ELECTROCHEMICAL DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)

This invention provides novel methods for the detection of single nucleotide polymorphisms (SNPs). In one embodiment, the method involves i) providing a nucleic acid primer complementary to a nucleic acid sequence adjacent to the location of the single nucleotide comprising said single nucleotide polymorphism wherein said primer is of sufficient length to initiate transcription by a nucleic acid polymerase; ii) performing a primer extension reaction using a nucleic acid polymerase, a chain terminator to produce one or more extension reaction products, wherein one or more components of said primer extension reaction are labeled with a redox-active label whereby one or more extension reaction products produced by the primer extension reaction are labeled with said redoxactive label; iii) detecting the extension reaction products by detecting the redox-active label, wherein detection of an extension reaction product longer than the nucleic acid primer indicates the presence of said single nucleotide polymorphism in said target nucleic acid.
ELECTROCHEMICAL DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs)

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of USSN 10/165,593, filed on June 7, 2002, which is incorporated herein by reference in its entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This work was supported by the National Institute of Health Grant 1R21HG01828-01. The Government of the United States of America has certain rights in this invention.

FIELD OF THE INVENTION

[0003] This invention pertains to the field of genomics. In particular, this invention provides novel methods for the detection of single nucleotide polymorphisms (SNPs).

BACKGROUND OF THE INVENTION

1.42 million SNPs present in the human genome (Sachidanandam et al. (2001) Nature, 409: 928-933). 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.


[0006] The most common techniques that fall into the first category include allele specific amplification (Saruta et al. (1995) Microbiol. Immunol., 39: 839-844; Howard et al.
The first two techniques mentioned above utilize two different primers, one with a sequence complimentary 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, where the primer containing the mutant site will only be extended 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 (Buetow et al. (2001) Proc. Natl. Acad. Sci., USA, 98: 581-584; Barta et al. (2001) Electrophoresis, 22: 779-782). 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 (Landegren et al. (1998) Genome Res., 8: 769-776).


**SUMMARY OF THE INVENTION**

[0009] This invention provides novel compositions and methods for the detection of single nucleotide polymorphisms (SNPs). In one embodiment, this invention provides a method of detecting a single nucleotide polymorphism in a target nucleic acid. The method
involves i) providing a nucleic acid primer complementary to a nucleic acid sequence adjacent to the location of the single nucleotide comprising the single nucleotide polymorphism where the primer is of sufficient length to initiate transcription by a nucleic acid polymerase; ii) performing a primer extension reaction using a nucleic acid polymerase and a chain terminator to produce one or more extension reaction products, where one or more components of the primer extension reaction are labeled with a redox-active label whereby one or more extension reaction products produced by the primer extension reaction are labeled with the redox-active label; and iii) detecting the extension reaction products by detecting the redox-active label, wherein detection of an extension reaction product longer than the nucleic acid primer indicates the presence of said single nucleotide polymorphism in said target nucleic acid. In certain embodiments, the primer is at least 10 or 20 nucleotides in length. In certain embodiments, the nucleic acid primer and/or the chain terminator bears the redox-active label. In certain embodiments the primer extension reaction is a sequencing reaction or an amplification reaction (e.g. PCR). The detecting can involve separating the reaction products (e.g. via electrophoresis). In certain embodiments the separation is by capillary gel electrophoresis.

[0010] The redox-active label(s) are typically detected using an electrochemical detection method (e.g. cyclic voltammetry). In certain embodiments, the electrochemical detection method is sinusoidal voltammetry (often using a single electrode). In certain embodiments the signal from the redox-active label is analyzed in the frequency domain (e.g. using a Fourier transform). The signal can be analyzed at a harmonic of the excitation frequency (e.g. a second or higher harmonic, third or higher harmonic of the excitation frequency, etc.). The detection system can involve detecting the signal for the redox-active label at a phase angle out of phase (e.g. about 45 degrees to about 90 degrees out of phase) with respect to the optimum phase angle for the redox-active label. In certain instances, the detecting comprises selecting voltammetric data detecting at a phase angle closest to 90 degrees out of phase with the optimum phase angle for the redox-active label(s).

[0011] In certain embodiments, the chain terminator is an acyclo nucleoside triphosphate (acyNTP) chain terminator and/or a a dideoxy chain terminator (e.g. 2',3'-dideoxyguanosine-5'-triphosphate, 7-deaza-2',3'-dideoxyguanosine-5'-triphosphate, 2',3'-dideoxyadenosine-5'-triphosphate, 2',3'-dideoxythymidine-5'-triphosphate, and 2',3'-
dideoxycytidine-5'-triphosphate, and the like). The chain terminator can be labeled with the redox-active label. One preferred chain terminator is a dideoxy-ferrocene-acycloATP.

[0012] Suitable redox-active labels include, but are not limited to porphyrin, an expanded porphyrin, a contracted porphyrin, a metalloocene, a linear porphyrin polymer, and a porphyrin array. Particularly suitable redox-active labels include, but are not limited to ferrocenes (e.g. an alkyl ferrocene, a ferrocene acetate, a ferrocene carboxylate, and an alkyl ferrocene dimethylcarboxamide), and/or a porphyrinic macrocycle substituted at a beta-position or at a meso-position. In certain embodiments, the assay is performed in a lab-on-a-chip format.

[0013] Also provided is a kit for detecting a single nucleotide polymorphism. The kit includes a container containing one or more moieties selected from the group consisting of a chain terminator labeled with a redox-active label; a primer labeled with a redox-active label, and nucleotide triphosphates labeled with a redox-active label. The kit can optionally include instructional materials teaching the use of said moieties to detect a single-nucleotide polymorphism. In certain embodiments, the primers chain terminators and redox active labels include any one or more of those described herein.

[0014] This invention also provides a method of identifying a single nucleotide polymorphism in a target nucleic acid. The method involves i) providing a nucleic acid primer complementary to a nucleic acid sequence adjacent to the location of the single nucleotide comprising the single nucleotide polymorphism wherein the primer is of sufficient length to initiate transcription by a nucleic acid polymerase; ii) performing a primer extension reaction using a nucleic acid polymerase, and chain terminators complementary to each base expected to comprise the single nucleotide polymorphism where each chain terminator is labeled with a redox-active label and different chain terminators are labeled with different and distinguishable redox-active labels; and iii) detecting the extension reaction products by detecting the redox-active labels, where identification of the redox-active label indicates the identity of the single nucleotide polymorphism present in the target nucleic acid.

DEFINITIONS

[0015] SNPs (single nucleotide polymorphisms) are differences, across the population, in a single base, within an otherwise conserved genomic sequence. They are
useful as indicators of variations in cellular functionality such as disease susceptibility, metabolism, protein production, etc. They are also becoming increasingly common in linkage and association studies.

[0016] The term "oxidation" refers to the loss of one or more electrons in an element, compound, or chemical substituent/subunit. In an oxidation reaction, electrons are typically lost by atoms of the element(s) involved in the reaction. The charge on these atoms then becomes more positive. The electrons are lost from the species undergoing oxidation and so electrons appear as products in an oxidation reaction. An oxidation is taking place in the reaction \( \text{Fe}^{2+}(aq) \rightarrow \text{Fe}^{3+}(aq) + e^- \) because electrons are lost from the species being oxidized, \( \text{Fe}^{2+}(aq) \), despite the apparent production of electrons as "free" entities in oxidation reactions. Conversely the term reduction refers to the gain of one or more electrons by an element, compound, or chemical substituent/subunit.

[0017] An "oxidation state" refers to the electrically neutral state or to the state produced by the gain or loss of electrons to an element, compound, or chemical substituent/subunit. In a preferred embodiment, the term "oxidation state" refers to states including the neutral state and any state other than a neutral state caused by the gain or loss of electrons (reduction or oxidation).

[0018] The terms "different and distinguishable" when referring to two or more oxidation states means that the net charge on the entity (atom, molecule, aggregate, subunit, etc.) can exist in two different states. The states are said to be "distinguishable" when the difference between the states is greater than thermal energy at room temperature (e.g. 0°C to about 40°C).

[0019] The term "electrode" refers to any medium capable of transporting charge (e.g. electrons) to and/or from a redox-active species. Preferred electrodes are metals or conductive organic molecules. The electrodes can be manufactured to virtually any 2-dimensional or 3-dimensional shape (e.g. discrete lines, pads, planes, spheres, cylinders, etc.).

[0020] A "redox-active" compound or molecule refers to a compound or molecule capable of being oxidized or reduced. A redox-active label or tag is a redox-active compound or molecule that can be or is attached to a moiety that is to be detected. The
redox-active tag provides a detectable signal or property (e.g. oxidation state) that provides an indication of the presence and/or amount of a moiety tagged with such a tag.

[0021] The term "E_{1/2}" refers to the practical definition of the formal potential (E°) of a redox process as defined by 
\[ E = E° + \frac{RT}{nF} \ln\left(\frac{D_{ox}}{D_{red}}\right) \]
where R is the gas constant, T is temperature in K (Kelvin), n is the number of electrons involved in the process, F is the Faraday constant (96,485 Coulomb/mole), D_{ox} is the diffusion coefficient of the oxidized species and D_{red} is the diffusion coefficient of the reduced species.

[0022] The term "optimum phase angle" for a redox active species (tag) refers to the phase angle of an electrochemical measurement in the frequency domain (e.g. a cyclic voltammetric measurement) that gives maximum current for the signal (i.e. greatest S/N ratio).

[0023] The phrase "drop out of signal at a phase angle" indicates a diminution or elimination of a signal at a particular phase angle as compared to that signal at a different phase angle. The diminution can be any detectable diminution, preferably a diminution of at least 5%, preferably of at least 10%, more preferably of at least 15% or 20%, most preferably of at least 30%, at least 50%, or at least 80%. In certain embodiments, the diminution is a statistically significant diminution (e.g. at the 10% confidence level, more preferably at the 5% confidence level and most preferably at the 1% confidence level). In certain embodiments, the diminution is relative to the signal at the optimum phase angle for that redox-active species. Where a collection of redox-active species is present, in particularly preferred embodiments, the diminution is as compared to the signal at the phase common response.

[0024] A "voltage source" is any source (e.g. molecule, device, circuit, etc.) capable of applying a voltage to a target (e.g. an electrode).

[0025] A "voltammetric device" is a device capable of measuring the current produced in an electrochemical cell as a result of the application of a voltage or change in voltage.

[0026] An "amperometric device" is a device capable of measuring the current produced in an electrochemical cell as a result of the application of a specific potential field ("voltage").
A "potentiometric device" is a device capable of measuring potential across an interface that results from a difference in the equilibrium concentrations of redox molecules in an electrochemical cell.

A "voltammogram" refers to the data set produced by a voltammetric measurement (e.g. a cyclic voltammogram is the data set produced by a cyclic voltammetric measurement). The data set can be permanently or transiently displayed in electronic or other forms. In certain embodiments, the voltammogram need not be display, but can simply exist, e.g. as a data set on a computer readable medium (e.g. dynamic memory, static memory, optical storage, magnetic storage, and the like) and be accessed for subsequent processing. The voltammogram can be the raw data from the measurement of a transform of such raw data (e.g. background subtracted, and/or Fourier transformed, and the like).

A "coulometric device" is a device capable of the net charge produced during the application of a potential field ("voltage") to a redox-active species.

A "cyclic voltameter" is a voltammetric device capable of determining the time and/or frequency domain properties of a redox-active species (i.e. a device capable of performing cyclic voltammetry).

Cyclic voltammetry, as used herein, refers to voltammetry using a periodic waveform (e.g. sine, cosine, triangle, or any combination thereof) as an excitation potential. Although often linear, such waveforms need not be so limited.

The term "sinusoidal voltammetry" refers to cyclic voltammetry using a periodic "excitation voltage" that is sinusoidal.

The term "porphyrinic macrocycle" refers to a porphyrin or porphyrin derivative. Such derivatives include porphyrins with extra rings ortho-fused, or orthoperifused, to the porphyrin nucleus, porphyrins having a replacement of one or more carbon atoms of the porphyrin ring by an atom of another element (skeletal replacement), derivatives having a replacement of a nitrogen atom of the porphyrin ring by an atom of another element (skeletal replacement of nitrogen), derivatives having substituents other than hydrogen located at the peripheral (meso-, β-) or core atoms of the porphyrin, derivatives with saturation of one or more bonds of the porphyrin (hydroporphyrins, e.g., chlorins, bacteriochlorins, isobacteriochlorins, decahydroporphyins, corphins, pyrrocorphins, etc.), derivatives obtained by coordination of one or more metals to one or
more porphyrin atoms (metalloporphyrins), derivatives having one or more atoms, including pyrrolic and pyrromethenyl units, inserted in the porphyrin ring (expanded porphyrins), derivatives having one or more groups removed from the porphyrin ring (contracted porphyrins, e.g., corrin, corrole) and combinations of the foregoing derivatives (e.g., phthalocyanines, sub-phthalocyanines, and porphyrin isomers). Preferred porphyrinic macrocycles comprise at least one 5-membered ring. A number of porphyrinic macrocycles are described in WO 01/03126.

[0034] The term "ferrocene" includes ferrocene and ferrocene derivatives, e.g. alkyl ferrocene, ferrocene acetate, ferrocene carboxylate, alkyl ferrocene dimethylcarboxamide, acetyl ferrocene, propioly ferrocene, butylferrocene, pentanoyl ferrocene, hexanoyl ferrocene, octanoyl ferrocene, benzoyl ferrocene, 1,1'diacetyl ferrocene, 1,1'-dibutyryl ferrocene, 1,1'-dihexanoyl ferrocene, ethyl ferrocene, propyl ferrocene, n-butyl ferrocene, pentyl ferrocene, hexyl ferrocene, 1,1'-diethyl ferrocene, 1,1'-dipropyl ferrocene, 1,1'-dibutyl ferrocene, 1,1'-dihexyl ferrocene, cyclopentenyl ferrocene, cyclohexenyl ferrocene, 3-ferrocenoyl propionic acid, 4-ferrocenoyl butyric acid, 4-ferrocenylbutyric acid, 5-ferrocenylvaleric acid, 3-ferrocenoyl propionic acid esters, 4-ferrocenoyl butyric acid esters, 4-ferrocenyl butyric acid esters, 5-ferrocenylvaleric acid esters, dimethylaminomethyl ferrocene, and the like.

[0035] The term "porphyrin" refers to a cyclic structure typically composed of four pyrrole rings together with four nitrogen atoms and two replaceable hydrogens for which various metal atoms can readily be substituted. A typical porphyrin is heme.

[0036] The term "working electrode" typically used to refer to one or more electrodes that are used to read the oxidation state of a redox-active species.

[0037] The term "reference electrode" is typically used to refer to one or more electrodes that provide a reference (e.g. a particular reference voltage) for measurements recorded from the working electrode. In certain embodiments, the excitation waveform is applied at the reference electrode.

[0038] The term "oligonucleotide primer" refers to an oligonucleotide or polynucleotide that, when annealed to a template nucleic acid, is capable of being extended from a 3'-end in the presence of primer extension reagents. Typically, an oligonucleotide primer will include a hydroxyl group at the 3'-position of a 3'-terminal nucleotide.
The term "phosphate analog" refers to analogs of phosphate wherein the phosphorous atom is in the +5 oxidation state and one or more of the oxygen atoms is with a non-oxygen moiety, exemplary analogs including phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, boronophosphates, and the like, including associated counterions, e.g., H, NH₄, Na, and the like if such counterions are present.

As used herein the term "primer-extension reagent" means a reagent including components necessary to effect the enzymatic template-mediated extension of a nucleic acid (e.g., an oligonucleotide) primer. Preferably, primer extension reagents include: (i) a polymerase enzyme, e.g., a thermostable polymerase enzyme such as Taq polymerase; (ii) a buffer; and (iii) 2'-deoxynucleotide triphosphates, e.g., 2'-deoxyuridine-5'-triphosphate, 2'-deoxyguanosine-5'-triphosphate, 2'-deoxy-7-deazadeyguanosine-5'-triphosphate, 2'-deoxyadenosine-5'-triphosphate, 2'-deoxythymidine-5'-triphosphate, 2'-deoxycytidine-5'-triphosphate.

As used herein, the terms "terminator" or "extension terminator" or "chain terminator" refers to a species that when incorporated into a primer extension product blocks further elongation of the product. Exemplary terminators include 2',3'-dideoxynucleotides, e.g., 2',3'-dideoxyguanosine-5'-triphosphate, 7-deaza-2',3'-dideoxyguanosine-5'-triphosphate, 2',3'-dideoxyadenosine-5'-triphosphate, 2',3'-dideoxythymidine-5'-triphosphate, and 2',3'-dideoxycytidine-5'-triphosphate.

As used herein, the term "template nucleic acid" refers to any nucleic acid which can be presented in a single stranded form and is capable of annealing with a nucleic acid primer. Exemplary template nucleic acids include DNA, RNA, which DNA or RNA can be single stranded or double stranded. More particularly, template nucleic acid can be genomic DNA, messenger RNA, cDNA, DNA amplification products from a PCR reaction, and the like. Methods for preparation of template DNA may be found elsewhere (ABI PRISM.TM. Dye Primer Cycle Sequencing Core Kit Protocol).

The terms "nucleic acid", or "oligonucleotide" or grammatical equivalents herein refer to at least two nucleotides covalently linked together. Nucleic acids of the present invention are single-stranded or double stranded and will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are
included that may have alternate backbones, comprising, for example, phosphoramidate
(Beaucage et al. (1993) Tetrahedron 49(10):1925) and references therein; Letsinger (1970)
5,644,048), phosphorodithioate (Briu et al. (1989) J. Am. Chem. Soc. 111 :2321, O-
methylphosphoromidite linkages (see Eckstein, Oligonucleotides and Analogues: A
Practical Approach, Oxford University Press), and peptide nucleic acid backbones and
207). Other analog nucleic acids include those with positive backbones (Denpcy et al.
Chem. Soc. 110:4470; Letsinger et al. (1994) Nucleoside & Nucleotide 13:1597; Chapters 2
and 3, ACS Symposium Series 580, "Carbohydrate Modifications in Antisense Research",
Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al. (1994), Bioorganic & Medicinal
37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos.
5,235,033 and 5,034,506, and Chapters 6 and 7, ACS Symposium Series 580, Carbohydrate
Modifications in Antisense Research, Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids
containing one or more carbocyclic sugars are also included within the definition of nucleic
are described in Rawls, C & E News June 2, 1997 page 35. These modifications of the
ribose-phosphate backbone may be done to facilitate the addition of additional moieties
such as labels, or to increase the stability and half-life of such molecules in physiological
environments.

BRIEF DESCRIPTION OF THE DRAWINGS

[0044] Figure 1 illustrates scheme 1, a model single base extension reaction system
utilizing a 5’ ferrocene labeled primer (SEQ ID NO:11) and a template (SEQ ID NO:10).
The SBE reaction is similar to PCR with the exception that dideoxynucleotide triphosphates
(ddNTPs) are used in place of the deoxynucleotide triphosphates (dNTPs). As a result, the
-12-
primer is extended by a single nucleotide complementary to the mutation site X (SEQ ID NO:12).

[0045] Figure 2 illustrates design/selection of an extension primer. The extension primer is complementary to the region adjacent to and upstream of the polymorphic site in the coding strand (e.g. SEQ ID NO:1) or its complement (SEQ ID NO:2).

[0046] Figure 3 illustrates a cyclic voltamogram (CV) recorded from a static solution of 100 μM FA-T3 in 1X 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.

[0047] Figures 4A and 4B illustrate matrix assisted laser desorption ionization-time of flight mass spectra recorded in linear positive mode with 3-hydroxypicolinic acid as the matrix. Figure 4A: 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. Figure 4B: Single base extension reaction product utilizing the FA-T3 primer (100 pmoles), 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 of 6495 ± 6 corresponds to the unextended FA-T3 primer. The second peak observed at a m/z of 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.

[0048] Figure 5 shows the results of capillary gel electrophoresis coupled to the sinusoidal voltammetric detection of the SBE extension product. The separation capillary, 25 μm i.d. and 25 cm in length, is dynamically coated with POP-4 polymer, which is also used as the separation medium. The separation conditions employed: injection for 6 sec. 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. The time courses shown in this figure are both 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, while the late eluting peak corresponds to the extended SBE product. The dotted trace is from the injection of the control SBE reaction utilizing ddCTP.

[0049] Figure 6 illustrates a MALDI-TOF spectrum obtained from the SBE reaction replacing ddATP with ddCTP, an uncomplimentary base. The spectrum for the SBE reaction with ddCTP, employing all the same experimental conditions and desalting as in Figure 3B, shows only a single peak at m/z 6495 ± 6. This peak corresponds to the unextended primer as expected.

[0050] Figure 7 shows the frequency responses for the three peaks observed in Figure 5 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 the experimental conditions are the same as in Figure 5. The three separate graphs are difficult to distinguish from one another, which further illustrates the reproducibility of phase angles in the SV measurement.

[0051] Figure 8 illustrates a model single base extension reaction system utilizing a ferrocene-acycloATP terminator. The SBE reaction shown utilizes a dideoxy-ferrocene labeled acyclic ATM at the chain terminator. As a result, the primer is extended by a single nucleotide complementary to the mutation site X. The incorporation of the nucleotide allows the extension product to be electrochemically detected.

[0052] Figures 9A and 9B show cyclic voltammetry of 100 μM ferrocene-acycloATP. Figure 9A: The chemical structure of the ferrocene-acycloATP chain terminator used in the model SBE reaction. Figure 9B: A cyclic voltamogram recorded from a static solution of 100 μM ferrocene acycloATP in 1x Genetic Analyzer buffer is shown. The CV was recorded at a 32 μm carbon fiber electrode scanning at 10 V/s from –200 to 800 mV vs. Ag/AgCl. No background subtraction was performed.

[0053] Figures 10A and 10B show capillary gel electrophoresis coupled to the sinusoidal voltammetric detection of the free ferrocene-acycloATP nucleotide. The separation capillary, 25 μm i.d. and 17 cm in length, was dynamically coated with POP-4 polymer. The separation conditions employed were injection for 6 sec. at -5 kV and a run voltage of -5 kV. The SV detection employed a 11 Hz sine wave scanning from –200 to 800 mV vs. Ag/AgCl. The time course shown in this figure is from the third harmonic or
33 Hz. The migration time for the free nucleotide under the employed experimental conditions was 10 minutes.

[0054] Figure 11A and 11B show MALDI-TOFMS of SBE extension product and control reaction using the acyclic terminator Matrix assisted laser desorption ionization-time of flight mass spectra (MALDI-TOFMS) recorded in linear positive mode with 3-hydroxypicolinic acid as the matrix. Figure 11A: The MALDI spectrum for 1 μl of the desalted SBE reaction mixture coevaporated with 2 μl of HPA matrix. The m/z of the first peak, 6269 ± 6, corresponds to the unincorporated T3 SBE primer. The second peak has a m/z of 6803 ± 6, which is within the experimental error of the calculated m/z (6809) of the primer extended at the 3' end with the ferrocene-acycloATP nucleotide. Figure 11B: The MALDI taken of the SBE control reaction. The primer used was specificity primer (see experimental section for the sequence) and should not be extended in the SBE reaction. The peak at a m/z of 6265 ± 6 corresponds to the control primer (non-extended). There are no additional peaks in the spectrum, confirming that no extension took place. The SBE reaction mixtures in both A and B were first desalted on a dialysis membrane then resuspended in 10 μl of water. 1 μl of desalted sample was mixed with 2 μl of 3-HPA matrix and allowed to air dry to generate the spectrum. The smaller peaks in both spectra correspond to either sodium or potassium adducts of the molecular ion peak.

[0055] Figure 12A and 12B show capillary gel electrophoresis coupled to the sinusoidal voltammetric detection of the SBE extension product and specificity control. The CGE conditions were identical to Figure 10. Figure 12A: The third harmonic (33 Hz) time course data for the SBE reaction analyzed in Figure 11A and B are shown. The solid line trace is the injection of the SBE reaction mixture utilizing the T3 primer. The first peak in the electropherogram is due to excess free ferrocene-acycloATP and the second peak is the 21-mer extension product with the electrochemically labeled nucleotide attached to its 3' end. Figure 12B: The frequency domain spectra for the extension product (open circles) and free electrochemically labeled nucleotide (filled squares). The two frequency spectra are hard to differentiate from one another, because the electrochemical signal arises from the same electrochemical tag.
DETAILED DESCRIPTION


Information about SNPs may be used in various ways in genetic analysis. First, SNPs can be used as genetic makers in mapping studies. For example, SNPs can be used for whole-genome scans in pedigree-based linkage analysis of families; for this purpose, a map of about 2000 SNPs has the same analytical power as a map of about 800 microsatellite markers, currently the most frequently used type of marker. Second, when disease genetics is studied in individuals in a population, rather than in families, the haplotype distributions and linkage disequilibria can be used to map genes by association methods. For this purpose, it has been estimated that 30,000 to as many as 300,000 mapped SNPs will be needed. Third, genetic analysis can be used in case-control studies to identify functional SNPs contributing to a particular phenotype. Most SNPs are located outside of coding sequences, because only three to five percent of the human DNA sequence encodes proteins. However, SNPs located within protein-coding sequences ("cSNPs") are of particular interest because they are more likely than a random SNP to have functional significance. It also is likely that some of the SNPs in noncoding DNA will have functional consequences, such as those in sequences that regulate gene expression.
Discovery of SNPs that affect biological function should become increasingly important over the next several years, and should be greatly facilitated by the availability of a large collection of SNPs, from which candidates for polymorphisms with functional significance can be identified. Accordingly, SNPs discovery is an important objective of SNPs research.

SNPs will be particularly important for mapping and discovering the genes associated with common diseases. Many processes and diseases are caused or influenced by complex interactions among multiple genes and environmental factors. These include processes such as development and aging, and diseases such as diabetes, cancer, cardiovascular and pulmonary disease, neurological diseases, autoimmune diseases, psychiatric illnesses, alcoholism, common birth defects, and susceptibility to infectious diseases, teratogens, and environmental agents. Many of the alleles associated with health problems are likely to have a low penetrance, meaning that only a small percentage of individuals carrying the alleles will develop disease. However, because such polymorphisms are likely to be very common in the population, they may make a significant contribution to the health burden of the population. Examples of common polymorphisms associated with an increased risk of disease include the ApoE4 allele and Alzheimer's disease, and the APC I1307K allele and colon cancer.

Most of the successes to date in identifying (a) the genes associated with diseases inherited in a Mendelian fashion, and (b) the genetic contribution to common diseases, e.g., BRCA1 and 2 for breast cancer, MODY 1, 2, and 3 for type 2 diabetes, and HNPCC for colon cancer, have been of genes with relatively rare, highly penetrant variant alleles. These genes are well-suited to discovery by linkage analysis and positional cloning techniques. However, the experimental techniques and strategies useful for finding low penetrance, high frequency alleles involved in disease are usually not the same, and not as well developed, as those that have been applied successfully in positional cloning. For example, pedigree analysis of families often does not have sufficient power to identify common, weakly contributing loci. The types of association studies that do have the power to identify such loci efficiently require new approaches, techniques, and scientific resources to make them as robust and powerful as positional cloning. Among the resources needed is a genetic map of much higher density than the existing, microsatellite-based map.
Association studies using a dense map should allow the identification of disease alleles even for complex diseases. SNPs are well suited to be the basis of such a map.

[0061] The identification of SNPs and their association with pathologies, with particular responses or susceptibilities to pharmaceuticals, with genetic predilections or abnormalities and the like facilitates basic research, the development of diagnostic assays, optimization of therapeutics and therapeutic regimen, selection of pharmaceuticals to minimize patient risk and optimize response, etc.

[0062] In order to effectively screen for hundreds to thousands of SNPs in a large sample population, the detection scheme is desirably rapid, inexpensive, accurate, and sensitive. This invention provides improved methods of detecting SNPs that afford a number of these advantages.

[0063] The SNP detection strategies of this invention exploit the accuracy of a nucleic acid polymerase (e.g. a DNA polymerase) and advantages of electrochemical detection. In one embodiment the methods of this invention utilize a single-base extension (SBE) reaction. In the single base extension reaction, an extension primer, that is designed so that its 3' end anneals adjacent to the polymorphic base-pair. The reaction is essentially a sequencing reaction containing only terminator (chain terminator) nucleotides. Consequently, all that can occur is the addition of a chain terminator which then cannot be extended further. Where a single polymorphism is to be detected, the chain terminator is selected to be complementary to the polymorphic nucleotide that is to be detected. When the particular polymorphic nucleotide is present, the primer is extended one nucleotide and detection of the "n+1" reaction product (where n is the length of the primer) indicates the presence of the polymorphic nucleotide. This method is illustrated schematically in Figure 1.

[0064] In certain embodiments, the, e.g. "n+1" extension product is detected by separating the reaction action products (e.g. by electrophoresis) and detecting a label associated with the separated reaction product(s).

[0065] In certain embodiments, where several different nucleotides can occur at the polymorphic site, the extension can be run with several (e.g. 4) different chain terminators (e.g. corresponding to A, C, G, and T). Each chain terminator can be differently labeled and
detection of reaction product(s) bearing a particular label indicates the presence and/or identity of the polymorphic nucleotide.

[0066] The primer extension reaction is typically run such that a detectable label is incorporated into the reaction products. This is readily accomplished by the use of a labeled extension primer, and/or by the use of a labeled chain terminator, and/or by the use of labeled nucleotide triphosphates. In preferred embodiments, the detectable label is a redox-active tag (e.g. a metallocene, a porphyrinic macrocycle, etc.) which can readily be detected using electrochemical methods (e.g. voltammetry, chronoamperometry, chronocoulometry impedance and/or capacitance measurements, and the like).

[0067] Electrochemical detection is well suited for high throughput mutation detection because of the low cost of instrumentation employed, adequate sensitivity, and ease of miniaturization. In addition, native DNA is not regarded as electrochemically active at modest potentials and traditional electrode materials and therefore does not interfere (presents low or no background) with electrochemical measurements. Electrochemical detection is also readily incorporated into highly multiplexed formats and/or "lab on a chip" formats.

[0068] In certain embodiments, the primer extension methods of this invention involve

[0069] i) providing a nucleic acid primer complementary to a nucleic acid sequence adjacent to (e.g. upstream from) the location of the single nucleotide comprising the single nucleotide polymorphism;

[0070] ii) performing a primer extension reaction using a nucleic acid polymerase and a chain terminator to produce one or more extension reaction products, where one or more components of the primer extension reaction are labeled with a redox-active label such that one or more extension reaction products produced by the primer extension reaction are labeled with the redox-active label; and

[0071] iii) detecting the extension reaction products by detecting the redox-active label, where detection of an extension reaction product longer than the nucleic acid primer indicates the presence of said single nucleotide polymorphism in the target nucleic acid.
I. Providing a nucleic acid primer.

[0072] Typically, the primer extension reaction is run using a nucleic acid primer that is complementary to a region of the "target nucleic acid" (the nucleic acid being probed for the presence of the SNP) immediately upstream of the location of the SNP that is to be detected. More specifically, the assay typically utilizes an internal extension primer, that is designed so that its 3' end anneals adjacent to the polymorphic base-pair (see, e.g., Figure 2).

[0073] The extension primer is selected/designated to anneal immediately upstream of the SNP to be typed. The extension primer should be long enough to initiate polymerization by a nucleic acid polymerase. In preferred embodiments, the extension primer is sufficiently long to specifically bind to the desired site (immediately upstream of the SNP). Typically extension primers are at least 8 nucleotides in length, preferably at least 10 or 15 nucleotides in length, more preferably at least 20 or 25 nucleotides in length.

[0074] In certain embodiments, the primer is designed/selected to have a T_m in the range of about 50°C to about 90°C, more preferably in the range of about 60°C to about 80°C (e.g. as determined using the the nearest neighbor calculation in Oligo software, or other standard software tools).

[0075] The primer is also designed/selected so that there are typically no stable dimers or hairpins at the very 3' end of the primer, otherwise it could extend on itself instead of on the target template in the single-base extension reaction. The extension primer can be chosen on either the sense or antisense strand, as long as the 3' end anneals immediately upstream of the SNP (see Figure 2').

[0076] There can be situations where the sequence flanking a SNP in the 5' direction on either strand does not offer a good choice for an extension primer. If there appears to be no way to avoid 3' dimers or hairpins, it may be possible in some cases to modify the primer sequence. The specificity of primer annealing comes primarily from base pairing at the 3' end. Substituting one or more nucleotides away from the 3' end may eliminate the problem of dimers or hairpins at the 3' end.

[0077] Sometimes designing a 20 nucleotide or longer primer will yield a T_m that is above 80°C. It is typically better to choose a primer slightly shorter than 20 nt than to greatly exceed 80°C in T_m. Oppositely, for primers with low T_mS, increasing their length to
increase their $T_m$ above 60°C is a good idea, as long as it does not introduce primer annealing with a recessed 3' end.

[0078] The primers can be made according to standard methods well known to those of skill in the art. Primers of this invention can be prepared by any suitable method as, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang et al. (1979) Meth. Enzymol. 68: 90-99; the phosphodiester method of Brown et al. (1979) Meth. Enzymol. 68: 109-151; the diethylphosphoramidite method of Beaucage et al. (1981) Tetra. Lett., 22: 1859-1862; and the solid support method of U.S. Patent No. 4,458,066. Typically the primers are chemically synthesized oligonucleotides.

[0079] Custom primers can also be purchased from a number of commercial vendors (see, e.g., Operon Technologies, Inc., Alameda, CA; Integrated DNA Technologies, Coralville, IA; MWG Biotech, High Point, NC; Sigma-Genosys, The Woodlands, TX; Synthetic Genetics, San Diego, CA; and the like).

[0080] Where the extension primer is to be labeled with a redox-active label, the label can be attached according to standard methods known to those of skill in the art (e.g. as described herein).

II. Performing a primer extension reaction.

[0081] In certain embodiments, a primer extension reaction is run (e.g. as described below and in the Examples) using the target nucleic acid(s) as template(s), for a nucleic acid polymerase and a chain terminator to produce one or more extension reaction products, where one or more components of the primer extension reaction are labeled with a redox-active label such that one or more extension reaction products produced by the primer extension reaction are labeled with the redox-active label;

A) Primer extension reaction.

[0082] The primer extension reaction is essentially a sequencing reaction containing only dye-terminator nucleotides. Since there are no typical nucleotides, all that can occur is the addition of a single chain terminator (e.g. dideoxynucleotide), which then cannot be extended further. Typically if the target nucleic acid(s) were generated using an amplification reaction (e.g. PCR), primers and dNTPs left over from the original PCR
should be removed or degraded before running the single-base extension reaction. Residual PCR primers are problematic because they can compete with the extension primer, effectively extending multiple targets, which would ruin the results. Residual dNTPs are problematic because they can allow extension to proceed beyond a single base.

5 [0083] The primer extension reaction is typically run with one or more chain terminators rather than "normal" nucleoside triphosphates. Suitable chain terminators are well known to those of skill in the art and include, but are not limited to dideoxynucleotide triphosphates (ddNTPs), acyclonucleotide triphosphates (acyNTPs), and the like. Various suitable terminators include, but are not limited to 2',3'-dideoxyguanosine-5'-triphosphate, 7-deaza-2',3'-dideoxyguanosine-5'-triphosphate, 2',3'-dideoxyadenosine-5'-triphosphate, 2',3'-dideoxythymidine-5'-triphosphate, and 2',3'-dideoxycytidine-5'-triphosphate.

10 [0084] The primer extension reaction can be a simple sequencing reaction (e.g. an *in vitro* template guided DNA polymerization) and/or an amplification reaction (e.g. Polymerase Chain Reaction transcription-based amplification system (TAS), self-sustained sequence replication system (SSR), etc.). Examples of suitable primer-extension reaction systems and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press, Inc., San Diego, CA; Sambrook et al. (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY; Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994); and the like. Examples 1 and 2 herein also illustrate suitable primer extension reaction systems.

B) Redox-active tags.

25 [0085] In certain embodiments, the primer extension reaction incorporates a redox-active tag into the reaction product(s). Typically this is accomplished by providing a primer bearing a redox active tag, and/or a chain terminator bearing a redox-active tag, and/or nucleoside triphosphates bearing redox-active tags (the latter where the extension is not to be immediately terminated).

30 [0086] A wide variety of molecules can be used as redox-active tags in this invention. Preferred molecules include, but are not limited to a porphyrinic macrocycle, a
metallocene, a linear polyene, a cyclic polyene, a heteroatom-substituted linear polyene, a heteroatom-substituted cyclic polyene, a tetraathiophenvalene, a tetraselenaphthalene, a metal coordination complex, a buckyball, a triarylamino, a 1,4-phenylenediamine, a xanthene, a flavin, a phenazine, a phenothiazine, an acridine, a quinoline, a 2,2'-bipyridyl, a 4,4'-bipyridyl, a tetrathiotetracene, and a peri-bridged naphthalene dichalcogenide. Even more preferred molecules include a porphyrin, an expanded porphyrin, a contracted porphyrin, a metalloocene (e.g. a ferrocene), a linear porphyrin polymer, and a porphyrin array. Certain particularly preferred redox-active tags include a porphyrinic macrocycle substituted at a β-position or at a meso-position (e.g. as described in WO 01/03126).

[0087] Particularly preferred redox-active tags include metalloccenes, more preferably ferrocenes having different substituents attached to the ferrocene ring, where the electron donating or withdrawing character of the substituent alters the half-wave potential of the modified metalloocene. Particularly preferred ferrocenes include, but are not limited to alkyl ferrocene, ferrocene acetate, ferrocene carboxylate, alkyl ferrocene dimethylcarboxamide, acetyl ferrocene, propioly ferrocene, butyryl ferrocene, pentanoyl ferrocene, hexanoyl ferrocene, octanoyl ferrocene, benzoyl ferrocene, 1,1'diacetyl ferrocene, 1,1'-dibutylferrocene, 1,1'-dihexanoyl ferrocene, ethyl ferrocene, propyl ferrocene, n-butyl ferrocene, pentyl ferrocene, hexyl ferrocene, 1,1'-diethyl ferrocene, 1,1'-dipropyl ferrocene, 1,1'-dibutyl ferrocene, 1,1'-dihexyl ferrocene, cyclopentenyl ferrocene, cyclohexenyl ferrocene, 3-ferrocenoyl propionic acid, 4-ferrocenoyl butyric acid, 4-ferrocenylbutyric acid, 5-ferrocenylvaleric acid, 3-ferrocenoyl propionic acid esters, 4-ferrocenoyl butyric acid esters, 4-ferrocenyl butyric acid esters, 5-ferrocenylvaleric acid esters, dimethylaminomethyl ferrocene, and the like.

[0088] Where the primer extension reaction is performed with a plurality of different redox-active tags, the redox-active tags are selected so that each tag that is to be present, e.g. in a mixture of tags, has a different and distinguishable oxidation state from the other tags in that mixture.

[0089] Control over the oxidation state(s) of the redox-active tags of this invention can be regulated through synthetic design. The oxidation (redox) potential can be tuned with precision by choice of base molecule(s), associated metals and peripheral substituents (Yang et al. (1999) J. Porphyrins Phthalocyanines, 3: 117-147).
[0090] For example, in the case of porphyrins, Mg porphyrins are more easily oxidized than Zn porphyrins, and electron withdrawing or electron releasing aryl groups can modulate the oxidation properties in predictable ways. The effects of metals on metalloporphyrin oxidation potentials are well known (Fuhrhop and Mauzerall (1969) J. Am. Chem. Soc., 91: 4174-4181) and provide a strong foundation for designing redox-active tags with oxidation states.

[0091] The design of compounds with predicted redox potentials is well known to those of ordinary skill in the art. In general, the oxidation potentials of redox-active units or subunits are well known to those of skill in the art and can be looked up (see, e.g., *Handbook of Electrochemistry of the Elements*). Moreover, in general, the effects of various substituents on the redox potentials of a molecule are generally additive. Thus, a theoretical oxidation potential can be readily predicted for any potential redox-active molecule. The actual oxidation potential, particularly the oxidation potential of the redox-active labels or the labeled moieties can be measured according to standard methods.

Typically the oxidation potential is predicted by comparison of the experimentally determined oxidation potential of a base molecule and that of a base molecule bearing one substituent in order to determine the shift in potential due to that particular substituent. The sum of such substituent-dependent potential shifts for the respective substituents then gives the predicted oxidation potential.


[0095] In particularly preferred embodiments, metalloocene redox-active tags (e.g. ferrocene tags) of this invention can be synthesized according to the procedure of Ihara et al. (see, e.g., Ihara et al. (1996) Nucleic Acids Research, 24: 4273-4280; Ihara et al. (1997) Chemical Communications, 1609-1610). Briefly, an activated N-hydroxysuccinimide (NHS) ester of ferrocene carboxylic acid and ferrocene acetic acid is synthesized using NHS and dicyclohexylcarbodiimide. The product can be purified on a column of silica gel (Merck 60, methylene chloride eluent). The activated ester (dissolved in dimethyl sulfoxide) can then be linked to an oligonucleotide, e.g., a 5’ amine terminated T3 primer in a 0.5 M NaHCO3/Na2CO3 (pH=9) buffer solution overnight. The reaction mixture can then be diluted with water and chromatographed on a NAP-10 column (Pharmacia Sephadex G-25). The absorbance was measured at 260 nm and used to determine which fractions contained the nucleic acid. Those fractions are combined, lyophilized, and purified by RP-HPLC.

Using the teachings provided in the references cited herein and illustrated in the Examples, one of skill can routinely synthesize a wide number of different and distinguishable redox-active tags.

The redox-active tags can be coupled to chain terminators, to nucleoside triphosphates, and/or to nucleic acid primers using standard methods well known to those of skill in the art. Means of coupling reactive moieties such as redox-active tags to a target are well known to those of skill in the art. Linkage of the redox-active tag to a moiety can be covalent, or by charge or other non-covalent interactions. The target moiety and/or the redox-active tag can be specifically derivatized to provide convenient linking groups (e.g. hydroxyl, amino, sulphhydryl, etc.). Covalent linkage of the redox-active tag to the target analyte can be direct or through a covalent linker. Where the moiety to be labeled is a protein, antibody, etc., it is noted that proteins contain a variety of functional groups; e.g., carboxylic acid (COOH) or free amine (-NH₂) groups, which are available for reaction with a suitable functional group on either the redox-active tag or on a linker attached to the surface. Similarly nucleic acids bear free hydroxyl groups suitable for coupling tags through the sugar moiety. In certain embodiments, the tag can be coupled to the nucleic acid through the base.

Generally linkers are either hetero- or homo-bifunctional molecules that contain two or more reactive sites that may each form a covalent bond with the respective binding partner (*i.e.* surface or ecotin variant). Linkers suitable for joining biological binding partners are well known to those of skill in the art. For example, a protein molecule may be linked by any of a variety of linkers including, but not limited to a peptide linker, a straight or branched chain carbon chain linker, or by a heterocyclic carbon linker. Heterobifunctional cross linking reagents such as active esters of N-ethylmaleimide have been widely used. See, for example, Lerner et al. (1981) *Proc. Nat. Acad. Sci. (USA)*, 78: 3403-3407 and Kitagawa et al. (1976) *J. Biochem.*, 79: 233-236, and Birch and Lennox (1995) Chapter 4 in *Monoclonal Antibodies: Principles and Applications*, Wiley-Liss, N.Y.).
III. **Detection of redox-active tags.**

[0100] The presence and/or identity of the extension reaction product(s) is detected by detecting the redox-active label(s). In certain embodiments the detection follows a separation reaction products.

5

A) **Separation of reaction products.**

[0101] In certain embodiments, the extension reaction product(s) are separated prior to or simultaneously with detection of the redox-active label(s). The separation can be by any of a number of convenient methods. Such methods include, but are not limited to electroosmotic methods, electrokinetic methods, electrophoretic methods, chromatographic methods (e.g. HPLC, FPLC, etc.), gel retardation columns, and the like. In preferred embodiments, the extension reaction product(s) are separated using electrophoretic methods. In particularly preferred embodiments, the extension reaction product(s) are separated using capillary electrophoresis.

[0102] Capillary electrophoresis offers a number of technical advantages: (i) capillaries have high surface-to-volume ratios which permit more efficient heat dissipation which, in turn, permit high electric fields to be used for more rapid separations; (ii) the technique requires minimal sample volumes; (iii) superior resolution of most analytes is attainable; and (iv) the technique is amenable to automation (see, e.g., Camilleri (1993) *Capillary Electrophoresis: Theory and Practice*, CRC Press, Boca Raton; and Grossman et al. (1992) *Capillary Electrophoresis*, Academic Press, San Diego; Drossman et al. (1990) *Anal. Chem.*, 62: 900-903; Huang et al., (1992) *Anal. Chem.*, 64: 2149-2154; and Swerdlow et al. (1990) *Nucleic Acids Research*, 18: 1415-1419).

B) **Detection and discrimination of redox-active tags.**

[0103] The redox-active labels used in the methods of this invention can be detected using any of a wide variety of electrochemical technologies including amperometric methods (e.g. chronoamperometry), coulometric methods (e.g. chronocoulometry), voltammetric methods (e.g., linear sweep voltammetry, cyclic voltammetry, pulse voltammetries, sinusoidal voltammetry, etc.), any of a variety of impedance and/or capacitance measurements, and the like. Such readouts can be performed in the time and/or frequency domain.
In preferred embodiments, cyclic voltammetric methods are used. Data acquisition in sinusoidal voltammetry is preferably performed as described by Brazill \textit{et al.} (2000) \textit{Anal. Chem.} 72: 5542-5548 and in the Examples described herein. Briefly, a computer-generated time-varying potential (\textit{e.g.} a triangle wave, a sine wave, etc) scans through the potential window of interest. The data is acquired using standard methods, \textit{e.g.} a data acquisition system, preferably at a frequency substantially higher (\textit{e.g.} preferably 50 fold higher or greater, more preferably 100 fold or greater, and most preferably 150- or 200-fold or greater), than the scan (excitation) frequency.

In particularly preferred embodiments, frequency domain information is obtained by continuous conversion of each scan, \textit{e.g.}, via the application of a Fast Fourier Transform (FFT). Other approaches can also be used to extract frequency domain information. Such approaches include, but are not limited to LaPlace transform, wavelet analysis, Wigner distribution, and the like. This frequency domain data provides voltammetric information characteristic of each redox active species (the SV frequency spectrum) and time course information (current versus time).

The frequency spectrum for a specific analyte consists of a series of vectors (represented as magnitude and phase) at each of a number of harmonics (\textit{e.g.} at least 2, preferably 5, more preferably 10 or more). To isolate the information particular to a given analyte, a background frequency spectrum can be subtracted from the entire data set. While definition of the background spectrum is somewhat arbitrary, a convenient measure is at least one scan, preferably the average of the two, more preferably first five, and most preferably the first scans, that typically represent the background processes (capacitive and faradaic) at the electrode surface (in the absence of analyte). It will be appreciated that the background scan can be varied to optimize signal to noise ratio or other parameters.

The frequency spectrum for the analyte of interest is simply defined as the background-subtracted current vector (magnitude and phase) at the highest part of the signal.

In addition to the SV frequency spectrum, time course data can be obtained at each harmonic frequency element by performing the “digital equivalent of a lock-in amplifier”. Analogous to an analog lock-in amplifier, the instantaneous current is monitored at the optimum phase angle for the signal of interest, thus increasing the
sensitivity and selectivity over traditional voltammetric techniques. Maximum sensitivity is achieved if the background phase angle is ± 90 degrees out of phase with the optimum phase angle for the signal. Therefore, the analyte is typically monitored at the phase angle that gives the maximum current for the signal and minimum background, which increases the S/N for the measurement. Finally, the phase-optimized time course data can, optionally, be digitally filtered, e.g., with a low-pass filter using a boxcar averaging routine or other routine.

[0109] This "digital lock-in approach" can also be used to distinguish between different molecules in the time domain. Selective discrimination between analytes with different electrochemical characteristics (formal potential, kinetics, etc.) is accomplished by identifying the frequency component where signal due to the analyte is closest to 90° out of phase with all other components. Using such a "phase nulling approach" with careful selection of the frequency and phase angle one can isolate the signal of the component of interest in almost any complex matrix.

[0110] In certain embodiments, where multiple labels are used, a frequency and phase angle are determined where all components (label signals) can be monitored with similar sensitivity (i.e. monitored at a single frequency and phase angle where each tag is equally represented). This “phase-common” signal can be obtained at any given frequency, simply by examining the difference between signals as a function of phase angle. The variance between each pair of components is calculated at each phase angle (e.g., \( i_{ab} = (i_a - i_b)^2 \)), and the overall variance (\( \text{VAR}_{\text{TOTAL}} = \Sigma i / n \), where \( i \) are the individual variances and \( n \) is the total number of combinations), representing the sum of all the individual variances, is calculated and minimized to find the optimum phase angle for all components. This allows rapid identification of a single phase angle that can be used to monitor all the subject tags with only slight variation from their maximum current response.

[0111] At the frequency and phase angle producing the "phase-common" signal, a large signal is observed for all tags, with typically minimal loss in signal compared to that observed at each of the optimum phase angles. The phase-nulled signal for each redox-active tag can then be obtained at the same frequency (e.g., third harmonic) by locking-in at a phase angle that is out of phase from the optimum for each tag. The phase angle is preferably either + or - 90 degrees out of phase. The signal corresponding to the phase-
nulled component is effectively diminished while the other three tags are relatively unaffected. When compared to the phase-common signal, it is easy to determine which peak has been removed. This method thus allows rapid "tag calling" on a single set of data simply by identifying the peaks that disappear. Such "tag calling" is illustrated in copending application USSN 09/945,238, filed on August 31, 2001.

Such selective nulling of each redox-active tag can be constantly performed over the course of a measurement. Thus, for example, where the output of a capillary electrophoresis experiment (e.g. nucleic acid sequencing reaction) is being assayed, a detection electrode is positioned at the end of the capillary tube or at one or more discrete points along the tube. The above-described phase nulling is performed for each tag expected to be present in the experiment. Dropout of the signal for the tag indicates the presence of that tag at that time.

Where only a single redox-active label is used, such "tag calling" is unnecessary. In this context, we utilize the optimum phase angle for the analyte of interest, obtained from the frequency domain, and monitor the raw signal at that particular phase angle. At this phase angle, the analyte's signal will be at its maximum, while only the background contribution at that particular phase angle will remain. The greatest elimination of background current occurs when its phase angle is either +/- 90 degrees different from the optimum phase angle for the analyte. We have recently demonstrated the utility of this same locking technique for selectively nulling out interfering signals as described above.

A wide variety selection of equipment for such electrochemical measurement is commercially available (see, e.g., Sycopel, Inc.; Solartron Analytic potentiostat; Princeton Applied Research, Pinnacle Technology, Inc. Multichannel Potentiostats, etc.). Commercial data acquisition systems often have integrated and/or associated data acquisitions systems and appropriate software that allows the programming of data analysis functions.

IV. Other assay formats.

While in certain embodiments, this invention pertains to methods of SNP detection that exploit a single base primer extension methodology, the methods contemplated herein are not so limited. Using the teachings provided herein, other assay formats are readily developed. For example, the use of redox-active labels, as described

V. Lab on a chip and HTS formats.

[0116] The methods of this invention are well suited to "lab on a chip" formats and/or for high throughput screening (HTS) systems. The miniaturization of chemical analysis systems, employing semiconductor processing methods, including photolithography and other wafer fabrication techniques borrowed from the microelectronics industry, has attracted increasing attention and has progressed rapidly. The so-called "lab-on-a-chip" technology enables sample preparation and analysis to be carried out on-board microfluidic-based cassettes. Moving fluids through a network of interconnecting enclosed microchannels of capillary dimensions is possible using electrokinetic, and other, transport methods.

[0117] Application of microfluidics technology embodied in the form of analytical devices has many attractive features. Advantages of miniaturization include greatly increased throughput and reduced costs, in addition to low consumption of both sample and reagents and system portability.

[0118] Various lab-on-a-chip formats are well known to those of skill in the art. For example, Burns et al. (1998) *Science*, 282: 484-487, describe a device that uses microfabricated fluidic channels, heaters, temperature sensors, and fluorescence detectors to analyze nanoliter-size DNA samples. The device is capable of measuring aqueous reagent and DNA-containing solutions, mixing the solutions together, amplifying or digesting the DNA to form discrete products, and separating and detecting those products. No external lenses, heaters, or mechanical pumps are necessary for complete sample processing and analysis. Because all of the components are made using conventional photolithographic
production techniques, they operate as a single closed system. The components have the potential for assembly into complex, low-power, integrated analysis systems at low unit cost. Using the teachings provided herein, the device described by Burns et al. can readily be modified to perform a single-base extension reaction and electrochemical detection of one or more redox-active labels. Other "lab on a chip" have been described (see, e.g., U.S. Patents 6,132,685, 6,123,798, 6,107,044, 6,100,541, 6,090,251, 6,086,825, 6,086,740, 6,074,725, 6,071,478, 6,068,752, 6,048,498, 6,046,056, 6,042,710, and 6,042,709) and can readily be adapted to the assays of this invention.

[0119] The methods of this invention are readily amenable to high throughput screening (HTS) systems in lab-on-a-chip and other formats. High-throughput screening systems typically permit screening of a plurality (e.g. at least 10, preferably at least 100, more preferably at least 500 or 1000, and most preferably at least 5,000 or 10,000) analytes at one time. High throughput screening is typically facilitated by the use of robotics for sample handling and processing and by various multiplexed assay strategies. It is noted, for example, that U.S. Patent 6,361,671 describes a microfabricated capillary electrophoresis chip that can be modified for the assays described herein. Other microfabricated CE devices and Capillary Array Electrophoresis (CAE) microplates been described by Woolley and Mathies (1994) Proc. Natl. Acad. Sci., USA, 91: 11348-11352, Woolley and Mathies (1995) Anal. Chem. 67: 3676-3680, Woolley et al. (1997) Anal. Chem. 69: 2256-2261, Simpson (1998) Proc. Natl. Acad. Sci., USA, 95: 2256-2261, and the like.

[0120] A number of well known robotic systems have been developed for solution phase chemistries. These systems include, but are not limited to, automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a chemist and the VentureTM platform, an ultra-high-throughput synthesizer that can run between 576 and 9,600 simultaneous reactions from start to finish (see Advanced ChemTech, Inc. Louisville, KY)). Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art.
VI. Kits.

[0121] In another embodiment, this invention contemplates kits for the practice of the methods described herein. Preferred kits include one or more redox-active labels. The labels can be provided alone, attached to linkers, or linked to oligonucleotide primers and/or to chain terminators, and/or to nucleoside triphosphates. In certain embodiments, the kits can comprise "lab-on-a-chip" cassettes for performing the methods of this invention.

[0001] In still another embodiment, this invention provides a computer readable medium comprising computer readable program code for directing a potentiostat in a cyclic voltammetric measurement to produce a cyclic voltammogram of the redox-active label(s); and/or to detecting the signal for each redox-active label it is desired to detect at a phase angle out of phase with the optimum phase angle for said redox-active label.

[0122] The kits can optionally include any reagents, buffers and/or apparatus for performing the methods of this invention.

[0123] In addition, the kits can, optionally, include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. Preferred instructional materials provide protocols utilizing the kit contents for detecting single nucleotide polymorphism. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention.

[0124] Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXCEPTIONS

[0124] The following examples are offered to illustrate, but not to limit the claimed invention.
Example 1

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

Abstract

A novel single nucleotide polymorphism (SNP) detection system is described, where 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 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.

Introduction

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 have previously demonstrated the sensitive and selective detection of ferrocene labeled DNA with SV detection (Brazill et al. (2001) Anal. Chem., 73: 4882-4890). The coupling of CGE with EC detection provides a fast, simple, inexpensive, and easily miniaturized SNP screening technique.
Experimental:

Reagents.

[0127] The ddNTPs were purchased from Fermentas (Hanover, MD). The AmpliTaq polymerase, GeneAmp PCR buffer, dNTPs, Performance Optimized polymerase-4 (POP-4), Genetic Analyzer Buffer, 5' amino linked T3 primer, T3 forward primer and T3 reverse primer were all provided by Applied Biosystems (Foster City, CA). Thermosequenase enzyme (USB Corporation, Cleveland, OH), pBluescript SK+ phagemid (Stratagene, La Jolla, CA), 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.

[0128] The synthetic procedure of Ihara et al. was followed and has been described previously (Ihara et al. (1996) Nucleic Acids Res., 24: 4273-4280). Briefly, an activated Nhydroxysuccinimide (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 7 produced a reddish-brown solid with a yield of 45%. The FA-NHS was confirmed by 1H-NMR in DMSO.

[0129] FA-NHS was dissolved in dimethylsulfoxide. To this solution was added the 5' amine terminated T3 primer dissolved in a 0.5 M NaHCO3/15Na2CO3 (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, Missouri). 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 (Brazill et al. (2001) Anal. Chem., 73: 4882-4890). 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: 1X GeneAmp PCR buffer (from a 10X stock solution), 0.2 mM dNTPs, 1 mM MgCl₂, 1 µM of each primer (Forward primer: 5’ AAT TAA CCC TCA CTA AAG GG 3’ (SEQ ID NO:3, T3 primer), Reverse primer: 5’ ACT CAC TAT AGG GCG AAT TG 3’, SEQ ID NO:4), 50 ng of pBluescript sK+ phagemid 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 8 cycles of 94 °C for 30 s, 41 °C for 30 s, and 72 °C for 60 s, ending with a 7 minute 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/15v agarose gel (Agarose for the Separation of GeneAmp PCR Products, Applied Biosystems, Foster City, CA) in 1X TBE buffer (diluted from 5X 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 x 10⁶ M⁻¹ cm⁻¹.

Single nucleotide primer extension:

The reaction volume used for SBE reactions was 20 µl and contained 100 pmoles of FA labeled T3 primer, approximately 40 ng of the 159 bp PCR amplicon
template, 200 μM of ddATP (complimentary to the mock SNP site) or ddCTP (used as a specificity control, see Scheme 1), 4U of Thermosequenase DNA polymerase, and 1X 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 minutes.

[0133] The SBE reaction was desalted prior to both MALDI-TOFMS and CGE/15SV analysis with float dialysis filter membranes (0.025 μm pore size; VSWP 047, Millipore, 9 Bedford, MA). The membrane was suspended on top of about 500 ml of water and the sample was placed in the center of the membrane. Float dialysis of the sample was performed for approximately 2 hours. The sample was then carefully removed from the membrane filter with a pipette 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/15SV detection.

MALDI-TOFMS.

[0134] 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 N2 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/15v solution of 3-HPA in acetonitrile/15water to diammonium citrate in water. 1 μl 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.

[0135] The fabrication of carbon cylinder microelectrodes has been described previously. 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 with 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 6-10 8 hours 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.**

[0136] 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 Volts. The fused silica capillary employed in this work was from Polymicro Technologies (Phoenix, AZ) with dimensions of 360 µm o.d. x 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 (Olefirowicz and Ewing (1990) Anal. Chem., 62: 1872-1876). The strong acid etched the capillary resulting in a slightly larger i.d., thus permitting the 32 µm carbon fiber to be placed just inside (about 10-20 µm) the end of the separation capillary with the aid of a microscope and micropositioners.

[0137] POP-4 was pumped into the capillary and used to both dynamically coat the capillary walls and to provide a sieving medium for DNA separation. The electrophoretic buffer used was Genetic Analyzer Buffer (1x). 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.**

presented, consisted of a 21 Hz sine wave with a potential window of −200 to 800 mV vs. Ag/15AgCl. The data was acquired in scans of 4 cycles 11 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 ten harmonics of the excitation frequency in order to limit the file sizes.

[0139] 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 has published work illustrating the dependence of the frequency domain “fingerprint” response on the electrochemical characteristics of the analyte and experimental parameters chosen (Brazill et al. (2000) Anal. Chem., 72: 5542-5548; Brazill et al. (2001) Anal. Chem., 73: 4882-4890; Singhal et al. (1997) Anal. Chem., 69: 1662-1668; Singhal and Kuhr (1997) Anal. Chem. 69: 4828-4832; Singhal and Kuhr (1997) Anal. Chem., 69: 3552-3557). 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 ten 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).

[0140] 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 lockin is similar to the analog version, where the signal is monitored at a specific frequency and phase angle. Briefly, this is accomplished by utilizing the optimum phase 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, while only the background contribution at that particular phase angle will remain. The greatest elimination of background current occurs when its phase angle is either +/- 90° different from the optimum phase angle for the analyte. We have recently demonstrated the utility of
this same locking technique for selectively nulling out interfering signals (see, USSR USSR 09/945,238, filed on August 31, 2001).

Results and Discussion.

Single Base Extension Reaction for SNP Analysis.


The SBE reaction is similar to the polymerase chain reaction (PCR) except dideoxynucleotide triphosphates are used and the amplification is linear rather than exponential. As illustrated in Scheme 1 (Figure 1), the sequence of steps is identical to PCR. First any double stranded DNA is denatured at a high temperature, then the template and 13 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
(Dubiley et al. (1999) Nucleic Acids Res., 27: e19). The accuracy of this method lies in the high fidelity of DNA polymerase to only incorporate the complimentary base.

The primer used in this example is a 20-mer with a ferrocene acetate label attached to the 5' end (FA-T3), Figure 3. The label allows the oligonucleotide to be detected with electrochemical detection, as 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 the incorrect or uncomplimentary 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 (Hayes et al. (1998) Biosens. Bioelectron., 13: 1297-1305). Figure 3 shows the static CV of a 100 µM solution of FA-T3 in genetic analyzer 1X buffer. The potential window applied was −200 to 650 mV vs. Ag/15AgCl at a scan rate of 15 V/15s. The oxidative and reductive peaks are split by about 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 was 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 nM to pM).

**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/15SV. MALDI is a very powerful technique that has been utilized in many genotyping assays 2,4,6, 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 (Fei and Smith (2000) Rapid Commun. Mass Spectrom., 14: 950-959). Initially, we did try to analyze the extension reaction without any 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 at desalting the sample.

[0146] As shown in Figure 4, the MALDI spectra for the purified FA-T3 primer used in the extension reaction (Figure 4A) and the SBE reaction sample incorporating the 15 complementary ddATP (Figure 4B) are consistent with the calculated m/15z values. The spectrum in Figure 4A is taken in linear positive ion mode, with an instrumental error of ±0.1%. The m/15z value found for FA-T3 was 6497 ± 6, which is consistent with the calculated m/15z 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/15z 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/15SV Analysis of SBE Reaction Products.**

[0147] 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 (Venter et al. (2001) Science, 291: 1304-1351). 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) (Tian et al. (2000) Genomics, 63: 25-34), heteroduplex analysis (HDA) (Tian et al. (2000) Genome Res., 10: 1403-1413; Tian et al. (2001) Clin. Chem. (Washington, DC, U. S.), 47: 173-185) constant

The SBE reaction mixture, which is characterized by MALDI in Figure 2B is electrokinetically injected into the CGE/15SV system. Figure 5 (solid line) shows the single base separation of the 20-mer FA-T3 primer from the 21-mer extension product at 16 the second harmonic (42 Hz). The separation medium used to accomplish this single base separation is a commercially available polymer (POP-4, Applied Biosystems). This polymer has been optimized for single base resolution out to about 250 nucleotides (Hawkins and Hoffman (1999) *Electrophoresis*, 20: 1171-1176). We have previously found that this polymer is compatible with SV detection and is easily pumped into the 20 μm i.d. capillary (Brazill et al. (2001) *Anal. Chem.*, 73: 4882-4890). 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 5 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 (Heller (2000) *Electrophoresis*, 21: 593-602). 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 have illustrated the impressive detection sensitivity of sinusoidal voltammetry and analysis in the frequency domain (Brazill et al. (2000) *Anal. Chem.*, 72: 5542-5548; Singhal et al. (1997) *Anal. Chem.*, 69: 1662-1668; Singhal and Kuhr (1997) *Anal. Chem.*, 69: 4828-4832; Singhal and Kuhr (1997) *Anal. Chem.*, 69: 3552-3557). 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 non-linear and has signal at higher harmonics of the excitation 17 frequency. It has been shown that utilization of the higher order harmonics can increase the sensitivity of a measurement by as much as three orders of magnitude (Singhal and Kuhr (1997) Anal. Chem. 69: 4828-4832). The amount of FA-T3 labeled primer utilized in the SBE reaction was 100 pmoles 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 approximately 10 µM.

[0150] The S/15N 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 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 half as intense as the second, and we assume there is no preferential injection between the two fragments, then the concentration of the primer detected is about 3 µM and the extension product is about 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.**

[0151] The accuracy of this assay, utilizing the SBE reaction, for mutation screening relies on the fidelity of the polymerase to only incorporate the complimentary 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 18 - no extension product is visible at a mass corresponding to the addition of a C (+272 m/15z). Additionally, the dotted line trace in Figure 5 shows the CGE/15SV 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 5. 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.

**Frequency Domain Consistency.**

[0152] The frequency domain response is characteristic of the electrochemical properties of the 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 (Brazill et al. (2001) Anal. Chem., 73: 4882-4890). The SV frequency domain spectra for the three peaks (2 FA-T3 primer peaks and 1 extension product peak) in Figure 5 is shown in Figure 7. 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 ±8° for each harmonic) (Brazill et al. (2001) Anal. Chem., 73: 4882-4890). As shown in Figure 7, 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.**

[0153] 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 (Sachidanandam et al. (2001) Nature, 409: 928-933). 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.

Example 2

The Use of Electrochemically Labeled Nucleotide Terminators for Known Point

[0154] A novel single nucleotide polymorphism (SNP) assay utilizing an electrochemically tagged chain terminator is described. The system employs the single base extension (SBE) technique coupled to capillary gel electrophoresis with end column electrochemical detection. A redox labeled chain terminator, ferrocene-acycloATP, is used in the SBE reaction. When the mutation site corresponds to the labeled chain terminator, the extension product is rendered electroactive. The reaction mixture is subsequently separated by capillary gel electrophoresis and the extension product detected at the separation anode with sinusoidal voltammetry. This work demonstrates the first known SNP assay utilizing redox active chain terminators coupled to electrochemical detection. The methodology presented could lead to a fast, simple, and cost effective SNP scoring system.

Introduction.

[0155] Single point variations in the human genome are called single nucleotide polymorphisms (SNPs) and are the most abundant mutation type. Their abundance makes them ideally suited to study genetic variability, individual drug metabolism and disease development risk (Cargill et al. (1999) Nature Genetics, 23: 373; Landegren et al. (1998) Genome Research, 8: 769-776; Sachidanandam et al. (2001) Nature, 409: 928-933). These mutations are highly conserved in the genome and are faithfully inherited throughout generations (Sachidanandam et al. (2001) Nature, 409: 928-933). The SNPs located in protein coding regions (cSNPs) are likely to affect gene function, protein structure and gene regulation. It is believed that about half of the cSNPs cause missense mutations in the expressed proteins, while the other half are silent (Cargill et al. (1999) Nature Genetics, 23: 373; Wang and Moul (2001) Human Mutation, 17: 263-270). The missense cSNPs are hypothesized to be involved in individualized drug response and predisposition to disease (Id.). Direct DNA sequencing is the preferred analysis method for SNP discovery because of its ability to provide complete information on the location and identity of the sequence variant (Lehnert et al. (2001) Human Mutation, 17: 243-254). Once a SNP site has been
fully characterized its routine screening will need to be applied to large sample populations. Sequencing methods are currently too labor intensive and time consuming for population studies and routine clinical analysis. Therefore, it is imperative to develop methods, which are rapid, accurate and cost effective to meet the demands of a high throughput SNP assay.

Although direct sequencing is considered the optimum method for screening a genome for possible single point mutations, several alternative screening methods have been developed. For example several researchers have used; single stranded conformation polymorphism (SSCP) (Ru et al. (2000) J. Chromatography A, 894: 171-177; Bogh et al. (1999) Parasitology, 118: 73-82; Nataraj et al. (1999) Electrophoresis, 20: 1177-1185; Tian et al. (2000) Genomics, 63: 25-34; Orita et al. (1989) Genomics, 5: 874-879), heteroduplex analysis (HDA) (Nataraj et al. (1999) Electrophoresis, 20: 1177-1185; Tian et al. (2001) Clinical Chemistry, 47: 173-185; Tian et al. (2000) Genome Research, 10: 1403-1413; Ganguly et al. (1993) Proc. Natl. Acad. Sci., USA, 90: 10325-10329), constant denaturing gel electrophoresis (CDGE) (Muniappan and Thilly (1999) Genet. Anal.: Biomol. Eng., 14: 221-227; Li-Sucholeiki et al. (1999) Electrophoresis, 20: 1224-1232), and temperature gradient gel electrophoresis (TGGE) (Gao and Yeung (2000) Analytical Chemistry, 72: 2499-2506; Gelfi et al. (1994) Electrophoresis, 15: 1506-1511) to screen for possible mutation sites and scan for known point mutations. SSCP has been reported to be the most commonly used mutation screening method, since its conception in 1989 (Orita et al. (1989) Genomics, 5: 874-879). This method has been applied in p53 mutation detection in colon tumor samples (Ru et al. (2000) J. Chromatography A, 894: 171-177) and adapted to capillary gel electrophoresis (CGE) (Kuypers et al. (1993) J. Chromatography-Biomedical Applications, 621: 149-156). Recently, Landers et al. used the HDA analysis platform in both a capillary and on a microfluidic device to screen for two breast cancer susceptibility genes, BRCA1 and BRCA2 (Tian et al. (2000) Genome Research, 10: 1403-1413). They demonstrated fast total analysis time, \( \sqrt{2.5} \) hours, however they also noted that this method is limited to the DNA fragment sizes between 200 and 300 bases. Yeung et al. have used TGGE, where a temperature gradient is used to produce various degrees of denaturation along the length of the separation capillary, to achieve a turn around time of 1 hour (Gao and Yeung (2000) Analytical Chemistry, 72: 2499-2506).

There has been tremendous work lately, in the development of techniques to screen for known biologically significant point mutations. The polymerase chain reaction


[0159] A major advance in fluorescence detection has been the development of fluorescently labeled ddNTPs. Fluorescent ddNTPs not only dramatically increased the throughput of DNA sequencing analysis, but have also been an integral advancement in mutation detection. The use of uniquely labeled fluorescent terminators eliminates the need for specialized probes and can be differentiated by their unique spectral signature (Nampalli et al. (2000) Tetrahedron Letters, 41: 8867-8871; Kumar et al. (1999) Nucleosides & Nucleotides, 18: 1101-1103). Additionally, by determining the fluorescently labeled ddNTP incorporated, the alleles present in the target DNA sample can be inferred.

ferrocene labeled ddUTP (Anne et al. (2001) Bioconjugate Chemistry, 12: 396-405). The researchers used the terminal deoxynucleotidyl transferase enzyme, which results in the template-independent DNA polymerase catalyzed incorporation of the redox labeled ddUTP into the 3’-OH termini of DNA. However, the incorporation was characterized with RP-HPLC and UV absorbance rather than electrochemical detection methods. Previous work done by our group has shown that unique ferrocene molecules could be attached to DNA primers and used to develop an electrochemical detection strategy for DNA sequencing (Brazill et al. (2001) Analytical Chemistry, 73: 4882-4890). Sinusoidal voltammetry (SV) and analysis in the frequency domain was used to selectively detect the four uniquely labeled primers. In this work, a dideoxy-ferrocene-acycloATP (FC-ddaATP) and the SBE technique is used in a model SNP analysis detection platform. The methodology presented in this work utilizes a ferrocene labeled chain terminator in a SBE reaction.

**Materials and methods.**

**Reagents.**

[0161] The unlabeled ddNTPs were purchased from Fermentas (Hanover, MD) and the dideoxy-ferrocene-acycloATP (FC-ddaATP) was provided by Motorola Life Sciences (Northbrook, IL.). 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 all provided by Applied Biosystems (Foster City, CA). Thermosequenase enzyme (USB Corporation, Cleveland, OH), pBluescript SK+ phagemid (Stratagene, La Jolla, CA), Phi-x 174 standard ladder (Promega, Madison, WI) were used as received. Float dialysis membrane filters, VSWP 0.025 <m pore size, were purchased from Millipore (Bedford, MA). Water was deionized through a Milli-Q water purification system (Millipore, Bedford, MA).

**Template PCR.**

[0162] The template used in the primer extension reaction was a 159 bp fragment generated from the pBluescript II sK+ phagemid (Stratagene, La Jolla, CA). To produce and amplify the model template to be used in the SBE reaction, was accomplished by a standard PCR reaction: in a total volume of 50 <l containing; 1X GeneAmp PCR buffer (from a 10X stock solution), 0.2 mM dNTPs, 1 mM MgCl₂, 1 μM of each primer (Forward primer: 5’-50-
AAT TAA CCC TCA CTA AAG GG 3’ (T3 primer, SEQ ID NO:5), Reverse primer: 5’ ACT CAC TAT AGG GCG AAT TG 3’, SEQ ID NO:6), 50 ng of pBluescript sK+ plasmid and 1.3 U AmpliTaq DNA polymerase. 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 minute incubation at 72 °C.

**Purification of PCR amplicons.**

[0163] 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/15v agarose gel (Agarose for the Separation of GeneAmp PCR Products, Applied Biosystems, Foster City, CA) in 1X TBE buffer (diluted from 5X Tris-Borate-EDTA buffer from Sigma) and stained 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 and the DNA was extracted using the 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 x 10^6 M⁻¹ cm⁻¹.

**Single nucleotide primer extension:**

[0164] The SBE reaction was run in 20 αl and contained 100 pmoles of primer (either the T3 primer (5’ AAT TAA CCC TCA CTA AAG GG 3’, SEQ ID NO:7) or the specificity primer (5’ TGG AGC TCC AGC TTT TGT TC 3’, SEQ ID NO:8), approximately 40 ng of the 159 bp PCR amplicon template, 200 µM of ferrocene acycloATP (complimentary to the mock SNP site, corresponding to a T in this case), 4U of Thermosequenase DNA polymerase, and 1X sequencing buffer from the Thermosequenase terminator cycle sequencing kit. The primer extension reaction was performed in the same thermocycler as the template PCR amplification mentioned in the above section. 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 minutes. The SBE reaction mixture was desalted prior to both MALDI-TOFMS and CGE/15SV analysis with float dialysis.
filter membranes (0.025 μm pore size; VSWP 047, Millipore, Bedford, MA). The membrane was suspended on top of about 500 ml of water and the sample was placed in the center of the membrane. Float dialysis of the sample was performed for approximately 2 hours. The sample was then carefully removed from the membrane filter with a pipette and dried down with the DNA concentration (LabConco). The sample was redissolved in 10 ml of water and analyzed either by MALDI-TOFMS or CGE/15SV detection.

**MALDI-TOFMS.**

[0165] 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 N₂ 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 diammmonium citrate was dissolved in 1 ml of water. The matrix used was an 8:1 v/v solution of 3-HPA in acetonitrile/15 water to diammmonium citrate in water. 1 μl 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.**

[0166] The fabrication of carbon cylinder microelectrodes has been described previously (Brazill et al. (2001) Analytical Chemistry, 73: 4882-4890). 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 with 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 hours and once cured the carbon cylinder was clipped to 100 μm or less with a scalpel. The electrode was sonicated in water.
**Capillary Gel Electrophoresis.**

[0167] Capillary gel electrophoresis (CGE) coupled to SV detection was used in this work. The system was run with 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 is capable of supplying up to +/15- 6000 Volts. The fused silica capillary employed in this work was from Polymicro Technologies (Phoenix, AZ) with dimensions of 360 \( \mu \text{m} \) o.d. x 20 \( \mu \text{m} \) i.d. and 17 cm in total length. The detection (anode) end of the capillary was etched with 40% hydrofluoric acid with nitrogen flowing through the capillary. The strong acid etched the capillary resulting in a slightly larger i.d. diameter, thus permitting the 32 \( \mu \text{m} \) carbon fiber to be placed just inside (about 10–20 \( \mu \text{m} \)) 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 to provide a sieving medium for DNA separation. The electrophoretic buffer used was Genetic Analyzer Buffer (1x).

**Sinusoidal Voltammetry.**

[0168] The SV detection system has been described in great detail in previous publications from our group (Brazill et al. (2001) *Analytical Chemistry*, 73: 4882-4890; Brazill et al. (2000) *Analytical Chemistry*, 72: 5542- 5548; Singhal and Kuhr (1997) *Analytical Chemistry*, 69: 3552-3557; Singhal et al. (1997) *Analytical Chemistry*, 69: 1662-1668). The waveform used in the present work, consisted of an 11 Hz sine wave with a potential window of –200 to 800 mV vs. Ag/15AgCl. The program was designed to save only the information from the first ten harmonics of the excitation frequency in order to limit the file sizes. The frequency domain data can be expressed either as 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. In previous work, the dependence of the frequency domain “fingerprint” response on the electrochemical characteristics of the analyte and experimental parameters chosen has been demonstrated (Brazill et al. (2001) *Analytical Chemistry*, 73: 4882-4890; Brazill et al. (2000) *Analytical Chemistry*, 72: 5542- 5548).
The three-dimensional frequency response for the analyte of interest is obtained from the scan in time where the background subtracted signal is at its maximum (i.e., the top of the analyte peak). The other possible data 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. The digital equivalent of a lock-in detector was thoroughly described in previous work. The optimum phase angle for the analyte of interest, obtained from the frequency domain, is used to monitor the raw signal. Therefore, the analyte’s signal will be at its maximum, while only the background contribution at that particular phase angle will remain.

Results and Discussion

Single Base Extension Reaction Utilizing Electrochemically Labeled Terminators.

The first reference sequence of the human genome and the human genome project has provided new techniques and information for DNA analysis. The identification of an increasing number of cSNPs has intensified the need to develop a fast, simple, cost effective assay for known point mutations. The identification and location of SNP sites is only the beginning step. The next task is to screen for known SNPs and correlate SNP occurrence frequency in the population with disease and drug side effects. The elucidation and screening of SNPs will dramatically impact health care through the early diagnosis of diseases and the development of patient tailored treatment options.

section that contains the mutation site is PCR amplified prior to SBE analysis utilizing standard reaction conditions. As shown in Scheme 1 (Figure 8), the template used in SBE reactions is typically a fragment anywhere from 50 to 500 base pairs. The template used in this work is a model 159 base pair fragment generated from the pBluescript SK+ phagemid. Also included in the SBE reaction used in this work, a thermostable polymerase and a dideoxy-ferrocene labeled chain terminator, dideoxy-ferrocene- acycloATP. In this model system the mock mutation site lies immediately adjacent to the 3' terminus of the T3 primer. When the Watson-Crick complimentary base, A in this case, is added to the reaction mixture the enzyme incorporates it into the 3'-OH end of the annealed primer. The acyclic ATP nucleotide analog has been shown to effective act as a chain terminator in enzymatic reactions (Shirokova et al. (1994) J. Med. Chem., 37: 3739-3748; Victorova et al. (1993) Mol. Biol., 27: 143-152; Gardner et al. (2002) Nucleic Acids Research, 30: 605-613). Therefore, only a single base corresponding to the mock SNP site is incorporated because the acyclic nucleotide analog lacks the 3' OH necessary for further base elongation.


**The Structure and Cyclic Voltammetry of the Free Ferrocene-AcycloATP**

The ferrocene acycloATP structure is shown in Figure 3A. The ferrocene molecule is attached through an alkynyl amino linker at the C7 position on the base 7-deaza adenine. This same linker was recently used by Anne et al. to attach a similar ferrocene derivative to the C5 position of uridine (Anne et al. (2001) Bioconjugate Chemistry, 12: 396-405). The use of a triple bond in the linker arm is present to keep the ferrocene
molecule away from the hydrogen bonding sites on the adenine base. Shown in Figure 1B is the standard static cyclic voltammogram for a 100 μM solution of the free ferrocene-acycloATP. The voltammogram was taken at 10 V/15s at a 32 μm carbon fiber electrode. The voltammetry of this molecule is very reversible with peak splitting of roughly 60 mV and a formal potential of 265 mV vs Ag/15AgCl. Therefore, appears that the attachment of the ferrocene to the nucleotide does not affect the fast reversible electron transfer kinetics of ferrocene. Cyclic voltammetry is a commonly used electrochemical scanning technique which provides a great deal of information on the electrochemical characteristics of the molecule, formal potential and electron transfer kinetics. However, the background current associated with scanning the applied potential is very large in comparison to the faradaic current of the electroactive molecule. It is often times difficult to discriminate the signal from the background when performing analysis in the time domain, as is traditionally done. The sensitivity of CV and analysis in the time domain cannot reach the sensitivity level necessary for analysis of electrochemically labeled oligonucleotides.

CGE coupled with Electrochemical Detection for the Detection of the Free Ferrocene-AcycloATP.

[0174] Capillary gel electrophoresis (CGE) has improved the speed and efficiency of DNA analysis compared to traditional gel based separation techniques (Gao and Yeung (2000) Analytical Chemistry, 72: 2499-2506). It has been used effectively in mutation analysis using SBE with fluoroescently labeled chain terminators (Barta et al. (2001) Electrophoresis, 22: 779-782; Shumaker et al. (1996) Human Mutation, 7: 346-354; Piggee et al. (1997) J. Chromatography A, 781: 367-375; Cai et al. (2000) Genomics, 69: 395). The compatibility of electrochemical detection with the capillary gel electrophoresis separation of ferrocene labeled oligonucleotides has been shown previously (Brazill et al. (2001) Analytical Chemistry, 73: 4882-4890). Sinusoidal voltammetry (SV) is analogous to cyclic voltammetry except instead of a triangle wave a sine wave is used as the excitation potential. Additionally, instead of analyzing the data in the more traditional time domain, in SV the analysis is performed in the frequency domain. Previously, we reported the use of SV coupled to CGE to present a concept sequencing strategy with electrochemically unique ferrocene tags (Id.). Each of the four unique tags attached to the 5’ end of the T3 primer,
would code for one of the four nucleotides. It was shown that the four oligonucleotides could be differentiated from one another based on the unique electrochemical labels used.

Figure 10A is the time course electropherogram of a 1 μM solution of free ferrocene acycloATP dissolved in water. The injection was performed electrokinetically for 6 seconds at −5 kV and the free nucleotide eluded from the capillary with a −5 kV separation voltage applied. The time course shown in Figure 10A is the faradaic signal present at the third harmonic (33 Hz). As can be seen from the time course profile, the free nucleotide elutes at a migration time of about 10 minutes under the imposed elution conditions. The extrapolated detection limit (S/15N =3) for this time course is roughly 5 nM. The electrochemical parameters have not yet been optimized for the detection of this tag. In a previous publication by our group a detection limit of 900 pM was achieved and it is reasonable to assume that a similar detection limit could be achieved for this tag under optimized conditions (Brazill et al. (2001) Analytical Chemistry, 73: 4882-4890).

In addition to the time course data, frequency domain information is also acquired in the SV experiment. In Figure 10B the frequency spectrum for the same injection shown in Figure 10A is shown. The frequency domain spectrum has been shown to be characteristic of the electrochemical properties of the molecule as well as the experimental parameters employed. Additionally, it has been demonstrated that the phase angle information gained in the frequency domain is characteristic of the electrochemical molecule and independent of analyte concentration, with relative standard deviations on average of ±8 degrees (Id.). The phase angle consistency for a given molecule has been used in previous work to selectively null out interfering signals and devise a potential sequencing scheme using SV detection.

**MALDI-TOFMS of the SBE Reaction Products.**

To validate the accuracy of the SBE reaction technique matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) was used. Shown in Figure 11A is the linear positive ion MALDI-TOFMS of the SBE reaction mixture using the T3 primer and ferroceneacycloATP. The spectrum shows the successful incorporation of the ferroceneacycloATP into the T3 primer. The peak at a m/15z of 6269 ± 6 corresponds to the [M + H]+ peak for the excess T3 primer, with a calculated m/15z of 6274. The peak at a m/15z of 6803 ± 6 is the [M + H]+ for the T3 primer with the ferrocene-
acycloATP incorporated at the 3’ end, with a calculated m/z of 6809. It appears that the electrochemically labeled acycloATP does not interfere with enzyme incorporation. The relative intensity for the unextended primer exceeds the intensity of the ferrocene-acycloATP incorporated product. This is probably due to the unoptimized SBE experimental reaction conditions used in this analysis. Experimental parameters like magnesium concentration, cycling temperatures, primer concentration, template concentration, DNA polymerase used and acyclic nucleotide concentration will be optimized in the future to try and increase the efficiency of electrochemical terminator incorporation.

[0178] The accuracy of the SBE technique relies on the high fidelity of DNA polymerase to incorporate only the Watson-Crick complement onto the 3’ end of the primer. A different “control” primer, 5’ TGG AGC TCC AGC TTT TGT TC 3’ (SEQ ID NO:9), was used as a control for the selectivity of the SBE reaction. The primer used has a complementary sequence on the same 159 base pair template, however the base adjacent to the 3’ end of the specificity primer is a G. Therefore, the primer should not be extended when the labeled acycloATP is used in the reaction mixture. Figure 11B is the MALDI-TOFMS of the control SBE reaction. The m/z of 6265 ± 6 corresponds to the m/15z of the unextended control primer used in the SBE reaction, with a calculated m/15z = 6270. To ensure that this primer could be properly extended by the complementary nucleotide, a ddCTP was run under the identical SBE reaction conditions. The primer was successfully extended by a single base corresponding to a C, m/15z found was 6540 ± 6, calculated m/15z of 6542 (spectrum not shown). MALDI is a very powerful analytical technique, however the instrumentation needed is fairly expensive for implementation in a clinical setting.

25 **CGE/15SV Detection of SNPs Using a Ferrocene-AcycloATP Chain Terminator.**

[0179] The use of an electrochemically labeled chain terminator in the SBE reaction simplifies the analysis by electrochemical detection compared dye-primer methods. The major advantage of labeled chain terminators is that separation conditions do not need to be optimized for single base resolution because the only electrochemically active oligonucleotide generated is through chain elongation. Thus, the only potential interference
is from the excess free ferrocene-acycloATP chain terminator still present in the reaction mixture. Figure 12A is the time course data at the third harmonic (33 Hz) for the SBE reaction using the T3 primer, solid line (corresponding to the MALDI sample shown in Figure 10A) and the SBE control reaction, dashed line (corresponding to the MALDI sample shown in Figure 10B).

The time course profile for the control reaction, utilizing the specificity primer in the SBE reaction, shows a single peak with a migration time of about 10 minutes. This peak is believed to correspond to the free ferroceneacycloATP nucleotide because of the similarity in migration times found under identical separation conditions. This determination was made based on the injection of the free electrochemically labeled chain terminator shown in Figure 11A. The peak shown in Figure 12A (dashed line) is very large because of the large concentration, 200 μM, of the free ferrocene-acycloATP added to the SBE reaction mixture. It is difficult to determine exactly how much of the ferrocene-acycloATP is detected because some of the molecule was probably lost during dialysis. Regardless, the concentration is probably somewhere between 400 nM and 1 μM however probably closer to the latter.

The time course profile for the injection of the model single point mutation SBE reaction, i.e. T3 primer with ferrocene-acycloATP incorporated onto the 3’ end, shows two peaks. The first small peak has a migration time of about 10 minutes and most likely corresponds to excess unincorporated ferrocene-acycloATP. The second peak exhibits a migration time of about 11.3 minutes and presumably represents the 21-mer with the ferrocene-acycloATP incorporated in its 3’ end.

**Consistency in Frequency Domain for Ferrocene-AcycloATP.**

As mentioned earlier in this work, the frequency domain is the spectral fingerprint for a particular electrochemical molecule (Brazill et al. (2001) *Analytical Chemistry*, 73: 4882-4890; Brazill et al. (2000) *Analytical Chemistry*, 72: 5542-5548; Singhal and Kuhr (1997) *Analytical Chemistry*, 69: 4828-4832; Singhal and Kuhr (1997) *Analytical Chemistry*, 69: 3552-3557; Singhal et al. (1997) *Analytical Chemistry*, 69: 1662-1668). The current magnitude is proportional to the concentration of the analyte, however the phase angle information is independent and consistent within a given set of experimental conditions. It is very hard to distinguish between the frequency spectra for the free
ferrocene-acycloATP and T3 primer with the ferrocene chain terminator incorporated at the 3’ end because they are almost completely overlapping one another. However, this is expected because the electrochemical signal is coming from the same ferrocene molecule and therefore the spectral properties should be consistent when incorporated into a larger molecule, 20-mer oligonucleotide. Table 1 gives the phase angle information for the peak shown in Figure 10A and the three peaks present in Figure 12A. The first column represents the ten harmonics collected while the last column in Table 1 is the standard deviation in phase angle for the four peaks. The standard deviation is around 8 degrees for all harmonics except the 6th, 8th, and 10a. In these three harmonics clear signal was not present in all cases and the phase angle data is most likely from noise rather than the faradaic signal from the ferrocene molecule.

<table>
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<tr>
<th>Harmonic (Hz)</th>
<th>Dideoxy Ferrocene- aATP Phase Angles</th>
<th>Dideoxy Ferrocene- aATP Control SBE Reaction</th>
<th>Dideoxy Ferrocene- aATP 1st SBE Peak</th>
<th>Dideoxy Ferrocene- aATP 2nd SBE Peak (21 mer)</th>
<th>Extended T3 Primer</th>
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The phase angle information can be used to genotype mutations by adding two chain terminators labeled with unique electrochemical labels. Therefore, the base incorporated can be probed and determined from the electrochemical frequency spectrum obtained. Additionally, the use of uniquely labeled chain terminators could be used to
multiplex this analysis platform to screen for multiple SNPs in a single separation device. This can be accomplished by using SBE primers that differ in length and can be easily separated by CGE.

Conclusions.

The model SNP assay system presented utilizes a ferrocene labeled chain terminator and subsequent analysis by CGE coupled with SV detection. Electrochemical detection offers the advantages of relatively inexpensive instrumentation, sensitivity, and portability. The analysis demonstrated in this work was simple and did not require optimization of the separation conditions for single base analysis. To the inventors knowledge this is the first demonstration of SNP analysis using SBE and an electrochemically labeled chain terminator. The CGE/SV detection platform is potentially a cost effective and simple SNP detection method amenable to routine screening in the clinical laboratory.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.
CLAIMS

What is claimed is:

1. A method of detecting a single nucleotide polymorphism in a target nucleic acid, said method comprising:

   i) providing a nucleic acid primer complementary to a nucleic acid sequence adjacent to the location of the single nucleotide comprising said single nucleotide polymorphism wherein said primer is of sufficient length to initiate transcription by a nucleic acid polymerase;

   ii) performing a primer extension reaction using a nucleic acid polymerase, a chain terminator to produce one or more extension reaction products, wherein one or more components of said primer extension reaction are labeled with a redox-active label whereby one or more extension reaction products produced by the primer extension reaction are labeled with said redox-active label;

   iii) detecting the extension reaction products by detecting the redox-active label, wherein detection of an extension reaction product longer than the nucleic acid primer indicates the presence of said single nucleotide polymorphism in said target nucleic acid.

2. The method of claim 1, wherein said primer is at least 10 nucleotides in length.

3. The method of claim 1, wherein said nucleic acid primer bears said redox-active label.

4. The method of claim 1, wherein said chain terminator bears said redox-active label.

5. The method of claim 1, wherein said primer extension reaction is a nucleic acid amplification reaction.

6. The method of claim 5, wherein said primer extension reaction is polymerase chain reaction (PCR) nucleic acid amplification reaction.
7. The method of claim 1, wherein said detecting comprises electrophoretically separating the extension reaction products.

8. The method of claim 1, wherein said detecting comprises electrophoretically separating the extension reaction products by capillary gel electrophoresis.

9. The method of claim 1, wherein said redox-active label is detected using an electrochemical detection method.

10. The method of claim 1, wherein said redox-active label is detected using cyclic voltammetry.

11. The method of claim 10, wherein said cyclic voltammetry is sinusoidal voltammetry.

12. The method of claim 10, wherein said voltammetry is performed at a single electrode.

13. The method of claim 10 or 11, wherein a signal from said redox-active label is analyzed in the frequency domain.

14. The method of claim 13, the analysis comprises performing a Fourier transform.

15. The method of claim 13, wherein said signal is analyzed at a harmonic of the excitation frequency.

16. The method of claim 15, wherein said cyclic voltammetry comprises selecting voltammetric data at a second or higher harmonic of the excitation frequency.

17. The method of claim 15, wherein said cyclic voltammetry comprises selecting voltammetric data at a third or higher harmonic of the excitation frequency.

18. The method of claim 1, wherein said detecting comprises detecting the signal for the redox-active label at a phase angle out of phase with respect to the optimum phase angle for said redox-active label.
19. The method of claim 18, wherein said detecting comprises selecting voltammetric data at a phase angle about 45 degrees to about 90 degrees out of phase with the optimum phase angle for the redox-active label.

20. The method of claim 18, wherein said detecting comprises selecting voltammetric data detecting at a phase angle closest to 90 degrees out of phase with the optimum phase angle for the redox-active label.

21. The method of claim 1, wherein said chain terminator is an acyclo nucleoside triphosphate (acyNTP) chain terminator.

22. The method of claim 1, wherein said chain terminator is a dideoxy chain terminator.

23. The method of claim 22, wherein said chain terminator is a dideoxy chain terminator selected from the group consisting of 2',3'-dideoxyguanosine-5'-triphosphate, 7-deaza-2',3'-dideoxyguanosine-5'-triphosphate, 2',3'-dideoxyadenosine-5'-triphosphate, 2',3'-dideoxycytidine-5'-triphosphate, and 2',3'-dideoxythymidine-5'-triphosphate.

24. The method of claim 23, wherein said chain terminator is labeled with said redox-active label.

25. The method of claim 22, wherein said chain terminator is a dideoxyferrocene-acycloATP.

26. The method of claim 1, wherein said redox-active label is selected from the group consisting of a porphyrin, an expanded porphyrin, a contracted porphyrin, a metalloocene, a linear porphyrin polymer, and a porphyrin array.

27. The method of claim 1, wherein said redox-active label comprises a ferrocene.

28. The method of claim 27, wherein said ferrocene is selected from the group consisting of an alkyl ferrocene, a ferrocene acetate, a ferrocene carboxylate, and an alkyl ferrocene dimethylcarboxamide.

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29. The method of claim 1, wherein said redox-active label comprises a porphyrinic macrocycle substituted at a β- position or at a meso- position.

30. The method of claim 1, wherein said detecting is performed on a chip.

31. A kit for detecting a single nucleotide polymorphism, said kit comprising:

a container containing one or more moieties selected from the group consisting of a chain terminator labeled with a redox-active label; a primer labeled with a redox-active label, and a nucleotide triphosphates labeled with a redox-active label.

32. The kit of claim 31, further comprising instructional materials teaching the use of said moieties to detect a single-nucleotide polymorphism.

33. The kit of claim 31, wherein said primer is at least 10 nucleotides in length.

34. The kit of claim 31, wherein said chain terminator is a dideoxy chain terminator.

35. The kit of claim 31, wherein said chain terminator is a dideoxy chain terminator selected from the group consisting of 2',3'-dideoxyguanosine-5'-triphosphate, 7-deaza-2',3'-dideoxyguanosine-5'-triphosphate, 2',3'-dideoxyadenosine-5'-triphosphate, 2',3'-dideoxycytidine-5'-triphosphate, and 2',3'-dideoxythymidine-5'-triphosphate.

36. The kit of claim 31, wherein said chain terminator is a dideoxy-ferrocene-acycloATP.

37. The kit of claim 31, wherein said redox-active label is selected from the group consisting of a porphyrin, an expanded porphyrin, a contracted porphyrin, a metallocene, a linear porphyrin polymer, and a porphyrin array.

38. The kit of claim 31, wherein said redox-active label comprises a ferrocene.
39. The kit of claim 38, wherein said ferrocene is selected from the group consisting of an alkyl ferrocene, a ferrocene acetate, a ferrocene carboxylate, and an alkyl ferrocene dimethylcarboxamide.

40. The kit of claim 31, wherein said redox-active label comprises a porphyrinic macrocycle substituted at a β- position or at a meso- position.

41. A method of identifying a single nucleotide polymorphism in a target nucleic acid, said method comprising:

i) providing a nucleic acid primer complementary to a nucleic acid sequence adjacent to the location of the single nucleotide comprising said single nucleotide polymorphism wherein said primer is of sufficient length to initiate transcription by a nucleic acid polymerase;

ii) performing a primer extension reaction using a nucleic acid polymerase, and chain terminators complementary to each base expected to comprise the single nucleotide polymorphism where each chain terminator is labeled with a redox-active label and different chain terminators are labeled with different and distinguishable redox-active labels; and

iii) detecting the extension reaction products by detecting the redox-active labels, wherein identification of the redox-active label indicates the identity of the single nucleotide polymorphism present in the target nucleic acid.
1. Denature

2. Anneal

\[ 5'\text{-A}TC\text{A}CA\text{T}A\text{T}A\text{G}GG\text{C}GA\text{A}T\text{G}G\text{G}TTA\text{ATT} - 3' \]

\[ 3'\text{-G}G\text{G}A\text{A}A\text{T}A\text{C}A\text{T}C\text{C}C\text{C}A\text{A}T\text{A}A\text{A} - 5' \]

Template

Primer

3. ddNTP and Thermo Sequenase

\[ \text{ddATP} \]

4. Extension of Primer

\[ 3'\text{-A}GG\text{G}A\text{A}A\text{T}A\text{C}A\text{T}C\text{C}C\text{A}\text{A}T\text{A}A - 5' \]

**Fig. 1**

**Fig. 2**
Fig. 3
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Fig. 4A
Fig. 4B
Fig. 5
Fig. 6
Fig. 7
**Fig. 8**

DNA Template 5' TTTGTCCCTTTAGTGAGGGTTTAATT 3'

Primer 3' GGGAAATCACCACCAATTAA 5'

ThermoSequenase +

Ferrocene-acycloATP +

Ferrocene-A 5' GGGAAATCACCACCAATTAA 3'

Extension Product 3' 5'
Fig. 9A
Fig. 9B
Fig. 10A
Fig. 11A
Fig. 11B
Fig. 12A
SEQUENCE LISTING

<KUHR, WERNER G.
BRAZILL, SARA A.>

LABELS

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<K1B> 407T-302200PC
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<K1D> 2002-06-07
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