** or **II**,[6] we chose to introduce a dimethylcarbamyl group that is both electron-withdrawing and free of an acidic proton. Dimethylcarbamyl ferrocene, **1**, was prepared from literature procedures[14] and reacted with 4-bromobutyryl chloride in the presence of aluminum chloride to yield 4-bromobutyryl dimethylcarbamyl ferrocene. Following Clemmensen reduction, the 4-bromobutyl dimethylcarbamyl ferrocene, **2**, was isolated in 62% yield (Scheme 1). The singly isolated product from the Friedel−Crafts acylation of compound **1** can be attributed to the ring deactivation by the dimethylcarbamyl group.

Preparation of Ferrocene-Containing Phosphoramidite. Deprotonation of adenosine was achieved by using sodium hydride followed by reaction with **2** to give **3** and **4** as an inseparable mixture (Scheme 2).[6] After protection of the exocylic amino group with benzoyl chloride, products **5** and **6** were isolated as a mixture. Following protection of the 5′-hydroxyl group with DMT, the 2′-isomer **8** and the 3′-isomer **7** were separated by silica gel chromatography in excellent yield. The acidic nature of the 2′-OH is responsible for the preferential formation of the isomer **8** (**8**/**7** = 2.7). The structures of **7** and **8** were assigned on the basis of ^{1}H NMR in DMSO-d_6 (Scheme 2). The ^{1}H NMR spectra of **7** show that the splitting pattern of the 2′-H is a quartet centered at 4.95 ppm with a coupling constant of 4.8 Hz indicating that no substitution of the 2′-OH (5.63 ppm) had occurred. In contrast, the 2′-H of **8** shows a triplet centered at 4.67 ppm with a coupling constant of 4.5 Hz and corresponds to the 2′-substituted isomer. Further, the chemical shift of the 3′-OH is shifted upfield to 5.28 ppm compared to that of the 2′-OH of compound **7**. Utilizing diisopropylamino cyanoethyl chlorophosphine as a phosphitylating reagent, **8** was converted to the corresponding phosphoramidite **9** in excellent yield.

Preparation of Ferrocene-Containing DNA Oligonucleotides. By using automated DNA/RNA synthesis techniques, phosphoramidite **9** was incorporated into DNA oligonucleotides with average coupling efficiency greater than 96% (DMT reading). Standard protocols were employed except for the prolonged coupling time (15 min). All DNA oligonucleotides were purified by HPLC on either a C6 reversed-phase column or an Oligo R3 (polystyrene-based column). Table 1 lists the sequences and numberings of the DNA oligonucleotides. The metal-containing DNA oligonucleotides **D3** (containing double **9**; calcd MS 5253.0, found 5255.0), **D5** (containing triple **I**, calcd MS 6563.1, found 6565), and **D6** (containing triple **9**, calcd MS 6760.2, found 6763) were characterized by MALDI-TOF mass spectral analyses, showing the successful incorporation of the ferroceneyl moiety into the DNA oligonucleotides.

Thermal Stability Study. A random 15 base-pair sequence [46.7% (G + C)] was designed to determine the thermal stability of the ferroceneyl-modified oligonucleotides. The thermal denaturation curves of two pairs of DNA hybrids **D1**:**D2** (perfect match) and **D1**:**D3** (**9** at the 12th and 14th positions) were obtained (Figure 1). Upon close examination of Figure 1 it can be seen that the melting curve of **D1**:**D3** duplex (ferrocence-containing DNA) is sharper than that of **D1**:**D2** duplex (nonmodified DNA). Data from Figure 1 clearly indicate that the two modifications in **D3** did not result in a decrease of the observed T_m value (**D1**:**D3**, 50.5 °C) as compared to that of nonmodified DNA duplex (**D1**:**D2**, 51.2 °C). The remarkably similar thermal stability of the modified-ferrocene DNA oligonucleotides and nonmodified ferrocene DNA oligonucleotides (**D1**:**D4**, 51.3 °C)[6] indicates that the dimethylcarbamyl ferrocence does not alter the hybridization properties of the corresponding DNA duplexes.

Detection of a Single-Base Mismatch. To test the ability of the ferrocene-modified oligonucleotides as signaling probes for the detection of nucleic acids, the electrochemical behavior of **D3** and **D6** was investigated by cyclic voltammetry (CV). The CV of **D3** in aqueous buffer is shown in Figure 2. A reversible wave associated with the oxidation and reduction of the pendant ferrocenyl complex was obtained with $E_{1/2}$ (0.324 V vs Ag/AgCl) and is very similar to that of nonmodified oligonucleotides in the shape of the CV response. A potential difference

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Scheme 2

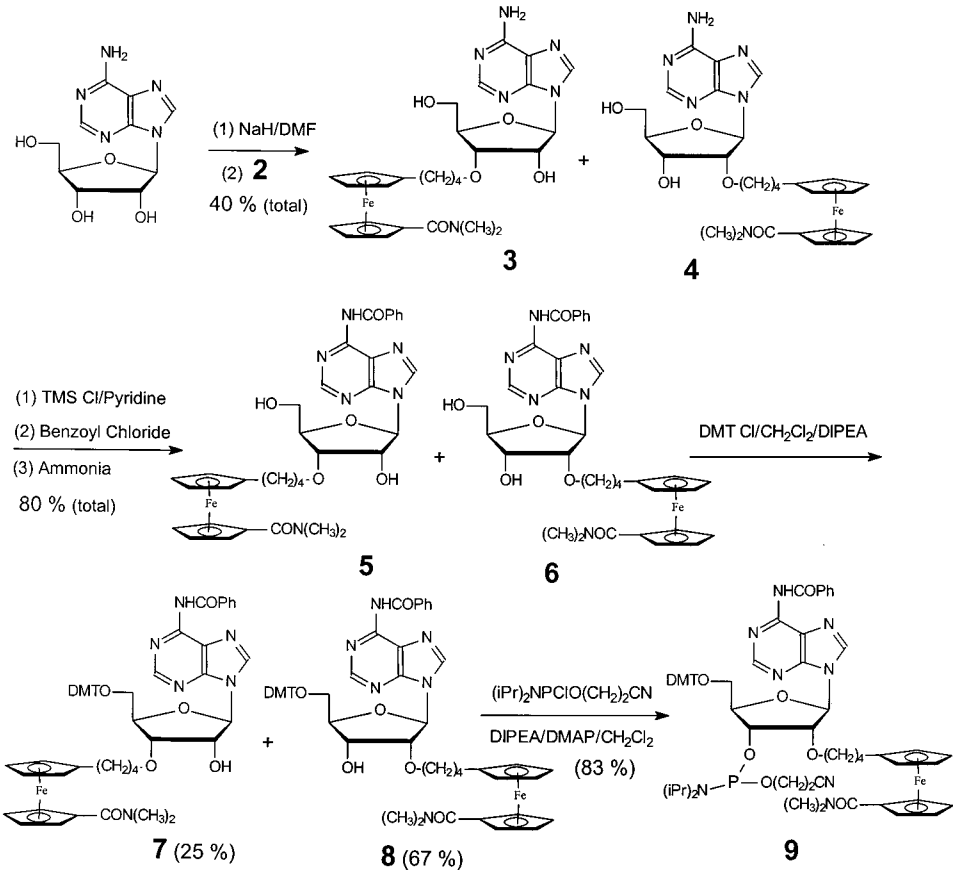

Table 1. DNA Sequences and Their Numberings

DNA Entry	DNA Oligonucleotides Sequences
D1	5'-ACC ATG GAC TCT GTT-3'
D2	5'-AAC AGA GTC CAT GGT-3'
D3	5'-A(**9**)C (**9**)GA GTC CAT GGT-3'
D4[8]	5'-A(**I**)C (**I**)GA GTC CAT GGT-3'
D5	5'-(**I**) (**I**) (**I**) GAC CT***G*** CCC TGT GCA G-3'
D6	5'-(**9**) (**9**) (**9**) GAC CT***A*** CCC TGT GCA G-3'
D7	5'-AAC CCA CAG CTG CAC AGG G***C***A GGT CTT GGC CAG TTG GCA AAA CAT CTT –3' (Target, Wild-type)
D8	5'-AAC CCA CAG CTG CAC AGG G***T***A GGT CTT GGC CAG TTG GCA AAA CAT CTT –3' (Target, Mutant)
D9	5'-AAG ATG TTT TGC CAA CTG GCC AA-Linker-3' (Capture probe on surface for this study)
D10	5'-CAG CAC ATG ACG GAG GTT GT-Linker-3' (Negative control)

of +0.166 V vs Ag/AgCl for the signaling probes containing **9** and **I** under identical conditions was observed.[6]

To determine if the two ferrocenyl complexes could be electrochemically differentiated, a mixture of **D3** and **D4** in similar concentrations (100 μM) in aqueous buffer was analyzed by using alternating current voltammetry (ACV)[4d,7,15] and monitored at the 1st harmonic (100 Hz) (Figure 3). Two clearly distinguishable peaks with similar heights were observed yielding E_p values of 0.350 and 0.180 V vs Ag/AgCl for **9** and **I**, respectively. These data demonstrate that the potential difference between the new ferrocenyl derivative **9** and **I** can be resolved and therefore used as electrochemical (dual) signaling probes.

The dual-signaling probes were incorporated into CMS-DNA chips[8] to facilitate single-base mismatch detection. A low potential ferrocenyl complex (containing **I**) and a high potential ferrocenyl complex (containing **9**) were inserted into signaling probes **D5** and **D6**, respectively. The CMS-DNA arrays were prepared on gold electrode surfaces by using self-assembled mixed monolayers coupled to an electronic detection system.[1a,7] The assay format (sandwich) used in this array is shown in Scheme 3. The single-base mismatches under investigation were located where the signaling probe hybridizes to the target.

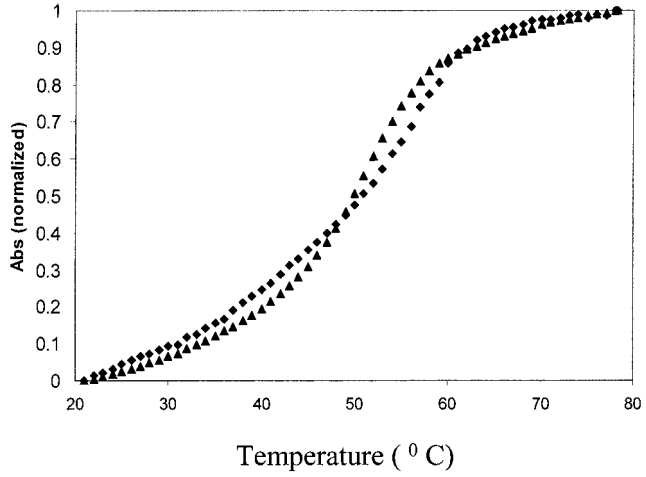

Figure 1. Thermal denaturation curves of **D1**:**D2** duplex (■, T_m = 51.2 °C) and **D1**:**D3** duplex (▲, T_m = 50.5 °C).

The CMS-DNA chip design (16 electrodes/chip) is shown in Figure 4. Six of the electrodes were coated with **D9** to capture the target mimics **D7** and **D8** (this sequence is associated with the p53 gene). Six electrodes were coated with **D10** as a negative control, and 4 electrodes were coated with the linker fragment terminated with three glycol units as the second negative control. The mismatch in the two target mimics is at position 20, where *T* in **D7** is mutated to dC in **D8** and vice versa. Finally, **D5** is

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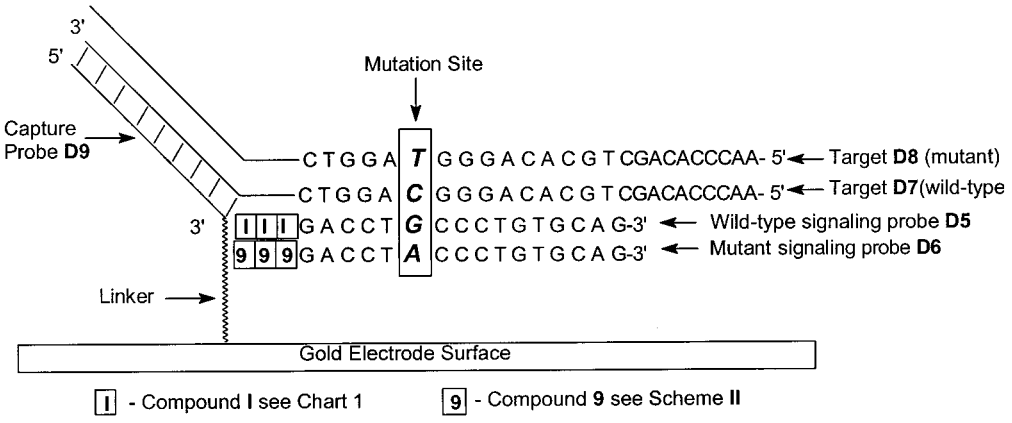
Scheme 3



designed to hybridize perfectly to **D7**, while **D6** is designed to hybridize perfectly to **D8**.

Three experiments were performed to test this system. In the first experiment a 1000 μL cocktail containing **D5** (250 nM) and **D6** (250 nM) in a hybridization buffer solution[7] was added to 4.24 μL of **D7** (11.78 μM, final concentration = 50 nM, wild-type). After thorough mixing, the resulting solution (85 μL) was injected into the chamber of two different CMS-DNA chips. After hybridization at 40 °C for 4 h the chips were interrogated on an electrochemical reader monitored at 1st harmonics (result not shown) and 4th harmonics (100 Hz fundamental).[15,16] Both 1st and 4th harmonics gave identical results; however, the ACV spectra have different appearances. Only electrodes coated with **D9** on both chips yielded electronic signals with similar peak heights (Figure 5).

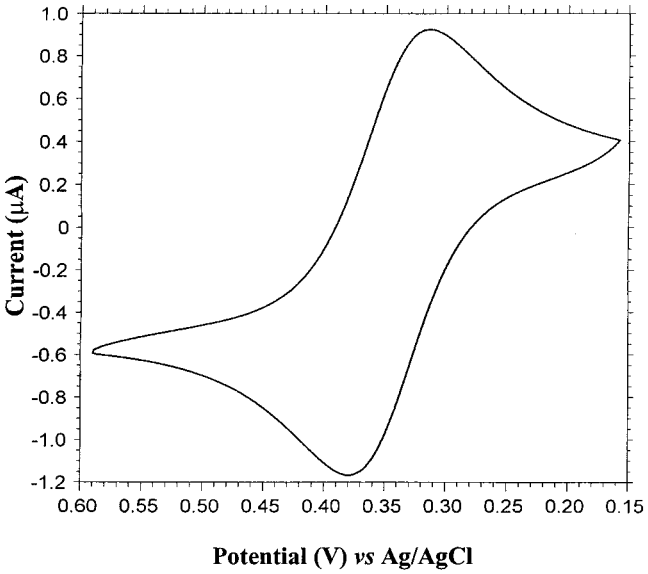


Figure 2. Cyclic voltammogram of **D3** in aqueous buffer solution.

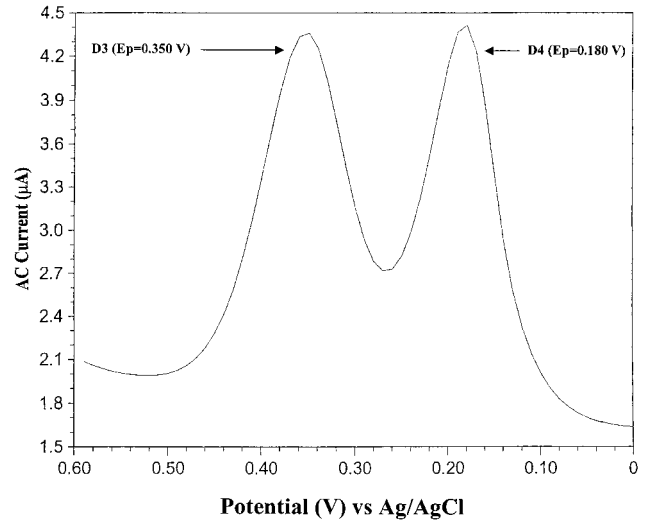


Figure 3. Alternating current voltammogram (ACV) of a mixture of **D3** and **D4** in aqueous buffer solution.

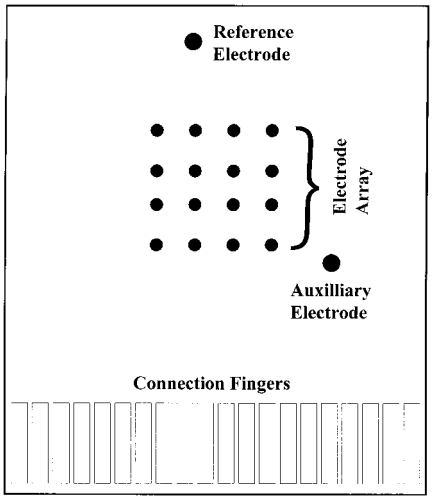


Figure 4. DNA chip design.

In the second experiment, 4.90 μL of **D8** (10.21 μM, final concentration = 50 nM, mutant) was added to 1000 μL of the cocktail. Following the procedure described above, the same electrodes in both chips were observed to have signals with similar currents (Figure 6). Finally, 4.24 μL of **D7** and 4.90 μL of **D8** were added to 1000 μL of the cocktail. This experiment serves as a model of a heterozygous test sample (Figure 7). In all experiments the ratio of the total concentration of signaling probes to target was 5- to 10-fold.

The results show that none of the negative control electrodes (with or without DNA probes) generate any detectable signal associated with nonspecific interactions. Examination of the data in Figures 5−7 reveals that only **D5** generated a signal ($E_p \approx$ 180 mV vs Ag/AgCl). There is no detectable signal from **D6**; however, the presence of target **D7** (wild-type) was indicated. In the second experiment, only **D6** generated a signal ($E_p \approx$

(16) The 4th harmonic is the 4th multiple of the primary excitation frequency. The 4th harmonic is used for background suppression and significantly enhances the signal-to-noise ratio. In the case of targets or target mimics at very low concentrations, the application of the 4th harmonic is clearly advantageous. Details are provided in the Experimental Section.

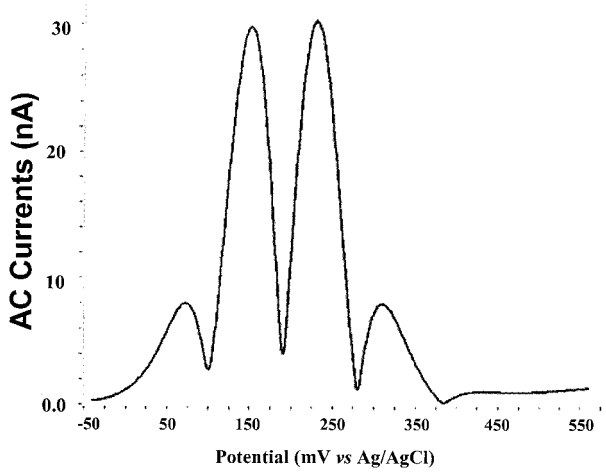


Figure 5. ACV spectrum for wild-type target mimic (**D7**).

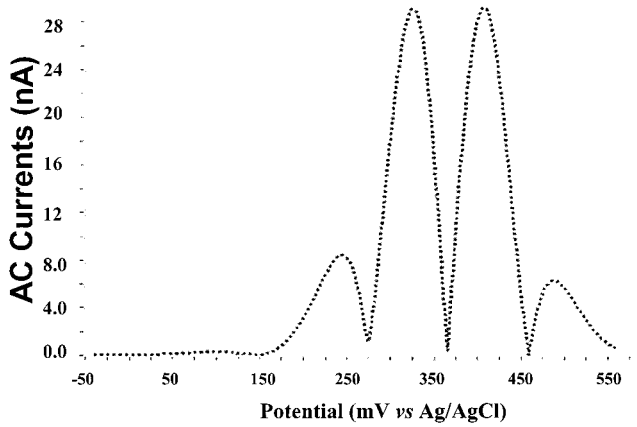


Figure 6. ACV spectrum for mutant target mimic (**D8**).

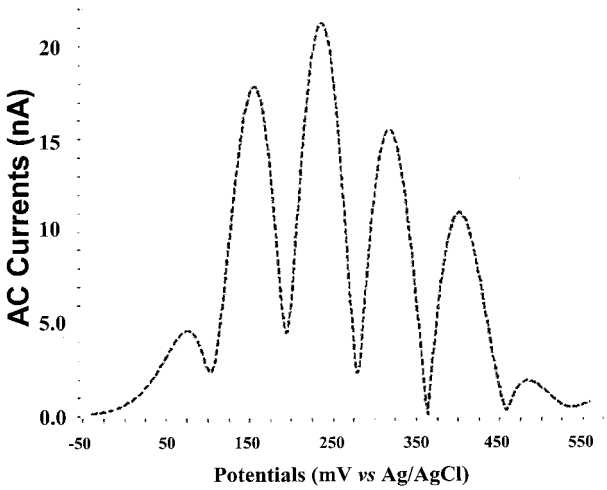


Figure 7. ACV spectra for a mixture of target mimics (**D7** and **D**8).

360 mV vs Ag/AgCl) with no detectable signal from **D5**. This result detects the presence of the target **D8** (mutant). It is important to note that in contrast to fluorescent DNA detection systems, no washing steps are required for this analysis.

The results shown in Figures 5 and 6 demonstrate that single-base mismatches are cleanly distinguished. An explanation for these results is that the T_m of the perfect DNA duplex on the chip surface (monitored by P^{32} labeled target mimics) is more than 14 deg lower than when measured in solution.[17] It is expected that a DNA duplex with a mismatch located in the middle of the sequence would be further destabilized. At an elevated temperature of 40 °C (where hybridization and scanning occur) the mismatched signaling probe will be more unstable. Under these conditions the perfectly matched signaling probe will completely replace the mismatched probe. This explanation is consistent with room-temperature data where a small amount of signal from the mismatched signaling probe can be detected (results not shown). Further experiments are underway to elucidate this mechanism.

In the third experiment, both **D5** and **D6** produced a signal. When partial overlap of the two peaks was observed we employed a software routine to deconvolute the data.[18] This revealed the presence of two signals of approximately equal magnitude centered at 180 and 360 mV (vs Ag/AgCl). The asymmetry of the voltammogram for the combined **D7** and **D8** is likely due to different heterogeneous electron transfer rate constants for the two ferrocenyl species at the modified electrode and may be attributable to their different hydrophobicities. These data clearly demonstrate that our approach of using dual electrochemical signaling probes efficiently detects the single-base mismatch (GT), and accurately genotypes the correct sequence. The application of detecting SNPs with the dual signaling probes for genotyping of human blood samples is the subject of a future publication.

In summary, we have designed and synthesized a new ferrocene-modified phosphoramidite **9** for the electronic detection of single-base mismatches in an array format. By employing automated DNA/RNA synthesis techniques the ferrocenyl complexes have been inserted into oligonucleotides at various positions. The thermal stability of the metal-containing DNA oligonucleotides has been investigated and indicates that the incorporation of **9** into DNA oligonucleotides causes little or no destabilization of the duplex. Electrochemical analysis of oligonucleotides containing **9** reveals that the derivative can function as a signaling probe for the electronic detection of nucleic acids. When incorporated into a CMS-DNA chip, results clearly show that dual-signaling oligonucleotide probes containing **9** and **I** detect single-base mismatches.

Experimental Section

Materials. Sodium hydride, *N*,*N*-dimethylaminopyridine (DMAP), triethylamine (TEA), trimethylchlorosilane (TMSCl), benzoyl chloride, ferrocene, diisopropylethylamine (DIPEA), aluminum trichloride ($AlCl_3$), dimethyl carbamyl chloride, and 4-bromobutyryl chloride were purchased from Aldrich and used as received. DMF (anhydrous), pyridine (anhydrous), dichloromethane, silica gel (240−400 mesh), acetonitrile (MeCN, HPLC grade), ethyl acetate, sodium bicarbonate, hexane, and methanol were purchased from EM Science and used as received. Acetonitrile (DNA synthesis grade) and dichloromethane (DNA synthesis grade) were purchased from Burdick & Jackson. 2-Cyanoethyl *N*,*N*-diisopropyl chlorophosphane, 4,4′-dimethoxytrityl chloride (DMT Cl), and andenosine were purchased from Chemgenes and used as received. All standard phosphoramidites and ancillary reagents were purchased from Glen Research and used as received.

Instrumentation. ^1H and ^{31}P NMR spectra were acquired on a 300 MHz (GE) and UV−vis spectra on a model HP 845X. Oligo. R3 HPLC

(17) The measurement of T_m of a DNA duplex on the chip surface was measured by attaching a 15 mer (5′-ACC ATG GAC TCA GCT-3′) to the chip via the 3′-end, and by labeling (^{32}P) the complementary strand (5′-AGC TGA GTC CAT GGT-3′) at the 5′-position. By measuring counts per minute (CPM) of the chip and the supernatant while incubating the chip at the appropriate temperatures, the melting curve was obtained and the T_m value extracted. The T_m of this duplex on the chip surface is 44 °C. However, in solution the T_m is 58.5 °C.

(18) The software was developed by Motorola Clinical Micro Sensors (Pasadena, CA).

columns were purchased from PerSeptive Biosystems, and C6 columns were acquired from Keystone Scientific. Mass spectra for organic compounds were obtained from Mass Consortium at San Diego with HP 1100 MSD for electrospray and high-resolution FAB Mass Spec were obtained from the Mass Spectrometry Laboratory for Biotechnology at North Carolina State University. MALDI-TOF mass spectra for DNA oligonucleotides, **D3**, **D5**, and **D6**, were obtained from Caltech Protein/Peptide Micro Analysis Lab.

All DNA oligonucleotides were synthesized with either an ABI 394 or an ABI 392 RNA/DNA synthesizer. HPLC analyses were performed on Hitachi D7000 systems equipped with a diode array, with a Betasil C_6 reversed-phase column (25 cm × 4.6 mm I.D.) and an Oligo R3 polystyrene column (10 cm × 4.6 mm I.D.). For the modified DNA oligonucleotides containing compounds **9** and **I** with a Betasil C_6 reversed-phase column, the gradient system is 10% to 35% MeCN over 32 min and 35% to 100% MeCN over 10 min in 100 mM TEAA (pH 6.5). For nonmodified DNA oligonucleotides with an Oligo R3 column, the gradient system is 0 to 25% MeCN over 32 min and 25% to 100% MeCN over 8 min in 100 mM TEAA (pH 10.0).

T_m values of DNA duplexes were measured under the following conditions: [DNA strand] = 2.0 μM; buffer, 1X SSC, with a temperature ramp from 20 to 80 °C at a rate of 1 °C per min.

CV data were acquired by using a computer-based CHI instrument (model 660) electrochemical workstation with a scan rate = 10 mV/s, and the following conditions: [**D3**] = 1.0 mM; buffer, 50 mM NaCl, 50 mM $MgCl_2$, 50 mM TRIS-HCl, pH 7.0; working electrode, gold wire; reference electrode, Ag/AgCl. ACV data were acquired by using the same instruments, the same buffer solutions, and the same electrodes with an amplitude of 25 mV and a frequency of 100 Hz, along with concentrations of [**D3**] = [**D4**] = 100 μM.

DNA chip analysis and ACV experiments were performed on an in-house electrochemical reader, which consists of a 500 MHz Pentium PC computer with a 16-bit, 100 kHz PCI-MIO-16XE-10 data acquisition (DAQ) card with an onboard digital-to-analog converter from National Instruments, a lock-in amplifier (Stanford Research Systems, SR830), and a custom summing amplifier/variable gain (10^4−10^7 V/A) current-to-voltage converter. The system is controlled via LABVIEW software (National Instruments). The reader is typically operated in the following fashion: A dc voltage is applied to the cell and ramped from −100 to + 500 mV (vs Ag/AgCl) at 50 mV/s. An ac voltage of 200 mV (peak to peak) is applied at a typical frequency of 100 or 1000 Hz in conjunction with the dc bias. For a fourth harmonic measurement, the 400 or 4000 Hz component of the output signal from the electrochemical cell is plotted as a function of DC voltage. DNA chips were manufactured by Motorola Clinical Micro Sensors.

Synthesis of Dimethylcarbamyl Ferrocene (1). To a solution of ferrocene (40.0 g, 0.22 mol) in 1,2-dichloroethane (250 mL) was added dimethylcarbamyl chloride (20 mL, 0.22 mol), followed by $AlCl_3$ (31.0 g, 0.24 mol) at room temperature. The reaction mixture was refluxed at 80 °C for 4 h. TLC revealed completion of the reaction (AcOEt: hexane = 70:30). The reaction mixture was cooled to room temperature and poured into ice−water with vigorous stirring. The organic layer was separated and the aqueous phase extracted once with CH_2Cl_2. The combined organic layers were washed with diluted sodium bicarbonate aqueous solution, then brine and dried over sodium sulfate and concentrated. The residue was purified by a 600 g silica gel column that was packed with 3% TEA/hexane and eluted with 3% TEA/20−60% ethyl acetate/hexane. The desired fractions were identified by TLC, pooled, and concentrated to give 30.0 g (54%) of the title compound as a brown solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.17 [br s, 6H, N(CH_3)$_2$], 4.27 (s, 5H, Fc), 4.35 (s, 2H, Fc), 4.66 (s, 2H, Fc). Anal. Calcd for $(C_{13}H_{15}NOFe + Na)^+$: 280.03. Found: 280.

Synthesis of 4-Bromobutyl Dimethylcarbamyl Ferrocene (2). To a solution of **1** (30.0 g, 0.11 mole) and 4-bromobutylryl chloride (13.5 mL, 0.11 mol) in dichloromethane (280 mL) was added $AlCl_3$ (37.0 g, 0.29 mol) in two portions. After being stirred for 2 h, the reaction mixture was poured into ice−water with vigorous stirring. The organic layer was separated and the aqueous phase was extracted with dichloromethane. The combined organic layer was washed with diluted sodium bicarbonate aqueous solution and brine, dried over sodium sulfate, and concentrated. The crude product was used for the next step of the reaction without further purification.

The crude product was dissolved in toluene (1.1 L). To this solution was added zinc dust (165.0 g, 2.5 mol), mercury chloride (16.0 g, 57 mmol), and water (300 mL), followed by dropwise addition of concentrated HCl (300 mL). After the mixture was stirred for 2 h, the toluene layer was separated and the aqueous phase was extracted twice with CH_2Cl_2. The combined organic layers were washed with 5% $NaHCO_3$ aqueous solution and brine, dried over Na_2SO_4, and concentrated. The crude product was purified on a 800 g silica gel column that was packed with hexane 1% TEA/hexane and eluted with 30−50% ethyl acetate/hexane. The desired fractions were pooled and concentrated to give 28.0 g (62% overall yield) of the title compound as a brown solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.64−1.71 (m, 2H, CH_2CH_2), 1.86−1.95 (m, 2H, CH_2CH_2), 2.40 (t, J = 7.5 Hz, 2H, $BrCH_2$), 3.14 [br s, 6H, N(CH_3)$_2$], 3.45 (t, J = 6.0 Hz, 2H, $FcCH_2$), 4.14−4.20 (m, 5H, Fc), 4.29 (s, 2H, Fc), 4.60 (s, 2H, Fc). Anal. Calcd for $(C_{17}H_{22}BrFeNO - Br)^+$: 311.12. Found: 311.

Synthesis of 3 and 4. A suspension of adenosine (95.0 g, 0.36 mol) in dried DMF (5 L) was heated until it became clear. To this suspension was added NaH (60% in mineral oil) (13.0 g, 0.33 mol) portion by portion. After the mixture was stirred for 4 h at room temperature, a solution of compound **2** (54.0 g, 0.14 mol) in 500 mL of DMF was added. Upon completing addition, the temperature of the reaction mixture was raised to 35 °C and the reaction mixture was stirred at this temperature for 48 h. The reaction was quenched by adding ice water (200 mL). After removing solvents in vacuo, the residue was dissolved in a mixture of ethyl acetate and water. The organic layer was separated and the aqueous layer was extracted once with ethyl acetate. The combined organic extracts were washed with 5% $NaHSO_3$ aqueous solution and brine, dried over Na_2SO_4, and concentrated. The crude product was purified on a 1400 g silica gel column which was packed with 2% TEA/50% of ethyl acetate/hexane and eluted with 80% of ethyl acetate/hexane and then 10% methanol/ethyl acetate. The desired fractions were pooled and concentrated to afford 32.0 g (40% total yield) of the title product as a brown solid, which contains both 2′- and 3′-isomers.

Synthesis of 5 and 6. To a solution of **3** and **4** (26.0 g, 45 mmol) in pyridine (210 mL) was added TMS Cl (28.0 mL, 0.22 mol) dropwise. After the mixture was stirred for 1 h at room temperature, benzoyl chloride (26.0 mL, 0.22 mol) was added dropwise. The reaction mixture was stirred for another 2 h and cooled in an ice−water bath. To this mixture was added 25 mL of water and after 30 min of stirring concentrated ammonia (50 mL) was added and the mixture was warmed to room temperature for 0.5 h. The reaction mixture was diluted by adding methylene chloride (1 L), and the reaction mixture was washed with water, 5% citric acid aqueous solution, and brine. The organic solution was dried over Na_2SO_4 and concentrated. The crude product was purified on a 700 g silica gel column which was packed with 2% TEA/50% ethyl acetate/hexane and eluted with 0−7% methanol/ethyl acetate. The desired fractions were pooled and concentrated to give 20.0 g (80% yield based on the consumption of starting materials) of the title product as an inseparable mixture, along with 3.2 g of the recovered starting materials. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30−1.36 (m, 4H, CH_2CH_2), 2.16 (t, J = 7.5 Hz, 2H, $FcCH_2$), 3.07 [br s, 6H, N(CH_3)$_2$], 3.40−3.78 (m, 4H, OCH_2 + 5′-H), 4.00−4.10 (m, 5H, 4′-H + 4 Fc), 4.25 (s, 2H, 2Fc), 4.32−4.37 (m, 1H, 3′-H), 4.52−4.58 (m, 3H, 2Fc + 2′-H), 5.15−5.25 (m, 1H, 2′- and 3′-OH), 6.17 (d, J = 6.0 Hz, 1H, 1′-H), 7.55−7.70 (m, 3H, aromatic), 8.08 (d, J = 7.8 Hz, 2H, aromatic), 8.77 (s, 2H, 2-H + 8-H), 11.25 (s, 1H, amide). Anal. Calcd for $(C_{34}H_{38}FeN_6O_6 + Na)^+$ and $(C_{34}H_{38}FeN_6O_6 - H)^-$: 705.54 and 681.55. Found: 705 and 681.

Synthesis of 7 and 8. To a solution of **5** and **6** (20.0 g, 29 mmol), DMAP (70.0 mg), and DIPEA (25.0 mL, 0.15 mol) in dried dichloromethane (240 mL) cooled into an ice−water bath was added DMT Cl (20.0 g, 58.6 mmol) in one portion. The reaction mixture was warmed to room temperature and stirred for 1 h, followed by adding dichloromethane (200 mL) and methanol (20 mL). This mixture was washed with 5% $NaHCO_3$ aqueous solution and brine, dried over Na_2SO_4, and concentrated. The crude product was purified on a 700 g silica gel column which was packed with 1% TEA/30% ethyl acetate/hexane

and eluted with 1% TEA/30−100% ethyl acetate/hexane and then 1% TEA/1−2% methanol/ethyl acetate. The desired fractions were pooled and concentrated to give 19.0 g (67%) of the pure **8** (2′-isomer) and 7.0 g (25%) of the pure compound **7** (3′-isomer). **7**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.43−1.53 (m, 4H, CH_2CH_2), 2.25 (t, J = 7.5 Hz, 2H, $FcCH_2$), 3.02 [br s, 6H, $N(CH_3)_2$], 3.22−3.65 (m, 4H, OCH_2 + 5′-H), 3.70 (s, 6H, 2 × OCH_3), 3.87−4.05 (m, 5H, 4Fc + 4′-H), 4.13−4.19 (m, 1H, 3′-H), 4.25 (t, J = 2.4 Hz, 2H, 2Fc), 4.53 (t, J = 1.8 Hz, 2H, 2Fc), 4.95 (q, J = 4.8 Hz, 1H, 2′-H), 5.63 (d, J = 6.0 Hz, 1H, 2′-OH), 6.05 (d, J = 4.8 Hz, 1H, 1′-H), 6.81−6.84 (m, 4H, aromatic), 7.18−7.62 (m, 10H, aromatic), 8.05 (d, J = 6.9 Hz, 2H, aromatic), 8.62 (s, 1H, 8-H), 8.68 (s, 1H, 2-H), 11.24 (s, 1H, amide). Anal. Calcd for $(C_{55}H_{56}FeN_6O_8 + Na)^+$: 1007.34. Found: 1007.

Compound **8**: ^{1}H NMR (300 MHz, DMSO-*d6*) δ 1.37−1.53 (m, 4H, CH_2CH_2), 2.18 (t, J = 6.0 Hz, 2H, $FcCH_2$), 3.02 [br s, 6H, $N(CH_3)_2$], 3.25−3.66 (m, 4H, OCH_2 +5′-H), 3.73 (s, 6H, 2 x OCH_3), 4.02 (br.s, 2H, 2Fc), 4.05 (br.s, 2H, 2Fc), 4.12−4.15 (m, 1H, 4′-H), 4.26 (s, 2H, 2Fc), 4.42−4.44 (m, 1H, 3′-H), 4.53 (s, 2H, 2Fc), 4.67 (t, J = 4.5 Hz, 1H, 2′-H), 5.28 (d, J = 5.7 Hz, 1H, 3′-OH), 6.17 (d, J = 4.8 Hz, 1H, 1′-H), 6.83−6.87 (m, 4H, aromatic), 7.18−7.69 (m, 10H, aromatic), 8.05 (d, J = 7.5 Hz, 2H, aromatic), 8.63 (s, 1H, 8-H), 8.69 (s, 1H, 2-H), 11.25 (s, 1H, amide). Anal. Calcd for $(C_{55}H_{56}FeN_6O_8 + Na)^+$: 1007.34. Found: 1007.

Synthesis of Phosphoramidite 9. To a solution of **8** (19.0 g, 19.3 mmol) and DMAP (100 mg) in dichloromethane (350 mL) was added DIPEA (27.0 mL, 0.15 mol), followed by 2-cyanoethyl *N*,*N*-diisopropyl chlorophosphane (8.6 mL, 38.6 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 3 h. After addition of dichloromethane (200 mL), the mixture was washed with 5% $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated. The crude product was purified on a 450 g silica gel column which was packed with 1% TEA/10% ethyl acetate/hexane and eluted with 1% TEA/50−90% ethyl acetate/hexane. The desired fractions were pooled and concentrated. After precipitating twice from hexane, 19.0 g (83%) of the title product as golden foam was obtained. ^{31}P NMR (121 MHz, DMSO-d_6) δ 150.01, 150.28. HRMS calcd for $(C_{64}H_{73}FeN_8O_9P)$: 1184.4588. Found: 1184.4602.

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