

METHODS FOR DETECTING NUCLEIC ACIDS OF INTEREST VIA CIS-CLEAVAGE OF CRISPR/CAS

REFERENCE TO A SEQUENCE LISTING

5 [0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled "P4415_SEQ_AF", created March 10, 2025, which is 30 KB in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

CROSS-REFERENCES TO RELATED APPLICATIONS

10 [0002] This application claims priority and the benefit of U.S. Provisional Patent Application No. 63/566,348, filed March 17, 2024, the entirety of which is incorporated herein by reference

BACKGROUND OF THE INVENTION

[0003] 1. FIELD OF THE INVENTION

15 [0004] The present disclosure relates to the field of disease diagnosis. More particularly, the disclosure invention relates to methods for identifying gene alterations associated with diseases via CRISPR/Cas EIS (electrochemical impedance spectroscopy) system.

[0005] 2. DESCRIPTION OF RELATED ART

[0006] In addition to being one of the most promising tools for therapeutic genome editing, CRISPR/Cas systems have significant potential for nucleic acid detection. However, the performance of this system usually requires the help of reporter systems. A CRISPR/Cas system monitors and quantifies a target in three steps, i.e. target recognition and binding, Cas protein activation, and reporter cleavage. The activated Cas proteins act like scissors to cleave the single stranded nucleic acid, leading to the separation of reporter segments. Cleavage of the reporter results in a direct readout or initiation of a secondary event generating detectable signals, such as fluorescence, colorimetric read-out etc.

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[0007] The present disclosure provides an improved method for detecting nucleic acid via CRISPR/Cas system, in which the detection is provided in signals that are read directly after cis-cleavage by CRISPR/Cas protein without using a reporter (e.g., a DNA or RNA molecule labeled with a fluorophore).

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SUMMARY

[0008] In one aspect, the present disclosure is directed to a method for detecting a nucleic acid of interest in a sample, such as a sample from a subject having a disease or suspected of having a disease. The method comprises steps of:

- 5 (i) producing a labeled nucleic acid having a first adapter at its 5'- or 3'-ends;
- (ii) applying the labeled nucleic acid of step (i) onto a substrate having an electrode disposed thereon, in which the electrode is coated with a second adapter configured to capture the first adapter, thereby immobilizing the nucleic acid of interest onto the electrode;
- 10 (iii) contacting the immobilized nucleic acid of interest of step (ii) with a composition comprising a CRISPR/Cas protein and a guide RNA specific to a segment of the nucleic acid of interest to initiate cis-cleavage of the immobilized nucleic acid of interest by the CRISPR/Cas protein; and
- (iv) measuring a signal of the electrode after the cis-cleavage.
- 15 wherein, the method is characterized in not using a reporter to detect the cleaved product after the cis-cleavage.

[0009] According to some embodiments of the present disclosure, in step (i), the nucleic acid of interest is labeled by,

- (i-1) extending the nucleic acid of interest with forward and reverse primers
- 20 respectively hybridized and annealed to 3'- and 5'-ends of the nucleic acid of interest via polymerase chain reaction (PCR), thereby producing the labeled nucleic acid, wherein the forward and reverse primers independently comprise the first adapter connected to its 5'-end.

[0010] According to some embodiments of the present disclosure, after step (i-1),

- 25 in step (ii), the first adapter at the 5'-end of the labeled nucleic acid is captured by the second adapter on the electrode; and
- in step (iv), the impedance increases after cis-cleavage as compared to that of a control, which is the signal measured before the immobilized nucleic acid of step (ii) contacting the composition of step (ii).

[0011] According to other embodiments of the present disclosure, after step (i-1),
in step (ii), the first adapter at the 3'-end of the labeled nucleic acid is captured by
the second adapter on the electrode; and
in step (iv), the signal decreases after cis-cleavage as compared to that of a control,
5 which is the signal measured before the immobilized nucleic acid of step (ii)
contacting the composition of step (iii).

[0012] According to some embodiments of the present disclosure, in step (i), the nucleic
acid of interest is labeled by,

(i-2) respectively connecting the 3'-end of a forward primer and the 5'-end of a reverse
10 primer to 5'- and 3'-ends of the nucleic acid of interest via nucleic acid ligation
thereby producing the labeled nucleic acid, wherein the forward and reverse
primers independently comprise a first adapter connected to its 5'- and 3'-ends.

[0013] According to some embodiments of the present disclosure, after step (i-2),
in step (ii), the first adapter of at the 5'-end of the labeled nucleic acid is captured by
15 the second adapter on the electrode; and
in step (iv), the impedance increases after cis-cleavage as compared to that of a
control, which is the signal measured before the immobilized nucleic acid of step
(ii) contacting the composition of step (iii).

[0014] According to other embodiments of the present disclosure, after step (i-2),
20 in step (ii), the first adapter of at the 3'-end of the labeled nucleic acid is captured by
the second adapter on the electrode; and
in step (iv), the signal decreases after cis-cleavage as compared to that of a control,
which is the signal measured before the immobilized nucleic acid of step (ii)
contacting the composition of step (iii).

25 [0015] According to some embodiments of the present disclosure, the electrode is made
of metal. Examples of the metal suitable for making the electrode include, but are not
limited to, gold, copper, platinum, aluminum etc. Preferably, the electrode is made of
gold.

[0016] According to some embodiments of the present disclosure, the electrode is made of a non-metal material, which may be carbon or poly(3,4-ethylenedioxythiophene).

[0017] According to embodiments of the present disclosure, the first and second adapters are respectively biotin and streptavidin, digoxigenin and anti-digoxigenin, or magnetic
5 beads and magnets. Preferably, the first adapter is biotin and the second adapter is streptavidin or *vice versa*.

[0018] Examples of Cas protein suitable for use in the present disclosure include, but are not limited to, Cas 9, Cas 12, Cas 13 and Cas 14. In some examples, the Cas protein is Cas 9. In other examples, the Cas protein is Cas 12.

10 [0019] According to embodiments of the present disclosure, in step (iv), the signal generated from the electrode may be impedance, current or voltage. Preferably, the signal is impedance.

[0020] Examples of the nucleic acid of interest suitable for use in the present method include, but are not limited to, single strand DNA (ssDNA), double strand DNA
15 (dsDNA), single strand RNA (ssRNA), double strand RNA (dsRNA), complementary DNA (cDNA), and DNA-RNA hybrid.

[0021] According to embodiments of the present disclosure, the nucleic acid of interest comprises a genomic alteration therein. Examples of genomic alteration that may be present in the nucleic acid of interest include, but are not limited to, a point mutation, a
20 deletion, an insertion, an inversion, a duplication, a fusion, and a combination thereof. According to alternative embodiments of the present disclosure, the nucleic acid of interest may be a foreign gene present in a sample, such as a viral nucleic acid in a blood sample.

[0022] In all embodiments, the sample is any one of blood, serum, oral mucosa, body
25 fluids, hair roots, tissues, feces, bodily secretions, medium, soil, water and plant.

[0023] Many of the attendant features and advantages of the present disclosure will become better understood with reference to the following detailed description considered in connection with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The present description will be better understood from the following detailed description read considering the accompanying drawings, where:

[0025] **FIG. 1** is a schematic drawing depicting steps of the present method **100** in accordance with preferred embodiments of the present disclosure;

[0026] **FIG. 2** depicts the detection of target DNA via impedance-increasing mode in accordance with Example 1.1 of the present disclosure;

[0027] **FIG. 3** depicts the detection of target DNA via impedance-decreasing mode in accordance with Example 1.1 of the present disclosure; and

10 [0028] **FIG. 4** depicts the detection of two point mutations in a target nucleic acid via impedance-increasing mode in accordance with Example 1.2 of the present disclosure.

DESCRIPTION

[0029] The detailed description provided below in connection with the appended drawings is intended as a description of the present examples and is not intended to represent the only forms in which the present example may be constructed or utilized. The description sets forth the functions of the example and the sequence of steps for constructing and operating the example. However, the same or equivalent functions and sequences may be accomplished by different examples.

[0030] **1. Definitions**

20 [0031] For convenience, certain terms employed in the specification, examples and appended claims are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of the ordinary skill in the art to which this invention belongs.

[0032] As used herein, the term “nucleic acid” refers to a polymer composed of nucleotides (i.e., deoxyribonucleotides or ribonucleotides) of any length, such as greater than 10 nucleotides, greater than 50 nucleotides, greater than 100 nucleotides, greater than 500 nucleotides, greater than 1,000 nucleotides, or greater than 5,000 nucleotides. Naturally occurring nucleotides include guanine (G), cytosine (C), adenine (A), and thymine (T)/uracil (U). According to embodiments of the present disclosure, the nucleic

acid may be mammalian, viral, fungal, or bacterial, or a mixture thereof. Further, the term “nucleic acid” encompasses a single strand DNA (ssDNA), a double strand DNA (dsDNA), a single strand RNA (ssRNA), a double strand RNA (dsRNA), a genomic DNA, a complementary DNA (cDNA), and a DNA-RNA hybrid.

5 [0033] As used herein, the term “nucleic acid of interest” refers to a nucleic acid to be analyzed. In some embodiments, the “nucleic acid of interest” comprises both a known or pre-determined sequence and adjacent nucleotides to be analyzed, which may be referred to as “an unknown sequence.” According to embodiments of the present disclosure, the “nucleic acid of interest” may have a length of 10 or more nucleotides, 20
10 or more nucleotides, 30 or more nucleotides, 40 or more nucleotides, 50 or more nucleotides, 60 or more nucleotides, 70 or more nucleotides, 80 or more nucleotides, 90 or more nucleotides, 100 or more nucleotides, 200 or more nucleotides, 300 or more nucleotides, 400 or more nucleotides, 500 or more nucleotides, or 1,000 or more nucleotides. In some embodiments, the “nucleic acid of interest” has a length of 10 to
15 100 nucleotides, 10 to 200 nucleotides, 10 to 300 nucleotides, 10 to 400 nucleotides, 10 to 500 nucleotides, 10 to 1,000 nucleotides, 50 to 100 nucleotides, 50 to 200 nucleotides, 50 to 300 nucleotides, 50 to 400 nucleotides, 50 to 500 nucleotides, or 50 to 1,000 nucleotides.

[0034] The term “hybridization”, “hybridizing” or “hybridize(s) to” refers to a process
20 where completely or partially complementary nucleic acid strands are joined by hydrogen bonds to form a double-stranded structure or region under specified conditions. Note that hydrogen bonds typically form between adenine (A) and thymine (T) or uracil (U) nucleotides, or cytosine (C) and guanine (G) nucleotides.

[0035] The term “anneal” refers to the formation of one or more complementary base
25 pairs between two nucleic acids. In some embodiments, annealing involves two completely or substantially complementary nucleic acid strands hybridizing together. In some embodiments, in the context of an extension reaction, annealing involves the hybridization of primer to a template such that a primer extension substrate for a template-dependent polymerase enzyme is formed. The condition for annealing may vary based on

the length and sequence of the primer. In some embodiments, conditions for annealing are based on a calculated T_m of a primer. In some embodiments, an annealing step of an extension reaction involves reducing the temperature after the strand separation step to a temperature based on the calculated T_m for a primer, for a time sufficient to permit such
5 annealing. In some embodiments, the calculated T_m can be determined by any known software, such as VENTRO NTTTM (Invitrogen, Inc. California), Primer3 (Premier Biosoft, Palo Alto, California) and the like. For most amplifications, the annealing temperature may be selected to be 5°C below the predicted T_m , though temperature closer to or above the predicted T_m can be used as well.

10 [0036] The term “primer” means an oligonucleotide capable of acting as a point of initiation of nucleic acid synthesis upon forming a duplex with a polynucleotide template and being extended from its 3'-end along the template so that an extended duplex is formed. The sequence of nucleotides being added during the extension process is determined by the sequence of the template polynucleotide. A primer serves as a point of
15 initiation of nucleic acid synthesis catalyzed by DNA polymerase, RNA polymerase or reverse transcriptase.

[0037] The term “adapters” as used herein refers to two substances acting as a pair and exhibiting high affinity toward each other, which thus are useful for purification or detection of various biomolecules (e.g., proteins, nucleic acids etc.). Various adapters are
20 known in the relevant field and can be used in the present disclosure. Examples of adapters acting as a pair include, but are not limited to, biotin and streptavidin, digoxigenin and anti-digoxigenin, and magnetic beads and magnets.

[0038] The term “genomic alteration(s)” as used herein refers to a point mutation, a deletion, an insertion, a duplication, or a fusion in one or more genes. The term “fusion”
25 when applies to a gene refers to a gene alteration including a hybrid gene formed from two previously independent genes or portions thereof or a particular gene formed from at least two independent exons thereof.

[0039] Ranges of values are disclosed herein. The ranges set out a lower limit value and an upper limit value. Unless otherwise stated, the ranges include all values to the

magnitude of the smallest values (either lower limit value or upper limit value) and ranges between the values of the stated ranges.

[0040] The singular forms “a”, “an”, and “the” are used herein to include plural referents unless the context clearly dictates otherwise.

5 [0041] **2. Methods for detecting genomic alterations via CRISPR/Cas electrochemical impedance spectroscopy (EIS) system**

[0042] An example of the method described herein is to detect unknown sequences, particularly those containing genetic alterations such as insertions, deletions, mutation, duplications, fusions or a combination thereof in a nucleic acid sample via CRISPR/Cas
10 system. The present method is characterized in that the detection is made by the direct readout of an electrode, thereby eliminating the need for a reporter for detecting the cleaved product after the cis-cleavage by CRISPR/Cas protein.

[0043] Reference is made to **Fig. 1**, which is a schematic drawing depicting steps of detecting a nucleic acid of interest in a biological sample. In general, the present method
15 **100** includes four stages, which are stage I: producing a labeled nucleic acid; stage II: capturing/immobilizing the labeled nucleic acid; stage III: cis-cleavage of the captured/immobilized nucleic acid via CRISPR/Cas protein; and stage IV: detecting signals.

[0044] *Stage I: producing a labeled nucleic acid*

20 [0045] The present method **100** commences by labeling the nucleic acid of interest **110** in the sample to produce a labeled nucleic acid **120**. In this embodiment, the labeling is achieved by use of a polymerase. In some embodiments, a forward primer **112** having a first adapter **115** connected to its 5'-end, and a reverse primer **114** without the first adapter **115** connected to its 5'-end are mixed with the nucleic acid of interest **110**, the forward
25 and reverse primers **112**, **114** will automatically hybridize and anneal to 5' and 3'-ends of the nucleic acid of interest **110**, respectively. The hybridized product is then extended by polymerase chain reaction (PCR) to produce the labeled nucleic acid **120**, which is a duplex with one strand having the first adapter **115** connected to its 5'-end. In other embodiments, a reverse primer **114** having a first adapter **115** connected to its 5'-end, and

a forward primer **112** without the first adapter **115** connected to its 5'-end are mixed with the nucleic acid of interest **110**. The forward and reverse primers **112**, **114** will automatically hybridize and anneal to 5' and 3'-ends of the nucleic acid of interest **110** and extended by PCR to produce the labeled nucleic acid **120**, which is a duplex with one strand having the first adapter **115** connected to its 3'-end. In the case where the nucleic acid of interest **110** is a DNA, DNA polymerase (e.g., Taq polymerase) is used for labeling purpose. In the case where the nucleic acid of interest **110** is an RNA, then reverse transcriptase is used for labeling purpose.

[0046] *Stage II: capturing/immobilizing the labeled nucleic acid*

[0047] To capture and/or detect the labeled nucleic acid **120** described above, an electrode **130** disposed on a substrate (e.g., a graphene layer) and comprises a second adapter **116** coated thereon is constructed according to procedures described in working examples of this present disclosure. The second adapter **116** is a substance that exhibits high affinity toward the first adapter **115** described above, thus will immediately form a strong binding upon contacting the first adapter **115**.

[0048] In this stage, the labeled nucleic acid **120** produced in stage I is applied onto the electrode **130**, accordingly, the first adapter **115** of the labeled nucleic acid **120** is immediately captured by the second adapter **116** coated on the electrode **130** resulting in the labeled nucleic acid **120** being immobilized on the surface of the electrode **130**.

[0049] According to embodiments of the present disclosure, the electrode may be made of metal or non-metal material. Examples of metal suitable for use as the electrode in the present disclosure include, but are not limited to, gold, copper, platinum, and aluminum. Preferably, the electrode is made of gold. Examples of the non-metal material suitable for use as the electrode in the present disclosure include, but are not limited to, carbon and poly (3,4-ethylenedioxythiophene).

[0050] For the nucleic acid of interest **110** being labeled by extending with the forward primer **112** via PCR in stage I, the first adapter **115** at the 5'-end of the forward primer **112** in the labeled nucleic acid duplex **120** is immobilized on the electrode **130** (**Fig 1**, left panel). For the nucleic acid of interest **110** being labeled by extending with the reverse

primer **114** via PCR in stage I, the first adapter **115** at the 5'-end of the reverse primer **114** in the labeled nucleic acid duplex **120** is immobilized on the electrode **130** (Fig. 1, right panel).

[0051] *Stage III: cis-cleavage of the captured/immobilized nucleic acid via CRISPR/Cas protein*

[0052] In this stage, the immobilized nucleic acid **120** described above is brought into contact with a composition, which comprises a CRISPR/Cas protein **140** and a single strand guide RNA (sgRNA) **150**. The sgRNA **150** comprises a sequence specific to a segment of the nucleic acid of interest **110** and may guide the CRISPR/Cas protein **140** to initiate cis-cleavage of the immobilized nucleic acid **120** thereby generating signals for subsequent detection.

[0053] *Stage IV: detecting signals*

[0054] Since the labeled nucleic acid **120** is immobilized on an electrode, accordingly, impedance, voltage or current generated from an electrode may be used as a signal for detection purpose.

[0055] For the labeled nucleic acid **120** extended by forward primer **112** in stage I, after cis-cleavage by Cas protein in stage III, the Cas/sgRNA complexes would remain bound to the electrode **130** thereby resulting in increases in signals as compared to those of a control (i.e., a DNA free of Cas protein bound thereto) (Fig. 1, left panels). According to some embodiments of the present disclosure, the signal (e.g., impedance) increases with an increase in the nucleic acid concentration in the sample. According to preferred embodiments of the present disclosure, the concentration of the nucleic acid of interest that could be detected by the present impedance-increasing mode is 10^{-16} M.

[0056] For the labeled nucleic acid **120** extended by reverse primer **114** in stage I, after cis-cleavage by Cas protein in stage III, the Cas/sgRNA complexes would be cut away after cis-cleavage by Cas thereby resulting in a decrease in signals as compared to those of a control (i.e., a DNA free of Cas protein bound thereto) (Fig. 1 right panels). According to some embodiments of the present disclosure, the signal (e.g., impedance) decreases with a decrease in the nucleic acid concentration in the sample. According to preferred

embodiments of the present disclosure, the concentration of the nucleic acid of interest that could be detected by the present impedance-decreasing mode is 10^{-9} M.

[0057] **3. Alternative embodiments of the present method**

[0058] The steps described in this alternative method are similar to those in Section 2 of the present disclosure, except the nucleic acid of interest **110** is labeled via the use of a ligase. In such cases, the 5'- and 3'-ends of nucleic acid of interest **110** are respectively ligated to 3'- and 5'-ends of forward and reverse primers **112**, **114** using a ligase, and the 5'-end of the forward primer **112** and the 3'-end of the reverse primer **114** independently have a first adapter **115** connected thereto. Accordingly, in stage I, the labeled nucleic acid **120** thus produced comprises, from 5'-end to 3'-end, the first adapter **115**, the forward primer **112**, and the nucleic acid of interest **110**; or the nucleic acid of interest **110**, the reverse primer **114**, and the first adapter **115**.

[0059] In stage II, the labeled nucleic acid **120** is also captured by the second adapter **116** coated on the electrode **130**. Specifically, for the nucleic acid of interest **110** being labeled by the forward primer **112** in the presence of a ligase, the first adapter **115** at the 5'-end of the forward primer **112** is captured by the second adapter **116** thereby immobilizing the labeled nucleic acid **120** on the electrode **130**. As to the nucleic acid of interest **110** being labeled by the reverse primer **114** in the presence of a ligase, the first adapter **115** at the 3'-end of the reverse primer **114** is captured by the second adapter **116**, thereby immobilizing the labeled nucleic acid **120** on the electrode **130**.

[0060] The immobilized nucleic acid **120** is then cis-cleaved via CRISPR/Cas protein (stage III) and the signal generated therefrom is detected as described above in Section 2 of the present disclosure (stage IV).

[0061] The present method described above is advantageous in that the detection of the cleaved product can be directly read from impedance signals of the electrode, thereby eliminating the need of a reporter system such as a fluorescence resonance energy transfer (FRET)-based reporter that comprises short DNA or RNA oligonucleotides labeled with a fluorophore and quencher, required in the conventional CRISPR/Cas system.

[0062] It is to be understood that this invention is not limited to particular embodiments described, as such may vary. It is also to be understood that the terminology used herein is for the purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention is limited only by the appended claims.

5 [0063] **Examples**

[0064] **Example 1: Detection of EGFR gene mutation by the present CRISPR/Cas electrochemical impedance spectroscopy (EIS) system**

[0065] **1.1 Detecting L858R point mutation in EGFR gene in both impedance-increasing and impedance-decreasing modes**

10 [0066] In this example, two biotin-labeled EGFR gene sequence derived nucleic acid containing L858R point mutation of about 156 kb (SEQ ID NO: 1, hereafter “target DNA 1”) and 157 kb (SEQ ID No: 5, hereafter “target DNA 2”) in length were used as the target DNA to demonstrate the feasibility of the present system and method in both impedance-increasing and decreasing modes. The target DNA 1 was extended with
15 forward primer 1 (SEQ ID NO: 2) and reverse primer 1 (SEQ ID NO: 3), while the target DNA 2 was extended with forward primer 2 (SEQ ID NO: 6) and reverse primer 2 (SEQ ID NO: 7) by polymerase chain reaction (PCR). The nucleic acid sequences of the target DNA and primers employed in this example are provided in Table 1.

[0067] To this purpose, a streptavidin-coated gold electrode was first constructed from
20 a commercial G3-Flat Gold electrode, which used gold as the working electrode, carbon as the counter electrode, and Ag/AgCl as the reference electrode. The gold of the working electrode was functionalized by reacting with 11-mercaptopundecanoic acid (11-MUA) (5mM of 11-MUA in 75% ethanol solution, 20 μ L) for more than 0.5 hours at 25°C; then, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) (0.1M) and N-
25 hydroxysuccinimide (NHS) (0.05M) solutions were applied onto the functionalized gold surface to turn the carboxy groups thereon into amine-reactive esters for subsequent coupling with streptavidin (1mg/mL) for 0.5 hours at room temperature. Finally, bovine serum albumin (BSA, 1 mg/mL) was added to remove non-specific binding, thereby producing the streptavidin-coated gold electrode.

[0068] The 5'-end of the biotin-labeled target DNA 1 (SEQ ID NO: 1, ranges from 10^{-9} , 10^{-11} , 10^{-13} , 10^{-15} , 10^{-16} M) (20 μ L), and 3'-end of the biotin-labeled target DNA 2 (SEQ ID NO: 5, ranges from 10^{-7} , 10^{-8} , 10^{-9} M)(20 μ L) were applied on to the streptavidin-coated gold electrode to allow the biotin-labeled target nucleic acid to be captured by the streptavidin-coated gold electrode. A segment of human chromosome Y gene similar in size as the target DNA was used as a control. Then, SpCas9 (NEB, M0386T) and sgRNA (SEQ ID NO: 4) were applied onto the electrode and incubated for 30 mins at room temperature, and the impedance signals generated therefrom were recorded by EIS, which were measured at the frequency ranged from 10 to 200 kHz, a bias voltage of 0 mV, and the amplitude of 10mV/s.

[0069] **Table 1:** Nucleotide sequence of the target DNA and primers

Name	Oligonucleotide Sequence (5' to 3')	length	SEQ ID NO
Target DNA 1 (L858R) Biotin labeled at 5'-end	CCCTCACAGCAGGGTCTTCTCTGTTTCA GGGCATGAACTACTTGGAGGACCGTCG CTTGGTGCACCGCGACCTGGCAGCCAGG AACGTACTGGTGAAAACACCGCAGCAT GTCAAGATCACAGATTTTGGGCTGGCCA AACGGCTGGGTGCGGAAG	156	1
Forward primer 1 (L858R) Biotin labeled at 5'-end	CCCTCACAGCAGGGTCTTCTC	21	2
Reverse primer 1 (L858R)	CTTCCGCACCCAGCAGTTTGG	21	3
sgRNA 1 (L858R)	UUUUGGGCUGGCCAAACUGCGUUUUAG AGCUAGAAAUAGCAAGUAAAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGU GGCACCGAGUCGGUGCUUUU	100	4
Target DNA 2 (L858R) Biotin labeled at 3'-end	CTTGGTGCACCGCGACCTGGCAGCCAGG AACGTACTGGTGAAAACACCGCAGCAT GTCAAGATCACAGATTTTGGGCTGGCCA AACGGCTGGGTGCGGAAGAGAAAGAAT ACCATGCAGAAGGAGGCAAAGTAAGGA GGTGGCTTTAGGTCAGCCAGC	157	5
Forward primer 2 (L858R)	CTTGGTGCACCGCGACCT	20	6
Reverse primer 2 (L858R) Biotin labeled at 5'-end	CTGGCTGACCTAAAGCCACCT	21	7

[0070] When the target DNAs were labeled with biotin to their 5'-ends, the Cas9/sgRNA complexes would remain bound to the gold electrode after cis-cleavage by Cas9, resulting in increases in the impedance values as compared to those of the free target DNAs in a dose-dependent manner (**Fig. 2**). Similarly, when the target DNAs were labeled with biotin to their 3'-ends, the Cas9/sgRNA complexes would be cut away after cis-cleavage by Cas9, resulting in decreasing in the impedance values as compared to those of the free target DNAs in a dose-dependent manner (**Fig. 3**). Note that the lowest concentration of the target DNA that could be detected by the gold electrode in the impedance-increasing mode was 10^{-16} M, whereas the lowest concentration was 10^{-9} M in the impedance-decreasing mode.

[0071] **1.2 Detecting L858R and T790M point mutations in EGFR gene in impedance-increasing mode**

[0072] In this example, two template DNAs respectively containing L858R point mutation (SEQ ID NO: 1, hereafter "Target DNA 1") and T790M point mutation (SEQ ID NO: 8, hereafter "Target DNA 3") were used as the target DNAs to demonstrate the feasibility of the present EIS system and method in impedance-increasing mode. The nucleic acid sequences of the target DNA and primers employed in this example are provided in Table 2. The detection was conducted in accordance with similar procedures described in Example 1.

[0073] Table 2: Nucleotide sequence of the target DNA and primers

Name	Oligonucleotide Sequence (5' to 3')	length	SEQ ID NO
Target DNA 1 (L858R) Biotin labeled at 5'-end	CCCTCACAGCAGGGTCTTCTCTGTTTCA GGGCATGAACTACTTGGAGGACCGTCG CTTGGTGCACCGCGACCTGGCAGCCAGG AACGTACTGGTGAAAACACCGCAGCAT GTCAAGATCACAGATTTTGGGCTGGCCA AACGGCTGGGTGCGGAAG	156	1
Forward primer 1 (L858R) Biotin labeled at 5'-end	CCCTCACAGCAGGGTCTTCTC	21	2
Reverse primer 1 (L858R)	CTTCCGCACCCAGCAGTTTGG	21	3
sgRNA 1 (L858R)	UUUUGGGCUGGCCAAACUGCGUUU UAGAGCUAGAAAUAGCAAGUAAA	100	4

	AUAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGGCACCGAGUCGGUGC UUUU		
Target DNA 3 (T790M) Biotin labeled at 5'- end	GTGCCCCTCCTTCTGGCCACCATGCGAA GCCACACTGACGTGCCTCTCCCTCCCTC CAGGAAGCCTACGTGATGGCCAGCGTG GACAACCCCCACGTGTGCCGCCTGCTGG GCATCTGCCTCACCTCCACCGTGCAGCT CATCATGCAGGTCATGCCC	158	8
Forward primer 3 (T790M) Biotin labeled at 5'- end	GTGCCCCTCCTTCTGGCCACCA	22	9
Reverse primer 3 (T790M)	GGGCATGACCTGCGTGATGAGC	22	10
sgRNA 2 (T790M)	CACCGUGCAGCUCAUCAUGCGUUUAG AGCUAGAAAUAGCAAGUAAAAUAAG GCUAGUCCGUUAUCAACUUGAAAAAGU GGCACCGAGUCGGUGCUUUU	100	11

[0074] It was found when biotin was labeled on the 5'-end of target DNA 2 (L858R) or target DNA 3 (T790M), these target DNAs could be recognized by CRISPR/Cas9 and cis-cleavage of a very small 3'-end. Further, the Cas9 protein sgRNA-L858R complex and the Cas9 protein sgRNA-T790M complex would sequentially bind to their respective target biotin-DNAs, which allowed for the measurement of impedance signals. The concentration of biotin-DNAs was 10⁻⁸M (**Fig. 4**). The impedance of biotin-DNA mix was 27,858 Ohm, the impedance of cis-cleavage-L858R was 37,422 Ohm, and the impedance of cis-cleavage-T790M was 48,578 Ohm. Thus, the increased impedance change for target DNA 2 (L858R) was 9,564 Ohm, and the increased impedance change for target DNA 3 (T790M) was 11,156 Ohm. The data substantiates the detection of two different mutant genes on the same chip.

[0075] It will be understood that the above description of embodiments is given by way of example only and that various modifications may be made by those with ordinary skills in the art. The above specification, examples, and data provide a complete description of the structure and use of exemplary embodiments of the invention. Although various embodiments of the invention have been described above with a certain degree of particularity, or with reference to one or more individual embodiments, those with

ordinary skills in the art could make numerous alterations to the disclosed embodiments without departing from the spirit or scope of this invention.

WHAT IS CLAIMED IS:

1. A method for detecting a nucleic acid of interest in a sample comprising:

- (i) producing a labeled nucleic acid having a first adapter at its 5'- or 3'-ends;
- (ii) applying the labeled nucleic acid of step (i) onto a substrate having an
5 electrode disposed thereon, in which the electrode is coated with a second
adapter configure to capture the first adapter thereby immobilizing the nucleic
acid of interest onto the electrode;
- (iii) contacting the immobilized nucleic acid of interest of step (ii) with a
composition comprising a CRISPR/Cas protein and a guide RNA specific to
10 a segment of the nucleic acid of interest to initiate cis-cleavage of the
immobilized nucleic acid of interest by the CRISPR/Cas protein; and
- (iv) measuring a signal of the electrode after the cis-cleavage.

wherein, the method is characterized in not using a reporter to detect the cleaved
product after cis-cleavage by CRISPR/Cas protein.

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2. The method of claim 1, wherein the labeled nucleic acid of step (i) is produced by,

- (i-1) extending the nucleic acid of interest with forward and reverse primers
respectively hybridize and anneal to 3'- and 5'-ends of the nucleic acid of interest
via polymerase chain reaction (PCR), thereby producing the labeled nucleic acid,
20 wherein the forward and reverse primers independently comprise the first adapter
connected to its 5'-end.

3. The method of claim 2, wherein in step (i-1), the nucleic acid of interest is extended
with two forward primers and two reverse primers respectively different from each
25 other.

4. The method of claim 2, wherein,

in step (ii), the first adapter at the 5-end of the labeled nucleic acid is captured by the
second adapter on the electrode; and

in step (iv), the impedance increases after cis-cleavage as compared to that of a control, which is the signal measured before the immobilized nucleic acid of step (ii) contacting the composition of step (iii).

5 5. The method of claim 4, wherein the method is capable of detecting the nucleic acid of interest in the sample at a concentration at or above 10^{-16} M.

6. The method of claim 2, wherein,
in step (ii), the first adapter at the 3'-end of the labeled nucleic acid is captured by the
10 second adapter on the electrode; and
in step (iv), the signal decreases after cis-cleavage as compared to that of a control, which is the signal measured before the immobilized nucleic acid of step (ii) contacting the composition of step (iii).

15 7. The method of claim 6, wherein the method is capable of detecting the nucleic acid of interest at a concentration at or above 10^{-9} M.

8. The method of claim 1, wherein in step (i), the nucleic acid of interest is labeled by,
(i-2) respectively connecting the 3'-end of forward primer and the 5'-end of reverse
20 primer to 5'- and 3'-ends of the nucleic acid of interest via nucleic acid ligation thereby producing the labeled nucleic acid, wherein the forward and reverse primers independently comprises the first adapter connected to its 5'- and 3'-ends.

9. The method of claim 8, wherein in step (i-2), two forward primers and two reverse
25 primers different from each other are respectively ligated to nucleic acid of interest to produce the labeled nucleic acid.

10. The method of claim 8, wherein

- in step (ii), the first adapter at the 5'-end of the labeled nucleic acid is captured by the second adapter on the electrode; and
- in step (iv), the impedance increases after cis-cleavage as compared to that of a control, which is the signal measured before the immobilized nucleic acid of step
- 5 (ii) contacting the composition of step (iii).
11. The method of claim 8, wherein,
- in step (ii), the first adapter at the 3'-end of the labeled nucleic acid at is captured by the second adapter on the electrode; and
- 10 in step (iv), the signal decreases after cis-cleavage as compared to that of a control, which is the signal measured before the immobilized nucleic acid of step (ii) contacting the composition of step (iii).
12. The method of claim 1, wherein the electrode is made of a metal selected from the
- 15 group consisting of gold, copper, platinum, and aluminum.
13. The method of claim 1, wherein the electrode is made of a non-metal material that is carbon or poly(3,4-ethylenedioxythiophene).
- 20 14. The method of claim 1, wherein the first and second adapters are respectively biotin and streptavidin, digoxigenin and anti-digoxigenin, or magnetic beads and magnets.
15. The method of claim 1, wherein the Cas protein is Cas 9, Cas 12, Cas 13 or Cas 14.
- 25 16. The method of claim 1, wherein in step (iv), the signal is impedance, current or voltage.
17. The method of claim 1, wherein the nucleic acid of interest is a single strand DNA (ssDNA), a double strand DNA (dsDNA), a single strand RNA (ssRNA), a double
- 30 strand RNA (dsRNA), a complementary DNA (cDNA), or a DNA-RNA hybrid.

18. The method of claim 17, wherein the nucleic acid of interest comprises a genomic alteration.
- 5 19. The method of claim 18, wherein the genomic alteration is a point mutation, a deletion, an insertion, an inversion, a duplication, a fusion, or a combination thereof.
20. The method of claim 1, wherein the sample is selected from the group consisting of blood, serum, oral mucosa, body fluids, hair roots, tissues, feces, bodily secretions,
10 medium, soil, water and plant.