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A Single Base Extension Technique for the Analysis of Known Mutations Utilizing Capillary Gel Electrophoreisis with Electrochemical Detection

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A novel single nucleotide polymorphism (SNP) detection system is described in which the accuracy of DNA polymerase and advantages of electrochemical detection are demonstrated. A model SNP system is presented to illustrate the potential advantages in coupling the single base extension (SBE) technique to capillary gel electrophoresis (CGE) with electrochemical detection. An electrochemically labeled primer, with a ferrocene acetate covalently attached to its 5′ end, is used in the extension reaction. When the Watson−Crick complementary ddNTP is added to the SBE reaction, the primer is extended by a single nucleotide. The reaction mixture is subsequently separated by CGE, and the ferrocene-tagged fragments are detected at the separation anode with sinusoidal voltammetry. This work demonstrates the first single base resolution separation of DNA coupled with electrochemical detection. The unextended primer (20-mer) and the 21-mer extension product are separated with a resolution of 0.8.

Single nucleotide polymorphisms (SNPs) are the most abundant variation in the human genome, occurring on average every kilobase. These single base changes are biallelic and highly conserved within the human genome, making them ideal markers for genetic variability.[1−4] Individual SNPs are already known to cause several genetic diseases, and are hypothesized to be involved in complex disease progression.[1,5−11] Recently, The International SNP Map Working Group published a map of 1.42 million SNPs present in the human genome.[5] The development of a detailed map of SNPs will help to identify the variations present in coding regions (cSNPs), which are important in disease progression and drug response variability. The need for an efficient, cost-effective, and high-throughput scanning method is necessary to screen large populations for important cSNPs.

To effectively screen for hundreds to thousands of SNPs in a large sample population, the detection scheme employed has to be rapid, inexpensive, accurate, and sensitive.[6,12] Direct DNA sequencing is the ultimate method to identify and fully characterize a single nucleotide variation within a genome.[13] However, once the location within the genome is identified, direct sequencing is too time-consuming and expensive to routinely screen large populations for a particular SNP. There are generally considered to be two different modes of SNP analysis that are more amenable to high-throughput assays. The first type of mutation analysis assay deals with the detection of specific well-characterized mutations whose position and base substitution are known, and the second involves scanning a genome for uncharacterized SNPs. Scanning the genome for possible SNP sites is performed by either direct sequencing, single-stranded conformational polymorphism (SSCP),[13−16] heteroduplex analysis (HDA),[15,17−19] denaturing gradient gel electrophoresis (DGGE),[20,21] or temperature gradient gel electrophoresis (TGGE).[22]

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The most common techniques that fall into the first category include allele-specific amplification,[23−25] allele-specific hybridization,[26−31] 5′ nuclease PCR assays,[32] and primer extension reactions.[6−9,33−38] The first two techniques mentioned above involve utilizing two different primers, one with a sequence complementary to the wild type and the other primer differing by a single base corresponding to the mutation site. The two unique primers are used either in a PCR amplification reaction in which the primer containing the mutant site will be extended only if the mutation is present, or in a hybridization assay. Recently, the hybridization assay has been incorporated into DNA chips that are able to screen for hundreds of SNPs on a single device.[39,40] These devices have extremely high throughput, but because of the hardwiring of oligonucleotide markers, they are difficult to redesign for the analysis of different SNP screening sets.[3]

Primer extension reactions or minisequencing involves the design of a primer whose 3′ end falls immediately adjacent to the SNP site of interest.[2,7,35] This primer is then used in a DNA polymerase extension reaction with dideoxynucleotide triphosphates (ddNTP). The use of ddNTP ensures that the polymerase will incorporate only a single nucleotide corresponding to the Watson−Crick compliment of the SNP site of interest. The single base extension (SBE) technique has been used in both solution[2,6−8,34−37] and solid-phase reactions.[12,33,38] The technique is also compatible with multiplexed analysis and has been found to be extremely accurate as a result of the high fidelity of DNA polymerase.

SNP assays have been employed with many different detection platforms. Laser-induced fluorescence (LIF) is the most commonly used detection method. LIF detection is very sensitive, almost completely replacing the need to use hazardous radioisotopes as labels. Fluorescence is used in the detection of known mutations through hybridization assays[29,32] and SBE reactions using fluorescently labeled ddNTPs.[12,33,34,38,41] Additionally, fluorescently tagged primers are employed in mutation analysis utilizing separation methods.[9,26,27,42,43] There has been tremendous research into other possible detection methods for SNP analysis, including flow cytometry with fluorescence detection,[36,41] mass spectrometry,[2,6,35,37] and luminescence detection.[10]

Recently, the electrochemical detection of SNPs has been explored.[44,45] The detection strategy utilized by many is to label either the primer[46−48] or ddNTPs[49] with an electroactive molecule in an analogous strategy currently used for labeling with fluorescent molecules. Yu et al. utilized oligonucleotides labeled with ferrocene derivatives in a SNP hybridization based assay.[44] Several other authors have developed synthetic strategies for linking electrochemical labels to oligonucleotides (1) at the 5′ end,[46,47] (2) at a predetermined location during solid-phase synthesis,[50,51] and (3) to ddNTPs.[49] Electrochemical detection is a simple, cost-effective method that is highly compatible with miniaturization and portability.

This work presents a feasibility study for using single base extension coupled to capillary gel electrophoresis with electrochemical detection for the screening of known SNPs. A SBE primer (20-mer) with a ferrocene molecule covalently attached to its 5′ end is used in the primer extension reaction along with the ddNTP complementary to the mutation site of interest. Capillary gel electrophoresis (CGE) is used to separate the remaining primer from the 21-mer extension product, and sinusoidal voltammetry (SV) is used to detect the ferrocene label in each case. We previously demonstrated the sensitive and selective detection of ferrocene labeled DNA with SV detection.[52] The coupling of CGE with EC detection should provide a fast, simple, inexpensive, and easily miniaturized SNP screening technique.

EXPERIMENTAL SECTION

Reagents. The ddNTPs were purchased from Fermentas (Hanover, MD). The AmpliTaq polymerase, GeneAmp PCR buffer, dNTPs, Performance Optimized Polymer-4 (POP-4), Genetic analyzer buffer, 5′ amino linked T3 primer, T3 forward primer, and T3 reverse primer were provided by Applied Biosystems

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(Foster City, CA). Thermosequenase enzyme (USB Corporation, Cleveland, OH), pBluescript SK+ phagemid (Stratagene, La Jolla, CA), and Phi-x 174 standard ladder (Promega, Madison, WI) were used as received. Ferrocene acetic acid, *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, and 3-hydroxypicolinic acid (3-HPA) were obtained from Sigma-Aldrich (Milwaukee, WI) and used as received. Float dialysis membrane filters, VSWP 0.025-μm pore size, were purchased from Millipore (Bedford, MA). Water was deionized through a Milli-Q water purification system (Millipore, Bedford, MA).

Synthesis of 5′ Ferrocene Acetate T3 Primer. The synthetic procedure of Ihara et al. was followed and has been described previously.[47] Briefly, an activated N-hydroxysuccinimide (NHS) ester of ferrocene acetic acid (FA-NHS) was synthesized utilizing NHS and dicyclohexylcarbodiimide. The reaction mixture was purified using flash chromatography with methylene chloride as the eluent. The FA-NHS reaction produced a reddish-brown solid with a yield of 45%. The FA-NHS was confirmed by ^{1}H NMR in DMSO.

FA-NHS was dissolved in dimethyl sulfoxide. To this solution was added the 5′ amine-terminated T3 primer dissolved in a 0.5 M $NaHCO_3/Na_2CO_3$ (pH = 9) buffer solution. The reaction was agitated overnight and then diluted to 1 mL with water. The diluted reaction mixture was chromatographed on a NAP-10 Sephadex G-25 column from Pharmacia. The fractions containing DNA were combined and lyophilized on a DNA concentrator (LabConco, Kansas City, MO). The labeled oligonucleotide was purified by RP-HPLC using a Thompson liquid chromatograph 100 column, C18 with 5-μm particles, 4.6-mm i.d., and 15 cm in length. The details of the HPLC purification have been described previously.[52] The collected fractions containing the FA-labeled T3 were combined, dried, and then characterized using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer from PerSeptive Biosystems (Voyager DE-STR, Foster City, CA).

Template PCR. The template used in the primer extension reaction was a 159-bp fragment generated from the pBluescript II sK+ phagemid (Stratagene, La Jolla, CA). PCR reactions to produce and amplify the model template for SBE reactions were carried out in a total volume of 50 μL containing 1× GeneAmp PCR buffer (from a 10× stock solution), 0.2 mM dNTPs, 1 mM $MgCl_2$, 1 μM of each primer (forward primer, 5′ AAT TAA CCC TCA CTA AAG GG 3′ (T3 primer); reverse primer, 5′ ACT CAC TAT AGG GCG AAT TG 3′), 50 ng of pBluescript sK+ phagmid, and 1.3 U Amplitaq DNA polymerase. The thermocycling was performed in an ABI GeneAmp PCR System 2400 (Applied Biosystems). The conditions employed were 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 41 °C for 30 s, and 72 °C for 60 s, ending with a 7-min incubation at 72 °C.

Purification of PCR Amplicons. After PCR amplification of the template for the single base extension reaction, it is necessary to remove the remaining primers, enzyme, and dNTPs. This was accomplished by running a 2.75% w/v agarose gel (Agarose for the Separation of GeneAmp PCR Products, Applied Biosystems, Foster City, CA) in 1× TBE buffer (diluted from 5× Tris−borate−EDTA buffer from Sigma) with ethidium bromide (Fisher Scientific, Fair Lawn, NJ). Phi-x 174 was used as the size standard, and the band corresponding to the 159 bp PCR amplicon was excised with a scalpel. The DNA from the band was extracted using a MiniElute Gel Extraction Kit from Qiagen (Valencia, CA). The DNA concentration was approximated by measuring the absorbance at 260 nm using an average extinction coefficient of 16 × 10^6 M^{-1} cm^{-1}.

Single Nucleotide Primer Extension. The reaction volume used for SBE reactions was 20 μL and contained 100 pmol of FA-labeled T3 primer, ∼40 ng of the 159 bp PCR amplicon template, 200 μM of ddATP (complementary to the mock SNP site) or ddCTP (used as a specificity control, see Scheme 1), 4U of Thermosequenase DNA polymerase, and 1× sequencing buffer from the Thermosequenase terminator cycle sequencing kit. The primer extension reaction was performed in the same thermocycler as the template PCR amplification. The cycling conditions were as follows: denaturation at 94 °C for 90 s and then the temperature was cycled 60 times through 94 °C for 30s, 41 °C for 30s, and 72 °C for 60s. The SBE reaction was then held at 72 °C for an additional 10 min. The SBE reaction was desalted prior to both MALDI-TOFMS and CGE/SV analysis with float dialysis filter membranes (0.025 μm pore size; VSWP 047, Millipore, Bedford, MA). The membrane was suspended on top of ∼500 mL of water, and the sample was placed in the center of the membrane. Float dialysis of the sample was performed for ∼2 h. The sample was then carefully removed from the membrane filter with a pipet and dried down with the DNA concentration (LabConco). The sample was redissolved in 10 μL of water and analyzed either by MALDI-TOFMS or CGE/SV detection.

MALDI-TOFMS. Mass spectra were taken with a Voyager DE-STR time-of-flight mass spectrometer (PerSeptive Biosystems, Foster City, CA). The TOFMS was equipped with a N_2 laser (337 nm) operated in linear positive ion mode. The acceleration voltage used was 20 kV with an 800 ns delay time. The MALDI matrix used for oligonucleotide analysis was prepared as follows: 50 mg of 3-HPA was dissolved in 1 mL of a 50:50 mixture of acetonitrile/water, and 50 mg of diammonium citrate was dissolved in 1 mL of water. The matrix used was an 8:1 v/v solution of 3-HPA in acetonitrile/water to diammonium citrate in water. A 1-μL portion of the desalted SBE reaction mixture in water was mixed with 2 μL of the MALDI matrix, spotted on a polished stainless steel MALDI plate, and allowed to air-dry.

Carbon Cylinder Microelectrodes. The fabrication of carbon cylinder microelectrodes has been described previously.[52] Briefly, 32-μm carbon fibers were aspirated into glass capillaries and pulled with a model PE-2 microelectrode puller (Narishige, Tokyo, Japan). The pulled end of the capillary was then cut under a microscope using a scalpel and sealed with epoxy by backfilling with EPO-TEK 314 epoxy (Epoxy Technology, Billerica, MA). Before the epoxy was cured, a 150-μm copper wire was inserted until it made physical contact with the carbon fiber electrode. The epoxy was cured in an oven at 130 °C for 6−8 h, and once cured, the carbon cylinder was clipped to 100 μm or less with a scalpel. The electrode was sonicated in water prior to use.

Capillary Gel Electrophoresis. The CGE coupled to SV detection setup has been recently reported.[52] The system used contains a battery-powered high voltage power supply built with a 12 V rechargeable battery and a G50 HV module from EMCO High Voltage Corp. (Sutter Creek, CA). The battery-powered high voltage power supply was capable of supplying up to ±5000 V.

Scheme 1. Model Single Base Extension Reaction System Utilizing a 5′ Ferrocene Labeled Primer.[a]

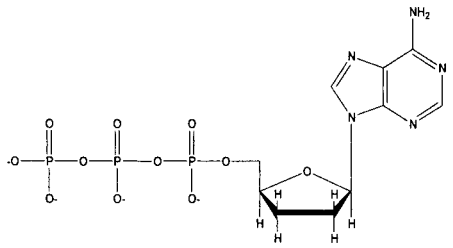
1. Denature

2. Anneal

5'-ACTCACTATAGGGCGAATTGG.....XCCCTTTAGTGAGGGTTAATT-3' **Template**

3'-GGGAAATCACTCCCAATTAA-ferrocene-5' **Primer**

3. ddNTP and ThermoSequenase



ddATP

4. Extension of Primer

3'-AGGGAAATCACTCCCAATTAA-ferrocene-5'

[a] The SBE reaction is similar to PCR with the exception that dideoxynucleotide triphosphates (ddNTPs) are used in place of the deoxynucleotide triphoshphates (dNTPs). As a result, the primer is extended by a single nucleotide complementary to the mutation site x.

The fused-silica capillary employed in this work was from Polymicro Technologies (Phoenix, AZ) and had dimensions of 360 μm o.d. × 20 μm i.d. and 25 cm in total length. The detection (anode) end of the capillary was etched with 40% hydrofluoric acid with nitrogen flowing through the capillary.[53] The strong acid etched the capillary, resulting in a slightly larger inside diameter, thus permitting the 32-μm carbon fiber to be placed just inside (~10−20 μm) the end of the separation capillary with the aid of a microscope and micropositioners. POP-4 was pumped into the capillary and used to both dynamically coat the capillary walls and provide a sieving medium for DNA separation. The electrophoretic buffer used was Genetic Analyzer Buffer (1×). The detection waste reservoir, where the reference and CGE anode were placed, was constructed by suspending a drop of buffer on top of a 0.5-mL polystyrene vial.

Sinusoidal Voltammetry. The SV detection system has been described in great detail in prior publications.[48,52,54−56] The excitation waveform utilized was generated with Labview software and a National Instruments data acquisition card (PCI-4451, Austin, TX). The waveform used in the work presented consisted of a 21 Hz sine wave with a potential window of −200 to 800 mV vs Ag/AgCl. The data were acquired in scans of 4 cycles consisting of 512 points. These 512 points were fast Fourier transformed in real time with a Labview program written in-house. The program was designed to save only the information from the first 10 harmonics of the excitation frequency in order to limit the file sizes.

The frequency domain data can be expressed as either a frequency spectrum or a time course profile at each of the collected harmonics. The former is represented as a three-dimensional plot with frequency on the *x* axis, current magnitude on the *z*-axis, and phase angle on the *y* axis. Previously, our group published work illustrating the dependence of the frequency domain "fingerprint" response on the electrochemical characteristics of the analyte and experimental parameters chosen.[48,52,54−56] To obtain the frequency response for the analyte signal of interest, a user-defined background vector was subtracted from the entire instantaneous current vector, resulting in a set of 10 harmonics where ideally the background component has been removed by the digital background subtraction routine. The three-dimensional frequency response for the analyte of interest was then obtained from the scan in time where the signal is at its maximum (i.e., the top of the analyte peak).

The other possible data representation format is a time course profile for each of the collected harmonics. This is merely the current magnitude of the scans collected throughout the duration of the experiment. To improve signal quality (signal/noise ratio) we employ the digital equivalent of a lock-in detector to our raw signal. The digital lock-in is similar to the analogue version in which the signal is monitored at a specific frequency and phase angle. Briefly, this is accomplished by utilizing the optimum phase

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angle for the analyte of interest, obtained from the frequency domain, and monitoring the raw signal at that particular phase angle. Therefore, the analyte's signal will be at its maximum, and only the background contribution at that particular phase angle will remain. The greatest elimination of background current occurs when its phase angle is ± 90° different from the optimum phase angle for the analyte. We recently demonstrated the utility of this same locking technique for selectively nulling out interfering signals.[52]

RESULTS AND DISCUSSION.

Single Base Extension Reaction for SNP Analysis. Single nucleotide primer extension, or single base extension (SBE), has been used by a number of researchers for SNP analysis.[6−9,12,33,34,36−38] It is relatively fast, easily automated, accurate, and compatible with miniaturization. In this work, a proof of concept scheme utilizing SBE and analysis performed with capillary gel electrophoresis coupled to electrochemical detection is illustrated. Scheme 1 depicts the mock SNP system used for this novel analysis technique. The template used, as shown in Scheme 1, is a 159-bp fragment generated from PCR amplification of the pBluescript sK+ phagemid using the appropriate forward and reverse primers (see Experimental Section for the sequences). The mock site of the SNP, labeled with an X in Scheme 1, lies immediately adjacent to the 3′ end of the SNP primer (T3 primer). The illustrated technique can be used in screening samples for a known SNP mutation.

The SBE reaction is similar to the polymerase chain reaction (PCR) except that dideoxynucleotide triphosphates are used, and the amplification is linear rather than exponential. As illustrated in Scheme 1, the sequence of steps is identical to PCR. First, any double-stranded DNA is denatured at a high temperature, then the template and primer are allowed to anneal, and finally, the polymerase incorporates the Watson−Crick complementary base into the 3′ end of the primer. As shown in Scheme 1, the nucleotide incorporated in our mock system is the base adenine. Dideoxynucleotide triphosphates, which lack the 3′ OH group necessary for further chain elongation, are used along with a thermostable polymerase, Thermosequenase. It has been shown that this DNA polymerase effectively incorporates the ddNTPs with similar efficiency compared to dNTPs.[33] The accuracy of this method lies in the high fidelity of DNA polymerase to only incorporate the complementary base.

The primer used in this work is a 20-mer with a ferrocene acetate label attached to the 5′ end (FA-T3) (Figure 1). The label will allow the oligonucleotide to be detected with electrochemical detection, because native DNA is not electroactive at the potentials applied in this work. Initially, the SBE reaction scheme used in the presented work is for screening known mutations only, because a single ddNTP is added to the reaction. To probe the mutation site, the complementary ddNTP is added to the reaction, resulting in extension of the primer by a single base. However, if an incorrect or uncomplementary ddNTP is added to the reaction, the polymerase will not incorporate the base, and the primer will not be extended.

Cyclic Voltammetric Characterization of the FA-T3 primer. Cyclic voltammetry (CV) is a common scanning electrochemical technique used to detect redox-active molecules. It is very useful in characterizing some of the electrochemical properties of a molecule, such as formal potential and kinetics.[57] Figure 1 shows the static CV of a 100 μM solution of FA-T3 in genetic analyzer 1× buffer. The potential window applied was −200 to 650 mV vs Ag/AgCl at a scan rate of 15 V/s. The oxidative and reductive peaks are split by ~60 mV, demonstrating the reversibility of the ferrocene tag, even when attached to a relatively large oligonucleotide. However, the faradaic response is almost completely masked by the large charging current that results from scanning the electrode surface. Ideally, if CV were coupled to a separation technique, some of the charging current could be removed by performing background subtraction. Unfortunately, background subtraction in the time domain often does not discriminate signal from background effectively enough to achieve the detection limits necessary for DNA analysis (low nanomolar to picomolar).

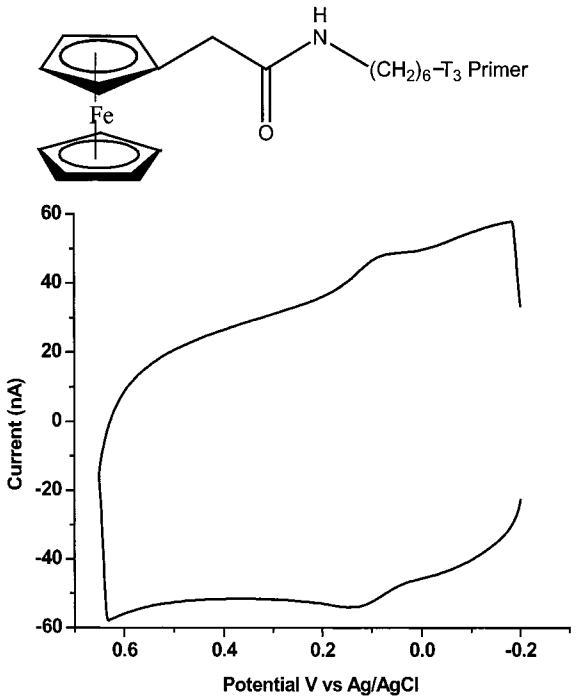

Figure 1. A cyclic voltamogram recorded from a static solution of 100 μM FA-T3 in 1× Genetic Analyzer Buffer is shown. The CV is recorded at a 32 μm carbon fiber electrode scanning at 15 V/s from −200 to 650 mV vs Ag/AgCl. No background subtraction is performed. The structure of the ferrocene acetate tag attached to the 5′ end of the T3 primer is shown on top of the voltammogram.

MALDI-TOFMS of the Primer and the Extension Reaction. Matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOFMS) was used to characterize the SBE reaction using the ferrocene labeled primer. It is important to determine if the ferrocene tag at the 5′ end of the primer interferes with the extension reaction before the product can be characterized with CGE/SV. MALDI is a very powerful technique that has been utilized in many genotyping assays [2,4,6,37] and is used here to validate proper extension of the FA-T3 primer. It is known that the high salt concentration present in the extension reaction mixture interferes with MALDI analysis by forming salt adducts, which broaden or cause the molecular ion peak to disappear.[6] Initially, we did try to analyze the extension reaction without any

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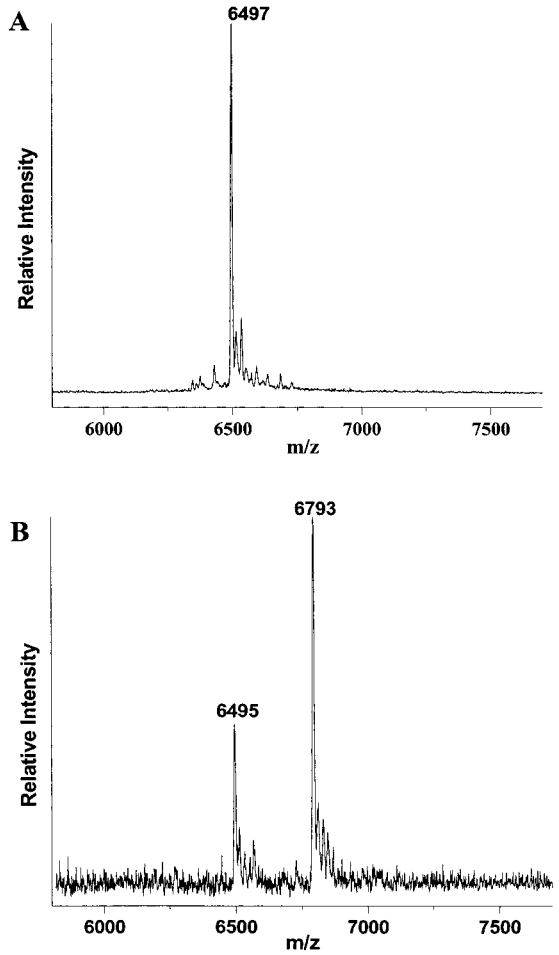

Figure 2. Matrix assisted laser desorption ionization-time-of-flight mass spectra recorded in linear positive mode with 3-hydroxypiccolinic acid as the matrix. (A) 100 pmol of purified ferrocene acetate-labeled T3 primer mixed 1:9 v/v with matrix and allowed to air-dry. The *m*/*z* found is 6497 ± 6. (B) Single base extension reaction product utilizing the FA-T3 primer (100 pmol), the Watson−Crick complement ddATP, and Thermosequenase DNA polymerase (4U). The SBE reaction mixture is first desalted on a dialysis membrane, then resuspended in 10 μL of water. To generate the spectrum, 1 μL of desalted sample is mixed with 2 μL of matrix and allowed to air-dry. Two peaks are observed in the MALDI spectrum. The first at *m*/*z* 6495 ± 6 corresponds to the unextended FA-T3 primer. The second peak observed at *m*/*z* 6793 ± 6 corresponds to the FA-T3 primer plus the addition of an A at the 3′ end. The smaller peaks in both spectra correspond to either sodium or potassium adducts of the molecular ion peak.

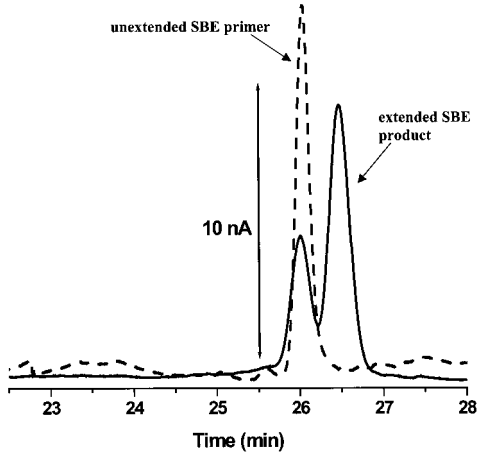

Figure 3. Capillary gel electrophoresis coupled to the sinusoidal voltammetric detection of the SBE extension product. The separation capillary, 25-μm i.d. and 25-cm length, is dynamically coated with POP-4 polymer, which is also used as the separation medium. Separation conditions employed: injection for 6 s at −4 kV and a run voltage of −4 kV. The SV detection employs a 21 Hz sine wave scanning from −200 to 800 mV vs Ag/AgCl. Both of the time courses shown in this figure are from the second harmonic or 42 Hz. The solid trace is from the injection of the successful SBE utilizing ddATP in the reaction. The first peak corresponds to elution of the unextended primer, whereas the late-eluting peak corresponds to the extended SBE product. The dotted trace is from the injection of the control SBE reaction utilizing ddCTP.

prior desalting steps; however, the quality of the peaks was very poor (data not shown). Therefore, the SBE reaction mixture is first desalted on float dialysis membranes, which are found to be very effective.

As shown in Figure 2, the MALDI spectra for the purified FA-T3 primer used in the extension reaction (Figure 2A) and the SBE reaction sample incorporating the complementary ddATP (Figure 2B) are consistent with the calculated *m*/*z* values. The spectrum in Figure 2A is taken in linear positive ion mode with an instrumental error of ±0.1%. The *m*/*z* value found for FA-T3 was 6497 ± 6, which is consistent with the calculated *m*/*z* of 6500. The incorporation of an A into the primer results in a mass change of 297. The spectrum of the SBE product, Figure 2B, shows two peaks that are separated by 298 *m*/*z* units, demonstrating the correct incorporation of an A onto the 3′ end of the primer. Therefore, it can be concluded that the ferrocene label on the primer does not interfere significantly with the enzyme's ability to elongate the primer by a single base, A. Although MALDI-TOFMS may be used to detect SNPs, the instrumentation required is rather costly and might not be attainable by a clinical lab for routine SNP analysis.

CGE/SV Analysis of SBE Reaction Products. CGE is a very powerful DNA separation technique. Recently, it has been utilized in the generation of a rough draft sequence of the human genome.[58] The advantages of CGE over traditionally used slab gel methods are its high throughput (shown to be over 200 times faster than slab gels), efficiency, and compatibility with automation. CGE has also recently been applied to SNP detection assays. Single strand conformation polymorphism (SSCP),[14] heteroduplex analysis (HDA),[17,18] constant denaturing capillary electrophoresis (CDCE)[59,60] and temperature gradient gel electrophoresis (TGGE)[43] are a few methods used to characterize mutations and have been successfully adapted to the capillary format.

The SBE reaction mixture, which is characterized by MALDI in Figure 2B, is electrokinetically injected into the CGE/SV system. Figure 3 (solid line) shows the single base separation of the 20-mer FA-T3 primer from the 21-mer extension product at the second harmonic (42 Hz). The separation medium used to accomplish this single base separation is a commercially available

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polymer (POP-4, Applied Biosystems). This polymer has been optimized for single base resolution out to ~250 nucleotides.[42] We have previously found that this polymer is compatible with SV detection and is easily pumped into the 20-μm-i.d. capillary.[52] Another advantage of using POP-4 as the sieving matrix is that it also acts to dynamically coat the capillary walls to eliminate electroosmotic flow. The highly negative charge on the capillary walls is masked, and the sieving medium is introduced simply by refilling the capillary after each run, leading to a very reproducible separation. The calculated resolution achieved for the separation shown in Figure 3 was 0.8. It has been reported that a resolution of 0.5 or better is sufficient for single base resolution in a DNA separation.[61] Although the 20 and 21-mer fragments were not baseline-resolved, the resolution was certainly sufficient to differentiate the extension product from the primer. Additionally, when this sample is spiked with excess 20-mer (primer), the first peak increases in magnitude (data not shown).

Previously, we illustrated the impressive detection sensitivity of sinusoidal voltammetry and analysis in the frequency domain.[48,54−56] Analysis in the frequency domain can discriminate against the background charging current more efficiently than traditional time domain analysis. The majority of the background signal is due to the double layer charging current. This process has been shown to be primarily linear in nature, and therefore, most of the resulting signal remains at the fundamental excitation frequency. The faradaic current resulting from the oxidation and reduction of the electroactive molecule is nonlinear and has signal at higher harmonics of the excitation frequency. It has been shown that utilization of the higher-order harmonics can increase the sensitivity of a measurement by as much as 3 orders of magnitude.[55] The amount of FA-T3-labeled primer utilized in the SBE reaction was 100 pmol, and after the extension reaction, the sample was desalted, dried, and redissolved in 10 μL of water. If we assume that no FA-T3-labeled primer or extension reaction product is lost during dialysis, then the concentration of the sample injected is ~10 μM. The S/N ratio in Figure 3 is 3000 for the FA-T3 peak and 6000 for the extension product (21-mer) peak. The primer peak is about one-half as intense as the extension product; since they are labeled with the same electroactive molecule, the signal intensities should scale similarly. If the initial concentration of the sample is 10 μM and the first peak is one-half as intense as the second, and if we assume there is no preferential injection between the two fragments, then the concentration of the primer detected is ~3 μM, and the extension product is ~6 μM. The extrapolated detection limit (S/N = 3) for the two FA-T3-labeled fragments is on the order of 3 nM. The assumption of complete recovery of the primer and extension product after dialysis is probably unlikely, and the actual amount injected is probably much less. However, these assumptions help to give an idea of the detection limits that can currently be achieved by SV detection.

Specificity of the SBE Reaction. The accuracy of this assay, utilizing the SBE reaction, for mutation screening relies on the fidelity of the polymerase to incorporate only the complementary base. To test the accuracy of Thermosequenase, ddCTP is added to the SBE reaction in place of ddATP with all other experimental parameters being identical. Figure 4 is the MALDI spectrum for the control SBE reaction with the addition of ddCTP. As expected, there is only one peak present due to the FA-T3 primer; no extension product is visible at a mass corresponding to the addition of a C (+272 m/z). Additionally, the dotted line trace in Figure 3 shows the CGE/SV electropherogram recorded at the second harmonic (42 Hz) for the SBE reaction using ddCTP. The same CGE and SV experimental conditions are used for both traces illustrated in Figure 3. The time course data for the control extension reaction with ddCTP is consistent with the MALDI data, showing only a single peak for the FA-T3 primer. The migration time for the FA-T3 primer in the control reaction is consistent with the migration time for the first peak, the unextended primer, in the extension reaction.

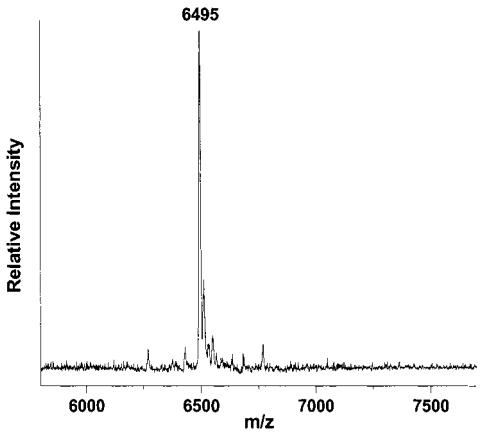

Figure 4. MALDI-TOF spectrum shown is obtained from the SBE reaction replacing ddATP with ddCTP, an uncomplementary base. The spectrum for the SBE reaction with ddCTP, employing all the same experimental conditions and desalting as in Figure 1B, shows only a single peak at m/z 6495 ± 6. This peak corresponds to the unextended primer as expected.

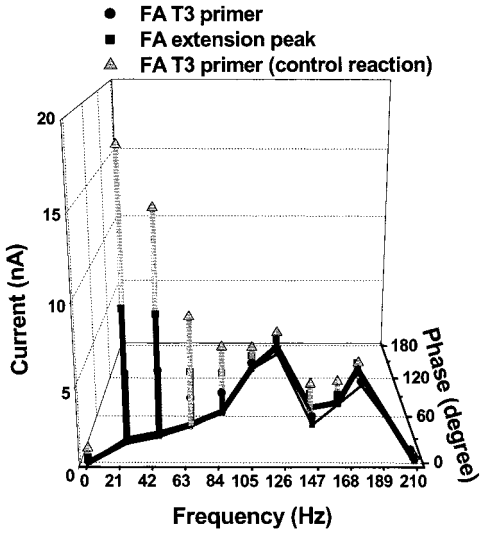

Figure 5. Frequency responses for the three peaks observed in Figure 3 plotted on the same graph. The gray triangle and gray circle are the FA-T3 primer peaks, and the black square is the frequency response for the 21-mer extension product. All of the experimental conditions are the same as in Figure 3. The three separate graphs are difficult to distinguish from one another, which further illustrates the reproducibility of phase angles in the SV measurement.

Frequency Domain Consistency. The frequency domain response is characteristic of the electrochemical properties of the

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redox molecule and experimental conditions employed. In prior publications from our group, we have demonstrated the advantages of SV detection in the frequency domain for the selective detection of oligonucleotides derivatized with unique ferrocene labels.[52] The SV frequency domain spectra for the three peaks (two FA-T3 primer peaks and one extension product peak) in Figure 3 is shown in Figure 5. The phase angle information is very useful in identifying a redox molecule, because it is independent of concentration and consistent within a given set of experimental parameters (average standard deviation ($n = 9$) of $\pm 8°$ for each harmonic).[52] As shown in Figure 5, the three signals arising from the same electrochemical tag have very similar phase responses. This advantage of SV will be utilized in the future to increase the throughput of the proposed assay by using SBE primers of different lengths and modified with unique electrochemical labels. Additionally, work is currently underway in our laboratory to use redox-labeled ddNTPs in SBE reactions for genotyping SNPs.

CONCLUSION

With the recent generation of a map containing the location of 1.42 million SNP sites, there is a greater need to develop simple, cost-effective, accurate, and high-throughput methods to score and screen for SNPs in large sample populations.[5] We have demonstrated a model SNP detection assay system based on the coupling of CGE with electrochemical detection. CGE is a very fast and efficient separation technique, which is easily automated and adapted to parallel microfluidic devices. The SV detection technique has the potential for achieving the requisite sensitivity and selectivity for DNA analysis. Additionally, electrochemical detection is compatible with miniaturization and can be easily incorporated into a fully portable detection system. The proof of concept SNP scoring assay presented may prove to be ideal for routine clinical analysis of known mutations.

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