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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	Q0052.70018US02
First Named Inventor	David Wilson
Title	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN, etc.
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [ ] **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. [ ] **Applicant asserts small entity status.** See 37 CFR 1.27
3. [ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. [X] **Specification** [*Total Pages* 66] Both the claims and abstract must start on a new page. *(See MPEP § 608.01(a) for information on the preferred arrangement)*
5. [X] **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 5]
6. **Inventor's Oath or Declaration** [*Total Pages* 2] *(including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))*
   a. [ ] Newly executed (original or copy)
   b. [X] A copy from a prior application (37 CFR 1.63(d))
7. [X] **Application Data Sheet** ** See note below.* See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program (*Appendix*)
   [ ] Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** (*if applicable, items a. – c. are required*)
   a. [ ] Computer Readable Form (CRF)
   b. [ ] Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**

ACCOMPANYING APPLICATION PAPERS

10. [ ] **Assignment Papers** (cover sheet & document(s))
    Name of Assignee
11. [ ] **37 CFR 3.73(c) Statement** (*when there is an assignee*) [X] **Power of Attorney**
12. [ ] **English Translation Document** (*if applicable*)
13. [ ] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
14. [ ] **Preliminary Amendment**
15. [ ] **Return Receipt Postcard** (*MPEP § 503*) (*Should be specifically itemized*)
16. [ ] **Certified Copy of Priority Document(s)** (*if foreign priority is claimed*)
17. [ ] **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. [X] **Other:** 37 CFR 3.73(b) Statement

***Note:** (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

19. CORRESPONDENCE ADDRESS

[X] The address associated with Customer Number: 23628 **OR** [ ] Correspondence address below

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Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Andrea J. Gabert/	Date	September 19, 2016
Name (Print/Type)	Andrea J. Gabert, Ph.D.	Registration No. (Attorney/Agent)	68,904

Application Data Sheet

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Postal or Zip Code of mailing address::	02021

Correspondence Information

Correspondence Customer Number::	23628

Application Information

Application Type::	Regular
Subject Matter::	Utility
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Title::	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Attorney Docket Number::	Q0052.70018US02
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	5
Small Entity?::	No
Petition included?::	No
Portions or all of the application associated with this	No

Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2::

This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013:: No

Representative Information

Representative Customer Number:: 23628

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Continuation of	14/111326	06/24/14
14/111326	National Stage of	PCT/US2012/033343	04/12/12
PCT/US2012/033343	An application claiming the benefit under 35 USC 119(e)	61/524693	08/17/11
PCT/US2012/033343	An application claiming the benefit under 35 USC 119(e)	61/474315	04/12/11

Foreign Priority Information

Applicant Information

Applicant Number:: 1

Applicant Type:: Assignee

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City of mailing address::	Lexington
State or Province of mailing address::	MA
Postal or Zip Code of mailing address::	02421-3125

Assignee Information Including Non-Applicant Assignee Information

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City of mailing address::	Lexington
State or Province of mailing address::	MA
Postal or Zip Code of mailing address::	02421-3125

Authorization or Opt-Out of Authorization to Permit Access

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

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A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

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[x] B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Andrea J. Gabert/	Date (YYYY-MM-DD)	2016-09-19
Name	Andrea J. Gabert, Ph.D.	Registration Number	68,904

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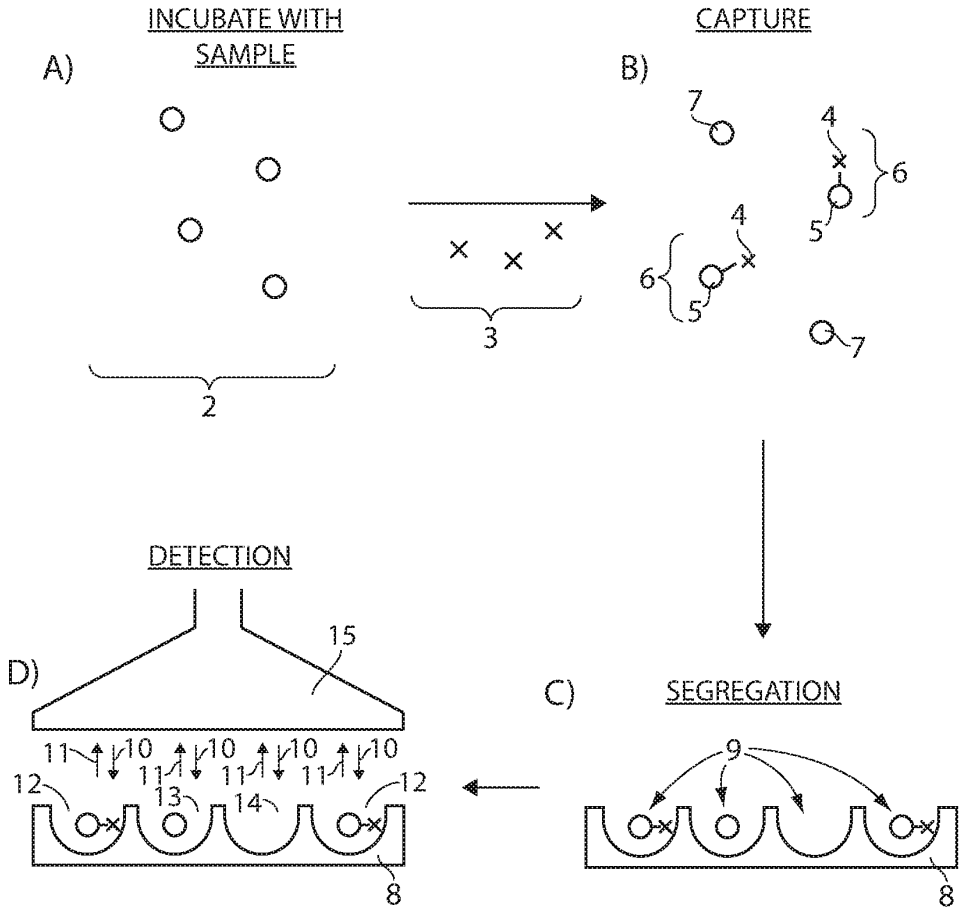

INCUBATE WITH SAMPLE
A)
2
3
CAPTURE
B)
7
4
6
5
4
6
5
7
C)
SEGREGATION
9
8
DETECTION
15
D)
11
10
12
13
14
8

Fig. 1A

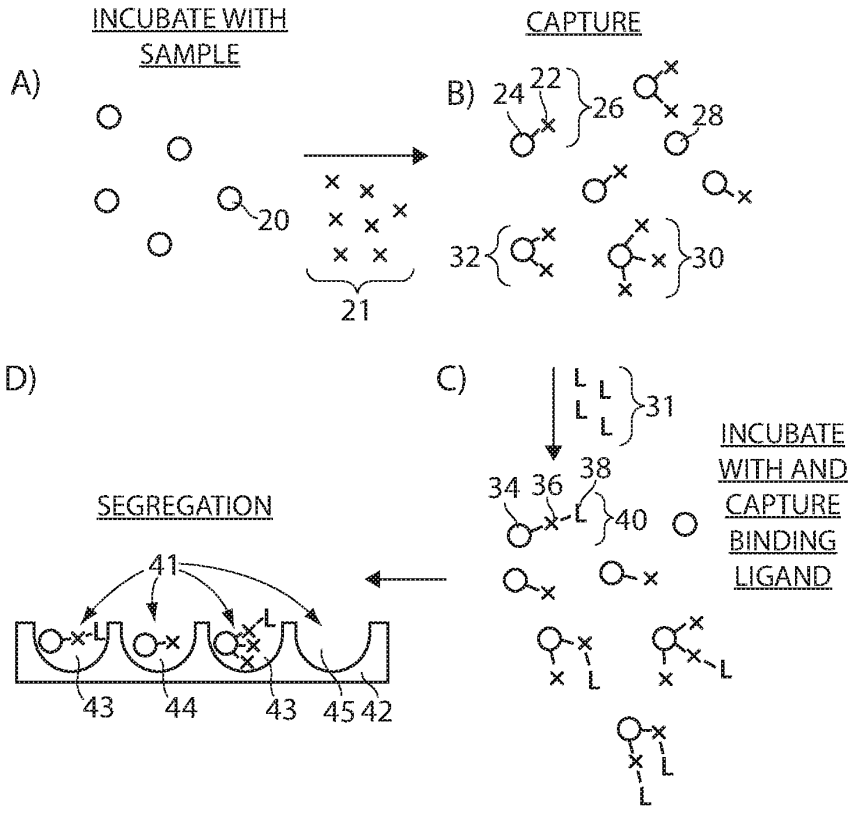

INCUBATE WITH SAMPLE
CAPTURE
A)
B)
20
21
22
24
26
28
30
32
C)
31
INCUBATE WITH AND CAPTURE BINDING LIGAND
34
36
38
40
D)
SEGREGATION
41
43
44
43
45
42
L

Fig. 1B

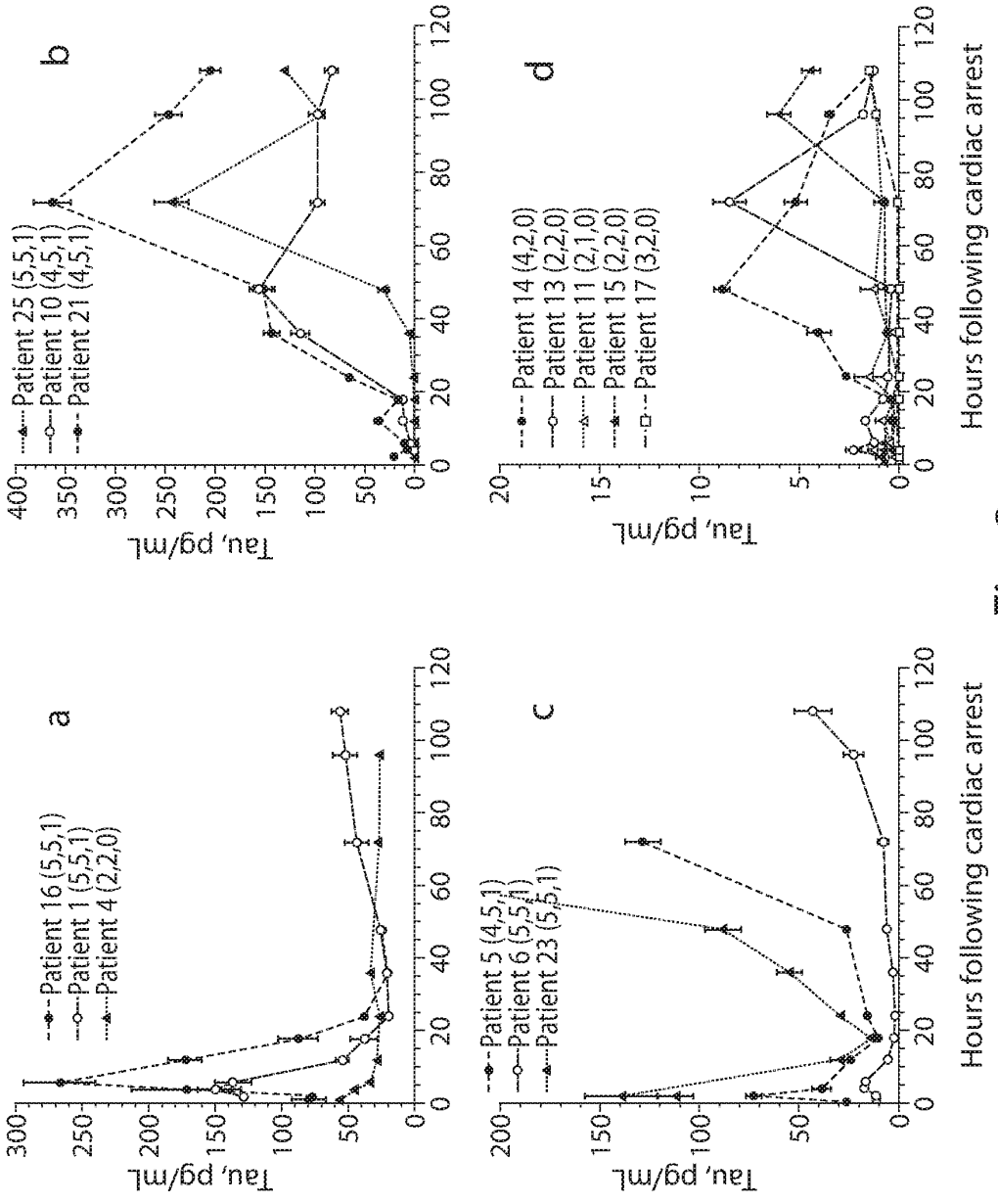

a
Patient 16 (5,5,1)
Patient 1 (5,5,1)
Patient 4 (2,2,0)
Tau, pg/mL
b
Patient 25 (5,5,1)
Patient 10 (4,5,1)
Patient 21 (4,5,1)
Tau, pg/mL
c
Patient 5 (4,5,1)
Patient 6 (5,5,1)
Patient 23 (5,5,1)
Tau, pg/mL
Hours following cardiac arrest
d
Patient 14 (4,2,0)
Patient 13 (2,2,0)
Patient 11 (2,1,0)
Patient 15 (2,2,0)
Patient 17 (3,2,0)
Tau, pg/mL
Hours following cardiac arrest

Fig. 2

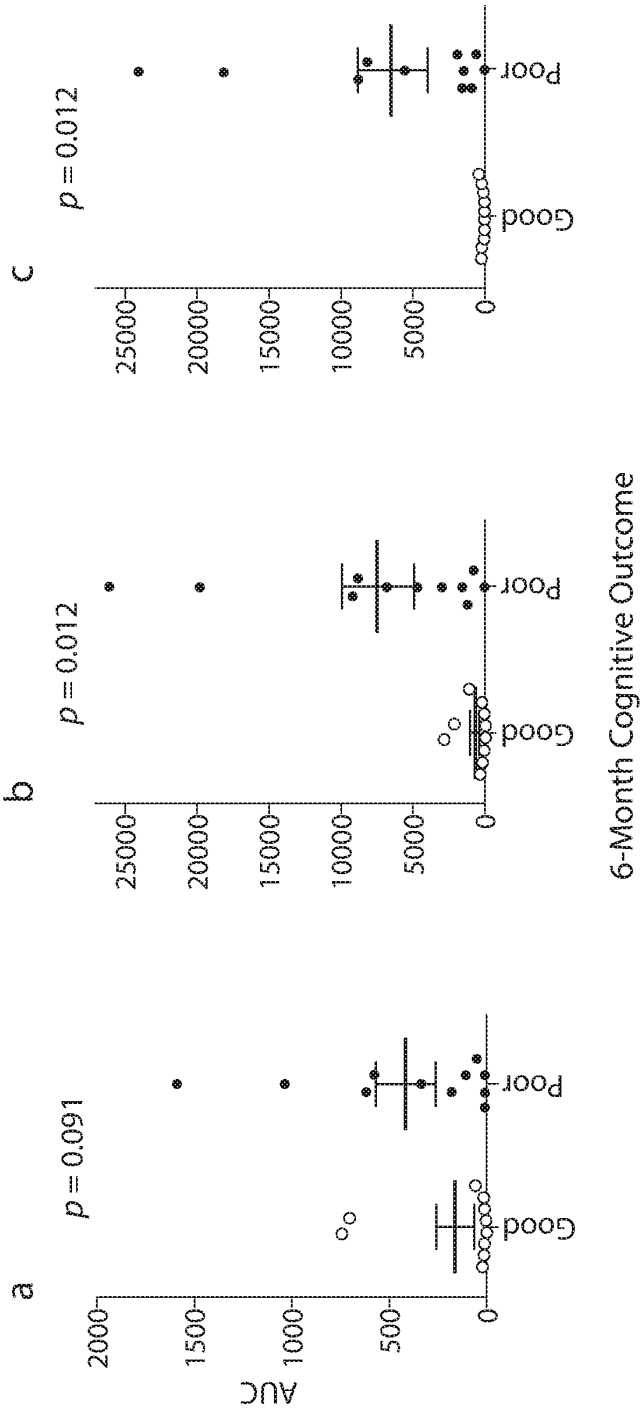

a
b
c
p = 0.091
p = 0.012
p = 0.012
AUC
2000
1500
1000
500
0
25000
20000
15000
10000
5000
Good
Poor
6-Month Cognitive Outcome

Fig. 3

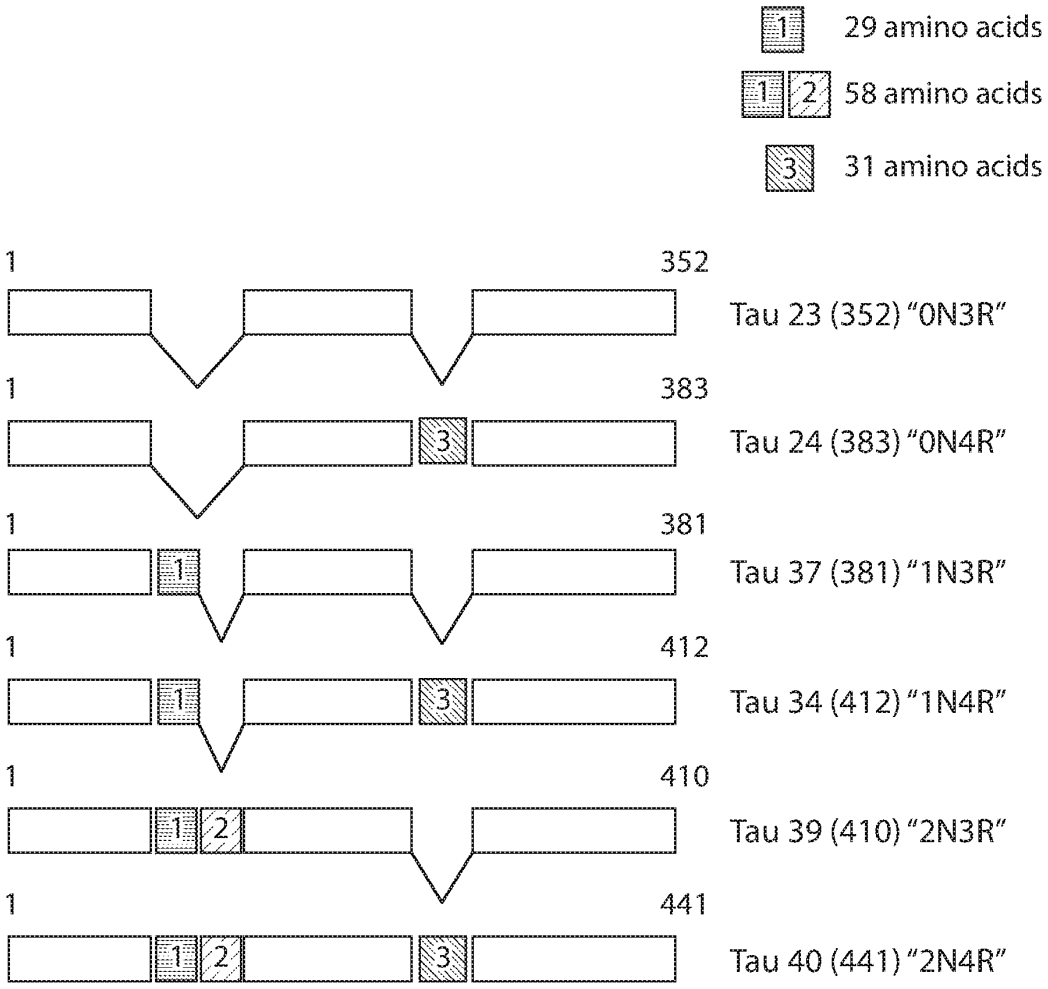

1 29 amino acids
1 2 58 amino acids
3 31 amino acids
1
352
Tau 23 (352) "0N3R"
1
383
3
Tau 24 (383) "0N4R"
1
381
1
Tau 37 (381) "1N3R"
1
412
1
3
Tau 34 (412) "1N4R"
1
410
1 2
Tau 39 (410) "2N3R"
1
441
1 2
3
Tau 40 (441) "2N4R"

Fig. 4

METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

Related Applications

This application is a continuation of U.S. Patent Application Serial No. 14/111,326, filed June 24, 2014, and entitled "METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY," which is a national stage of International Patent Application Serial No. PCT/US2012/033343, filed April 12, 2012, and entitled "METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY," which claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application Serial No. 61/474,315, filed April 12, 2011, and entitled "METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY RESULTING FROM A HYPOXIC EVENT," and claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application Serial No. 61/524,693, filed August 17, 2011, and entitled "METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY," each of which are incorporated herein by reference in their entireties for all purposes.

Field of the Invention

The present invention generally relates, in some embodiments, to methods of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury. In some embodiments, the brain injury results from a hypoxic event. In some embodiments, methods are provided for determining a measure of the concentration of tau protein in a patient sample containing or suspected of containing tau protein.

Background of the Invention

A brain injury in a human may be caused by any number of events or conditions. In some cases, a brain injury may be caused by external mechanical force, such as rapid acceleration or deceleration, impact, blast waves, or penetration by a projectile. This type of acquired brain injury is generally known as traumatic brain

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injury. Another type of acquired brain injury involves biochemical forces, such as oxygen deprivation (hypoxia). Hypoxia generally refers to a deficiency in the amount of oxygen reaching body tissues or a condition of insufficient levels of oxygen in tissue or blood. Oxygen deprivation to the brain results in neuronal damage and death, which is in turn related to the extent of long term brain dysfunction. The concentration of certain biomarkers may become elevated as a result of neuronal damage and death. For example, tau proteins are associated with microtubules and localized in the axonal compartment of neurons. Tau is known to be elevated in the cerebrospinal fluid (CSF) of patients with neurodegenerative disease and head injuries. However, since such biomarkers must diffuse across the blood brain barrier, they may be present in the blood in proportion in extremely low concentrations that are not reliably measurable by typical conventional immunoassays. While the concentration of some biomarkers in the brain and central nervous system are known to increase with hypoxic events, the increased concentration has not been correlated with specific diagnostic indications and/or methods of treatment. In addition, while some methods exist for determining a brain injury in a patient and/or determining a course of treatment following a brain injury, many of the known methods are costly (e.g., magnetic resonance imaging) and/or provide unclear results and/or predictors. Accordingly, improved methods are needed.

Summary of the Invention

In some embodiments, a method for determining a measure of the concentration of tau protein in a patient sample containing or suspected of containing tau protein is provided comprising performing an assay to determine a measure of the concentration of tau protein in the sample, wherein the limit of detection of tau protein of the assay is less than about 0.2 pg/mL.

In some embodiments, a method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury is provided comprising performing an assay on a blood sample from the patient and/or plasma and/or serum derived from the blood sample to determine a measure of the concentration of tau protein in the sample; and determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measured concentration of tau protein present in the sample.

In some embodiments, a method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury is provided comprising determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on a measured concentration of tau protein present in a patient sample, wherein the measured concentration has been determined by performing an assay on the patient sample, which comprises a blood sample from the patient and/or plasma and/or serum derived from the blood sample, to determine the measure of the concentration of tau protein in the sample.

In some embodiments, a method for performing an assay and providing data for determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury is provided comprising performing an assay on a blood sample from the patient and/or plasma and/or serum derived from the blood sample to determine a measure of the concentration of tau protein in the sample; and providing data from the assay to enable determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measured concentration of tau protein present in the sample.

In some embodiments, a method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury is provided comprising determining a measure of the concentration of tau protein in each of a plurality of samples obtained from the patient following the brain injury; and determining a prognostic of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measured concentration tau protein present in the sample.

In some embodiments, a method of determining a method of treatment for and/or a prognosis of a patient's recovery from a brain injury is provided comprising (a) performing an assay on each of a plurality of samples obtained from the patient following the brain injury to determine the measured concentration of tau protein in each of the samples, wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours; (b) determining the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time, wherein the area is determined for the entire time period and/or for a second peak in the tau protein concentration; and (c) determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on

the area under the curve for the entire time period and/or the second peak in the tau protein concentration determined in step (b).

In some embodiments, a method of determining a method of treatment for and/or a prognosis of a patient's recovery from a brain injury is provided comprising determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time, wherein the area is determined for the entire time period and/or for a second peak in the tau protein concentration, which has been determined by (a) performing an assay on each of a plurality of samples obtained from the patient following the brain injury to determine the measured concentration of tau protein in each of the samples, wherein the plurality of samples have been obtained from the patient over a period of time of at least about 48 hours; and (b) determining the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time for the entire time period and/or for a second peak in the tau protein.

In some embodiments, a method for performing an assay and providing data for determining a method of treatment for and/or a prognosis of a patient's recovery from a brain injury is provided comprising (a) performing an assay on each of a plurality of samples obtained from the patient following the brain injury to determine the measured concentration of tau protein in each of the samples, wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours; (b) determining the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time, wherein the area under the curve is determined for the entire time period and/or for a second peak in the tau protein concentration; and (c) providing data derived in steps (a) and (b) to enable determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the area under the curve determined for the entire time period and/or for a second peak in the tau protein concentration.

Brief Description of the Drawings

Figure 1a is a schematic flow diagram depicting one embodiment of steps (A-D) for performing an exemplary method of the present invention;

Figure 1b is a schematic flow diagram depicting one embodiment of steps (A-D) for performing an exemplary method of the present invention;

Figure 2 show plots of serum tau concentrations for numerous patients following resuscitation from cardiac arrest having a-c) a poor outcome and d) a good outcome;

Figure 3 shows plots of receiver operating characteristics (ROC) curves and areas under the curve values of tau protein concentration versus time for a) the first 24 hours, b) all serial samplings, and c) the secondary tau peak only;

Figure 4 illustrates six naturally occurring isoforms of tau proteins.

Other aspects, embodiments, and features of the invention will become apparent from the following detailed description when considered in conjunction with the accompanying drawings. The accompanying figures are schematic and are not intended to be drawn to scale. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. All patent applications and patents incorporated herein by reference are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Detailed Description

The present invention generally relates to methods of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury. In some embodiments, the brain injury may result from a hypoxic event. In some embodiments, methods are provided for determining a measure of the concentration of tau protein in a patient sample containing or suspected of containing tau protein. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In some embodiments, a method of the present invention comprises determining a measure of the concentration of at least one biomarker in one or more samples obtained from a patient following a brain injury. In some embodiments, a method of the present invention comprises determining a measure of the concentration of at least one biomarker in one or more samples obtained from a patient following the brain injury (e.g., optionally resulting from a hypoxic event). A prognostic indication of the patient's recovery and/or determining a course of treatment (e.g., from the brain injury, optionally resulting from a hypoxic event) may be based at least in part on the measure of the concentration of the at least one biomarker present in the one or more samples. It should be understood, that while much of the discussion below is directed to methods involving the analysis of more than one sample, this is by way of example only, and similar methods may be employed wherein only a single sample is employed.

As will be known to those of ordinary skill in the art, the term "hypoxia" generally refers to a deficiency in the amount of oxygen reaching body tissues or a condition of insufficient levels of oxygen in tissue or blood. Hypoxia at a cellular level develops when delivery of oxygen to cell mitochondria slows as the partial pressure gradient from capillaries to tissues decreases. As the delivery of oxygen decreases, aerobic metabolism stops and less efficient anaerobic pathways of glycolysis become responsible for the production of cellular energy. The end result is an increase in cellular concentrations of sodium, calcium, and hydrogen ions which may lead to cell death.

Oxygen deprivation to the brain results in neuronal damage and death. The extent of neuronal damage and death in turn relates to the extent of long term brain dysfunction, as can be assessed using standard criteria (such as Cerebral Performance

Category, CPC rating, or like criteria). Severe hypoxia can result in a patient's death and/or an irreversible brain injury (e.g., resulting in the patient being in a vegetative state). Hypoxic events may be global (e.g., due to low oxygen content in the blood) or focused (e.g., affecting only an area of the brain). Causes of hypoxia include, but are not limited to, local asphyxia (e.g., caused by smoke inhalation), carbon monoxide poisoning and/or toxicity, cardiac arrest, choking, drowning, high altitudes, strangulation, an ischemic event, thrombosis, arterial embolism, hemorrhage, swelling of the brain, stroke, physical trauma and/or physical injury (e.g., blunt trauma to the head), arteriosclerosis, and/or atherosclerosis. In some cases, the event may be myocardial infarction, myocardial ischemia, and/or transient ischemic attack.

Hypoxic conditions can lead to the production and/or change in the concentration of certain biomarkers. That is, the concentration of certain biomarkers increase or decrease following the hypoxic event. For example, the production of the proteolytic products of β-amyloid precursor protein has been found to become elevated in the brain and central nervous system under hypoxic condition. The increased concentration is theorized to be due to a hypoxia-inducible factor (HIF-1) that promotes the production of beta-amyloid peptides from amyloid precursor protein, a membrane protein concentrated in neuronal synapses. A cascade of biomarkers, such as tau proteins, is generated in the brain in proportion to the extent of hypoxia. Such biomarkers could in turn diffuse across the blood brain barrier and into the blood in proportion to the extent of hypoxia, and may be generally found in low abundance. The ability to determine a change in the concentration of a biomarker in a plurality of samples (or a single sample) obtained from a patient following a hypoxic event can, in some embodiments, be correlated with a prognostic indication of the patient's recovery from a brain injury and/or used to determine a method of treatment. In some cases, sample(s) of the patient's cerebrospinal fluid (CSF) may be obtained and analyzed to determine the concentration and/or a change in the concentration of the biomarker. In some cases, however, it is advantageous to determine the level of a biomarker in the blood of a patient as compared to CSF, as blood sampling is generally less invasive and may result in fewer complications as compared to CSF sampling. However, many of the biomarkers that are present in the CSF have a slow rate of transmission across and/or a high barrier of transportation across the blood-brain barrier (BBB) and thus, the concentration of the biomarker in

the patient's blood may be sufficient lowered as compared to the concentration in CSF to make it difficult or impossible to accurately determine using typically employed conventional immunoassays. Accordingly, assay methods which have very low limits of quantification (LOQ) and/or limits of detection (LOD) are generally necessary to determine a measure of the concentration of a biomarker in the patient's blood to provide statistically significant and/or meaningful results. In some embodiments, the methods of the present invention make use of methods having very low LODs and/or LOQs (e.g., in the low pg/mL range) to determine a measure of the concentration of a biomarker in a sample(s) obtained from a patient following a hypoxic event. Various parameters related to the changes in the concentration of the biomarker in the samples (e.g., blood samples) may be correlated with a prognostic indication and/or a method of treatment following a hypoxic event. Correlations (e.g., between the concentration and prognostic indication(s) and/or between the concentration and method(s) of treatment) have been discovered and/or are now discoverable due to recent advancements in technology which allow for the determination of the low concentrations of biomarkers in bodily fluids with sufficient accuracy and precision, thus allowing for the variations in concentration to be statistically significant and therefore, diagnostic.

It should be noted, that while many of the embodiments described herein focus on brain injuries caused by hypoxic events, this is by no way limiting, and in some embodiments, the brain injury may be caused by other events, for example, traumatic brain injuries wherein the force is such that the skull fractures causing mechanical damage to the brain. In some cases, a traumatic brain injury may be caused by external mechanical force, such as rapid acceleration or deceleration, impact, blast waves, or penetration by a projectile.

In embodiments where a plurality of samples are obtained from a patient, the samples may be obtained from a patient over any suitable period of time. Generally, the period of time may be selected such that the concentration of a biomarker in the samples becomes statistically significant and/or a trend is observable (e.g., an increase and/or decrease in the concentration). For biomarkers which are analyzed in blood samples, the period of time over which a plurality of samples are obtained from the patient may account for any lag time required for the biomarker to cross the BBB. Non-limiting examples of suitable periods of time in which the samples may be obtained from the patient include 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 8 hours,

10 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 4 days, 5 days, 6 days, 7 days, or more. In some cases, the duration of time of sample collection time is at least 60 hours, or at least 72 hours. In some cases, the duration of time of sample collection is between 12 hours and 7 days, or between 24 hours and 4 days, or between 2 days and 4 days, or between 3 days and 4 days. The first sample may be obtained from the patient without a short timeframe following the brain injury. For example, the first sample may be obtained from the patient within 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, 10 hours, or 12 hours of the brain injury. In some cases, the first sample is obtained within 6 hours of the brain injury. In some embodiments, a first sample is obtained from the patient within 6 hours of the suspected brain injury, and at about 1, about 2, about 6, about 12, about 24, about 48, and about 72 hours, following the first sampling. In some embodiments, additional samples are obtained at about 96 and/or at about 108 hours following the first sampling.

Any number of samples (e.g., one or more) may be obtained from the patient over the time period of sample collection. Generally, the minimum number of samples obtained is such that a trend (e.g., an increase or decrease) in the concentration of the biomarker is observable. Non-limiting examples of the number of samples that are obtained from the patient (e.g., during the prescribed collection time) is at least about bout 1, about 2, at least about 3, at least about 4, at least abut 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 12, at least about 15 or more. In some cases, the number of samples obtained from the patient is between 2 and 20, between 5 and 15, or between 5 and 10.

The sample(s) obtained from the patient may be from any suitable bodily source. In some cases, the samples are CSF fluid samples. In some cases, the samples are not CSF fluid samples. In some cases, the samples are blood or blood products (e.g., whole blood, plasma, serum, etc.). In other cases, the samples may be urine or saliva samples. In some embodiments, the samples may be analyzed directly (e.g., without the need for extraction of the biomarker from the fluid sample) and/or with dilution (e.g., addition of a buffer or agent to the sample). Generally, each of the samples obtained from the patient is collected using substantially similar procedures (e.g., to ensure minimal variation between samples based on sample collection

methods). Those of ordinary skill in the art will be aware of suitable systems and methods for obtaining a sample from a patient.

Each of or substantially all of the samples may be analyzed using an assay method (e.g., as described herein) to determine a measure of the concentration of at least one biomarker in each, a subset of or substantially all of the samples. In some cases, the methods comprise determining a measure of the concentration of a single biomarker in the samples. In other cases, a method comprises determining a measure of the concentration of more than one biomarker in each, a subset of or substantially all of the samples. For example, a measure of the concentration of 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, biomarkers may be determined in the samples.

In some embodiments, the methods of the present invention comprise determining a prognostic indication based at least in part on the measured concentration of the at least one biomarker in the samples. In some cases, the prognostic indication may be correlated with standard criteria employed to define long-term brain dysfunction and/or injury. Those of ordinary skill in the art will be aware of such criteria, for example, cerebral performance category ratings ("CPC rating"), or more specifically, Glasgow-Pittsburgh cerebral performance category ratings (or scale) (e.g., see Teasdale G, Jennett B (1974); *Assessment of coma and impaired consciousness*; Lancet 2 (7872): 81–84). The CPC scale ranges from 1 to 5, with 1 representing a slight possibility of neurological deficit and 5 representing severe deficit and/or death. In some methods of the present invention, the prognostic indication of the patient's recovery from the brain injury is classified as either "good" (e.g., correlating to a CPC score of 1 or 2) corresponding to a high likelihood of recovery and/or returning to independent living, or "poor" (e.g., correlating to a CPC score of 3, 4, or 5) corresponding to little possibility of a full recovery and resulting in assisted living and/or death.

In the CPC scale, a rating of 1 is generally classified as good cerebral performance. The patient is conscious and alert, is able to work, but may have mild neurological or psychological deficit. A rating of 2 is generally classified as having moderate cerebral disability. The patient is conscious and has sufficient cerebral function for independent activities of daily life, and is generally able to work in sheltered environment. A rating of 3 corresponds to severe cerebral disability. While the patient is conscious, they generally depend on others for daily support because of impaired brain function. The patient may have abilities ranging from ambulatory

state to severe dementia or paralysis. A rating of 4 corresponds to a coma or vegetative state. The patient is generally unaware, even if they appear awake (e.g., the patient is in a vegetative state) without interaction with environment and is cerebral unresponsive. A rating of 5 refers to brain death, associated with apnea, areflexia, and/or EEG silence. The CPC scale is summarized in Table 1.

Table 1: CPC Scale Summary

CPC Score	Description
1	Conscious and alert with normal function or only slight disability
2	Conscious and alert with moderate disability
3	Conscious with severe disability
4	Comatose or persistent vegetative state
5	Brain dead or death from other causes

Other non-limiting examples of suitable criteria include the "scale g" criteria (e.g., see Dekaban AS, Robinson CE.; Application of a new rating scale of brain dysfunction to monitoring rehabilitation in 65 patients with severe head injury, Bull Clin Neurosci., 1984; 49, 82-92), the "Rancho Los Amigos Scale," and the "Disabilities Rating Scale."

In addition to the biomarkers specifically mentioned herein, those of ordinary skill will be aware of other suitable biomarkers to use in connection with the methods described herein. As described herein, the biomarker generally undergoes a change in concentration as a result of a hypoxic event. For example, the concentration of the biomarker may increase or decrease as a result of the brain injury. Non-limiting examples of biomarkers include neuron specific neuronal enolase (NSE), β-site aPP-cleaving enzyme 1 (BACe1), S100B, myelin basic protein (MBP), growth associated protein 43, glutamine synthetase, glial fibrillary acid protein (GFAP), glycine transporter (e.g., GLYT1, GLYT2), neuron specific glycoprotein (e.g., GP50), calpain, neurofibrillary protein, heat shock protein 72, beta-amyloid precursor proteins, calbindin D-28K, proteolipid protein, myeline associated glycoprotein, neurofilament H, creatine kinase protein (e.g., CK-BB), tau proteins (including phosphorylated taus such as p-tau-81 or p-tau-231), and endothelium membrane proteins (e.g., thrombomodulin).

In some cases, the biomarker is a tau protein. Various forms and/or combinations of tau proteins may be contemplated for use as a target biomarker with

the methods described herein, include isoforms and short isoforms, for example, ranging from tau 23 (352a.a, "0N3R", wherein R indicates the number of repeats and N indicates the number or amino terminal inserts, as will be understood by those of ordinary skill in the art) to tau 40 (441a.a,"2N4R"). There are six known naturally-occurring tau proteins, the sequences of which are well known in the art. The six tau proteins include tau 23 (352, 0N3R), tau 24 (383, 0N4R), tau 37 (381, 1N3R), tau 34 (412, 1N4R), tau 39 (410, 2N3R), tau 40 (441, 2N4R), and/or combinations thereof (e.g., see FIG. 4). In some cases, at least some of the tau proteins may be phosphorylated.

Those of ordinary skill in the art will understand that determination of a biomarker in a sample may comprise determining the concentration a single isoform of a biomarker, or alternatively, may comprise determining the concentration of a plurality of isoforms of the biomarker. For example, with respect to tau proteins, in some cases, the concentration of tau protein employed in the algorithms and methods described herein may be the concentration of a single isoform of tau protein in the sample, or alternatively, the concentration of tau protein employed in the algorithms and methods described herein may be the concentration of a plurality of forms of tau proteins in the sample.

The plurality of samples obtained from the patient may be analyzed (e.g., using an assay method as described herein) to determine a measure of the concentration of the at least one biomarker in each of the samples (or a single sample). A prognostic indication and/or a method of treatment may be determined based at least in part on the measure of the concentration of the biomarker in each of the plurality of samples. The data may be analyzed using a variety of techniques, as described herein, and a prognostic indication for recovery from a brain injury (e.g., a "good" outcome or a "poor" outcome), or a specific method of treatment may be determined based on the results.

In some embodiments, the measure of the concentration of the at least one biomarker for each of the plurality of samples obtained from the patient may be plotted on a graph of concentration versus time (e.g., in hours), and one or more parameters can be obtained from the graph and used to determine the method of treatment and/or the prognostic indication. Non-limiting examples of parameters that may be determined and/or employed include baseline biomarker concentration, increase in biomarker concentration, duration of rise of biomarker concentration,

maximum slope of increasing biomarker concentration, rate of change of biomarker concentration, area under the curve and/or magnitude of the fold increase of biomarker concentration. Each of the parameters and their determination will now be described in detail, followed by a description of possible data analysis and methods useful or potentially useful to determine suitable correlations between the measure of the concentration determined in the samples and a treatment/prognostic indication. The measure of the concentration of the biomarker may be determined/displayed in any suitable unit. In some cases, the measure of the concentration is determined/displayed in pg/mL.

The term "baseline biomarker concentration" refers to the concentration of the biomarker generally present in a fluid sample from a normal patient (e.g., prior to or unexposed to a hypoxic event). The baseline biomarker concentration can be determined using a variety of methods which will be commonly known and understood by those of ordinary skill in the art. In some cases, the baseline biomarker concentration can be determined by averaging the value of a biomarker present in a population of control patients (e.g., patients who have not experienced a hypoxic event). This may be useful in embodiments where the baseline concentration of the biomarker is substantially the same for a given population of individuals (e.g., based on age, gender, medical condition, etc.). However, many of the methods described herein require accurate determination of extremely low concentrations of the at least one biomarker in samples obtained from a patient, and accordingly, a general baseline concentration of a biomarker (e.g., based on a sampling of a population) may not provide enough accuracy and/or precision to give useful results. Accordingly, a measure of the baseline biomarker concentration, in some embodiments, may be determined for each individual patient. In some embodiments, the baseline biomarker concentration may be set to zero, e.g., in embodiments where the concentration of the biomarker is zero or essentially zero in normal patients.

In some cases, the baseline biomarker concentration for each individual is equal to the measure of the concentration of the biomarker in the first sample obtained from the patient following the hypoxic event. In other cases, if available, a baseline biomarker concentration is the measure of the concentration of the biomarker present in a sample obtained from the patient prior to the hypoxic event. In yet other cases, the baseline biomarker concentration is the average concentration of the biomarker determined in a plurality of samples taken from the patient immediately or

substantially immediately following the hypoxic event (e.g., prior to the concentration of the biomarker increasing due to the hypoxic event). That is, the concentration of the biomarker in the plurality of samples obtained from the patient in a selected time period following the hypoxic event may be averaged to determine the baseline biomarker concentration for that patient. As will be understood by those of ordinary skill in the art, in such cases, any sample which has a concentration level that significantly differs from the other concentration levels during that period of time (e.g., a data point/outlier having a concentration which deviates significantly from the other concentrations measured during the time frame) may be excluded from the averaging calculation. In some cases, if a sample has a concentration which differs by greater than about 50% from the calculated average, that sample may be excluded from the averaging calculation. In some cases, if a sample has a concentration which differs by more than about 0.5 pg/mL, about 1 pg/mL, about 2 pg/mL, about 5 pg/mL, or about 10 pg/mL from the calculated average, that sample may be excluded from the averaging calculation. An anomalous data point may be observed and/or caused by administration of drugs to the patient. The baseline biomarker concentration may be determined by averaging the concentration of the biomarker in the samples obtained from the patient between the first sample (e.g., obtained within 6 hours of the hypoxic event) and 24 hours following the first collection of a sample, or between the first sample and 18 hours following the first collection of a sample, or between the first sample, and 12 hours following the first collection of a sample, or between the first sample, and 6 hours following the first collection of a sample (e.g., not including any outliers).

The term "area under the curve," (or AUC) is a common parameter used to analyze data, and such calculations will be well known and understood by those of ordinary skill in the art. In context with the methods of this disclosure, the AUC refers to the area under the biomarker concentration versus time curve. Generally the calculation takes into account the baseline biomarker concentration. Those of ordinary skill it the art will be aware of suitable algorithms and/or computer programs capable of determining the area under the curve for a selected set of data. In some cases, the area under the curve may be determined for more than one portion of the data. For example, a first area under the curve value may be determined for a first range of data, and a second area under the curve value may be determined for a second range of data. The first and the second ranges of data may or may not be

overlapping (e.g., may comprise some overlapping data points or may comprise different data points). It should be understood, that determining the area under the curve can be accomplished using a variety of techniques which will be known to those of ordinary skill in the art, including but not limited to, plotting the concentration versus time on a graph and determining the area under the line/curve (e.g., optionally with use of a computer program) and/or determining a functional relationship between the concentration and time and integrating the data without requiring physically plotting or drawing of a graph. As used herein, the phrase "determining the area under the curve of a graph of a biomarker concentration versus time" covers all of the above techniques and others applicable for determining the area or equivalent, and is not limited to physically or electronically plotting the concentration versus time on a graph and determining the area under the line/curve.

The term "change in biomarker concentration" refers to the change (e.g., increase or decrease) in concentration of the biomarker over the time period in which the samples are collected. An increase in biomarker concentration may be calculated by subtracting the baseline biomarker concentration (e.g., as described herein) from the maximum biomarker concentration. The "maximum biomarker concentration" is the maximum measured concentration of the biomarker measured in a single sample collected over the duration of the sample collection. For example, if the duration of the sample collection is 72 hours, the maximum concentration is equal to the maximum measured concentration in a single sample which was obtained from the patient in the 72 hour period. A decrease in biomarker concentration may be calculated by subtracting an elevated biomarker concentration (e.g., as described herein) from the minimum
biomarker concentration.

The term "magnitude of the fold increase of biomarker concentration" refers to the magnitude of the fold-increase in concentration over the duration of sample collection, and may be calculated by dividing the maximum biomarker concentration (e.g., as described herein) by the baseline biomarker concentration (e.g., as described herein).

The term "duration of rise of biomarker concentration" refers to the time over which the concentration of the biomarker is increasing during the time period over which the samples are collected. In some cases, the duration of rise of biomarker concentration can be determined by subtracting the time at which the last sample was

collected at approximately the baseline biomarker concentration (e.g., starting time of the rise) from the time at which the maximum biomarker concentration was observed (e.g., ending time of the rise). That is, the duration of the rise may be equal to the ending time of the rise minus the starting time of the rise. The duration of rise is generally provided in hours.

The term "maximum slope of increasing biomarker concentration" refers to the slope at which the maximum increase in concentration occurred over the time period in which the samples were collected. Those of ordinary skill will be aware of methods to calculate this value using common graphical analysis methods. For example, a plot may be prepared showing concentration versus time, and the maximum slope may be calculated based on this plot. In some cases, the maximum slope is equal to the slope between two data points, whereas in other cases, the maximum slope may be determined based on the average slope between a plurality of data points.

The above parameters, alone or in combination, may be correlated to a prognostic indication and/or a method of treatment for a patient following a brain injury (e.g., caused by a hypoxic event). Following determination of a correlation between biomarker concentrations and a prognostic indication and/or a method of treatment (e.g., using a plurality of samples obtained from a plurality of test patients with known outcomes), the correlation can be used in connection with methods to determine prognosis (e.g., prognostic indications) and/or methods of treatment for patients with unknown outcomes. That is, an algorithm can be developed relating changes in a biomarker concentration and specific methods of treatment and/or prognostic indications using samples from test patients having been subject to known methods of treatment and/or having a known prognosis. Once the algorithm has been developed, it can be used to determine methods of treatment and/or prognostic indications for patients with unknown outcomes.

To determine a correlation between a biomarker and a prognostic indication and/or a method of treatment (e.g., to develop an algorithm relating the measured biomarker concentration to preferred methods of treatment and/or prognostic indications), a plurality of samples from a plurality of test patients may be obtained. A "test patient" is a patient who has a known outcome (e.g., a good or a poor CPC score) and/or has received a certain treatment. The plurality of the samples obtained from each of the test patients may be analyzed to determine the measure of the

concentration of at least one biomarker in the each of the samples. Some or all of the parameters described herein may be determined for each patient, and the data may be analyzed to determine correlations between the parameters and the patient outcomes and/or treatments, which can in turn be used to develop an algorithm. The algorithm may then be applied to the concentration of the at least one biomarker in samples obtained from a patient having an unknown outcome to determine a method of treatment and/or a prognostic indication for that patient. A specific example of such an analysis is described below, and further details are provided in Example 1.

Similar analysis and methods may be used to correlate suitable methods of treatment based upon the measured concentration(s) of a biomarker in a plurality of samples obtained from a patient. In some cases, the method of treatment may comprise administering at least one therapeutic agent to the patient. For example, the therapeutic agent may be a neuroprotective drug. Other non-limiting methods of treatment include administration of anti-oxidants, hypothermia, blood thinning, and administration of steroids (e.g., to help reduce brain swelling) (e.g., see T. S. Richmond, Cerebral Resuscitation After Global Brain Ischemia: Linking Research to Practice, American Association of Critical-Care Nurses Journal, May 1997, Volume 8, Number 2).

A therapeutic agent is generally administered in an amount effective to provide a medically desirable result. The effective amount will vary with the particular condition being treated, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration and the like factors within the knowledge and expertise of the health care practitioner. In some cases, a therapeutic agent may reduce brain injuries resulting from the hypoxic event. In some cases, the method of treatment may involve a change in treatment, such as an increase or decrease in the dose of a therapeutic agent, a switch from one therapeutic agent to another therapeutic agent, an addition of another therapeutic agent to the existing therapeutic agent, or a combination thereof. A switch from one therapeutic agent to another may involve a switch to a therapeutic agent with a high risk profile but where the likelihood of expected benefit is increased.

In one embodiment, a method of the present invention for determining a treatment protocol for and/or a prognostic indication of a patient's recovery from a brain injury (e.g., resulting from a hypoxic event) comprises performing an assay on a

plurality of samples to determine a measure of the concentration of tau protein in each sample and determining a prognostic indication of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measure of the concentration of tau protein present in the samples. The sample, in some embodiments, is a blood sample from the patient and/or plasma and/or serum derived from the blood sample. In some cases, the concentration of the tau protein in the samples is less than about or about 1000 pg/mL, less than about or about 900 pg/mL, less than about or about 800 pg/mL, less than about or about 700 pg/mL, less than about or about 600 pg/mL, less than about or about 500 pg/mL, less than about or about 400 pg/mL, less than about or about 300 pg/mL, less than about or about 200 pg/mL, less than about or about 100 pg/mL, less than about or about 50 pg/mL, less than about or about 30 pg/mL, less than about or about 20 pg/mL, less than about or about 10 pg/mL, less than about or about 5 pg/mL, less than about or about 1 pg/mL, or less. In some cases, the assay has a limit of quantification of less than about or about 100 pg/mL, less than about or about 50 pg/mL, less than about or about 40 pg/mL, less than about or about 30 pg/mL, less than about or about 20 pg/mL, less than about or about 10 pg/mL, less than about or about 5 pg/mL, less than about or about 4 pg/mL, less than about or about 3 pg/mL, less than about or about 2 pg/mL, less than about or about 1 pg/mL, less than about or about 0.8 pg/mL, less than about or about 0.7 pg/mL, less than about or about 0.6 pg/mL, less than about or about 0.5 pg/mL, less than about or about 0.4 pg/mL, less than about or about 0.3 pg/mL, less than about or about 0.2 pg/mL, less than about or about 0.1 pg/mL, less than about or about 0.05 pg/mL, less than about or about 0.04 pg/mL, less than about or about 0.02 pg/mL, less than about or about 0.01 pg/mL, or less. In some cases, the assay has a limit of detection of less than about or about 100 pg/mL, less than about or about 50 pg/mL, less than about or about 40 pg/mL, less than about or about 30 pg/mL, less than about or about 20 pg/mL, less than about or about 10 pg/mL, less than about or about 5 pg/mL, less than about or about 4 pg/mL, less than about or about 3 pg/mL, less than about or about 2 pg/mL, less than about or about 1 pg/mL, less than about or about 0.8 pg/mL, less than about or about 0.7 pg/mL, less than about or about 0.6 pg/mL, less than about or about 0.5 pg/mL, less than about or about 0.4 pg/mL, less than about or about 0.3 pg/mL, less than about or about 0.2 pg/mL, less than about or about 0.1 pg/mL, less than about or about 0.05 pg/mL, less than about or about 0.04

pg/mL, less than about or about 0.02 pg/mL, less than about or about 0.01 pg/mL, or less.

In one embodiment, a correlation was determined between certain parameters (e.g., area under the curve of a plot of tau protein concentration versus time) and a good (e.g., CPC rating 1 or 2) or a poor (e.g., CPC rating of 3, 4, or 5) prognostic indication. To determine the correlation, an assay was carried out on each of the samples obtained from a plurality of test patients having undergone a brain injury (e.g., resulting from a hypoxic event) (e.g., patients having a known outcome and/or having undergone a certain method of treatment following a brain injury). The first sample was taken within 6 hours of the brain injury (e.g., resulting from a hypoxic event), and additional samples were generally obtained at about 1, 2, 6, 12, 24, 48, and 72 hours following the first sample (and optionally, at 96 and/or 108 hours). Each of the test patients had a known prognostic outcome according to the CPC scale. For each test patient, a plot of the measured concentration of tau protein in pg/mL time in hours was prepared. The data was analyzed and the area under the curve of the tau protein concentration (in pg/mL) versus time (in hours) was determined for a variety of time ranges, and was determined to correlate with a "good" or "poor" prognosis for the patients. In some embodiments, the plot of the concentration of the tau protein versus time showed two peaks, one occurring mostly in the first 24 hours following the hypoxic event, and one occurring at some point following the first 24 hours following the hypoxic event. Thus, the area under the curve was determined for each patient for three ranges of time: 1) for the entire duration of the data collection, 2) for the first 24 hours, and 3) for the second peak in tau protein concentration (if present). Generally, the baseline used to determine the area under the curve for 1) and 2) was set to zero, whereas the baseline used to determine the area under the curve for 3) was set as the concentration of tau protein determined at the beginning of the rise of the second peak. For the total area under the curve, a value of greater than about 800 correlated with a poor prognosis (e.g., a CPC score of 3, 4, or 5) and a value of less than about 800 correlated with a good prognosis (e.g., a CPC score of 1 or 2). For the area under the curve of the second peak, a value of greater than about 500 correlated with a poor prognosis (e.g., a CPC score of 3, 4, or 5) and a value of less than about 500 correlated with a good prognosis (e.g., a CPC score of 1 or 2). Accordingly, a correlation/algorithm was established between the varying parameters relating to concentration and a prognostic indication.

The developed correlation/algorithm can be applied to patients with unknown outcomes. That is, samples may be obtained from a patient and the concentration of tau protein in each of the samples can be determined. The data may be analyzed to determine the total area under the curve and/or the area under the curve of the second tau protein peak. If the sum of the total area under the curve and/or the area under the curve of the second peak is greater than 800 or 500, respectively, the prognostic indication for that patient is "poor," and if the sum is less than 800 or 500, respectively, the prognostic indication is "good." Using similar techniques, a variety of correlations may be determined for this tau protein (e.g., increase in tau protein concentration, duration of the increase of tau protein concentration, and/or the magnitude of the fold increase of tau protein) and/or other biomarkers.

Exemplary Assay Methods and Systems

Those of ordinary skill in the art will be aware of a variety of assay methods and systems that may be used in connection with the methods of the present invention. Generally, the methods employed have low limits of detection and/or limits of quantification as compared to bulk analysis techniques (e.g., ELISA methods). The use of assay methods that have low limits of detection and/or limits of quantification allows for correlations to be made between the various parameters discussed above and a method of treatment and/or diagnostic indication that may otherwise not be determinable and/or apparent. For example, in the method described above which correlates the total area under the curve and/or the area under the curve of a second peak of tau protein concentration to a prognostic indication of brain injury, the limits of detection, and/or limits of quantification needs to be substantially lower than the LOD and/or LOQ provided by common ELISA techniques.

The terms "limit of detection" (or LOD) and "limit of quantification" (or LOQ) are given their ordinary meaning in the art. The LOD refers to the lowest analyte concentration likely to be reliably distinguished from background noise and at which detection is feasible. The LOD as used herein is defined as three standard deviations (SD) above background noise. The LOQ refers to the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. Generally, as is used herein, the LOQ refers to the lowest concentration above the LOD wherein the coefficient of variation (CV) of the measured concentrations less than about 20%.

In some cases, an assay method employed has a limit of detection and/or a limit of quantification of less than about or about 500 pg/mL, less than about or about 250 pg/mL, less than about or about 100 pg/mL, less than about or about 50 pg/mL, less than about or about 40 pg/mL, less than about or about 30 pg/mL, less than about or about 20 pg/mL, less than about or about 10 pg/mL, less than about or about 5 pg/mL, less than about or about 4 pg/mL, less than about or about 3 pg/mL, less than about or about 2 pg/mL, less than about or about 1 pg/mL, less than about or about 0.8 pg/mL, less than about or about 0.7 pg/mL, less than about or about 0.6 pg/mL, less than about or about 0.5 pg/mL, less than about or about 0.4 pg/mL, less than about or about 0.3 pg/mL, less than about or about 0.2 pg/mL, less than about or about 0.1 pg/mL, less than about or about 0.05 pg/mL, less than about or about 0.04 pg/mL, less than about or about 0.02 pg/mL, less than about or about 0.01 pg/mL, or less. In some cases, an assay method employed has a limit of quantification and/or a limit of detection between about 100 pg/mL and about 0.01 pg/mL, between about 50 pg/mL and about 0.02 pg/mL, between about 25 pg/mL and about 0.02 pg/mL, between about 10 pg/mL and about 0.02 pg/mL. As will be understood by those of ordinary skill the art, the LOQ and/or LOD may differ for each assay method and/or each biomarker determined with the same assay. In some embodiments, the LOD of an assay employed for detecting tau protein is about equal to or less than 0.02 pg/mL. In some embodiments, the LOQ for an assay employed for detecting tau protein is equal to or less than 0.04 pg/mL

In some embodiments, the concentration of biomarker molecules in the fluid sample that may be substantially accurately determined is less than about or about 5000 fM, less than about or about 3000 fM, less than about or about 2000 fM, less than about or about 1000 fM, less than about or about 500 fM, less than about or about 300 fM, less than about or about 200 fM, less than about or about 100 fM, less than about or about 50 fM, less than about or about 25 fM, less than about or about 10 fM, less than about or about 5 fM, less than about or about 2 fM, less than about or about 1 fM, less than about or about 0.5 fM, less than about or about 0.1 fM, or less. In some embodiments, the concentration of biomarker molecules in the fluid sample that may be substantially accurately determined is between about 5000 fM and about 0.1 fM, between about 3000 fM and about 0.1 fM, between about 1000 fM and about 0.1 fM, between about 1000 fM and about 1 fM, between about 100 fM and about 1 fM, between about 100 fM and about 0.1 fM, or the like. The concentration of

analyte molecules or particles in a fluid sample may be considered to be substantially accurately determined if the measured concentration of the biomarker molecules in the fluid sample is within about 10% of the actual (e.g., true) concentration of the biomarker molecules in the fluid sample. In certain embodiments, the measured concentration of the biomarker molecules in the fluid sample may be within about 5%, within about 4%, within about 3%, within about 2%, within about 1%, within about 0.5%, within about 0.4%, within about 0.3%, within about 0.2% or within about 0.1%, of the actual concentration of the biomarker molecules in the fluid sample. In some cases, the measure of the concentration determined differs from the true (e.g., actual) concentration by no greater than about 20%, no greater than about 15%, no greater than 10%, no greater than 5%, no greater than 4%, no greater than 3%, no greater than 2%, no greater than 1%, or no greater than 0.5%. The accuracy of the assay method may be determined, in some embodiments, by determining the concentration of biomarker molecules in a fluid sample of a known concentration using the selected assay method.

In some embodiments, an assay method employs a step of spatially segregating biomarker molecules into a plurality of locations to facilitate detection/quantification, such that each location comprises/contains either zero or one or more biomarker molecules. Additionally, in some embodiments, the locations may be configured in a manner such that each location can be individually addressed. In some embodiments, a measure of the concentration of biomarker molecules in a fluid sample may be determined by detecting biomarker molecules immobilized with respect to a binding surface having affinity for at least one type of biomarker molecule. In certain embodiments the binding surface may form (e.g., a surface of a well/reaction vessel on a substrate) or be contained within (e.g., a surface of a capture object, such as a bead, contained within a well) one of a plurality of locations (e.g., a plurality of wells/reaction vessels) on a substrate (e.g., plate, dish, chip, optical fiber end, etc.). At least a portion of the locations may be addressed and a measure indicative of the number/percentage/fraction of the locations containing at least one biomarker molecule may be made. In some cases, based upon the number/percentage/fraction, a measure of the concentration of biomarker molecules in the fluid sample may be determined. The measure of the concentration of biomarker molecules in the fluid sample may be determined by a digital analysis method/system optionally employing Poisson distribution adjustment and/or based at least in part on a

measured intensity of a signal, as will be known to those of ordinary skill in the art. In some cases, the assay methods and/or systems may be automated.

Certain methods and systems which employ spatially segregating analyte molecules (e.g., biomarkers) are known in the art, and are described in U.S. Patent Application Publication No. US-2007-0259448 (Serial No. 11/707,385), filed February 16, 2007, entitled "METHODS AND ARRAYS FOR TARGET ANALYTE DETECTION AND DETERMINATION OF TARGET ANALYTE CONCENTRATION IN SOLUTION," by Walt et al.; U.S. Patent Application Publication No. US-2007-0259385 (Serial No. 11/707,383), filed February 16, 2007, entitled "METHODS AND ARRAYS FOR DETECTING CELLS AND CELLULAR COMPONENTS IN SMALL DEFINED VOLUMES," by Walt et al.; U.S. Patent Application Publication No. US-2007-0259381 (Serial No. 11/707,384), filed February 16, 2007, entitled "METHODS AND ARRAYS FOR TARGET ANALYTE DETECTION AND DETERMINATION OF REACTION COMPONENTS THAT AFFECT A REACTION," by Walt et al.; International Patent Publication No. WO 2009/029073 (International Patent Application No. PCT/US2007/019184), filed August 30, 2007, entitled "METHODS OF DETERMINING THE CONCENTRATION OF AN ANALYTE IN SOLUTION," by Walt et al.; U.S. Patent Application Publication No. US-2010-0075862 (Serial No. 12/236484), filed September 23, 2008, entitled "HIGH SENSITIVITY DETERMINATION OF THE CONCENTRATION OF ANALYTE MOLECULES OR PARTICLES IN A FLUID SAMPLE," by Duffy et al.; U.S. Patent Application Publication No. US-2010-00754072 (Serial No. 12/236,486), filed September 23, 2008, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES ON SINGLE MOLECULE ARRAYS," by Duffy et al.; U.S. Patent Application Publication No. US-2010-0075439 (Serial No. 12/236488), filed September 23, 2008, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES BY CAPTURE-AND-RELEASE USING REDUCING AGENTS FOLLOWED BY QUANTIFICATION," by Duffy et al.; International Patent Publication No. WO2010/039179 (International Patent Application No. PCT/US2009/005248), filed September 22, 2009, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES OR ENZYMES," by Duffy et al.; U.S. Patent Application Publication No. US-2010-0075355 (Serial No. 12/236490), filed September 23, 2008, entitled "ULTRA-SENSITIVE DETECTION OF ENZYMES BY CAPTURE-AND-RELEASE FOLLOWED BY QUANTIFICATION," by Duffy

et al.; U.S. Patent Application Serial No. 12/731,130, filed March 24, 2010, published as US-2011-0212848 on September 1, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS," by Duffy et al.; International Patent Application No. PCT/US2011/026645, filed March 1, 2011, published as WO 2011/109364 on September 9, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS," by Duffy et al.; International Patent Application No. PCT/US2011/026657, filed March 1, 2011, published as WO 2011/109372 on September 9, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES USING DUAL DETECTION METHODS," by Duffy et al.; U.S. Patent Application Serial No. 12/731135, filed March 24, 2010, published as US-2011-0212462 on September 1, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES USING DUAL DETECTION METHODS," by Duffy et al.; International Patent Application No. PCT/US2011/026665, filed March 1, 2011, published as WO 2011/109379 on September 9, 2011, entitled "METHODS AND SYSTEMS FOR EXTENDING DYNAMIC RANGE IN ASSAYS FOR THE DETECTION OF MOLECULES OR PARTICLES," by Rissin et al.; U.S. Patent Application Serial No. 12/731136, filed March 24, 2010, published as US-2011-0212537 on September 1, 2011, entitled "METHODS AND SYSTEMS FOR EXTENDING DYNAMIC RANGE IN ASSAYS FOR THE DETECTION OF MOLECULES OR PARTICLES," by Duffy et al.; U.S. Patent Application Serial No. 13/035,472, filed February 25, 2011, entitled "SYSTEMS, DEVICES, AND METHODS FOR ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES," by Fournier et al.; U.S. Patent Application Serial No. 13/037,987, filed March 1, 2011, published as US-2011-0245097 on October 6, 2011, entitled "METHODS AND SYSTEMS FOR EXTENDING DYNAMIC RANGE IN ASSAYS FOR THE DETECTION OF MOLECULES OR PARTICLES," by Rissin et al.; each herein incorporated by reference.

Additional details of exemplary, non-limiting assay methods which comprise one or more steps of spatially segregating biomarker molecules will now be described. In certain embodiments, a method for detection and/or quantifying biomarker molecules in a sample comprises immobilizing a plurality of biomarker molecules with respect to a plurality of capture objects (e.g., beads) that each include a binding surface having affinity for at least one type of biomarker. For example, the

capture objects may comprise a plurality of beads comprising a plurality of capture components (e.g., an antibody having specific affinity for a biomarker of interest, etc.). At least some of the capture objects (e.g., at least some associated with at least one biomarker molecule) may be spatially separated/segregated into a plurality of locations, and at least some of the locations may be addressed/interrogated (e.g., using an imaging system). A measure of the concentration of biomarker molecules in the fluid sample may be determined based on the information received when addressing the locations (e.g., using the information received from the imaging system and/or processed using a computer implemented control system). In some cases, a measure of the concentration may be based at least in part on the number of locations determined to contain a capture object that is or was associated with at least one biomarker molecule. In other cases and/or under differing conditions, a measure of the concentration may be based at least in part on an intensity level of at least one signal indicative of the presence of a plurality of biomarker molecules and/or capture objects associated with a biomarker molecule at one or more of the addressed locations.

In some embodiments, the number/percentage/fraction of locations containing a capture object but not containing a biomarker molecule may also be determined and/or the number/percentage/fraction of locations not containing any capture object may also be determined. In such embodiments, a measure of the concentration of biomarker molecules in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with a biomarker molecule to the total number of locations determined to contain a capture object not associated with a biomarker molecule, and/or a measure of the concentration of biomarker molecule in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with a biomarker molecule to the number of locations determined to not contain any capture objects, and/or a measure of the concentration of biomarker molecule in the fluid sample may be based at least in part on the ratio of the number of locations determined to contain a capture object associated with a biomarker molecule to the number of locations determined to contain a capture object. In yet other embodiments, a measure of the concentration of biomarker molecules in a fluid sample may be based at least in part on the ratio of the number of locations

determined to contain a capture object and a biomarker molecule to the total number of locations addressed and/or analyzed.

In certain embodiments, at least some of the plurality of capture objects (e.g., at least some associated with at least one biomarker molecule) are spatially separated into a plurality of locations, for example, a plurality of reaction vessels in an array format. The plurality of reaction vessels may be formed in, on and/or of any suitable material, and in some cases, the reaction vessels can be sealed or may be formed upon the mating of a substrate with a sealing component, as discussed in more detail below. In certain embodiments, especially where quantization of the capture objects associated with at least one biomarker molecule is desired, the partitioning of the capture objects can be performed such that at least some (e.g., a statistically significant fraction; e.g., as described in International Patent Application No. PCT/US2011/026645, filed March 1, 2011, published as WO 2011/109364 on September 9, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS," by Duffy et al) of the reaction vessels comprise at least one or, in certain cases, only one capture object associated with at least one biomarker molecule and at least some (e.g., a statistically significant fraction) of the reaction vessels comprise an capture object not associated with any biomarker molecules. The capture objects associated with at least one biomarker molecule may be quantified in certain embodiments, thereby allowing for the detection and/or quantification of biomarker molecules in the fluid sample by techniques described in more detail herein.

An exemplary assay method may proceed as follows. A sample fluid containing or suspected of containing biomarker molecules is provided. An assay consumable comprising a plurality of assay sites is exposed to the sample fluid. In some cases, the biomarker molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the assay sites contain a single biomarker molecule and a statistically significant fraction of the assay sites do not contain any biomarker molecules. The assay sites may optionally be exposed to a variety of reagents (e.g., using a reagent loader) and or rinsed. The assay sites may then optionally be sealed and imaged (see, for example, U.S. Patent Application Serial No. 13/035,472, filed February 25, 2011, entitled "SYSTEMS, DEVICES, AND METHODS FOR ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES," by Fournier et al.). The images are then analyzed (e.g., using a

computer implemented control system) such that a measure of the concentration of the biomarker molecules in the fluid sample may be obtained, based at least in part, by determination of the number/fraction/percentage of assay sites which contain a biomarker molecule and/or the number/fraction/percentage of sites which do not contain any biomarkers molecules. In some cases, the biomarker molecules are provided in a manner (e.g., at a concentration) such that at least some assay sites comprise more than one biomarker molecule. In such embodiments, a measure of the concentration of biomarker molecules in the fluid sample may be obtained at least in part on an intensity level of at least one signal indicative of the presence of a plurality of biomarkers molecules at one or more of the assay sites

In some cases, the methods optionally comprise exposing the fluid sample to a plurality of capture objects, for example, beads. At least some of the biomarker molecules are immobilized with respect to a bead. In some cases, the biomarker molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the beads associate with a single biomarker molecule and a statistically significant fraction of the beads do not associate with any biomarker molecules. At least some of the plurality of beads (e.g., those associated with a single biomarker molecule or not associated with any biomarker molecules) may then be spatially separated/segregated into a plurality of assay sites (e.g., of an assay consumable). The assay sites may optionally be exposed to a variety of reagents and/or rinsed. At least some of the assay sites may then be addressed to determine the number of assay sites containing a biomarker molecule. In some cases, the number of assay sites containing a bead not associated with a biomarker molecule, the number of assay sites not containing a bead and/or the total number of assay sites addressed may also be determined. Such determination(s) may then be used to determine a measure of the concentration of biomarker molecules in the fluid sample. In some cases, more than one biomarker molecule may associate with a bead and/or more than one bead may be present in an assay site. In some cases, the plurality biomarker molecules may be exposed to at least one additional reaction component prior to, concurrent with, and/or following spatially separating at least some of the biomarker molecules into a plurality of locations.

The biomarker molecules may be directly detected or indirectly detected. In the case of direct detection, a biomarker molecule may comprise a molecule or moiety that may be directly interrogated and/or detected (e.g., a fluorescent entity).

In the case of indirect detection, an additional component is used for determining the presence of the biomarker molecule. For example, the biomarker molecules (e.g., optionally associated with a bead) may be exposed to at least one type of binding ligand. A "binding ligand," is any molecule, particle, or the like which specifically binds to or otherwise specifically associates with a biomarker molecule to aid in the detection of the biomarker molecule. In certain embodiments, a binding ligand may be adapted to be directly detected (e.g., the binding ligand comprises a detectable molecule or moiety) or may be adapted to be indirectly detected (e.g., including a component that can convert a precursor labeling agent into a labeling agent). A component of a binding ligand may be adapted to be directly detected in embodiments where the component comprises a measurable property (e.g., a fluorescence emission, a color, etc.). A component of a binding ligand may facilitate indirect detection, for example, by converting a precursor labeling agent into a labeling agent (e.g., an agent that is detected in an assay). A "precursor labeling agent" is any molecule, particle, or the like, that can be converted to a labeling agent upon exposure to a suitable converting agent (e.g., an enzymatic component). A "labeling agent" is any molecule, particle, or the like, that facilitates detection, by acting as the detected entity, using a chosen detection technique. In some embodiments, the binding ligand may comprise an enzymatic component (e.g., horseradish peroxidase, beta-galactosidase, alkaline phosphatase, etc.). A first type of binding ligand may or may not be used in conjunction with additional binding ligands (e.g., second type, etc.).

More than one type of binding may be employed in any given assay method, for example, a first type of binding ligand and a second type of binding ligand. In one example, the first type of binding ligand is able to associate with a first type of biomarker molecule and the second type of binding ligand is able to associate with the first binding ligand. In another example, both a first type of binding ligand and a second type of binding ligand may associate with the same or different epitopes of a single biomarker molecule, as described herein. In some embodiments, at least one binding ligand comprises an enzymatic component.

In some embodiments, a binding ligand and/or a biomarker may comprise an enzymatic component. The enzymatic component may convert a precursor labeling agent (e.g., an enzymatic substrate) into a labeling agent (e.g., a detectable product). A measure of the concentration of biomarker molecules in the fluid sample can then be determined based at least in part by determining the number of locations

containing a labeling agent (e.g., by relating the number of locations containing a labeling agent to the number of locations containing a biomarker molecule (or number of capture objects associated with at least one biomarker molecule to total number of capture objects)). Non-limiting examples of enzymes or enzymatic components include horseradish peroxidase, beta-galactosidase, and alkaline phosphatase. Other non-limiting examples of systems or methods for detection include embodiments where nucleic acid precursors are replicated into multiple copies or converted to a nucleic acid that can be detected readily, such as the polymerase chain reaction (PCR), rolling circle amplification (RCA), ligation, Loop-Mediated Isothermal Amplification (LAMP), etc. Such systems and methods will be known to those of ordinary skill in the art, for example, as described in "DNA Amplification: Current Technologies and Applications," Vadim Demidov et al., 2004.

Another exemplary embodiment of indirect detection is as follows. In some cases, the biomarker molecules may be exposed to a precursor labeling agent (e.g., enzymatic substrate) and the enzymatic substrate may be converted to a detectable product (e.g., fluorescent molecule) upon exposure to a biomarker molecule.

The assay methods and systems may employ a variety of different components, steps, and/or other aspects that will be known and understood by those of ordinary skill in the art. For example, a method may further comprise determining at least one background signal determination (e.g., and further comprising subtracting the background signal from other determinations), wash steps, and the like. In some cases, the assays or systems may include the use of at least one binding ligand, as described herein. In some cases, the measure of the concentration of biomarker molecules in a fluid sample is based at least in part on comparison of a measured parameter to a calibration curve. In some instances, the calibration curve is formed at least in part by determination at least one calibration factor, as described above.

As will be understood by those of ordinary skill in the art, a system and/or method may be calibrated using natural and/or synthetic forms of the target biomarker, and/or analogues thereof. In embodiments where the target analyte is a tau protein, the system and/or method may be calibrated using one or more natural and/or synthetic isoforms of tau protein. Naturally occurring tau proteins are described herein. Synthetic isoforms of tau proteins include two nearest neighbor antibody epitope synthetic peptides (<20 amino acids) to long synthetic peptides (80-100 amino acids in length. In some cases, short isoforms of the naturally occurring

tau proteins may be employed. For example, tau protein isoforms can have varying lengths of amino acids selected from the tau 441 sequence. Non-limiting examples of short forms of tau proteins include tau 50-mers (e.g., comprising residues 187-237, 190-240, or 155-205 of tau 441) and tau 64-mers (e.g., comprising residues 155-235 of tau 441, RGAAP PGQKG QTPPA PKpTPP SSKSG DRSGY SSPGS PGTSR TPSLP TPPTR EPKKV AVVRT PPKS-NH_2 (SEQ ID NO.: 1)). In some cases, at least a portion of the tau protein(s) used may be phosphorylated (e.g., RGAAP PGQKG QTPPA PKpTPP SSKSG DRSGY SSPGS PGTSR TPSLP TPPTR EPKKV AVVRpT PPKS-NH2 (SEQ ID NO.: 2)).

In certain embodiments, solubilized, or suspended precursor labeling agents may be employed, wherein the precursor labeling agents are converted to labeling agents which are insoluble in the liquid and/or which become immobilized within/near the location (e.g., within the reaction vessel in which the labeling agent is formed). Such precursor labeling agents and labeling agents and their use is described in commonly owned U.S. Patent Application Publication No. US-2010-0075862 (Serial No. 12/236484), filed September 23, 2008, entitled "HIGH SENSITIVITY DETERMINATION OF THE CONCENTRATION OF ANALYTE MOLECULES OR PARTICLES IN A FLUID SAMPLE," by Duffy et al., incorporated herein by reference.

An exemplary embodiment of an assay method that may be used in certain embodiments of the invention is illustrated in Figure 1a. A plurality of capture objects 2, are provided (step (A)). In this particular example, the plurality of capture objects comprises a plurality of beads. The beads are exposed to a fluid sample containing a plurality of biomarker molecules 3 (e.g., beads 2 are incubated with biomarker molecules 3). At least some of the biomarker molecules are immobilized with respect to a bead. In this example, the biomarker molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the beads associate with a single biomarker molecule and a statistically significant fraction of the beads do not associate with any biomarker molecules. For example, as shown in step (B), biomarker molecule 4 is immobilized with respect to bead 5, thereby forming complex 6, whereas some beads 7 are not associated with any biomarker molecules. It should be understood, in some embodiments, more than one biomarker molecule may associate with at least some of the beads, as described herein. At least some of the plurality of beads (e.g., those associated with a single

biomarker molecule or not associated with any biomarker molecules) may then be spatially separated/segregated into a plurality of locations. As shown in step (C), the plurality of locations is illustrated as substrate 8 comprising a plurality of wells/reaction vessels 9. In this example, each reaction vessel comprises either zero or one beads. At least some of the reaction vessels may then be addressed (e.g., optically or via other detection means) to determine the number of locations containing a biomarker molecule. For example, as shown in step (D), the plurality of reaction vessels are interrogated optically using light source 15, wherein each reaction vessel is exposed to electromagnetic radiation (represented by arrows 10) from light source 15. The light emitted (represented by arrows 11) from each reaction vessel is determined (and/or recorded) by detector 15 (in this example, housed in the same system as light source 15). The number of reaction vessels containing a biomarker molecule (e.g., reaction vessels 12) is determined based on the light detected from the reaction vessels. In some cases, the number of reaction vessels containing a bead not associated with a biomarker molecule (e.g., reaction vessel 13), the number of wells not containing a bead (e.g., reaction vessel 14) and/or the total number of wells addressed may also be determined. Such determination(s) may then be used to determine a measure of the concentration of biomarker molecules in the fluid sample.

A non-limiting example of an embodiment where a capture object is associated with more than one biomarker molecule is illustrated in Figure 1b. A plurality of capture objects 20 are provided (step (A)). In this example, the plurality of capture objects comprises a plurality of beads. The plurality of beads is exposed to a fluid sample containing plurality of biomarker molecules 21 (e.g., beads 20 are incubated with biomarker molecules 21). At least some of the biomarker molecules are immobilized with respect to a bead. For example, as shown in step (B), biomarker molecule 22 is immobilized with respect to bead 24, thereby forming complex 26. Also illustrated is complex 30 comprising a bead immobilized with respect to three biomarker molecules and complex 32 comprising a bead immobilized with respect to two biomarker molecules. Additionally, in some cases, some of the beads may not associate with any biomarker molecules (e.g., bead 28). The plurality of beads from step (B) is exposed to a plurality of binding ligands 31. As shown in step (C), a binding ligand associates with some of the biomarker molecules immobilized with respect to a bead. For example, complex 40 comprises bead 34, biomarker molecule 36, and binding ligand 38. The binding ligands are provided in a manner such that a

statistically significant fraction of the beads comprising at least one biomarker molecule become associated with at least one binding ligand (e.g., one, two, three, etc.) and a statistically significant fraction of the beads comprising at least one biomarker molecule do not become associated with any binding ligands. At least a portion of the plurality of beads from step (C) are then spatially separated into a plurality of locations. As shown in step (D), in this example, the locations comprise a plurality of reaction vessels 41 on a substrate 42. The plurality of reaction vessels may be exposed to the plurality of beads from step (C) such at each reaction vessel contains zero or one beads. The substrate may then be analyzed to determine the number of reaction vessels containing a binding ligand (e.g., reaction vessels 43), wherein in the number may be related to a measure of the concentration of biomarker molecules in the fluid sample. In some cases, the number of reaction vessels containing a bead and not containing a binding ligand (e.g., reaction vessel 44), the number of reaction vessels not containing a bead (e.g., reaction vessel 45), and/or the total number of reaction vessels addressed/analyzed may also be determined. Such determination(s) may then be used to determine a measure of the concentration of biomarker molecules in the fluid sample.

In some embodiments, a plurality of locations may be addressed and/or a plurality of capture objects and/or species/molecules/particles of interest may be detected substantially simultaneously. "Substantially simultaneously" when used in this context, refers to addressing/detection of the locations/capture objects/species/molecules/particles of interest at approximately the same time such that the time periods during which at least two locations/capture objects/species/molecules/particles of interest are addressed/detected overlap, as opposed to being sequentially addressed/detected, where they would not. Simultaneous addressing/detection can be accomplished by using various techniques, including optical techniques (e.g., CCD detector). Spatially segregating capture objects/species/molecules/particles into a plurality of discrete, resolvable locations, according to some embodiments facilitates substantially simultaneous detection by allowing multiple locations to be addressed substantially simultaneously. For example, for embodiments where individual species/molecules/particles are associated with capture objects that are spatially segregated with respect to the other capture objects into a plurality of discrete, separately resolvable locations during detection, substantially simultaneously addressing the plurality of discrete, separately

resolvable locations permits individual capture objects, and thus individual species/molecules/particles (e.g., biomarker molecules) to be resolved. For example, in certain embodiments, individual molecules/particles of a plurality of molecules/particles are partitioned across a plurality of reaction vessels such that each reaction vessel contains zero or only one species/molecule/particle. In some cases, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5% of all species/molecules/particles are spatially separated with respect to other species/molecules/particles during detection. A plurality of species/molecules/particles may be detected substantially simultaneously within a time period of less than about 1 second, less than about 500 milliseconds, less than about 100 milliseconds, less than about 50 milliseconds, less than about 10 milliseconds, less than about 1 millisecond, less than about 500 microseconds, less than about 100 microseconds, less than about 50 microseconds, less than about 10 microseconds, less than about 1 microsecond, less than about 0.5 microseconds, less than about 0.1 microseconds, or less than about 0.01 microseconds, less than about 0.001 microseconds, or less. In some embodiments, the plurality of species/molecules/particles may be detected substantially simultaneously within a time period of between about 100 microseconds and about 0.001 microseconds, between about 10 microseconds and about 0.01 microseconds, or less.

In some embodiments, the locations are optically interrogated. The locations exhibiting changes in their optical signature may be identified by a conventional optical train and optical detection system. Depending on the detected species (e.g., type of fluorescence entity, etc.) and the operative wavelengths, optical filters designed for a particular wavelength may be employed for optical interrogation of the locations. In embodiments where optical interrogation is used, the system may comprise more than one light source and/or a plurality of filters to adjust the wavelength and/or intensity of the light source. In some embodiments, the optical signal from a plurality of locations is captured using a CCD camera.

In some embodiments of the present invention, the plurality of reaction vessels may be sealed (e.g., after the introduction of the biomarker molecules, binding ligands, and/or precursor labeling agent), for example, through the mating of the second substrate and a sealing component. The sealing of the reaction vessels may be such that the contents of each reaction vessel cannot escape the reaction vessel during

the remainder of the assay. In some cases, the reaction vessels may be sealed after the addition of the biomarker molecules and, optionally, at least one type of precursor labeling agent to facilitate detection of the biomarker molecules. For embodiments employing precursor labeling agents, by sealing the contents in some or each reaction vessel, a reaction to produce the detectable labeling agents can proceed within the sealed reaction vessels, thereby producing a detectable amount of labeling agents that is retained in the reaction vessel for detection purposes.

The plurality of locations may be formed may be formed using a variety of methods and/or materials. In some embodiments, the plurality of locations comprises a plurality of reaction vessels/wells on a substrate. In some cases, the plurality of reaction vessels is formed as an array of depressions on a first surface. In other cases, however, the plurality of reaction vessels may be formed by mating a sealing component comprising a plurality of depressions with a substrate that may either have a featureless surface or include depressions aligned with those on the sealing component. Any of the device components, for example, the substrate or sealing component, may be fabricated from a compliant material, e.g., an elastomeric polymer material, to aid in sealing. The surfaces may be or made to be hydrophobic or contain hydrophobic regions to minimize leakage of aqueous samples from the microwells. The reactions vessels, in certain embodiments, may be configured to receive and contain only a single capture object.

In some embodiments, the reaction vessels may all have approximately the same volume. In other embodiments, the reaction vessels may have differing volumes. The volume of each individual reaction vessel may be selected to be appropriate to facilitate any particular assay protocol. For example, in one set of embodiments where it is desirable to limit the number of capture objects used for biomarker capture contained in each vessel to a small number, the volume of the reaction vessels may range from attoliters or smaller to nanoliters or larger depending upon the nature of the capture objects, the detection technique and equipment employed, the number and density of the wells on the substrate and the expected concentration of capture objects in the fluid applied to the substrate containing the wells. In one embodiment, the size of the reaction vessel may be selected such only a single capture object used for biomarker capture can be fully contained within the reaction vessel (see, for example, U.S. Patent Application Serial No. 12/731,130, filed March 24, 2010, published as US-2011-0212848 on September 1, 2011, entitled

"ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS," by Duffy et al.; International Patent Application No. PCT/US2011/026645, filed March 1, 2011, published as WO 2011/109364 on September 9, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS ," by Duffy et al., each herein incorporated by reference).

In some embodiments, the reaction vessels may have a volume between about 1 femtoliter and about 1 picoliter, between about 1 femtoliters and about 100 femtoliters, between about 10 attoliters and about 100 picoliters, between about 1 picoliter and about 100 picoliters, between about 1 femtoliter and about 1 picoliter, or between about 30 femtoliters and about 60 femtoliters. In some cases, the reaction vessels have a volume of less than about 1 picoliter, less than about 500 femtoliters, less than about 100 femtoliters, less than about 50 femtoliters, or less than about 1 femtoliter. In some cases, the reaction vessels have a volume of about 10 femtoliters, about 20 femtoliters, about 30 femtoliters, about 40 femtoliters, about 50 femtoliters, about 60 femtoliters, about 70 femtoliters, about 80 femtoliters, about 90 femtoliters, or about 100 femtoliters.

The total number of locations and/or density of the locations employed in an assay (e.g., the number/density of reaction vessels in an array) can depend on the composition and end use of the array. For example, the number of reaction vessels employed may depend on the number of types of biomarker molecule and/or binding ligand employed, the suspected concentration range of the assay, the method of detection, the size of the capture objects, the type of detection entity (e.g., free labeling agent in solution, precipitating labeling agent, etc.). Arrays containing from about 2 to many billions of reaction vessels (or total number of reaction vessels) can be made by utilizing a variety of techniques and materials. Increasing the number of reaction vessels in the array can be used to increase the dynamic range of an assay or to allow multiple samples or multiple types of biomarker molecules to be assayed in parallel. The array may comprise between one thousand and one million reaction vessels per sample to be analyzed. In some cases, the array comprises greater than one million reaction vessels. In some embodiments, the array comprises between about 1,000 and about 50,000, between about 1,000 and about 1,000,000, between about 1,000 and about 10,000, between about 10,000 and about 100,000, between about 100,000 and about 1,000,000, between about 100,000 and about 500,000,

between about 1,000 and about 100,000, between about 50,000 and about 100,000, between about 20,000 and about 80,000, between about 30,000 and about 70,000, between about 40,000 and about 60,000 reaction vessels. In some embodiments, the array comprises about 10,000, about 20,000, about 50,000, about 100,000, about 150,000, about 200,000, about 300,000, about 500,000, about 1,000,000, or more, reaction vessels.

The array of reaction vessels may be arranged on a substantially planar surface or in a non-planar three-dimensional arrangement. The reaction vessels may be arrayed in a regular pattern or may be randomly distributed. In a specific embodiment, the array is a regular pattern of sites on a substantially planar surface permitting the sites to be addressed in the X-Y coordinate plane.

In some embodiments, the reaction vessels are formed in a solid material. As will be appreciated by those in the art, the number of potentially suitable materials in which the reaction vessels can be formed is very large, and includes, but is not limited to, glass (including modified and/or functionalized glass), plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, cyclic olefin copolymer (COC), cyclic olefin polymer (COP), Teflon®, polysaccharides, nylon or nitrocellulose, etc.), elastomers (such as poly(dimethyl siloxane) and poly urethanes), composite materials, ceramics, silica or silica-based materials (including silicon and modified silicon), carbon, metals, optical fiber bundles, or the like. In general, the substrate material may be selected to allow for optical detection without appreciable autofluorescence. In certain embodiments, the reaction vessels may be formed in a flexible material.

A reaction vessel in a surface (e.g., substrate or sealing component) may be formed using a variety of techniques known in the art, including, but not limited to, photolithography, stamping techniques, molding techniques, etching techniques, or the like. As will be appreciated by those of the ordinary skill in the art, the technique used can depend on the composition and shape of the supporting material and the size and number of reaction vessels.

In a particular embodiment, an array of reaction vessels is formed by creating microwells on one end of a fiber optic bundle and utilizing a planar compliant surface as a sealing component. In certain such embodiments, an array of reaction vessels in the end of a fiber optic bundle may be formed as follows. First, an array of microwells is etched into the end of a polished fiber optic bundle. Techniques and

materials for forming and etching a fiber optic bundle are known to those of ordinary skill in the art. For example, the diameter of the optical fibers, the presence, size and composition of core and cladding regions of the fiber, and the depth and specificity of the etch may be varied by the etching technique chosen so that microwells of the desired volume may be formed. In certain embodiments, the etching process creates microwells by preferentially etching the core material of the individual glass fibers in the bundle such that each well is approximately aligned with a single fiber and isolated from adjacent wells by the cladding material. Potential advantages of the fiber optic array format is that it can produce thousands to millions of reaction vessels without complicated microfabrication procedures and that it can provide the ability to observe and optically address many reaction vessels simultaneously.

Each microwell may be aligned with an optical fiber in the bundle so that the fiber optic bundle can carry both excitation and emission light to and from the wells, enabling remote interrogation of the well contents. Further, an array of optical fibers may provide the capability for simultaneous or non-simultaneous excitation of molecules in adjacent vessels, without signal "cross-talk" between fibers. That is, excitation light transmitted in one fiber does not escape to a neighboring fiber.

Alternatively, the equivalent structures of a plurality of reaction vessels may be fabricated using other methods and materials that do not utilize the ends of an optical fiber bundle as a substrate. For example, the array may be a spotted, printed or photolithographically fabricated substrate produced by techniques known in the art; see for example WO95/25116; WO95/35505; PCT US98/09163; U.S. Patent Nos. 5,700,637, 5,807,522, 5,445,934, 6,406,845, and 6,482,593. In some cases, the array may be produced using molding, embossing, and/or etching techniques as will be known to those of ordinary skill in the art.

In some embodiments, the plurality of locations may not comprise a plurality of reaction vessels/wells. For example, in embodiments where capture objects are employed, a patterned substantially planar surface may be employed and the patterned areas form a plurality of locations. In some cases, the patterned areas may comprise substantially hydrophilic surfaces which are substantially surrounded by substantially hydrophobic surfaces. In certain embodiments, a plurality of capture objects (e.g., beads) may be substantially surrounded by a substantially hydrophilic medium (e.g., comprising water), and the beads may be exposed to the patterned surface such that the beads associate in the patterned areas (e.g., the hydrophilic locations on the

surface), thereby spatially segregating the plurality of beads. For example, in one such embodiment, a substrate may be or include a gel or other material able to provide a sufficient barrier to mass transport (e.g., convective and/or diffusional barrier) to prevent capture objects used for biomarker capture and/or precursor labeling agent and/or labeling agent from moving from one location on or in the material to another location so as to cause interference or cross-talk between spatial locations containing different capture objects during the time frame required to address the locations and complete the assay. For example, in one embodiment, a plurality of capture objects is spatially separated by dispersing the capture objects on and/or in a hydrogel material. In some cases, a precursor labeling agent may be already present in the hydrogel, thereby facilitating development of a local concentration of the labeling agent (e.g., upon exposure to a binding ligand or biomarker molecule carrying an enzymatic component). As still yet another embodiment, the capture objects may be confined in one or more capillaries. In some cases, the plurality of capture objects may be absorbed or localized on a porous or fibrous substrate, for example, filter paper. In some embodiments, the capture objects may be spatially segregated on a uniform surface (e.g., a planar surface), and the capture objects may be detected using precursor labeling agents which are converted to substantially insoluble or precipitating labeling agents that remain localized at or near the location of where the corresponding capture object is localized. The use of such substantially insoluble or precipitating labeling agents is described herein. In some cases, single biomarker molecules may be spatially segregated into a plurality of droplets. That is, single biomarker molecules may be substantially contained in a droplet containing a first fluid. The droplet may be substantially surrounded by a second fluid, wherein the second fluid is substantially immiscible with the first fluid.

In some embodiments, during the assay, at least one washing step may be carried out. In certain embodiments, the wash solution is selected so that it does not cause appreciable change to the configuration of the capture objects and/or biomarker molecules and/or does not disrupt any specific binding interaction between at least two components of the assay (e.g., a capture component and a biomarker molecule). In other cases, the wash solution may be a solution that is selected to chemically interact with one or more assay components. As will be understood by those of ordinary skill in the art, a wash step may be performed at any appropriate time point during the inventive methods. For example, a plurality of capture objects may be

washed after exposing the capture objects to one or more solutions comprising biomarker molecules, binding ligands, precursor labeling agents, or the like. As another example, following immobilization of the biomarker molecules with respect to a plurality of capture objects, the plurality of capture objects may be subjected to a washing step thereby removing any biomarker molecules not specifically immobilized with respect to a capture object.

Other assay methods in addition to those described herein are known in the art and may be used in connection with the inventive methods. For example, various analyzers are commercially available for the determination of the concentration of biomarkers. The assay methods employed should meet the algorithm requirements for LOD and LOQ.

The following examples are included to demonstrate various features of the invention. Those of ordinary skill in the art should, in light of the present disclosure, will appreciate that many changes can be made in the specific embodiments which are disclosed while still obtaining a like or similar result without departing from the scope of the invention as defined by the appended claims. Accordingly, the following examples are intended only to illustrate certain features of the present invention, but do not necessarily exemplify the full scope of the invention.

Example 1

The following example provides experimental details relating to prognostication of neurological outcome in comatose survivors of oxygen deprivation, using tau proteins

Objective: Use of peripheral tau protein measurements as an indicator of the presence of brain injury has remained elusive, in large part due to the lack of adequate sensitivity for reliable measurement of the protein in serum or plasma using common technologies. Additionally, little was known about the time-dependence of tau protein release from the central nervous system into peripheral circulation. Using methods described herein, which, in certain embodiments are capable of ultra sensitive tau protein measurement, serial serum samples from 25 resuscitated cardiac arrest patients were analyzed to longitudinally study tau protein release into serum and determine prognostic significance of tau protein elevation for prediction of long-term cognitive impairment from hypoxic insult.

Summary of Methods: 25 unconscious patients with cardiac arrest were resuscitated with restoration of spontaneous circulation and admitted to a hospital intensive care unit (ICU). Patients were treated with hypothermia and repeated blood samplings were obtained during the first five days in the ICU. Serum levels of total tau protein were measured with a digital immunoassay described in Example 2. Cognitive assessments were made using Cerebral Performance Categorization (CPC) at discharge from the ICU and six months later. Tau protein data were analyzed in the context of six-month cognitive outcome.

Summary of Results: Tau protein elevations ranged from modest to very high, and exhibited unexpected bi-modal kinetic profiles in many patients. Total area-under-the-curve (AUC) was highly prognostic for six-month cognitive outcome. AUC of the secondary tau protein peak exhibited 100% sensitivity and 91% specificity for predicting 6-month outcome.

Summary of Conclusions: The data indicate that sufficiently sensitive peripheral tau protein measurements in conjunction with an understanding of tau protein release kinetics have clinical utility for brain injury assessment and prognostication of cognitive outcome.

Additional Background: Tau proteins, with a molecular mass of 48 to 67 kd depending on isoform, are associated with microtubules and localized in the axonal compartment of neurons. Tau proteins plays a structural role in the assembly of tubulin monomers into axonal microtubule bundles, which are important for maintaining the cytoskeleton and axonal transport. Tau protein is generally elevated in the cerebrospinal fluid (CSF) of patients with neurodegenerative disease and severe head injuries, making it a candidate for peripheral measurement as a biomarker of acquired or traumatic brain injury (ABI, TBI). However, studies on peripheral tau protein have been hampered by its low abundance in serum and plasma (typically low pg/mL), making its measurement difficult.

While CSF tau protein elevation is known to correlate with 1-year outcome in severe TBI patients, no such correlation has been made to serum tau protein, in part because most common assays cannot accurately detect the low levels of tau protein in serum. In addition, the clinical value of serum tau protein for assessment of minor head injury has been questioned. A previous study looked at serum tau protein elevation measured in 24 ischemic stroke patients studied using an immunoassay with a limit of detection of 60 pg/mL, but no correlation was made or found between tau

protein appearance to stroke severity. A recent rat TBI model indicated serum tau protein elevation peaked rapidly and declined after six hours, with no significant additional tau protein elevation over 7 days. Little else has been reported about the kinetics of tau protein movement across the blood brain barrier (BBB), nor the potential prognostic significance of peripheral tau protein appearance with ABI or TBI.

Methods: This example employed the protocol described in Example 2 below, which is capable in certain embodiments of three logs greater sensitivity than typical conventional methods. The assay was utilized to examine serial serum samples from 25 resuscitated cardiac arrest patients to longitudinally study tau protein release into serum and probe for prognostic significance of tau protein elevation for prediction of long-term cognitive impairment due to hypoxic insult.

The study was performed at the general intensive care unit at Uppsala University Hospital, Sweden, and approved by the Human Ethics Committee of Uppsala, Sweden. Twenty-five unconscious patients with cardiac arrest were resuscitated with restoration of spontaneous circulation (ROSC). Patients were >18 yrs old, exhibited systolic blood pressure >80mmHg after ROSC, and a Glasgow Coma Scale $\leq$7.

Upon admission, hypothermia treatment was started immediately after resuscitation. Ventilation was administered during the coma period, with a target PaO_2 of $\geq$12 kPa (90 mmHg) and $PaCO_2$ between 5.0 and 5.5 kPa (38–41 mmHg). Targeted mean arterial pressure was 65–100 mmHg, with application of inotropic/vasopressor support, if required, using dobutamine as the first line medication, followed by noradrenaline (norepinephrine) or adrenaline (epinephrine), if necessary. If the patient was considered euvolaemic but had a diuresis of less than 0.5 ml/kg/h, furosemide was administered. Furosemide was also given if the intensive care physician considered that the patient had a fluid overload. All patients received an arterial line in the radial or femoral artery for blood sampling. Serial blood samples were collected, starting as soon as possible in the emergency phase (within 6 h after cardiac arrest), and continuing at 1, 2, 6, 12, 24, 48, 72, 96, and 108 h after cardiac arrest. Serum aliquots were frozen at -70°C until analysis.

Patient outcome was assessed in accordance with the Glasgow-Pittsburgh cerebral performance category (CPC) scale at discharge from the intensive care unit and 6 months later. The CPC scale ranges from 1 to 5, with 1 representing mildest

possible neurological deficit (patient is able to return to work), and 4-5 representing the most severe deficit (vegetative) and death. A CPC of 1 or 2 was considered a "good" outcome and a CPC score of 3–5 a "poor" outcome. For patients who died after ICU discharge, the better of the two scores was used, as recommended by the Utstein templates.

Patient serum samples were measured in triplicate by a single molecule digital immunoassay for tau protein (see Example 2 for more details). The technique involves performing a paramagnetic bead-based ELISA using beta-galactosidase as a reporter, followed by isolation of individual capture beads within femtoliter-sized reaction wells in a microarray. Isolation of individual beads permits the buildup of fluorescent substrate in the presence of tau protein, such that wells containing a single immunocomplex can be detected. The limit of detection of the assay is 0.02 pg/mL, making it approximately 1000-fold more sensitive than typical conventional immunoassays. The assay was calibrated from 0 pg/mL to 100 pg/mL total tau protein and was able to precisely measure serum tau protein in the patient samples. The extreme sensitivity of the method permits pre-dilution of the samples prior to assay, reducing potential endogenous interferences. All samples were pre-diluted 1:4 with a PBS-tween diluent prior to assay.

Tau protein elevation profiles were analyzed for area-under-the-curve (AUC) with GraphPad Prism 5.0d (GraphPad Software, La Jolla, CA). Four of the 25 patients died 24-48 hours after admission, and these patients were excluded from AUC analysis. AUC was evaluated during the first 24-hour period as well as over the full time course of samples (to 108 hours) using a baseline of zero. In addition, the AUC of secondary tau protein elevation peaks were estimated assuming a baseline corresponding to the tau protein concentration measured at the initial time point of the secondary peak. Statistical significance between 'good' and 'poor' 6-month outcome was determined by student t-test, with significance taken as $p < 0.05$.

Results: Representative elevation profiles for patients with good and poor 6-month outcomes are depicted in Figure 2**.** Figure 2 shows serum tau following resuscitation from cardiac arrest. CPC scores are listing for each patient in the legends. The first two numbers correspond to the CPC assessments upon discharge from the ICU and six months later. The third number represents overall 6-month outcome assessment (1=poor, 0=good). Figures 2a-2c depict representative groupings of patients exhibiting different profiles of tau elevations: a) initial 24-hour peaks; b)

delayed peaks, and c) both initial 24-hour and delayed peaks. Figure 2d depicts representative profiles from patients with good outcomes. Note the difference in scales. Error bars depict SD of triplicate measurements.

Tau protein expression ranged from almost undetectable to large elevations approaching 700 pg/mL. There was a strong general association between tau protein elevation and patient outcome: the more tau protein expressed, the greater the likelihood for poor 6-month outcome. Tau protein elevation also showed clear bi-modal tendencies, with the appearance of one or both peaks varying with patient. The initial peak was generally fully expressed during the first 24 hours following cardiac arrest, while the secondary peak generally expressed after 24-48 hours. Some patients exhibited only the first peak (Figure 2a), some exhibited only the second peak (Figure 2b), and some exhibited both peaks (Figure 2c).

Table 2.

Patient	AUC </= 24 hr		AUC all		AUC 2nd peak only	
	Good	Poor	Good	Poor	Good	Poor
2		1590		4753		1440
3	706.9		2145		0	
4	748.3		2861		154.2	
5		525.2		3007		1690
6		179.9		1232		897.3
7	9.72		92.86		64.5	
8		11.19		1646		1545
9	59		1141		0	
10		109.7		9336		8208
11	14.63		84.56		0	
12		52.15		846.2		587.8
13	26.01		289.5		215.6	
14	12.93		431.5		393	
15	12.31		184.4		168.2	
17	0.02		30.68		20.9	
18	15.78		46.62		0	
20		334.6		6827		6511
21		580.8		19875		18051
23		1036		26150		23935
24		12.92		72.9		35.94
25		9.37		8087		8642
AVG	160.26	413.17	730.61	7521.83	101.66	6447.39
t-test (p)	0.090609467		0.011726946		0.01197362	

To compare the significance of the primary and secondary elevation profiles for 6-month outcome, AUCs were calculated for the first 24 hours (referred to as the "first peak"), the full time course, and the secondary peak only. Table 2 exhibits AUCs for each patient, which are plotted in Figure 3. More specifically, Table 2 tabulates characteristics of serum tau elevation profiles from resuscitated survivors of cardiac arrest during the first 96-108 hours following admission to the ICD.

Parameters were sorted on the basis of good or poor 6-month cerebral outcome and compared by student t-test. Four patients (all with good outcome) exhibited no discernable secondary peaks. Figure 3 plots AUC results a) across the first 24 hours, b) for all serial samplings, and c) the secondary tau peak only. Error bars depict standard error of the means.

Weak correlation was observed between the initial 24 hours and 6-month outcome ($p > 0.05$). Notably, there was a strong correlation between the secondary peak and 6-month outcome ($p = 0.01$). The weak correlation between initial tau protein appearance and outcome reflects inter-patient differences in primary peak expression rather than lack of significance between tau protein elevation and outcome. Calculation of overall AUC gave similar statistical significance with outcome as the AUC of the second peak only. Patients with good outcome generally had very low serum tau protein, and secondary peaks where either absent or weak (Figure 2d).

The appearance and magnitude of the secondary tau protein peak was highly prognostic for 6-month outcome. Bifurcating the data with an AUC cut point of 500 resulted in 100% sensitivity (10/10 patients) and 91% specificity (10/11 patients) for predicting good and poor 6-month outcomes respectively.

Discussion: These data represent a high-resolution longitudinal examination of serum tau protein elevation following an acute ABI event. The bimodal profile elevation kinetics are consistent with two modes of neuronal damage: initially upon acute oxygen deprivation, followed by delayed cell death due to apoptosis and/or cerebral swelling. With ROSC as an inclusion criterion, the elevation kinetics should be unrelated to reperfusion differences between patients. Patients were all treated with hypothermia, and were not treated with drugs known to significantly affect BBB permeability. It seems likely the bimodal profiles are related to neuronal damage rather than BBB or reperfusion variables.

Inter-patient differences in expression one or both elevation peaks could be related to the extent and duration of the hypoxia. Global cerebral ischemia could trigger rapid necrosis in addition to longer-term apoptosis cascades. Sub lethal hypoxic encephalopathy can set a series of toxic reactions in motion that finish off injured neurons and kill additional ones over hours or days following the insult. While early tau protein peaks were less prognostics for 6-month outcome than the secondary elevation, they are nonetheless deadly when high levels of tau protein are measured. Among the four patients who did not survive the first 48 hours, two

patients had prominent initial tau protein peaks of well over 200 pg/mL that had dropped 10-fold by 24 hours (not shown). In these patients, it may have been that the acute initial necrosis was sufficiently lethal, and it might be anticipated that survival would have witnessed prominent secondary peaks.

It is noted that the serial sampling in this study was concluded at 108 hours. It may be that additional tau protein elevation occurs beyond 108 hours. Studies of tau protein elevation in CSF following severe TBI have revealed temporal elevations well beyond five days. It is possible that patients in the present study exhibiting tau protein peaks in the first 24 hours with minimal secondary elevation could go on to express significant additional tau protein beyond 108 hours.

Since the magnitude of cognitive impairment should reflect the magnitude of hypoxia, correlation of serum tau protein elevation with cognitive outcome indicates released tau protein reflects the extent of hypoxia and associated neuronal damage. Serum tau protein appearance as measured by digital immunoassay exhibited considerable prognostic significance for 6-month cognitive outcome, with a sensitivity and specificity of 100% using an AUC cut point of 500. This example demonstrates that serial blood measurements of tau protein in the ICU following resuscitation from cardiac arrest has a clinical value for stratifying likely cognitive outcome.

Example 2

The following example describes the tau protein ultra-sensitive digital immunoassay for plasma tau using single molecule arrays that was employed in Example 1.

Reagents were developed for a paramagnetic bead-based ELISA. Tau protein molecules in plasma were captured on antibody-coated paramagnetic capture beads and labeled with an enzyme conjugate. The beads were loaded into arrays of 50,000, 50-femtoliter reaction wells etched into bundles of optical fibers. Single capture beads trapped in each well were sealed in the presence of enzyme substrate and imaged using a fluorescence microscope fitted with a CCD camera. At low concentrations, the images were analyzed for the presence or absence of single immunocomplexes of labeled tau protein, resulting in a digital signal. At high concentrations, the analog intensity of the beads was normalized to the digital signals, extending the dynamic range of the assay to over four logs. Analytical performance

of the assay was evaluated, and serum samples from hypoxia patients were tested for tau protein.

The assay described in this example has a detection limit (i.e., LOD) of 0.02 pg/mL and was linear to 100 pg/mL tau protein (R2 > 0.996, Patient I.J cal curve). Results using serum samples from hypoxia patients showed a bi-phasic response across the time course post hypoxic insult.

Concentrations of tau protein in serum and plasma are believed to be over 100-fold lower than in cerebrospinal fluid.

This example describes the development and validation of a digital immunoassay using single molecule array technology that is capable of measuring tau protein in hypoxia induced serum without biomarker enrichment or sample pretreatment procedures. The assay exhibits over 1000-fold greater sensitivity than validated commercially available ELISAs. The assay can be used for directly measuring and monitoring serum tau protein in therapeutic trials aimed at altering and lowering levels of this protein, down to sub-femtomolar levels.

The single molecule array technology employed two primary steps: an initial analyte capture step conducted with paramagnetic beads, followed by isolation of individual beads in arrays of femtoliter-sized reaction wells for digital imaging. Isolation of the individual beads in microwells permits the buildup of fluorescent product from the enzyme label such that signal from a single immunocomplex is readily detected using a CCD camera. This approach permits counting of single molecules when tau protein concentrations are low enough that the ratio of bound labeled peptide per bead is much less than one. In this concentration realm, Poisson statistics predict that bead-containing microwells in the array will contain either a single labeled tau protein molecule or no labeled tau protein molecules, resulting in a binary signal. Due to the amplified sensitivity for detecting label molecules afforded by confining fluorescent product buildup to the microwells, concentrations of label (detector anti-Tau antibody and enzyme label) can be reduced relative to standard ELISAs. Lowered concentrations of labeling reagents reduces their interaction with capture beads, resulting in reduced nonspecific binding enabling high signal to background ratios, even at extremely low concentrations of biomarker. For higher biomarker concentrations where all beads contain one or more labeled immunocomplexes, digital signals from the Poisson realm are used to calibrate analog intensity measurements, extending the dynamic range to over four logs.

Three reagents were developed for this tau protein immunoassay: capture beads, biotinylated detector, and a conjugate of streptavidin:beta-galactosidase The capture bead reagent comprised of a commercially available monoclonal anti-tau antibody (Covance) directed to an epitope (amino acids 210-230). The antibody was covalently attached by standard coupling chemistry to 2.7 μm carboxy paramagnetic microbeads (Varian). Because individual beads were captured in array wells 4.5 μm wide x 3.25 μm deep, it was advantageous that the capture beads remain monomeric. The antibody-coated beads were diluted to a working concentration of 6 x 106 beads/mL in Tris buffer with a surfactant and BSA. The biotinylated detector reagent was comprised of two commercially available monoclonal anti-tau antibodys (Pierce) directed to the N-terminus of the Capture Detector (amino acids 159-163 and 194-198). The antibodies were biotinylated using standard methods (Solulink), and the biotinylation level was confirmed spectrophotometrically per manufacturer's instructions. The monomeric state of the detector antibody before and after biotinylation was confirmed by size exclusion HPLC. The biotinylated detector antibodies were diluted to individual concentration of 0.2 μg/ml for assay in a PBS diluent containing a surfactant and newborn calf serum, NCS (PBS/NCS). The enzyme conjugate—streptavidin: β-galactosidase (SβG)—was prepared by covalent conjugation of purified streptavidin (Thermo Scientific) and β-galactosidase (Sigma) using standard coupling chemistry (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Thermo Scientific). Aliquots of a concentrated stock solution of SβG were prepared in PBS with 50% glycerol and transferred to -20°C for storage. Prior to assay, an aliquot was thawed and diluted to 25 pM in PBS/NCS with MgCl2. Purified Tau 381 antigen for calibrators and for specificity testing were from Millipore.

Assay calibrators and controls were prepared by dilution of Tau 381 stock in PBS diluent containing a surfactant and BSA (PBS/BSA). In some cases, a stock solution prepared by dilution to 1000 ng/mL in PBS/10G. For dynamic range/linearity characterization, a series of calibrators were prepared by serial 3-fold dilution to give a calibration range of 0 – 100 pg/mL. To evaluate assay day-to-day reproducibility at low concentrations, three low controls were prepared at 0.1, 5.0, and 50.0 pg/mL in PBS/BSA.

50,000 well optical fiber microarrays were prepared as previously described. In brief, optical fiber bundles (Schott North America) were cut into 5 cm lengths and sequentially polished with 30, 9, and 1 um-sized diamond lapping films. One end of

each bundle was etched in a 0.025 N HCl solution for 2 minutes and then submerged into water. The differential etch rate of the core and cladding glass of the fiber bundles caused an array of 4.5 um diameter wells to be formed in the core fibers.

Bead-plasma incubations and labeling of immunocomplexes in conical 96 well plates (Axygen) were conducted using a robotic liquid handling system (Tecan EVO 150). Conical wells are used to facilitate magnetic attraction of the beads to the sides of the wells for efficient removal of reaction mixtures and bead washing. For magnetic attraction, a microplate bar magnet (Invitrogen) was used. Incubation periods were conducted with shaking on a microplate shaker (VWR) to keep beads suspended in the wells. The assays were initiated by mixing 100 uL of sample with 600,000 capture beads, and the mixtures were incubated with shaking for two hours. Serum samples were pre-diluted 1:4 prior to assay with PBS/BSA as a precaution for sample quality and interference effects. Following serum incubation in the presence of biotinylated detector antibody, the beads were washed 3 times with a wash buffer of 5-fold concentrated PBS with a surfactant (5xPBS). After a the wash step, 100 µL of streptavidin-β-galactosidase was incubated with the beads for 30 minutes to form the final enzyme-labeled immunocomplex. The beads were then washed eight times per above, and concentrated to 2 x 107 beads/ml with the addition of a reduced volume (30 µL) of array loading buffer comprised of PBS with a surfactant. Beads were then loaded onto the arrays. 10 µL of the concentrated bead solution (2 x 106 beads) were pipetted onto the arrays and the arrays were centrifuged at 1,300 g for 10 minutes. Excess beads were removed by a PBS rinse and swabbing with deionized water. With this technique, array filling by the beads was generally 50-60%, which was adequate for minimizing contributions to imprecision from Poisson noise. Wells containing tau protein-labeled beads were detected utilizing beta-galactosidase catalyzed hydrolysis of resorufin β-D-galactopyranoside (RGP, Invitrogen) into fluorescent product (resorufin, excitation 558nm, emission 577nm). To introduce RGP substrate to the array wells, droplets of substrate were placed on a silicone gasket and introduced into the array wells with a mechanical platform. This step resulted in an array of sealed femtoliter wells in which enzyme-containing beads developed a concentrated fluorescence signal.

Imaging was accomplished via a custom-built fluorescence imaging system containing a light source, objectives, filter cubes and a CCD camera. For each sample, five fluorescent images of one second each were acquired (to identify wells

containing an enzyme) and one white light image was acquired (to identify wells containing a bead). Background fluorescence and any contaminating artifacts were discriminated from true 'positive' wells by analyzing for signal growth over the multiple images. These images were analyzed to determine the average number of enzymes per bead (AEB) across the concentration range. In some cases, at <50% active beads, the system was determined to be in the digital realm, so AEB was be determined from the fraction of beads that contain at least one enzyme and the Poisson distribution; and at >50% active beads, the average fluorescence intensity of the beads was normalized to the average fluorescence intensity of beads containing a single enzyme to yield AEB. In other cases, at <70% active beads relative to total beads, AEB was determined as a count of active beads corrected for a low statistical probability of multiple enzymes per bead; and at >70% active beads, the probability of multiple enzymes/bead increases such that all wells contain multiple enzymes and all are growing in signal and in this realm, the signal is was no longer digital, and average fluorescence intensities of the wells were converted to AEB based on the average intensities of wells containing single enzymes as determined at lower Aβ42 concentrations. The AEB unit worked continuously across the digital and analog realms.

For description of various details associate with this assay, see, for example, U.S. Patent Application Serial No. 12/731,130, filed March 24, 2010, published as US-2011-0212848 on September 1, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS," by Duffy et al.; International Patent Application No. PCT/US2011/026645, filed March 1, 2011, published as WO 2011/109364 on September 9, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS," by Duffy et al.; International Patent Application No. PCT/US2011/026657, filed March 1, 2011, published as WO 2011/109372 on September 9, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES USING DUAL DETECTION METHODS," by Duffy et al.; U.S. Patent Application Serial No. 12/731135, filed March 24, 2010, published as US-2011-0212462 on September 1, 2011, entitled "ULTRA-SENSITIVE DETECTION OF MOLECULES USING DUAL DETECTION METHODS," by Duffy et al.; International Patent Application No. PCT/US2011/026665, filed

March 1, 2011, published as WO 2011/109379 on September 9, 2011, entitled "METHODS AND SYSTEMS FOR EXTENDING DYNAMIC RANGE IN ASSAYS FOR THE DETECTION OF MOLECULES OR PARTICLES," by Rissin et al.; U.S. Patent Application Serial No. 12/731136, filed March 24, 2010, published as US-2011-0212537 on September 1, 2011, entitled "METHODS AND SYSTEMS FOR EXTENDING DYNAMIC RANGE IN ASSAYS FOR THE DETECTION OF MOLECULES OR PARTICLES," by Duffy et al.; U.S. Patent Application Serial No. 13/035,472, filed February 25, 2011, entitled "SYSTEMS, DEVICES, AND METHODS FOR ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES," by Fournier et al.; U.S. Patent Application Serial No. 13/037,987, filed March 1, 2011, published as US-2011-0245097 on October 6, 2011, entitled "METHODS AND SYSTEMS FOR EXTENDING DYNAMIC RANGE IN ASSAYS FOR THE DETECTION OF MOLECULES OR PARTICLES," by Rissin et al.; each herein incorporated by reference. While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified unless clearly indicated to the contrary. Thus, as a non-limiting example, a reference to "A and/or B," when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A without B (optionally including elements other than B); in another embodiment, to B without A (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically

identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

What is claimed:

Claims

1. A method for determining a measure of the concentration of tau protein in a patient sample containing or suspected of containing tau protein, comprising:

performing an assay to determine a measure of the concentration of tau protein in the sample, wherein the limit of detection of tau protein of the assay is less than about 0.2 pg/mL.

2. The method of claim 1, wherein the tau protein is tau 23 (352, 0N3R), tau 24 (383, 0N4R), tau 37 (381, 1N3R), tau 34 (412, 1N4R), tau 39 (410, 2N3R), and/or tau 40 (441, 2N4R), optionally phosphorylated.

3. The method of claim 1, wherein the patient is suspected to have a brain injury, optionally resulting from a hypoxic event.

4. The method of claim 1, wherein the sample is a bodily fluid.

5. The method of claim 4, wherein the bodily fluid is blood or a blood component.

6. The method of claim 4, wherein the bodily fluid is CSF.

7. The method of claim 5, wherein the blood component is plasma or serum.

8. The method of claim 1, wherein the measure of the concentration of tau protein is determined in a plurality of samples taken from the patient over a period of time.

9. The method of claim 8, wherein the plurality of samples are collected within 96 hours after the patient has experienced the brain injury or suspected brain injury, optionally resulting from a hypoxic event.

10. A method of determining a patient's prognosis for recovery from, and/or determining a course of treatment for, a brain injury, wherein the prognosis and/or the

course of treatment is based at least in part on the measure of the concentration of tau protein determined in the patient sample according to the method of any one of claims 1-9.

11. The method of claim 10, wherein the brain injury results from a hypoxic event.

12. A method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury comprising:

performing an assay on a blood sample from the patient and/or plasma and/or serum derived from the blood sample to determine a measure of the concentration of tau protein in the sample; and

determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measured concentration of tau protein present in the sample.

13. The method of claim 12, wherein the brain injury results from a hypoxic event.

14. A method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury, comprising:

determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on a measured concentration of tau protein present in a patient sample, wherein the measured concentration has been determined by performing an assay on the patient sample, which comprises a blood sample from the patient and/or plasma and/or serum derived from the blood sample, to determine the measure of the concentration of tau protein in the sample.

15. The method of claim 14, wherein the brain injury results from a hypoxic event.

16. A method for performing an assay and providing data for determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury, comprising:

performing an assay on a blood sample from the patient and/or plasma and/or serum derived from the blood sample to determine a measure of the concentration of tau protein in the sample; and

providing data from the assay to enable determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measured concentration of tau protein present in the sample.

17. The method of claim 16, wherein the brain injury results from a hypoxic event.

18. A method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury, comprising:

determining a measure of the concentration of tau protein in each of a plurality of samples obtained from the patient following the brain injury; and

determining a prognostic of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measured concentration tau protein present in the sample.

19. The method of claim 18, wherein the brain injury results from a hypoxic event.

20. The method of any one of claims 12-19, wherein the limit of detection for the assay is less than about 10 pg/mL, 9 pg/mL, 8 pg/mL, 7 pg/mL, 6 pg/mL, 5 pg/mL, 4 pg/mL, 3 pg/mL, 2 pg/mL, 1 pg/mL, 0.5 pg/mL, 0.1 pg/mL, 0.05 pg/mL, 0.02 pg/mL.

21. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by cardiac arrest.

22. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by stroke.

23. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by an ischemic event.

24. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by a thrombosis.

25. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by arterial embolism.

26. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by a hemorrhage.

27. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by a high altitude.

28. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by asphyxia.

29. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by an asthmatic event.

30. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by swelling of the brain.

31. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by physical trauma and/or physical injury.

32. The method of any one of claims 12-20, wherein the brain injury comprises a hypoxic event caused by arteriosclerosis or atherosclerosis.

33. The method of any one of claims 12-20, wherein the patient's prognosis is correlated with a Cerebral Performance Category (CPC) score.

34. The method of claim 33, wherein the CPC score includes classifications of good or poor.

fpg35. The method of claim 34, wherein a good CPC score indicates a prognosis of the patient being conscious and alert with normal function or only slight disability or conscious and alert with moderate disability after a selected period of time.

36. The method of claim 34, wherein a poor CPC score indicates a prognosis of the patient being conscious with the patient having a severe disability, being comatose or in a persistent vegetative state, or being brain dead or suffering death from other causes after a selected period of time.

37. The method of claim 34 or 35, wherein the selected period of time is about 1 week, or about 2 weeks, or about 4 weeks, or about 2 months, or about 3 months, or about 4 months, or about 5 months, or about 6 months, or about 9 months, or about 1 year, or about 1.5 years, or about 2 years.

38. The method of any one of claims 12-20, wherein the plurality of samples are obtained from the patient within 2 days, or 2.5 days, or 3 days, or 3.5 days, or 4 days, or 5 days, or 6 days, or 7 days following the brain injury.

39. The method of any one of claims 12-20, wherein 2, or 3, or 4, or 5, or 6, or 7, or 8, or 9, or 10, or 11, or 12, or 13, or 14, or 15, or more, samples are obtained from the patient.

40. The method of any one of claims 12-20, wherein the measure of tau protein concentration in the sample is less than about 1000 pg/mL, or less than about 900 pg/mL, or less than about 800 pg/mL, or less than about 700 pg/mL, or less than about 600 pg/mL, or less than about 500 pg/mL, or less than about 400 pg/mL, or less than about 300 pg/mL, or less than about 200 pg/mL, or less than about 100 pg/mL, or less than about 50 pg/mL, or less than about 30 pg/mL, or less than about 20 pg/mL, or less than about 10 pg/mL, or less than about 5 pg/mL, or less than about 1 pg/mL.

41. The method of any one of claims 12-20, wherein the tau protein is tau 23 (352, 0N3R), tau 24 (383, 0N4R), tau 37 (381, 1N3R), tau 34 (412, 1N4R), tau 39 (410, 2N3R), and/or tau 40 (441, 2N4R), optionally phosphorylated.

42. The method of any one of claims 12-20, wherein a change in the concentration of tau protein in the plurality of samples is determined as a function of time.

43. The method of claim 42, wherein the concentration of tau protein in the plurality of samples is plotted on a graph as concentration versus time.

44. The method of claim 42, wherein the prognosis and/or course or treatment is based at least in part on at least a portion of the area under the curve plotted on the graph.

45. The method of claim 44, wherein the prognosis and/or course or treatment is based at least in part on the area under the curve plotted on the graph for samples obtained in the first 2 days, or 2.5 days, or 3 days, or 3.5 days, or 4 days, or 5 days, or 6 days, or 7 days following the brain injury.

46. The method of claim 42, wherein the prognosis and/or course of treatment is based at least in part on the area under the curve plotted on the graph for a second peak of tau protein concentration.

47. The method of claim 46, wherein the prognosis is good for the recovery from the brain injury if the area under the curve is less than about 800.

48. The method of claim 46, wherein the prognosis is poor for the recovery from the brain injury if the area under the curve is greater than about 800.

49. The method of claim 46, wherein the prognosis is good for the recovery from the brain injury if the area under the curve is less than about 500.

50. The method of claim 46, wherein the prognosis is poor for the recovery from the brain injury if the area under the curve is greater than about 500.

51. The method of any one of claims 12-20, wherein the biomarker or tau protein is measured in bodily fluid.

52. The method of claim 51, wherein the bodily fluid is blood.

53. The method of claim 51, wherein the bodily fluid is plasma and/or serum derived from a blood sample.

54. The method of any one of claims 12-20, wherein the concentration of only a single biomarker is determined.

55. A method of determining a method of treatment for and/or a prognosis of a patient's recovery from a brain injury, comprising:

(a) performing an assay on each of a plurality of samples obtained from the patient following the brain injury to determine the measured concentration of tau protein in each of the samples, wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours;

(b) determining the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time, wherein the area is determined for the entire time period and/or for a second peak in the tau protein concentration; and

(c) determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the area under the curve for the entire time period and/or the second peak in the tau protein concentration determined in step (b).

56. The method of claim 55, wherein the brain injury results from a hypoxic event.

57. A method of determining a method of treatment for and/or a prognosis of a patient's recovery from a brain injury, comprising:

determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time, wherein the area is determined for the entire time period and/or for a second peak in the tau protein concentration, which has been determined by:

(a) performing an assay on each of a plurality of samples obtained from the patient following the brain injury to determine the measured concentration of tau protein in each of the samples, wherein the plurality of samples have been obtained from the patient over a period of time of at least about 48 hours; and

(b) determining the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time for the entire time period and/or for a second peak in the tau protein.

58. The method of claim 57, wherein the brain injury results from a hypoxic event.

59. A method for performing an assay and providing data for determining a method of treatment for and/or a prognosis of a patient's recovery from a brain injury, comprising:

(a) performing an assay on each of a plurality of samples obtained from the patient following the brain injury to determine the measured concentration of tau protein in each of the samples, wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours;

(b) determining the area under the curve of a graph of the tau protein concentration in the plurality of samples versus time, wherein the area under the curve is determined for the entire time period and/or for a second peak in the tau protein concentration; and

(c) providing data derived in steps (a) and (b) to enable determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the area under the curve determined for the entire time period and/or for a second peak in the tau protein concentration.

60. The method of any one of claims 55-59, wherein the limit of detection for the assay is less than about 10 pg/mL, or less than about 9 pg/mL, or less than about 8 pg/mL, or less than about 7 pg/mL, or less than about 6 pg/mL, or less than about 5 pg/mL, or less than about 4 pg/mL, or less than about 3 pg/mL, or less than about 2 pg/mL, or less than about 1 pg/mL, or less than about 0.5 pg/mL, or less than about 0.1 pg/mL, or less than about 0.05 pg/mL, or less than about 0.02 pg/mL.

61. The method of any one of claims 55-60, wherein when the area under the curve for the entire time period is less than about 800, the prognosis is good based on the CPC scale.

62. The method of any one of claims 55-60, wherein when the area under the curve for the entire time period is greater than about 800, the prognosis is poor based on the CPC scale.

63. The method of any one of claims 55-60, wherein when the area under the curve for the second peak in the tau protein concentration is less than about 500, the prognosis is good based on the CPC scale.

64. The method of any one of claim 55-60, wherein when the area under the curve for the second peak in the tau protein concentration is greater than about 500, the prognosis is poor based on the CPC scale.

65. The method of any one of claims 55-60, wherein the baseline concentration for determining the area under the curve for the entire time period is equal to zero.

66. The method of any one of claims 55-60, wherein the baseline concentration for determining the area under the curve of the second peak of tau protein concentration is equal to the concentration of tau protein present in the sample obtained from the patient substantially immediately prior to a second increase in tau protein concentration.

67. The method of any preceding claim, wherein the tau protein is tau 23 (352, 0N3R), tau 24 (383, 0N4R), tau 37 (381, 1N3R), tau 34 (412, 1N4R), tau 39 (410, 2N3R), and/or tau 40 (441, 2N4R), optionally phosphorylated.

68. The method of any preceding claim, wherein tau protein is measured in bodily fluid.

69. The method of claim 68, wherein the bodily fluid is blood.

70. The method of claim 68, wherein the bodily fluid is not CSF.

71. The method of any preceding claim, wherein the assay comprises:

spatially segregating at least a portion of the biomarkers into a plurality of separate locations;

addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing a biomarker molecule; and

determining a measure of the concentration of biomarker in the sample based at least in part on the number of locations determined to contain a biomarker molecule.

72. The method of claim 71, further comprising exposing the plurality of biomarker molecules to a plurality of binding ligands such that at least some of the biomarker molecules associate with a single binding ligand and a statistically significant fraction of the biomarker molecules do not associate with any binding ligand;

73. The method of any preceding claim, wherein the assay comprises:

immobilizing biomarker molecules with respect to a plurality of capture objects such that at least some of the capture objects associate with at least one biomarker molecule and a statistically significant fraction of the capture objects do not associate with any biomarker molecules;

spatially segregating at least a portion of the capture objects subjected to the immobilizing step into a plurality of separate locations;

addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing a biomarker molecule; and

determining a measure of the concentration of biomarker in the sample based at least in part on the number of locations determined to contain a biomarker molecule.

74. The method of any preceding claim, wherein the assay comprises:

exposing a plurality of capture objects that each include a binding surface having affinity for at least one biomarker, to a solution containing or suspected of containing the at least one type of biomarker;

immobilizing biomarker molecules with respect to the plurality of capture objects such that at least some of the capture objects associate with at least one biomarker molecule and a statistically significant fraction of the capture objects do not associate with any biomarker molecules;

spatially segregating at least a portion of the capture objects subjected to the immobilizing step into a plurality of separate locations;

addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing a biomarker molecule; and

determining a measure of the concentration of biomarker in the sample based at least in part on the number of locations determined to contain a biomarker molecule.

75. A method of any preceding claim, wherein the assay comprises:

exposing a plurality of capture objects that each include a binding surface having affinity for at least one type of biomarker, to a solution containing or suspected of containing the at least one type of biomarker to form capture objects comprising at least one immobilized biomarker molecule;

mixing the capture objects prepared in the exposing step to a plurality of binding ligands such that at least some of the capture objects associate with a single binding ligand and a statistically significant fraction of the capture objects do not associate with any binding ligand;

spatially segregating at least a portion of the capture objects subjected to the mixing step into a plurality of locations;

addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of locations containing a binding ligand; and

determining a measure of the concentration of biomarker in the sample based at least in part on the number of locations determined to contain a binding ligand.

76. The method of any preceding claim, wherein in the addressing step, the number of said locations containing a capture object that includes a binding surface having affinity for at least one type of biomarker not containing a biomarker molecule or a binding ligand is determined.

77. The method of claim 76, wherein the measure of the concentration of biomarker in the sample is based at least in part on the ratio of the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of biomarker containing a biomarker molecule or a binding ligand, to the total number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of biomarker.

78. The method of any preceding claim, wherein the measure of the concentration of biomarker molecules in the fluid sample is based at least in part on the ratio of the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of biomarker molecules containing a biomarker molecule or a binding ligand, to the number of locations addressed in the addressing step determined to contain a capture object that includes a binding surface having affinity for at least one type of biomarker molecule but not to contain any capture objects that include a binding surface having affinity for at least one type of biomarker containing a biomarker molecule or a binding ligand.

79. The method of any preceding claim, wherein the plurality of capture objects that include a binding surface having affinity for at least one type of biomarker comprises a plurality of beads.

80. The method of any preceding claim, wherein the plurality of locations comprises a plurality of reaction vessels.

81. The method of any preceding claim, wherein the binding ligand comprises an enzymatic component.

82. The method of any preceding claim, wherein the measure of the concentration of biomarker molecules in the fluid sample is determined at least in part by comparison of a measured parameter to a calibration standard.

83. The method of any preceding claim, wherein following the spatially segregating step, the locations are exposed to precursor labeling agent.

84. The method of claim 83, wherein the precursor labeling agent is converted to a labeling agent upon exposure to a binding ligand.

85. The method of claim 84, wherein the number of locations containing a capture object containing a biomarker molecule or a labeling agent is determined by determining the number of locations comprising a labeling agent.

86. The method of any preceding claim, wherein the average volume of the plurality of reaction vessels is between about 10 attoliters and about 100 picoliters, or between about 1 femtoliter and about 1 picoliter.

87. The method of any preceding claim, wherein the average diameter of the plurality of beads is between about 0.1 micrometer and about 100 micrometers, or between about 1 micrometer and about 10 micrometers.

88. The method of any preceding claim, further comprising performing at least one wash step.

89. The method of any preceding claim, further comprising sealing the plurality of reaction vessels.

90. The method of any preceding claim, wherein the plurality of locations is addressed using optical techniques.

91. The method of any preceding claim, wherein the binding surface comprises a plurality of capture components.

Abstract

The present invention, in some embodiments, generally relates to methods of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury. In some embodiments, the brain injury results from a hypoxic event. In some embodiments, methods are provided for determining a measure of the concentration of tau protein in a patient sample containing or suspected of containing tau protein.

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Applicant/Patent Owner: Quanterix Corporation

Application No./Patent No.: 14/111,326 Filed/Issue Date: October 11, 2013

Titled: METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

Quanterix Corporation (Name of Assignee), a Corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. [X] the assignee of the entire right, title, and interest in;

2. [ ] an assignee of less than the entire right, title, and interest in
(The extent (by percentage) of its ownership interest is _____ %); or

3. [ ] an assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made) the patent application/patent identified above by virtue of either:

A. [X] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 033044, Frame 0544, or for which a copy thereof is attached.

OR

B. [ ] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

3. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

[ ] Additional documents in the chain of title are listed on a supplemental sheet(s).

[X] As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

[signature] Signature	June 24, 2014 Date
Paul Chapman Printed or Typed Name	Chief Executive Officer Title

3606721.1

PTO/SB/81 (01-09)
Approved for use through 11/30/2011. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

POWER OF ATTORNEY OR REVOCATION OF POWER OF ATTORNEY WITH A NEW POWER OF ATTORNEY AND CHANGE OF CORRESPONDENCE ADDRESS

Application Number	14/111,326-Conf. #3274
Filing Date	October 11, 2013
First Named Inventor	David Wilson
Title	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket No.	Q0052.70018US01

I hereby revoke all previous powers of attorney given in the above-identified application.

☐ A Power of Attorney is submitted herewith.

OR

☒ I hereby appoint Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith: 23628

OR

☐ I hereby appoint Practitioner(s) named below as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith:

Practitioner(s) Name	Registration Number	Practitioner(s) Name	Registration Number

Please recognize or change the correspondence address for the above-identified application to:

☒ The address associated with the above-mentioned Customer Number:

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name

Address					
City		State		Zip	
Country		Telephone		Email	

I am the:

☐ Applicant/Inventor.

OR

☒ Assignee of record of the entire interest. See 37 CFR 3.71.

Statement under 37 CFR 3.73(b) (Form PTO/SB/96) submitted herewith or filed on _____

SIGNATURE of Applicant or Assignee of Record

Signature		Date	June 24, 2014
Name	Paul Chapman	Telephone	617-301-9921
Title and Company	Chief Executive Officer, Quanterix Corporation		

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of 1 forms are submitted.

3606722.1

Electronic Acknowledgement Receipt

EFS ID:	26965369
Application Number:	15269142
International Application Number:	
Confirmation Number:	6662
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
Filer:	Andrea J. Gabert/Ellen Paul
Filer Authorized By:	Andrea J. Gabert
Attorney Docket Number:	Q0052.70018US02
Receipt Date:	19-SEP-2016
Filing Date:	
Time Stamp:	15:27:27
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	Q005270018US02-ADS-AJG.pdf	30751 8dd79b09be0fe70850d9770cdb54b7c7dce78b5d	no	7

Warnings:

This is not an USPTO supplied ADS fillable form

Information:					
2	Drawings-other than black and white line drawings	Q005270018US02-DRW-AJG.pdf	57022 b844dcd4b303c4770a8eb5fecc6055ef7e046a59	no	5
Warnings:					
Information:					
3	Oath or Declaration filed	Q005270018US02-INVDEC-AJG.pdf	159175 2bc74f2251d1a754929983874b3b66f3ce9215cb	no	2
Warnings:					
Information:					
4		Q005270018US02-POA373-AJG.pdf	186302 f1256cff536c7214b33c9c08e50738395a0aed9b	yes	2

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Power of Attorney	1	1
	Assignee showing of ownership per 37 CFR 3.73	2	2

Warnings:					
Information:					
5	Transmittal of New Application	Q005270018US02-UTLTRN-AJG.pdf	32455 32490f07a9ee9281a939101e2f0f5ec3efe94684	no	1
Warnings:					
Information:					
6		Q005270018US02-UTLAPL-AJG.pdf	249300 9bf70aff81f1e1b0939e6f2f430d181dd65bed74	yes	66

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Specification	1	52
	Claims	53	65
	Abstract	66	66

Warnings:	
Information:	
Total Files Size (in bytes):	715005

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DECLARATION (37 C.F.R. § 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 C.F.R. § 1.76)

Title of Invention	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

As the below named inventor, I declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/US2012/033343 filed on April 12, 2012

☒ As amended on October 11, 2013 (if applicable);

The above-identified application was made or authorized to be made by me.

I believe the inventor(s) named below to be the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. § 1.56.

All statements made herein of my own knowledge are true, and all statements made herein on information and belief are believed to be true.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. § 1001 by fine or imprisonment of not more than five (5) years, or both.

LEGAL NAME OF SOLE OR FIRST INVENTOR

Inventor one: David Wilson Date: 6/2/14

Signature: [signature] Citizen of: US

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor two: John Henrik Zetterberg Date:

Signature: Citizen of: Sweden

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor three: Kaj Blennow Date:

Signature: Citizen of: Sweden

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor four: Jeffrey D. Randall Date: 6/2/14

Signature: [signature] Citizen of: US

☐ Additional inventors are being named on additional form(s) attached hereto.

3566004.1

DECLARATION (37 C.F.R. § 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 C.F.R. § 1.76)

Title of Invention	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

As the below named inventor, I declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/US2012/033343 filed on April 12, 2012

☒ As amended on October 11, 2013 (if applicable);

The above-identified application was made or authorized to be made by me.

I believe the inventor(s) named below to be the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. § 1.56.

All statements made herein of my own knowledge are true, and all statements made herein on information and belief are believed to be true.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. § 1001 by fine or imprisonment of not more than five (5) years, or both.

LEGAL NAME OF SOLE OR FIRST INVENTOR

Inventor one: David Wilson Date: _____

Signature: _____ Citizen of: US

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor two: John Henrik Zetterberg Date: 01/22/2014

Signature: _____ Citizen of: Sweden

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor three: Kaj Blennow Date: 21/02/2014

Signature: _____ Citizen of: Sweden

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor four: Jeffrey D. Randall Date: _____

Signature: _____ Citizen of: US

☐ Additional inventors are being named on _____ additional form(s) attached hereto.

3566004.1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/269,142	09/19/2016	1629	0.00	Q0052.70018US02	91	8

CONFIRMATION NO. 6662

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

FILING RECEIPT

OC000000088127687

Date Mailed: 09/29/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

David Wilson, Boxborough, MA;
John Henrik Zetterberg, Molnlycke, SWEDEN;
Kaj Blennow, Kungsbacka, SWEDEN;
Jeffrey D. Randall, Canton, MA;

Applicant(s)

Quanterix Corporation, Lexington, MA;

Assignment For Published Patent Application

Quanterix Corporation, Lexington, MA

Power of Attorney: The patent practitioners associated with Customer Number 23628

Domestic Priority data as claimed by applicant

This application is a CON of 14/111,326 06/24/2014
which is a 371 of PCT/US2012/033343 04/12/2012
which claims benefit of 61/524,693 08/17/2011
and claims benefit of 61/474,315 04/12/2011

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: No

Permission to Access Search Results: No

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/28/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/269,142**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No

Title

METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENTÂ#S RECOVERY FROM A BRAIN INJURY

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1044
91

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/269,142	09/19/2016	David Wilson	Q0052.70018US02

CONFIRMATION NO. 6662

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

FORMALITIES LETTER

OC000000086127668

Date Mailed: 09/29/2016

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
- The application search fee must be submitted.
- The application examination fee must be submitted.
- Additional claim fees of $ **10720** as an undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
  The surcharge is due for any one of:
  - late submission of the basic filing fee, search fee, or examination fee,
  - late submission of inventor's oath or declaration,
  - filing an application that does not contain at least one claim on filing, or
  - submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **280** basic filing fee.
- $ **140** surcharge.
- $ **600** search fee.
- $ **720** examination fee.
- $ **2100** for **5** independent claims over 3.
- $ **7840** for **98** total claims over 20.

- $ **780** for multiple dependent claim surcharge.
- $(**0**) previous unapplied payment amount.
- $ **12460** TOTAL FEE BALANCE DUE.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/mabebe/

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
15269142	

Applicant(s) David Wilson

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1					
2		1				
3		1				
4		1				
5		1				
6		1				
7		1				
8		1				
9		1				
10		9				
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12	1					
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15		1				
16	1					
17		1				
18	1					
19		1				
20		8				
21		(1)				
22		(1)				
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24		(1)				
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28		(1)				
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45		(1)				
46		(1)				
47		(1)				
48		(1)				
49		(1)				
50		(1)				
Total Indep	8		0		0	
Total Depend	110	↵	0	↵	0	↵
Total Claims	118		0		0	

	*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend
51		(1)				
52		(1)				
53		(1)				
54		(1)				
55	1					
56		1				
57	1					
58		1				
59	1					
60		5				
61		(1)				
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100						

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/269,142

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	118 minus 20 =	* 98			OR	x 80 =	7840
INDEPENDENT CLAIMS (37 CFR 1.16(h))	8 minus 3 =	* 5				x 420 =	2100
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							780
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	12320

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

Docket No.: Q0052.70018US02
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	15/269,142
Confirmation No.:	6662
Filed:	September 19, 2016
For:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Examiner:	Not Yet Assigned
Art Unit:	1629

Certificate of Electronic Filing Under 37 CFR 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: _____February 28, 2017_____ Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 5 of this paper.

AMENDMENTS TO THE CLAIMS

Please replace all prior versions, and listings, of claims in the application with the following list of claims:

1. (Original) A method for determining a measure of the concentration of tau protein in a patient sample containing or suspected of containing tau protein, comprising:

performing an assay to determine a measure of the concentration of tau protein in the sample, wherein the limit of detection of tau protein of the assay is less than about 0.2 pg/mL.

2. (Original) The method of claim 1, wherein the tau protein is tau 23 (352, 0N3R), tau 24 (383, 0N4R), tau 37 (381, 1N3R), tau 34 (412, 1N4R), tau 39 (410, 2N3R), and/or tau 40 (441, 2N4R), optionally phosphorylated.

3. (Original) The method of claim 1, wherein the patient is suspected to have a brain injury, optionally resulting from a hypoxic event.

4. (Original) The method of claim 1, wherein the sample is a bodily fluid.

5. (Original) The method of claim 4, wherein the bodily fluid is blood or a blood component.

6. (Original) The method of claim 4, wherein the bodily fluid is CSF.

7. (Original) The method of claim 5, wherein the blood component is plasma or serum.

8. (Original) The method of claim 1, wherein the measure of the concentration of tau protein is determined in a plurality of samples taken from the patient over a period of time.

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9. (Original) The method of claim 8, wherein the plurality of samples are collected within 96 hours after the patient has experienced the brain injury or suspected brain injury, optionally resulting from a hypoxic event.

10. (Currently Amended) A method of determining a patient's prognosis for recovery from, and/or determining a course of treatment for, a brain injury, wherein the prognosis and/or the course of treatment is based at least in part on the measure of the concentration of tau protein determined in the patient sample according to the method of ~~any one of claims 1-9~~ claim 1.

11. (Original) The method of claim 10, wherein the brain injury results from a hypoxic event.

12. (Original) A method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury comprising:

performing an assay on a blood sample from the patient and/or plasma and/or serum derived from the blood sample to determine a measure of the concentration of tau protein in the sample; and

determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on the measured concentration of tau protein present in the sample.

13. (Original) The method of claim 12, wherein the brain injury results from a hypoxic event.

14. (Original) A method of determining a treatment protocol for and/or a prognosis of a patient's recovery from a brain injury, comprising:

determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based at least in part on a measured concentration of tau protein present in a patient sample, wherein the measured concentration has been determined by performing an assay on the patient sample, which comprises a blood sample from the patient and/or plasma and/or serum derived from the blood sample, to determine the measure of the concentration of tau protein in the sample.

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15. (Original) The method of claim 14, wherein the brain injury results from a hypoxic event.

16-19. (Cancelled)

20. (Currently Amended) The method of ~~any one of claims 12-19~~ claim 12, wherein the limit of detection for the assay is less than about 10 pg/mL, 9 pg/mL, 8 pg/mL, 7 pg/mL, 6 pg/mL, 5 pg/mL, 4 pg/mL, 3 pg/mL, 2 pg/mL, 1 pg/mL, 0.5 pg/mL, 0.1 pg/mL, 0.05 pg/mL, 0.02 pg/mL.

21. (Currently Amended) The method of ~~any one of claims 12-20~~ claim 12, wherein the brain injury comprises a hypoxic event caused by cardiac arrest.

22. (Currently Amended) The method of ~~any one of claims 12-20~~ claim 12, wherein the brain injury comprises a hypoxic event caused by stroke.

23. (Currently Amended) The method ~~of any one of claims 12-20~~claim 12, wherein the brain injury comprises a hypoxic event caused by an ischemic event.

24. (Currently Amended) The method of ~~any one of claims 12-20~~claim 12, wherein the brain injury comprises a hypoxic event caused by a thrombosis.

25- 91. (Cancelled)

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REMARKS

Claims 1-91 were pending in the application. Claims 16-19 and 25-91 have been cancelled. Claims 10 and 20-24 have been amended. The claims are being cancelled or amended to remove multiple dependencies and/or for the purpose of reducing the excess claims fee and without prejudice or disclaimer. The right to pursue the cancelled subject matter in this application or one or more continuing applications is hereby reserved. No new matter has been added. Claims 1-15 and 20-24 are now pending in this application with claims 1, 12, and 14 being independent.

In view of the above amendment, the pending application is believed to be in condition for examination.

If there is a fee occasioned by this amendment, including an extension fee, the Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith to Deposit Account No. 23/2825 under Docket No. Q0052.70018US02 from which the undersigned is authorized to draw.

Dated: February 28, 2017

Respectfully submitted,

Electronic signature: /Andrea J. Gabert/
Andrea J. Gabert, Ph.D.
Registration No.: 68,904
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Docket No.: Q0052.70018US02
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	15/269,142
Confirmation No.:	6662
Filed:	September 19, 2016
For:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Examiner:	Not Yet Assigned
Art Unit:	1629

Certificate of Electronic Filing Under 37 CFR 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: ____February 28, 2017_____ Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

RESPONSE TO NOTICE TO FILE MISSING PARTS OF APPLICATION

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Notice to File Missing Parts of Application – Filing Date Granted mailed September 29, 2016, Applicant respectfully submits a Preliminary Amendment and the Filing Fee for the Application.

Please charge our Credit Card in the amount of $3,140.00 covering the fees set forth in 37 C.F.R. § 1.16(a), 1.16(f), 1.16(k), and 1.16(o), and 1.17(a). The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith (including any necessary extension of time) to our Deposit Account No. 23/2825, under Docket No. Q0052.70018US02.

5065030.1

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith to our Deposit Account No. 23/2825, under Docket No. Q0052.70018US02.

Dated: February 28, 2017

Respectfully submitted,

Electronic signature: /Andrea J. Gabert/
Andrea J. Gabert, Ph.D.
Registration No.: 68,904
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	15/269,142-Conf. #6662
Filing Date	September 19, 2016
First Named Inventor	David Wilson
Art Unit	1629
Examiner Name	Not Yet Assigned
Attorney Docket Number	Q0052.70018US02

ENCLOSURES (*Check all that apply*)

- [x] Fee Transmittal Form
- [ ] Fee Attached
- [x] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [x] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [x] Reply to Missing Parts/ Incomplete Application
- [x] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
- [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

-Request for Corrected Filing Receipt
-Corrected Filing Receipt showing changes

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Andrea J. Gabert/		
Printed name	Andrea J. Gabert, Ph.D.		
Date	February 28, 2017	Reg. No.	68,904

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: ___February 28, 2017_____ Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

5280128.1

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) Q0052.70018US02
Application Number 15/269,142-Conf. #6662	Filed September 19, 2016
For METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY	
Art Unit 1629	Examiner Not Yet Assigned

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
X	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$ 1,400.00
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed.

X Payment by credit card. ~~Form PTO-2038 is attached~~.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

X The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 23/2825 .

X Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

X attorney or agent of record. Registration number 68,904 .

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Andrea J. Gabert/	February 28, 2017
Signature	Date
Andrea J. Gabert, Ph.D.	617.646.8000
Typed or printed name	Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of 1 forms are submitted.

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: February 28, 2017 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

5280129.1

PTO/SB/17 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995 no persons are required to respond to a collection of information unless it displays a valid OMB control number

FEE TRANSMITTAL

	Complete if known
	Application Number: 15/269,142-Conf. #6662
	Filing Date: September 19, 2016
☐ Applicant asserts small entity status. See 37 CFR 1.27.	First Named Inventor: David Wilson
☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.	Examiner Name: Not Yet Assigned
	Art Unit: 1629
TOTAL AMOUNT OF PAYMENT ($) 3,140.00	Practitioner Docket No.: Q0052.70018US02

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☐ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☐ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	280	140*	70	600	300	150	720	360	180	1,600.00
Design	180	90	45	120	60	30	460	230	115	
Plant	180	90	45	380	190	95	580	290	145	
Reissue	280	140	70	600	300	150	2,160	1,080	540	
Provisional	260	130	65	0	0	0	0	0	0	

* The $140 small entity status filing fee for a utility application is further reduced to $70 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	80	40	20
Each independent claim over 3 (including Reissues)	420	210	105
Multiple dependent claims	780	390	195

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**	**Multiple Dependent Claims**	
				Fee ($)	**Fee Paid ($)**
0 - 20 or HP =	x	=	0		

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
0 - 3 or HP =	x	=	0

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
- 100 =	/50 =	(round **up** to a whole number) x	=	

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1253 Extension for response within third month	1,400.00
1051 Surcharge - Late filing fee, search fee, ...	140.00

SUBMITTED BY

Signature	/Andrea J. Gabert/	Registration No. (Attorney/Agent)	68,904	Telephone	617.646.8000
Name (Print/Type)	Andrea J. Gabert, Ph.D.			Date	February 28, 2017

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: February 28, 2017 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

5280127.1

Docket No.: Q0052.70018US02
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	15/269,142
Confirmation No.:	6662
Filed:	September 19, 2016
For:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Examiner:	Not Yet Assigned
Art Unit:	1629

Certificate of Electronic Filing Under 37 CFR 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: _____February 28, 2017_____ Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

REQUEST FOR CORRECTED FILING RECEIPT

Office of Initial Patent Examination's Filing Receipt Corrections
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant hereby requests that a corrected Filing Receipt be issued in the above-identified patent application. The official Filing Receipt received by Applicant, a copy of which is attached hereto, has an error in the title.

On page 2 of the filing receipt, the title should read "METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A ~~PATIENTÂ#S~~ PATIENT'S RECOVERY FROM A BRAIN INJURY."

Applicant additionally requests that all pertinent U.S. Patent and Trademark Office records relating to the subject application be changed to reflect this correction.

Applicant believes no fee is due with this request. However, if a fee is due, please charge our Deposit Account No. 23/2825 under Docket No. Q0052.70018US02 from which the undersigned is authorized to draw.

Dated: February 28, 2017

Respectfully submitted,

Electronic signature: /Andrea J. Gabert/
Andrea J. Gabert, Ph.D.
Registration No.: 68,904
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1044
107

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/269,142	09/19/2016	1629	0.00	Q0052.70018US02	91	8

CONFIRMATION NO. 6662

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

FILING RECEIPT

OC000000088127687

Date Mailed: 09/29/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

David Wilson, Boxborough, MA;
John Henrik Zetterberg, Molnlycke, SWEDEN;
Kaj Blennow, Kungsbacka, SWEDEN;
Jeffrey D. Randall, Canton, MA;

Applicant(s)

Quanterix Corporation, Lexington, MA;

Assignment For Published Patent Application

Quanterix Corporation, Lexington, MA

Power of Attorney: The patent practitioners associated with Customer Number 23628

Domestic Priority data as claimed by applicant

This application is a CON of 14/111,326 06/24/2014
which is a 371 of PCT/US2012/033343 04/12/2012
which claims benefit of 61/524,693 08/17/2011
and claims benefit of 61/474,315 04/12/2011

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: No

Permission to Access Search Results: No

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/28/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/269,142**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No

Title

METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S ~~PATIENTÂ#S~~ RECOVERY FROM A BRAIN INJURY

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

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countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1044
111

Electronic Acknowledgement Receipt	
EFS ID:	28492657
Application Number:	15269142
International Application Number:	
Confirmation Number:	6662
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENTÂ'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
Filer:	Andrea J. Gabert/Ellen Paul
Filer Authorized By:	Andrea J. Gabert
Attorney Docket Number:	Q0052.70018US02
Receipt Date:	28-FEB-2017
Filing Date:	19-SEP-2016
Time Stamp:	17:57:59
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$3140
RAM confirmation Number	030117INTEFSW17591400
Deposit Account	232825
Authorized User	Wolf Greenfield
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.17 (Patent application and reexamination processing fees) 37 CFR 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Corrected Filing Receipt	Q005270018US02-CFR-AJG.pdf	460021 81c958913505f88101f68ef1e27d55974935bc9a	no	4
Warnings:					
Information:					
2	Extension of Time	Q005270018US02-EXT-AJG.pdf	23855 9d12cfc2ecfbb09412ebaab1fcec8d6912cce5b9	no	1
Warnings:					
Information:					
3	Fee Worksheet (SB06)	Q005270018US02-FEE-AJG.pdf	32072 e4d5b82bf1631b267546d5091fac1a62592feedf	no	1
Warnings:					
Information:					
4	Applicant Response to Pre-Exam Formalities Notice	Q005270018US02-NTMP-AJG.pdf	24564 2c100d2b91e17201268afe316dd6053c32e50c9c	no	2
Warnings:					
Information:					
5		Q005270018US02-PAMN-AJG.pdf	33612 394ce81efea55e320635a833bc9234e01602b945	yes	5

Multipart Description/PDF files in .zip description

Document Description	Start	End
Applicant Arguments/Remarks Made in an Amendment	5	5
Claims	2	4
Preliminary Amendment	1	1

Warnings:					
Information:					
6	Request for Corrected Filing Receipt	Q005270018US02-RCFR-AJG.pdf	24897 c0700f3abdf62779a68323641217294fe56fa7d56	no	2
Warnings:					
Information:					
7	Miscellaneous Incoming Letter	Q005270018US02-TRN-AJG.pdf	25395 8076e2c3d6610f98b215063844b2fd1ae135315d	no	1
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	39558 5d14399f91a3916a26339b3f2c24e7531a7616b4	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	663974		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Patent Application Fee Transmittal

Application Number:	15269142
Filing Date:	19-Sep-2016
Title of Invention:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENTÂ'S RECOVERY FROM A BRAIN INJURY
First Named Inventor/Applicant Name:	David Wilson
Filer:	Andrea J. Gabert/Ellen Paul
Attorney Docket Number:	Q0052.70018US02

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	280	280
UTILITY SEARCH FEE	1111	1	600	600
UTILITY EXAMINATION FEE	1311	1	720	720
Pages:				
Claims:				
Miscellaneous-Filing:				
LATE FILING FEE FOR OATH OR DECLARATION	1051	1	140	140
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1400	1400
Miscellaneous:				
		Total in USD ($)		**3140**

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United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/269,142	09/19/2016	1629	1740	Q0052.70018US02	20	3

CONFIRMATION NO. 6662

UPDATED FILING RECEIPT

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

OC000000089382481

Date Mailed: 03/02/2017

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

David Wilson, Boxborough, MA;
John Henrik Zetterberg, Molnlycke, SWEDEN;
Kaj Blennow, Kungsbacka, SWEDEN;
Jeffrey D. Randall, Canton, MA;

Applicant(s)

Quanterix Corporation, Lexington, MA;

Assignment For Published Patent Application

Quanterix Corporation, Lexington, MA

Power of Attorney: The patent practitioners associated with Customer Number 23628

Domestic Priority data as claimed by applicant

This application is a CON of 14/111,326 06/24/2014
which is a 371 of PCT/US2012/033343 04/12/2012
which claims benefit of 61/524,693 08/17/2011
and claims benefit of 61/474,315 04/12/2011

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: No

Permission to Access Search Results: No

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 09/28/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/269,142**

Projected Publication Date: 06/08/2017

Non-Publication Request: No

Early Publication Request: No

Title

METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENTÂ'S RECOVERY FROM A BRAIN INJURY

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1044
120

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/269,142

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*			OR	x 80 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1600

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/269,142	09/19/2016	David Wilson	Q0052.70018US02	6662

23628 7590 03/27/2017

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER
WEIDNER, ADAM M

ART UNIT	PAPER NUMBER
1649	

NOTIFICATION DATE	DELIVERY MODE
03/27/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	Application No. 15/269,142	Applicant(s) WILSON ET AL.	
	Examiner ADAM M. WEIDNER	Art Unit 1649	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *2/28/17*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-15 and 20-24* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-15 and 20-24* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

DETAILED ACTION

This action is in response to claim amendments filed 2/28/17. Claims 1-15 and 20-24 are pending and under examination.

Claim Objections

Claims 2-3 and 9 are objected to because of the following informalities:

The claim(s) currently use exemplary language (e.g. such as, optionally, preferably, in particular, etc.). Such language does not further limit the claim but potentially causes confusion over the claim scope. See MPEP § 2173.05(d) which states that "[d]escription of examples or preferences is properly set forth in the specification rather than the claims. If stated in the claims, examples and preferences may lead to confusion over the intended scope of a claim". Therefore, exemplary language should either be positively recited as a claim limitation or removed from the claim altogether.

Claim 20 is objected to because the list of detection limits should include the word "or" prior to the last member of the list.

Appropriate correction is required.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 9-11 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Claim 10 requires that a decision is made based at least in part on the measurement according to claim 1. However, it is unclear if the claim requires performing the method as part of claim 10 or if it is sufficient for one practicing the method of claim 10 to simply obtain these measurements, the measurement itself having been performed by another (similar to claim 14, which clearly does not require the artisan to actually perform the measurements to infringe the claim).

Claim 9 recites the limitation "the brain injury or suspected brain injury" in 2. There is insufficient antecedent basis for this limitation in the claim. Neither claim 8 nor claim 1 recite or require a patient with a brain injury or suspected brain injury and thus it is unclear to what this limitation is referring.

Therefore, claims 9-11 are indefinite.

The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-13 and 20-24 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled

in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claim 1 is a "method for determining a measure of the concentration of tau protein" wherein the only step is "performing an assay to determine a measure of the concentration of tau protein". The assay has no objective, repeatable steps and can be any possible method save the functional requirement of the assay to detect less than "about 0.2 pg/mL".

Thus, the breadth of the claim encompasses any and all methods that detect tau at the recited threshold. However, the instant specification discloses one such assay (e.g., p.46-49) with several documents cited in support of details "associate[d] with **this** assay" (p.48; emphasis added), supporting the conclusion that only one such assay is disclosed. However, a single example of one assay is insufficient to represent the full genus of all assays capable of the required sensitivity nor do the specification or claims set forth the common structures or steps required to define this genus.

Vas-Cath Inc. v. Mahurkar, 19USPQ2d 1111, clearly states "applicant must convey with reasonable clarity to those skilled in the art that, as of filing date sought, he or she was in possession of the invention. The invention is, for purposes of the 'written description' inquiry, whatever is now claimed." (See page 1117). The specification does not "clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed." (See Vas-Cath at page 1116). As discussed above, the artisan cannot envision the details of such assays commensurate with what is now claimed nor assays beyond the one disclosed in the instant specification, and therefore conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the method of isolation. Adequate written description requires more than a mere statement that it is part of the invention and reference to a potential method of isolating it. See Fiers v. Revel, 25 USPQ2d 1601 at 1606 (CAFC 1993) and Amgen Inc. v. Chugai Pharmaceutical Co. Ltd., 18 USPQ2d 1016. In the same way, adequate written description requires more than a statement that all assays are part of the invention coupled with an invitation for others to discover such assays.

One cannot describe what one has not conceived. See Fiddes v. Baird, 30 USPQ2d 1481 at 1483. In Fiddes, claims directed to mammalian FGF's were found to be unpatentable due to lack of

written description for that broad class. The specification provided only the bovine sequence. Here, there is no evidence that Applicant conceived of any method other than the one disclosed.

Thus, claim 1 does not meet the written description requirement. Dependent claims do not set forth any additional steps of this "assay" and are deficient for the same reason. Note that while claims 8 and 9 indicate multiple samples are taken or that the samples are collected within a certain period of time, this does not define the assay being used to actually measure the samples nor do these steps address the deficiencies as set forth above.

Claim 12 is even broader in that the "assay" does not require any particular sensitivity and is deficient for the same reasons as above. Dependent claims thereof do not address this deficiency.

Essentially, the claim is defined by a result to be achieved. Applicant claims a method of measuring tau whereby the only claim step is "measure tau" and defines such measurement by a detection threshold rather than any objective steps or structure. See MPEP §2163(I)(A) which states:

> "The claimed invention as a whole may not be adequately described where an invention is described solely in terms of a method of its making coupled with its function and there is no described or art recognized correlation or relationship between the structure of the invention and its function. A biomolecule sequence described only by a functional characteristic, without any known or disclosed correlation between that function and the structure of the sequence, normally is not a sufficient identifying characteristic for written description purposes, even when accompanied by a method of obtaining the claimed sequence."

Such is the case here, where the claims are described wholly by a sensitivity to be achieved; this is insufficient to meet the written description requirement.

Therefore, claims 1-13 and 20-24 do not meet the written description requirement.

Claims 1-13 and 20-24 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for measuring tau using the disclosed assay, does not reasonably provide enablement for the breadth of utilizing all possible assays that measure tau, even when limited to those assays with a specific limit of detection. The specification does not enable

any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

Claim 1 is a "method for determining a measure of the concentration of tau protein" wherein the only step is "performing an assay to determine a measure of the concentration of tau protein". The assay has no objective, repeatable steps and can be any possible method save the functional requirement of the assay to detect less than "about 0.2 pg/mL".

Thus, the breadth of the claim encompasses any and all methods that detect tau at the recited threshold. However, the instant specification discloses one such assay (e.g., p.46-49) with several documents cited in support of details "associate[d] with **this** assay" (p.48; emphasis added), supporting the conclusion that only one such assay is disclosed. Thus, the instant claims cover all possible methods while offering no more than a single example and an invitation to discover other methods which might have the required limit of detection; this is undue experimentation.

The claims are interpreted as "single means" claims. See MPEP § 2181 (V). In particular, this section of the MPEP notes that a single means claim cannot invoke §112, sixth paragraph and as the instant claims are not an explicit "means-plus-function" claim, the claims are not limited by the structure, material, or act disclosed in the specification. The instant fact pattern is similar to that in *In re Hyatt*, 798 F.2d 712, 218 USPQ 195 (Fed. Cir. 1983), wherein a single means claim which covered every conceivable means for achieving the stated purpose was held nonenabling for the scope of the claim because the specification at most disclosed only those means known to the inventors. When claims depend on a recited property, a fact situation comparable to Hyatt is possible, where the claim covers every conceivable structure (means) for achieving the stated property (result) while the specification discloses at most only those known to the inventor. Note the statement in *Hyatt*

> "the language [of the final paragraph of §112] does not go so far as to permit a so-called single means claim, that is a claim which recites merely one means plus a statement of function and nothing else. Attempts to evade this by adding purely nominal elements to such a claim will undoubtedly be condemned."

In this case, the claim recites "merely one means" ("assay to" does not imply any structure and is a generic placeholder; see MPEP §2181) and a function (measure tau with a detection limit of about 0.2 pg/mL) as the only limitations of the claim. Such a single means claim fails to meet the enablement requirement in full.

See also Fiers v. Sugano, 984 F.2d 164, 25 USPQ2d 1601 (Fed. Cir. 1993) and MPEP §2164.08(a). There is an unknown multitude of other specific methods that potentially could accomplish the claimed function and no structural requirement or particular steps is recited for any of the assays in the instant claims. Therefore, the specification fails to provide enough guidance for one skilled in the art on how to practice the instant method, thereby requiring trial and error experimentation to identify methods meeting the functional limitations of the claims. The general knowledge and level of skill in the art do not supplement the omitted description because specific, not general, guidance is what is needed.

Therefore, claims 1-13 and 20-24 are not enabled for the full scope of the claims.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 10-15 and 20-24 are rejected under 35 U.S.C. § 101 because the claims as a whole are not directed to patent eligible subject matter. Based upon an analysis with respect to the claim as a whole, these claims do not recite something significantly more than a judicial exception. The rationale for this determination is explained below:

Guidance with respect to patent eligibility under 35 U.S.C. § 101 has been provided, effective 16 December 2014 and published in the Federal Register V 79 No 241 (please visit http://www.uspto.gov/patents/law/exam/interim_guidance_subject_matter_eligibility.jsp for the published guidance). This guidance has been provided in light of recent court decisions such as *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 569 U.S. __, 133 S. Ct. 2107, 2116, 106, USPQ2d 1972

(2013), *Mayo Collaborative Services v. Prometheus Laboratories, Inc.*, 566 U.S. __, 132 S. Ct. 1289, 101 USPQ2d 1961 (2012), and *Alice Corporation PTY. LTD v CLS Bank International*.

The first step under this guidance is determining if the claim is directed to one of the four statutory categories (process, machine, manufacture, or composition of matter). In this case, the claims are methods (a process). The second step is determining if the claims recite or involve judicial exception(s), such as laws of nature, natural phenomena, natural products, or an abstract idea. In this case, the claims are drawn to judicial exceptions: "determining a prognosis…based at least in part on the measure concentration of tau protein".

Thus, it must be determined if the claim as a whole recites something significantly more than the judicial exceptions.

Regarding claims 14-15 (and claims 10-11 under one interpretation; see above), the claim is entirely abstract. The method clearly sets forth that the measuring of tau has already been completed prior to the start of the claimed method and such measurement in not required to be performed by the artisan as part of the claimed method, e.g., "the measured concentration has been determined" (emphasis added). All that is required to infringe on the claim is for, by way of example, an individual to read a report in which these tau numbers are reported and make a mental determination. Abstract ideas, including "mental processes" (MPEP §2106(II)) are not patent eligible.

Regarding the remaining rejected claims, where a claim describes a judicial exception, such a claim "requires closer scrutiny for eligibility because of the risk that it will 'tie-up' the excepted subject matter and pre-empt others from using [the judicial exception]" (federal register, p.74622, C1). While all inventions to some degree involve natural laws, products, and other judicial exceptions, the new guidance regarding patent eligibility makes clear that a practical application of these exceptions is necessary, offering "significantly more" than the exception itself. Limitations that were found not to be enough to qualify as "significantly more" include:

- Mere instructions to implement an abstract idea on a computer
- Adding generic instructions that the judicial exception should be used ("apply it")

- Simply appending well-understood, routine and conventional activities previously known to the industry, specified at a high level of generality
- Adding insignificant extrasolution activity to the exception ("mere data gathering")
- Generally linking the use of the exception to a particular technological environment or field of use

Claim 1 is an independent claim. Based on the newest life sciences guidance regarding §101 (published May 2016), claim 1 is eligible as it follows the fact pattern of the Julitis example claim 1, where the claim is no more than measuring/detecting the protein. However, claim 10 and independent claim 12 introduce the judicial exception of calling out the natural correlation between this protein and determining a treatment protocol/prognosis. Such limitations follow the pattern of Julitis claim 2, which is ineligible. This is also similar to the fact pattern of the claims at issue in *Mayo*, where a drug was administered, the natural metabolites were measured, and a conclusion was made about treatment efficacy; this was deemed ineligible by the Supreme Court.

In Mayo, a level of the metabolite "less than about 230 pmol per $8x10^8$ red blood cells [indicated] a need to increase the amount of said drug". Such claim limitations were found to be a description of the correlation itself and not "significantly more". It was also found that steps prior to this constituted routine data gathering, instructing the artisan to engage in conventional, well-known practices and then "warning" the user of the implications of the data; this was not eligible in Mayo.

The step of "determining" (mental) as well as the correlation between tau levels to prognosis/treatment protocol are judicial exceptions which must be set aside to determine if the claims are significantly more than a recitation of these exceptions.

For claim 12, the only other element of the claim is a generic indication that tau should be measured in a blood sample and encompasses any and all means of doing so. However, this represents a necessary step for anyone seeking to utilize the natural correlation of tau in blood to the diagnosis/treatment protocol and thus "ties up" the exception itself. Moreover, "mere data gathering" is generally recognized as insufficient, being characterized as "insignificant extrasolution activity to the exception".

Claims 13 and 21-24 simply describe the population on which to perform this method. This is a further description of the correlation (where the correlation exists) and must likewise be set aside and cannot offer "significantly more".

Claim 20 sets forth detection limits for the assay. However, this does not convey any structural requirements for the assay. In one interpretation, this is merely a description of the natural correlation itself, informing the artisan of the ranges in which the correlation is observable. In another, it is generic instructions to use assays of high sensitivity; using sensitive assays to detect proteins represents no more than conventional wisdom in the art. In either case, such a description does not offer significantly more.

Claim 10 recites solely the judicial exception, relying on claim 1 for the method. However, the method of claim 1 is "measure tau", which is required and unfairly ties up the judicial exception. The limitation that the assay has a detection limit of 0.2 pg/mL does not add significantly more for the same reasons as claim 20.

Claim 11 further describes the correlation and must set aside for the same reasons as above.

Finally, note that in the Julitis example, a generic, well-known assay was not patent eligible but an assay using a novel antibody (mAbD33) was. The instant claims are not limited to any particular assay; however, assays of the required specificity were well-known and routinely used to measure proteins. See for example Rissin (“single molecule enzyme linked immunosorbent assay detects serum proteins as subfemtomolar concentrations”; cited on form 892). Here, Rissin describes antibodies attached to beads, which then bind a target antigen, followed by binding the antigen with a biotin-labeled antibody, where only some of the beads bind the biotin-antibody, dividing these beads into wells, and imaging them to determine the number of locations containing the biotin-antibody by exposure to a streptavidin-ß-gal (streptavidin-enzyme) conjugate in order to determine the concentration of the antigen (e.g., figure 1). While Rissin does not explicitly use the technique to measure tau, Rissin teaches the technology is broadly applicable to detecting single protein molecules in blood for sensitive diagnostic assays (abstract). The instant claims do not require any particular, novel antibody or other unique reagent instead only defining the assay by a detection limit. Thus, once informed of the relevant correlation, the "digital ELISA" technique would have been a well-known choice using conventional, commercially available

monoclonal antibodies in order to measure tau concentration at the required sensitivity. Thus, such an assay does not provide significantly more than instructions to a relevant audience to use known techniques to observe a natural phenomenon and to "apply it". This is not patent eligible.

See also (all cited on 892):

- Harma 2000 *Clinical Chemistry*
- Gorris 2010 "analytical chemistry on the femtoliter scale"
- Ekins 2010 "*Clinical Chemistry*
- Duffy 2011 "immunoassays with broad dynamic ranges based on combining digital and digitally enhanced analog detection of enzyme labels"
- *Chen 2009 *AIChE*
- Xie 2009 *JACS*
- Burton 2010 *Lab Chip*
- Agrawal 2007 *J Agric Food Chem1*
- Quaternix 2010 *IQT Technology Focus Day*
- 2011 "Quanterix discovers link between heart attack-induced hypoxia suspected Alzheimer's disease pathway"
- US 20110245097
- US 20100075407
- US 20110212537
- US 20110212848

This list is not exhaustive, but illustrates that at the time of or before the instant invention, an assay meeting the criteria of the instantly claimed assay was already publicly known and had been disclosed as measuring numerous different proteins from cancer antigens to Aß to tau. For example, the Quaternix document above ("single molecule arrays for digital detection in complex samples" *IQT technology focus day* presented 25 March 2010, more than one year before the instant priority date of 12 April 2011) describes using antibodies bound to beads to capture a "target protein", followed by a biotin-labeled

detection antibody, performed in a plurality of wells (p.13), suggests its use relative to neurological diseases (p.19), and explicitly states that the method was used to measure tau in blood of AD patients and age-matched controls (p.22). The document also teaches measuring tau in relation to TBI (p.29).

The instant claims are similar to the claims at issue in Mayo, in which the Supreme Court indicated that a correlation that is a consequence of how a certain compound is metabolized was patent ineligible. Observation that the natural expression of tau is somehow correlated to brain injury with general instructions that the information should be somehow "applied" does not add significantly more to the exception itself and is thus patent ineligible. In Mayo, the data was also gathered in a way already known and used in the prior art and it was only the observation of the correlation that was “inventive”, which the Court deemed was not enough to render a claim patent eligible.

In summary, the above analysis conducted in line with all current guidance issued by the USPTO sets forth the rationale for deeming the above claims patent ineligible. It is appreciated that all inventions at some level embody, use, reflect, rest upon, or apply a law of nature, natural phenomenon, or abstract idea. However, the instant claims do not improve another technology, apply the judicial exception with any specific machine, transform the gathered data into a different state or thing, add a limitation that is anything more than what is well-understood, conventional, or routine in the field, or any other meaningful limitation beyond generally linking the use of the exception to a particular technological environment. In other words, the claims as a whole instruct a user to observe tau using conventional samples and a known methodology and then informs this relevant audience about a correlation that exists between the gathered data and a prognosis/treatment protocol. The added steps of determining the prognosis “based at least in part” on the gathered data is no more than instructions to “apply it”.

Therefore, claims 10-15 and 20-24 are not patent eligible.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claim(s) 1-5 and 7 are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Quaternix 2010 IQT Technology Focus Day (hereinafter Quaternix 2010a).

Regarding claim 1, Quaternix 2010a teaches an assay to measure tau in blood of AD patients and age matched controls (p.22). Thus, Quaternix 2010a performs an assay to determine a measure of the concentration of tau protein in a patient sample containing tau protein. Regarding the detection limit of the assay, Quaternix 2010a teaches detection of single molecules (p.5) and detection in the aM range (p. 4, 10, 11, 14). Thus, the Quaternix 2010a assay clearly has a detection limit at or below "about 0.2 pg/mL".

Regarding claim 2, Quaternix 2010a teaches measurement of total tau in a human blood sample and thus necessarily includes measurement of the recited isoforms of tau.

Regarding claim 3, Quaternix 2010a teaches measuring tau in blood of patients suffering traumatic brain injury (p.29: "You want a nice serum marker for TBI, you do it through tau").

Regarding claim 4, the sample is a body fluid (blood).

Regarding claim 5, the sample is blood.

Regarding claim 7, Quaternix 2010a teaches the sample is plasma or serum (p.13).

Therefore, claims 1-5 and 7 are anticipated by Quaternix 2010a.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 1-9 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Quaternix 2010a.

Claims 1-5 and 7 are anticipated by Quaternix 2010a and obvious for the same reasons. Further, selection of a specific tau isoform would have been a predictable variation of the disclosure of Quaternix 2010a given the robust nature of the technology to accurately measure tau and a variety of other proteins with such sensitivity. In the same way, it would have been a predictable variation of the disclosure to measure tau in any sample suspected of containing tau. Quaternix 2010a teaches the assay detects tau in blood of healthy controls and thus one of ordinary skill in the art at the time of the invention would have had a reasonable expectation of success when using the method to measure tau in any subject irrespective of that subject's health/disease, including those suffering a hypoxic event.

Regarding claim 6, Quaternix 2010a also teaches that tau is altered in TBI patients in the CSF. Thus, while not explicitly measuring tau in such a sample, one of ordinary skill in the art would have had a reasonable expectation of success when using a CSF sample to detect tau.

Regarding claim 8, Quaternix 2010a teaches measuring a plurality of samples (e.g., p.22) but does not explicitly teach these are from the same subject. As noted by the United States Supreme Court, if a person of ordinary skill can implement a predictable variation, § 103 likely bars its patentability. As Quaternix 2010a teaches the method measures tau in a sample for multiple subjects including healthy

and AD patients, repeating the test on another sample from the same subject is no more than a predictable, obvious variation of the method.

Regarding claim 9, the claim is indefinite as above. Inasmuch as the claim requires collection of a second sample within 96 hours of the first sample taken, it is noted that "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." In re Aller, 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955). See MPEP §2144.05(II). In this case, Quaternix 2010a discloses the whole of the methodology including taking a plurality of samples; thus, the difference in claim 9 is only in that Quaternix 2010a does not explicitly state that samples are taken from the same patient within 96 hours of each other. However, this is a predictable variation dependent on the results to be obtained. Instructions which optimize the timing of the samples to be taken does not make the claim non-obvious where all other elements were already known and disclosed in the prior art.

Therefore, claims 1-9 would have been obvious over Quaternix 2010a.

Claims 1-15, 20, and 22-24 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Quaternix "single molecule arrays for digital detection in complex samples" *IQT technology Focus Day* (hereinafter Quaternix 2010a) in view of Wunderlich (cited on form 892).

Claims 1-9 would have been obvious over Quaternix 2010a as above.

Quaternix 2010a describes the bead-based SiMoA (single molecule array) immunoassay (p.13). Quaternix 2010a teaches a monomeric bead attached to an antibody (see bead at left of page 13) exposed to blood plasma ("100 µl of serum or plasma as test sample") to form a bead comprising at least one immobilized protein molecule (second bead image on page 13). Quaternix 2010a then discloses exposing (mixing) the bead to an antibody/biotin conjugate followed by exposure to an antibody-enzyme complex. This is followed by using the enzyme to determine the concentration of the protein by spatially segregating the reporter. This can also be seen on page 5 where samples are partitioned into a plurality of well and each reporter is individually counted (where a statistically significant number of well are not associated), page 12 where the reporters are spatially separated and on page 14 where the number of

"active wells" is measured. Quaternix 2010a teaches using this technology for diagnostic use in neurological diseases such as Alzheimer's (p.19) and explicitly uses this assay to measure tau in blood of AD patients and age matched controls (p.22). Thus, Quanternix 2010a would have rendered obvious claims 1-9 as discussed above. Quaternix 2010a states "You want a nice serum marker for TBI, you do it through tau" (p.29). While Quaternix 2010a suggests the use of tau as a diagnostic marker for TBI, Quaternix 2010a follows this with "you may have a use" and "you may be able to get". These qualified statements in conjunction with a lack of experimental evidence of this relationship would not be sufficient on its own to provide a reasonable expectation of success, a best representing a hope that such a correlation exists.

Wunderlich measures tau in multiple samples from the same patient (66 such patients) over the course of 120 hours after stroke, eight out of nine measurements being made at 96 hours or less after stroke onset. Wunderlich teaches that tau protein values were significantly correlated with functional outcome at 3 months, is associated with clinical deficits and infarct volume, and can be used as an additional predictor of the early course of stroke and functional outcomes (abstract).

Regarding claim 9, under an interpretation of the claim where the patient actually has a brain injury, Wunderlich provides motivation for one of ordinary skill in the art at the time of the invention to measure tau in a subject with a brain injury at multiple times within 96 hours after said injury as Wunderlich demonstrates that tau values in this window are correlated with prognosis after stroke.

Regarding claim 10, Wunderlich teaches that tau serum levels are correlated with stroke prognosis. Thus, one of ordinary skill in the art at the time of the invention would have found it obvious to utilize such tau concentrations at least in part for determining a patient's prognosis. Obtaining those values using the methodology of Quaternix 2010a would have been obvious for the same reasons that claim 1 would have been obvious above. In the reverse, Quaternix 2010a suggests without evidence that tau is correlated to stroke and Wunderlich provides the experimental evidence to support that conclusion, thus providing the motivation to the ordinary artisan to reasonably expect the method of Quaternix 2010a to provide useful information when determining a patient's prognosis after brain injury.

Regarding claim 11, stroke is a hypoxic event (e.g., instant claim 22).

Regarding claim 12, measuring tau in blood/ serum and using this information to make a determination of patient prognosis would have been obvious for the same reasons above.

Regarding claim 13, stroke is a hypoxic event.

Regarding claim 14, using information about tau concentration in a patient's serum to determine a patient prognosis would have been obvious as above.

Regarding claim 15, stroke is a hypoxic event.

Regarding claim 20, the detection limit of the method of Quaternix 2010a is less than about 10 pg/ml as discussed above.

Regarding claim 22, Wunderlich teaches the correlation between tau and stroke prognosis as above.

Regarding claim 23, Wunderlich teaches the stroke is ischemic stroke.

Regarding claim 24, Wunderlich teaches performing thrombolysis on the patients with ischemic stroke (p.560 C1; table 1). Thus, Wunderlich clearly includes thrombolic stroke (where the hypoxic event is caused by a thrombosis). Further, thrombosis causing such hypoxic events is well known and one of ordinary skill in the art at the time of the invention would have had a reasonable expectation that the findings of Wunderlich could be predictably applied to such subject, particularly as Wunderlich notes that thrombolysis did not significantly lower tau values.

Therefore, claims 1-15, 20, and 22-24 would have been obvious over Quaternix 2010a and Wunderlich.

Claims 1-15, 20-21, and 23 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Quaternix 2010a in view of Mortberg (cited on form 892).

Claims 1-9 would have been obvious over Quaternix 2010a as above.

Regarding claim 10, Mortberg teaches measuring tau in plasma after cardiac arrest at 2, 6, 12, 24, 48, and 96 hours (p.28). Mortberg teaches there is a significant difference in tau levels between patients with a good outcome versus a poor outcome (p.48).

Thus, one of ordinary skill in the art at the time of the invention would have found it obvious to utilize such tau concentrations at least in part for determining a patient's prognosis as Mortberg teaches tau levels are correlated with prognosis. Obtaining those values using the methodology of Quaternix 2010a would have been obvious for the same reasons that claim 1 would have been obvious above.

Regarding claim 11, cardiac arrest is a hypoxic event that causes brain injury.

Regarding claim 12, measuring tau in blood/ serum and using this information to make a determination of patient prognosis would have been obvious for the same reasons above.

Regarding claim 13, cardiac arrest is a hypoxic event.

Regarding claim 14, using information about tau concentration in a patient's serum to determine a patient prognosis would have been obvious as above.

Regarding claim 15, cardiac arrest is a hypoxic event.

Regarding claim 20, the detection limit of the method of Quaternix 2010a is less than about 10 pg/ml as discussed above.

Regarding claim 21, Mortberg teaches the correlation between tau and cardiac arrest prognosis as above.

Regarding claim 23, cardiac arrest is an ischemic event.

Therefore, claims 1-15, 20-21, and 23 would have been obvious over Quaternix 2010a and Mortberg.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re*

Goodman, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1-15 and 20-24 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 1-11, 15, and 23-30 of copending Application No. 14111331 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the reference application is directed to the same methodology:

- detection limit less than 0.2 pg/mL, e.g., claim 1
- plurality of samples within 96 hours, e.g., claim 9
- same samples, e.g., claims 6,7

The difference is in the reference application being drawn to a "first biomarker" (claim 15) rather than explicitly tau. However, the specification of the reference application clearly indicates tau is one such biomarker (paragraph 39) indicating that one of ordinary skill would be aware of such biomarkers.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Claims 1-15 and 20-24 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 55, 92-102, 108-115 of copending Application No. 14111326 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because the reference claims are more specific and fully anticipate the instant claims.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Conclusion

No claim is allowed.

US 20100075407, US 20100075862, US 20110212462, US 20110212537, US 20110212848, and US 20110245097 (see form 892) all appear to be relevant to the assay steps, i.e., to the data gathering steps of the method. However, these documents fail to correlate tau levels with brain injury.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ADAM M. WEIDNER whose telephone number is (571)272-3045. The examiner can normally be reached on Monday - Friday, 800 - 1630 EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jeffrey Stucker can be reached on 571-272-0911. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/ADAM M WEIDNER/

Primary Examiner, Art Unit 1649

Notice of References Cited	Application/Control No. 15/269,142	Applicant(s)/Patent Under Reexamination WILSON ET AL.	
	Examiner ADAM M. WEIDNER	Art Unit 1649	Page 1 of 4

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Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 15/269,142	Applicant(s)/Patent Under Reexamination WILSON ET AL.	
	Examiner ADAM M. WEIDNER	Art Unit 1649	Page 2 of 4

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	L	US-				
	M	US-				

FOREIGN PATENT DOCUMENTS

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Notice of References Cited	Application/Control No. 15/269,142	Applicant(s)/Patent Under Reexamination WILSON ET AL.	
	Examiner ADAM M. WEIDNER	Art Unit 1649	Page 3 of 4

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FOREIGN PATENT DOCUMENTS

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	Examiner ADAM M. WEIDNER	Art Unit 1649	Page 4 of 4

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FOREIGN PATENT DOCUMENTS

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	N					
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15269142	WILSON ET AL.
	Examiner	Art Unit
	ADAM M WEIDNER	1649

CPC- SEARCHED		
Symbol	Date	Examiner

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
inventor/assignee east	3/17/17	amw
text/claims east/internet	3/17/17	amw

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S82	20	S80 and (tbi or stroke or hypoxic or (cardiac arrest) or thrombosis)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:52
S81	6	S79 and tau.clm.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:51
S80	62	S79 and tau	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:50
S79	7139	S76 or S78	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:50
S78	7117	((wilson near2 david) or (john near2 zetterber) or (kaj near2 blennow) or (jeffrey near2 randall)).in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:50
S77	10073	S75 or S76 or S74	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:50
S76	30	quanterix.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:50
S75	8920	duffy.in. or rissen.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:50
S74	1153	tufts.as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO	ADJ	ON	2017/03/17 10:50

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CONFIRMATION NO. 6662

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/269,142	09/19/2016 RULE	435	1649	Q0052.70018US02

APPLICANTS

Quanterix Corporation, Lexington, MA;

INVENTORS

David Wilson, Boxborough, MA;
John Henrik Zetterberg, Molnlycke, SWEDEN;
Kaj Blennow, Kungsbacka, SWEDEN;
Jeffrey D. Randall, Canton, MA;

**** CONTINUING DATA ***************************

This application is a CON of 14/111,326 06/24/2014
which is a 371 of PCT/US2012/033343 04/12/2012
which claims benefit of 61/524,693 08/17/2011
and claims benefit of 61/474,315 04/12/2011

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

09/28/2016

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /ADAM M WEIDNER/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		MA	5	20	3

ADDRESS

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

TITLE

METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENTÂ#S RECOVERY FROM A BRAIN INJURY

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/269,142	09/19/2016	1649	1740	Q0052.70018US02	20	3

CONFIRMATION NO. 6662

CORRECTED FILING RECEIPT

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

OC000000090472153

Date Mailed: 04/10/2017

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

David Wilson, Boxborough, MA;
John Henrik Zetterberg, Molnlycke, SWEDEN;
Kaj Blennow, Kungsbacka, SWEDEN;
Jeffrey D. Randall, Canton, MA;

Applicant(s)

Quanterix Corporation, Lexington, MA;

Assignment For Published Patent Application

Quanterix Corporation, Lexington, MA

Power of Attorney: The patent practitioners associated with Customer Number 23628

Domestic Priority data as claimed by applicant

This application is a CON of 14/111,326 06/24/2014
which is a 371 of PCT/US2012/033343 04/12/2012
which claims benefit of 61/524,693 08/17/2011
and claims benefit of 61/474,315 04/12/2011

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/269,142**

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Title

METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
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WOLF GREENFIELD & SACKS, P.C.
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The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

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Docket No.: Q0052.70018US02
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	15/269,142
Confirmation No.:	6662
Filed:	September 19, 2016
For:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Examiner:	A. M. Weidner
Art Unit:	1649

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AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. § 1.111

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

In response to the Office Action dated March 27, 2017, please amend the above-identified U.S. patent application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 7 of this paper.

AMENDMENTS TO THE CLAIMS

Please replace all prior versions, and listings, of claims in the application with the following list of claims:

Please replace all prior versions, and listings, of claims in the application with the following list of claims:

1-11. (Cancelled)

12. (Currently Amended) A method of determining ~~a treatment protocol for and/or~~ a prognosis of a patient's recovery from a brain injury comprising:

(a) performing an assay on each of a plurality of blood samples obtained from the patient following the brain injury and/or plasma and/or serum derived from each of the blood samples to determine a measure of the tau protein molecule concentration ~~of tau protein~~ in each ~~the~~ sample,

wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours,

wherein at least one of the plurality of samples is obtained within 12 hours of the brain injury,

wherein the assay comprises spatially segregating and detecting single tau protein molecules, and

wherein the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL;

(b) determining an area under a curve of a graph of the measure of the tau protein molecule concentration in the plurality of samples versus time, wherein the area is determined for the total period of time; and

(c) determining a prognosis of the patient's recovery from the brain injury ~~and/or a method of treatment~~ based ~~at least in part~~ on the area under the curve determined in step (b)~~the measured concentration of tau protein present in the sample~~.

13. (Original) The method of claim 12, wherein the brain injury results from a hypoxic event.

14-20. (Cancelled)

21. (Previously presented) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by cardiac arrest.

22. (Previously presented) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by stroke.

23. (Currently Amended) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by an ischemic event.

24. (Previously presented) The method of claim 12, wherein the brain injury comprises a hypoxic event caused by a thrombosis.

25- 91. (Cancelled)

92. (New) The method of claim 12, further comprising determine a method of treatment based at least in part on the area under the curve determined in step (b).

93. (New) The method of claim 12, wherein tau protein molecules are spatially segregated into a plurality of locations, wherein a statistically significant fraction of the locations contain a single tau protein molecule and a statistically significant fraction of the locations do not contain any tau protein molecules

94. (New) The method of claim 93, wherein the plurality of locations comprises a plurality of reaction vessels.

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95. (New) The method of claim 93, wherein the plurality of locations comprises plurality of droplets.

96. (New) The method of claim 12, wherein the assay further comprises:

exposing a plurality of capture objects that each include a binding surface having affinity for tau protein molecules to the blood sample;

immobilizing at least a portion of the tau protein molecules with respect to the plurality of capture objects such that at least some of the capture objects associate with at least one tau protein molecule and a statistically significant fraction of the capture objects do not associate with any tau protein molecules;

spatially segregating at least a portion of the capture objects subjected to the immobilizing step into a plurality of locations;

addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing a tau protein molecule; and

determining the measure of the tau protein molecule concentration in the blood sample based at least in part on the number of locations determined to contain a tau protein molecule.

97. (New) The method of claim 96, wherein the plurality of capture objects comprises a plurality of beads.

98. (New) The method of claim 12, further comprising plotting the measure of the tau protein molecule concentration versus time on a graph to determine the area under the curve.

99. (New) The method of claim 12, wherein each of the tau protein molecule is selected from the group consisting of tau 23 (352, 0N3R), tau 24 (383, 0N4R), tau 37 (381, 1N3R), tau 34 (412, 1N4R), tau 39 (410, 2N3R), and tau 40 (441, 2N4R).

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100. (New) The method of claim 99, wherein at least a portion of the tau protein molecules are phosphorylated.

101. (New) The method of claim 12, wherein the patient's prognosis is correlated with a Cerebral Performance Category (CPC) score including classifications of good or poor,

wherein a good CPC score indicates a prognosis of the patient being conscious and alert with normal function or only slight disability or conscious and alert with moderate disability after a selected period of time, and

wherein a poor CPC score indicates a prognosis of the patient being conscious with the patient having a severe disability, being comatose or in a persistent vegetative state, or being brain dead or suffering death from other causes after a selected period of time.

102. (New) The method of claim 101, wherein the prognosis is good for the recovery from the brain injury if the area under the curve is less than about 800.

103. (New) The method of claim 101, wherein the prognosis is poor for the recovery from the brain injury if the area under the curve is greater than about 800.

104. (New) A method of determining a prognosis of a patient's recovery from a brain injury comprising:

(a) performing an assay on each of a plurality of blood samples obtained from the patient following the brain injury and/or plasma and/or serum derived from each of the blood samples to determine a measure of the tau protein molecule concentration in each sample,

wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours,

wherein at least one of the plurality of samples is obtained within 12 hours of the brain injury,

wherein the assay comprises spatially segregating and detecting single tau protein molecules, and

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wherein the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL;

(b) determining the area under the curve of a graph of the measure of the tau protein concentration in the plurality of samples versus time, wherein the area is determined for a second peak in the tau protein molecule concentration; and

(c) determining a prognosis of the patient's recovery from the brain injury and/or a method of treatment based on the area under the curve determined in step (b).

105. (New) A method of determining a prognosis of a patient's recovery from a brain injury comprising:

(a) performing an assay on a blood sample obtained from the patient following the brain injury and/or plasma and/or serum derived from the blood sample to determine a measure of the tau protein molecule concentration in the sample,

wherein the assay comprises spatially segregating and detecting single tau protein molecules,

wherein the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL; and

(b) determining a prognosis of the patient's recovery from the brain injury based on the measure of the tau protein molecule concentration in the sample determined in step (a).

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REMARKS

Applicant respectfully requests reconsideration. Claims 1-15 and 20-24 were previously pending in this application. By this amendment, Applicant is cancelling claims 1-11, 14, 15, and 20 without prejudice or disclaimer. Claims 12 and 23 have been amended. New claims 92-105 have been added. The right to pursue the cancelled subject matter in one or more continuing applications is hereby reserved. As a result, claims 12-13, 21-24, and 92-105 are pending for examination with claims 12, 104, and 105 being independent claims. No new matter has been added.

Objections to the Claims

The Examiner objected to claims 2-3 and 9 because of informalities. Without acceding to the merits of the objections, claims 2-3 and 9 have been cancelled rendering the objection moot.

Rejections under 35 U.S.C. §112

The Examiner rejected claims 9-11 under 35 U.S.C. §112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the applicant regards as the invention.

Without acceding to the merits of the rejections, claims 9-11 have been cancelled rendering this rejection moot.

The Examiner rejected claims 1-13 and 20-24 under 35 U.S.C. §112 (pre-AIA), first paragraph, as allegedly failing to comply with the written description requirement. The Examiner also rejected these claims under 35 U.S.C. §112 (pre-AIA), first paragraph, alleging that the specification, while being enabling for measuring tau using the disclosed assay, does not reasonably provide enablement for the breadth of utilizing all possible assays that measure tau, even when limited to those assays with a specific limit of detection.

Without acceding to the merits of the rejections, independent claim 1 has been cancelled without prejudice or disclaimer. Accordingly, the rejection of this claim and its dependent claims 2-11, also cancelled, has been rendered moot.

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Regarding independent claim 12 and its dependent claims, without acceding to the merits of the rejection and solely to expedite allowance, this claim has been amended to recite specific acts further refining the claimed assay, specifically, that the assay comprises spatially segregating and detecting single tau protein molecules, and that the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL. Claim 12 as amended thus now even more clearly satisfies the written description requirements. In particular, the specification makes it clear that the inventors possessed and described the invention commensurate with the scope as presently claimed and did not limit the assay techniques to a single narrowly defined assay as alleged in the Office action. Indeed, the specification as filed provides numerous examples and variations of assay methods that may be employed in connection with invention as presently claimed (e.g., see pages 22-33 of the specification as filed). As just one example, the tau molecules may be spatially segregated into reaction vessels or droplets (e.g., see page 33-38 of the specification as filed). This is in stark contrast to the Examiner's statement that the instant specification only discloses one assay (Office Action, page 5). Furthermore, the patent and patent applications incorporated by reference provide a wide gamut of variations of the methods in which assays having the claimed limitations can be conducted. Accordingly, the rejections of the claims as failing to meet the written description requirement has been overcome and should be withdrawn.

Furthermore, with regard to the Examiner's alternative basis of rejection under the enablement requirement of 35 U.S.C. §112 (pre-AIA), first paragraph, it is not required that the claims recite all of the limitations of specific preferred embodiments,[1] nor to enable a claim is it required that the specification disclose every detail of every assay or every possible condition or combination of conditions for every possible type of assay that could be used in the claimed methods and that would fall within the scope of the methods as claimed so long as one skilled in the art could make and use the invention over a reasonable scope correlated with the scope of the

[1] MPEP Section 2164.08 – "[T]o provide effective incentives, claims must adequately protect inventors. To demand that the first to disclose shall limit his claims to what he has found will work or to materials which meet the guidelines specified for "preferred" materials in a process such as the one herein involved would not serve the constitutional purpose of promoting progress in the useful arts. Quoting In re Goffe, 542 F.2d 564, 567, 191 USPQ 429, 431 (CCPA 1976).

invention as claimed without undue experimentation.[2] It would be impossible to meet such a standard without restricting the scope of claims only to the specific preferred examples disclosed in the specification. It is a fundamental tenet of black letter patent law that claim scope need not be limited to only the exemplary embodiments disclosed in the specification to meet the enablement requirement.[3] As noted above, claim 12 has been amended to add specific limitations of the assay comprising spatially segregating spatially segregating and detecting single tau protein molecules, and the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL, even further guiding the person of ordinary skill and refining and limiting the scope of protection sought. Given that the Patent Office, with the exception of broad general allegations in the Office Action, has not explained why practicing the invention over a scope reasonably correlated with the scope of the invention as presently claimed would require undue experimentation – and it is the position of the applicant that that no undue experimentation would be required – the Patent Office has not established that scope of enablement does not bear a reasonable correlation to the scope of the claims.[4] Since nothing more is required to meet the enablement requirement, this rejection should be withdrawn.

Rejections Under 35 U.S.C. §101

The Examiner rejected claims 10-15 and 20-24 under 35 U.S.C. §101 because the claims as a whole are alleged to not be directed to patent eligible subject matter.

In the Office Action on page 8, the Examiner alleges that these claims are directed to a judicial exception to patent eligibility – the correlation of measured tau protein concentration to patient prognosis, presumably a "natural phenomenon" or "law of nature" – and that the remainder of the claims does not recite "something significantly more than the judicial exception(s)," thus rendering the claims ineligible. Applicant traverses on the basis of the above amendments and

[2] MPEP Section 2164.08 – "All that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art. Further the scope of enablement must only bear a "reasonable correlation" to the scope of the claims."
[3] In re Goffe, 542 F.2d 564, 567, 191 USPQ 429, 431 (CCPA 1976) – see quote from footnote 1 above.
[4] MPEP Section 2164.04 – "According to In re Bowen, 492 F.2d 859, 862-63, 181 USPQ 48, 51 (CCPA 1974), the minimal requirement is for the examiner to give reasons explaining the uncertainty of the enablement. This standard is applicable even when there is no evidence in the record of operability without undue experimentation beyond the disclosed embodiments."

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remarks below. However, regarding independent claim 12 and its dependent claims, without acceding to the merits of the rejection and solely in order to expedite allowance, independent claim 12 has been amended to recite a plurality of steps that more specifically describe the assay method for determining tau protein concentration and the process for determining a patient's prognosis. Without acceding to the merits of the rejection, independent claim 14 and its dependent claims have been cancelled rendering the rejection as applied to those claims moot.

The 2014 Interim Guidance on Patent Subject Matter Eligibility ("Guidelines") released by the Patent Office on December 16, 2014 set forth a two-step test for analyzing subject-matter eligibility of a claim under §101. The first step of the two-part test is whether the claims is directed to a judicial exception such as an abstract idea (Step 2A), for example, a "law of nature". If so, the second step involves determining whether the claims recite additional elements that amount to significantly more than the judicial exception (Step 2B).

The United States Supreme Court decision in *Mayo Collaborative Services v. Prometheus Laboratories, Inc.*, 132 S.Ct. 1289 (2012) set forth considerations for determining patent eligibility of claims that directed to a "law of nature." A patent-eligible claim must transform a process into an inventive application of the "law of nature" which is not merely an obvious application of purely conventional techniques. *Mayo at 1299*. The claim must also do more than simply instruct users to take the law of nature and simple "apply it." *Id. at 1300*. Rather, the claim should recite how the principal is implemented in a new and unconventional way. *Id*. Amended claim 12 meet the all of foregoing criteria and is thus patent eligible under the USPTO Guidelines and the controlling case law it embodies

Specifically, amended claim 12 is patent-eligible at least because - to the extent that under Step 2A of the Guidelines the claim is "directed to" a law of nature or natural correlation judicial exception, which is not conceded - the process steps in the context of the claimed method clearly recite something "significantly more" than the judicial exception (Step 2B), and thus, the claims are directed towards eligible subject matter. The steps do not simply instruct the skilled artisan to take the law of nature and simply "apply it," but rather involve an application of the judicial exception in a way that was unconventional and unprecedented at the time the invention was made. Arguably, the judicial exception at issue is a "law of nature" or "natural correlation" involving the relationship

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between tau measurement in blood or serum or plasma derived from the blood, and the prognosis of the patient's recovery from a brain injury. Notably, the link between the measured tau levels in blood or serum or plasma derived from the blood and the prognosis was not known at the time the invention was made. The inventors of the subject application discovered that, using certain assay techniques that were unconventional for use in measuring tau at the time of the invention, very low levels of tau in blood or serum or plasma derived from the blood could be determined, and were correlated to the extent of brain injury, such that that these low levels could be used to determine a patient's prognosis following the brain injury. Without this link discovered by the inventors, and the innovative and unconventional methods they developed and employed to facilitate reliable measurement, it would not be possible to use tau levels to determine a patient's prognosis as claimed.

While the inventors discovery of the correlation was a significant and important advance in the field, the current claims recite "significantly more' than that, thereby making them patent eligible. Indeed, they are patent-eligible at least because they do significantly more than merely restate the natural correlation in terms that instruct the person skilled in the art to just "apply it" using conventional techniques in the art. The claims by contrast recite assay techniques involving single molecule partitioning and counting for determining tau protein molecule concentration in blood derived samples and the use of this concentration data together with a specifically recited area under the curve analysis to determine a patient's prognosis, both of which were unconventional, unprecedented, and unappreciated as being useful in the context of the indication as claimed.

Regarding the specific points alleging lack of patent eligibility raised in the Office Action, Applicant respectfully disagrees with the Examiner's assertion that the described assays would have been "a well-known choice," and thus, "do[es] not provide significantly more than instructions to a relevant audience to use known techniques to observe a natural phenomenon and 'apply it'" (Office Action, page 10-11). By contrast, and as explained above, the techniques currently claimed were not "well-known" choices in the context and indications to which the present claims are directed. The mere description of an assay procedure generally in the prior art does not alone render it "a well-known choice" if it has not been previously engaged in by researchers in the field in the context of

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the invention as claimed.[5] In claim 12, both the steps for measuring tau in blood derived samples at the recited LOD and the area under the curve protocol for analyzing the concentration measurement data were not, at the time the invention was made and the application was filed, simply "appending (sic) well-understood, routine, and conventional activities previously known to the industry" (Office Action, page 9), but were unconventional, and significantly more than just a routine application of the natural correlation, and their use, as demonstrated by the inventors for the first time, provides a new, useful and unprecedented diagnostic and treatment tool to improve outcomes for patients suffering a brain injury. Thus, the claim 12 and its dependent claims are patent-eligible. Reconsideration and withdrawal of this rejection are respectfully requested.

Rejections Under 35 U.S.C. §102

The Examiner rejected claims 1-5 and 7 under pre-AIA 35 U.S.C. §102(b) as being anticipated by Quanterix 2010 IQT Technology Focus Day (hereinafter "Quanterix"). Without acceding to the merits of the rejections and in order to expedite allowance, claims 1-5 and 7 have been cancelled rendering this rejection moot.

Rejections Under 35 U.S.C. §103

The Examiner rejected claims 1-9 under pre-AIA 35 U.S.C. §103(a) as being unpatentable over Quanterix. Without acceding to the merits of the rejections and in order to expedite allowance, claims 1-9 have been cancelled rendering this rejection moot.

The Examiner rejected claims 1-15, 20 and 22-24 under pre-AIA 35 U.S.C. §103(a) as being unpatentable over Quanterix in view of Wunderlich.

[5] See, e.g. commentary in the USPTO's May 2016 "Subject Matter Eligibility Examples: Life Sciences" related to Example 31, Claim 70 (p. 26) – "Although SNOM was known to scientists at the time the invention was made and the application was filed, e.g. because it had been discussed in several widely-read scientific journals, mere knowledge of this technique does not make the use of SNOM to detect DNA hybridization routine or conventional in this field . . . Thus, the claim's recitation of using SNOM is more than mere instruction to "apply" the abstract idea using a well-understood, routine or conventional techniques in the field."

Without acceding to the merits of the rejections, independent claims 1 and 14 have been cancelled rendering the rejection with respect to these claims and the claims that depend therefrom moot.

Without acceding to the merits of the rejections and in order to expedite allowance, independent 12 has been amended to recite that the steps of (a) performing as assay on each of a plurality of blood samples obtained from the patient following the brain injury and/or plasma and/or serum derived from each of the blood samples to determine a measure of the tau protein molecule concentration in each sample, wherein the plurality of samples are obtained from the patient over a period of time of at least about 48 hours, wherein at least one of the plurality of samples is obtained within 12 hours of the brain injury, wherein the assay comprises spatially segregating and detecting single tau protein molecules, and wherein the limit of detection of tau protein molecules of the assay is less than about 0.2 pg/mL; (b) determining an area under a curve of a graph of the measure of the tau protein molecule concentration in the plurality of samples versus time, wherein the area is determined for the total period of time; and (c) determining a prognosis of the patient's recovery from the brain injury based on the area under the curve determined in step (b).

Nowhere does Quanterix teach or suggest the steps recited in this method. In contrast, Quanterix simply speculates that tau would be a "nice serum marker," but does not provide any direction or teachings as to how a person of ordinary skill in the art may reliably measure tau in such samples nor use such measurements to determine a prognosis of the patient's recovery from the brain injury.

Nowhere does Wunderlich cure the deficiencies of Quanterix. In Wunderlich, the concentration of tau protein in the samples was obtained using a basic ELISA assay (Wunderlich, page 599, first column, "Subjects and Methods"). Nowhere does Wunderlich teach or suggest the assay methods as recited in amended independent claim 12. Furthermore, Wunderlich does not describe nor provide any correlations between samples taken within 12 hours of the brain injury and any correlation with patient prognosis, as presently recited in claim 12, as amended. In contrast, Wunderlich notes only a correlation from 72 hours after stroke onset (see Wunderlich, page 561, first column, first full paragraph).

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In view of the above remarks, it is believed that the rejection of independent claim 12 as being unpatentable in view of Quanterix and Wunderlich has been overcome, and that the patentability of the claims has been established. The claims that depend from claim 12 are patentable for at least the same reasons. Thus, withdrawal of the rejection of the claims on this ground is respectfully requested.

The Examiner rejected claims 1-15, 20-21 and 23 under pre-AIA 35 U.S.C. §103(a) as being unpatentable over Quanterix in view of Mortberg.

Without acceding to the merits of the rejections, independent claims 1 and 14 have been cancelled rendering the rejection with respect to these claims and the claims that depend therefrom moot.

Regarding independent claim 12 and the claims that depend therefrom, as noted above in connection with the §103 rejection over Quanterix in view of Wunderlich, without acceding to the merits of the rejection, independent claim 12 has been amended to recited a plurality of steps. Nowhere does Quanterix teach or suggest the steps recited in this method and Quanterix simple speculates that tau would be a "nice serum marker".

Nowhere does Mortberg cure the deficiencies of Quanterix. The concentration of tau protein in the samples in Mortberg were determined using a colormetric reading (Mortberg, page 29, first paragraph). Mortberg teaches that there were only a few cases in which detectable levels of tau were present at less than 48 hours (Mortberg, page 48, first paragraph). Nowhere does Mortberg teach or suggest the assay methods as recited in amended independent claim 12. Furthermore, a person of ordinary skill in the art, when reading Mortberg, would not be able to determine whether tau is present in any level, let alone a detectable level, within 12 hours of the brain injury. Accordingly, Mortberg does not teach or suggest the claimed methods.

In view of the above remarks, it is believed that the rejection of independent claim 12 as being unpatentable in view of Quanterix and Mortberg has been overcome, and that the patentability of the claims has been established. The claims that depend from claim 12 are patentable for at least the same reasons. Thus, withdrawal of the rejection of the claims on this ground is respectfully requested.

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Double Patenting Rejection

The Examiner provisionally rejected claims 1-15 and 20-24 on the ground of nonstatutory double patenting as being unpatentable over claims 1-11, 15 and 23-30 of co-pending Application No. 14/111,331. The Examiner provisionally rejected claims 1-15 and 20-24 on the ground of nonstatutory double patenting as being unpatentable over claims 55, 92-102 and 108-115 of co-pending Application No. 14/111,326.

It is respectfully requested that these rejections be deferred until the claims of this application are otherwise in condition for allowance.

New Claims

New independent claims 103 and 104 have been added. For at least the reasons described above in connection with independent claim 12, it is respectfully asserted that these new claims meet all of the requirements of 35 U.S.C. §§101, 112, and 103.

CONCLUSION

A Notice of Allowance is respectfully requested. The Examiner is requested to call the undersigned at the telephone number listed below if this communication does not place the case in condition for allowance.

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If this response is not considered timely filed and if a request for an extension of time is otherwise absent, Applicant hereby requests any necessary extension of time. If there is a fee occasioned by this response, including an extension fee, the Director is hereby authorized to charge any deficiency or credit any overpayment in the fees filed, asserted to be filed or which should have been filed herewith to our Deposit Account No. 23/2825, under Docket No. Q0052.70018US02.

Dated: September 27, 2017

Respectfully submitted,

By /Andrea J. Gabert/
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DOCKET NO.: Q0052.70018US02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	15/269,142
Confirmation No.:	6662
Filed:	September 19, 2016
For:	METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'S RECOVERY FROM A BRAIN INJURY
Examiner:	A. M. Weidner
Art Unit:	1649

CERTIFICATE OF ELECTRONIC FILING UNDER 37 C.F.R. § 1.8

The undersigned hereby certifies that this paper, along with any paper referred to as being attached or enclosed, is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4), on:

Dated: September 27, 2017 Electronic Signature for: /Eileen M. MacKenzie/

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed more than three months after the filing date of this application and after the mailing date of a first Office Action, but before the mailing date of any of a Final Action under 37 C.F.R. § 1.113, a Notice of Allowance under 37 C.F.R. § 1.311, or an action that otherwise closes prosecution in this application.

Please charge our Credit Card in the amount of $180.00 covering the fee set forth in 37 C.F.R. § 1.17(p).

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

The undersigned hereby makes the following additional information of record in the above-identified application.

The undersigned would like to bring to the Examiner's attention the following co-pending application that may contain subject matter related to this application:

Serial No.	Filing Date	Inventor(s)	Docket No.
*14/111,331	12-18-2013	Wilson et al.	Q0052.70017US01

*A copy of this reference is not provided as the Office has waived the requirement under 37 C.F.R. § 1.98(a)(2)(iii) for submitting a copy of a cited U.S. patent application if it is scanned to the Image File Wrapper system and is available on Private PAIR.

The undersigned would like to bring to the Examiner's attention the enclosed search report or other communication from a corresponding or related International or Foreign National Application:

Serial No.	Date of Mailing	Type(s) of Communication	Docket No.
PCT/US2012/033338	**11-29-2012**	****International Search Report and Written Opinion**	**Q0052.70017WO00**
PCT/US2012/033338	**10-24-2013**	****International Preliminary Report on Patentability**	**Q0052.70017WO00**
PCT/US2012/033343	**10-29-2012**	****International Search Report and Written Opinion**	**Q0052.70018WO00**
PCT/US2012/033343	**10-24-2013**	****International Preliminary Report on Patentability**	**Q0052.70018WO00**

**a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. 14/111,326, filed June 24, 2014, and relied upon for an earlier filing date under 35 U.S.C. 120 (continuation, continuation-in-part, and divisional applications).

PART III: Explanation of Non-English Language References

The following is a concise explanation of the relevance of each non-English language reference listed on the attached form PTO-1449 (modified PTO/SB/08):

The relevance of **CN 1635146 A may be understood by its English language abstract and figures.

The relevance of **CN 1950520 A may be understood by its English language abstract and figures.

The relevance of CN 101351564 A may be understood by its English language abstract and figures.

The relevance of CN 101529227 A may be understood by its English language abstract and figures.

The relevance of CN 101541974 A may be understood by its English language abstract and figures.

The relevance of **DE 19540098 A1 may be understood by its English language abstract and figures.

The relevance of **EP 1 259 810 B1 may be understood by the English language abstract of what is believed to be its counterpart, **WO 01/57520 A1, cited on the attached form PTO-1449.

The relevance of **JP 2001-269196 A may be understood by its English language translation. The undersigned makes no representation as to the accuracy of the translation, but is unaware of any inaccuracies.

The relevance of **JP 2002-506200 A may be understood by what is believed to be its English language counterpart, **WO 99/45357, cited on the attached form PTO-1449.

The relevance of **JP 2002-525587 A may be understood by what is believed to be its English language counterpart, **AU 199956253, cited on the attached form PTO-1449.

The relevance of **JP 2002-526743 A may be understood by what is believed to be its English language counterpart, **WO 99/58948, cited on the attached form PTO-1449.

The relevance of **JP 2004-354164 A may be understood by its English language translation. The undersigned makes no representation as to the accuracy of the translation, but is unaware of any inaccuracies.

The relevance of **JP 2005-518553 A may be understood by what is believed to be its English language counterpart, **WO 03/073817, cited on the attached form PTO-1449.

The relevance of **JP 2006-511792 A may be understood by what is believed to be its English language counterpart, US 2004-0253624, cited on the attached form PTO-1449.

The relevance of **WO 01/57520 A2 may be understood by its English language abstract and figures.

**a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. 14/111,326, filed June 24, 2014, and relied upon for an earlier filing date under 35 U.S.C. 120 (continuation, continuation-in-part, and divisional applications).

PART IV: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. Q0052.70018US02 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Andrea J. Gabert/

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Docket No.: Q0052.70018US02
Date: September 27, 2017
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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/269,142	ATTY. DOCKET NO.: Q0052.70018US02
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				FIRST NAMED INVENTOR: David Wilson	
Sheet	1	of	22	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		3,712,986		Collings	01-23-1973
		4,200,110		Peterson et al.	04-29-1980
		4,232,119		Carlsson et al.	11-04-1980
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		4,962,037		Jett et al.	10-09-1990
		5,026,159		Allen et al.	06-25-1991
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		5,441,870		Seubert et al.	08-15-1995
		5,445,934		Fodor et al.	08-29-1995

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

**a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. 14/111,326, filed June 24, 2014, and relied upon for an earlier filing date under 35 U.S.C. 120 (continuation, continuation-in-part, and divisional applications).

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/269,142	ATTY. DOCKET NO.: Q0052.70018US02
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				FIRST NAMED INVENTOR: David Wilson	
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Bibliographic data: CN101351564 (A) — 2009-01-21

Detection of nucleic acids by target-specific hybrid capture method

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Applicant(s): DIGENE CORP [US] + (DIGENE CORP, ; QIAGEN GAITHERSBURG, INC)

Classification: **- international:** ***C07H21/04; C12N15/09; C12P19/34; C12Q1/68; G01N33/53; G01N33/566***
- cooperative: **C12Q1/6804**; **C12Q1/6834**; **C12Q1/6876**; **C12Q1/708** more

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Also published as: CN101351564 (B) US2006051809 (A1) US7601497 (B2) US2013203632 (A1) US9115410 (B2) more

Abstract of CN101351564 (A)

Target-specific hybrid capture (TSHC) provides a nucleic acid detection method that is not only rapid and sensitive, but is also highly specific and capable of discriminating highly homologous nucleic acid sequences. The method produces DNA:RNA hybrids which can be detected by a variety of methods.

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权利要求书 11 页 说明书 54 页 序列表 16 页

[54] 发明名称

通过靶特异性杂交捕获方法检测核酸

[57] 摘要

靶特异性杂交捕获(TSHC)提供了检测核酸的方法，该方法不仅快速、灵敏还高度特异，并且能够区分高度同源的核酸序列。 所述方法产生能够被多种方法检测的 DNA：RNA 杂交体。

1. 一种检测多个靶核酸中的每一个的方法，其包括：

a）通过将所述多个靶核酸、与所述靶核酸的至少一部分互补的核酸探针和固体载体混合来将所述多个靶核酸捕获至所述固体载体上，形成多个富集的靶核酸，其中所述靶核酸及核算探针一个为 RNA 另一个为 DNA；

b）扩增富集的核酸或核酸探针，形成多个扩增的靶；和

c）通过将所述多个扩增的靶、与所述扩增的靶的第一部分杂交的可选择性寡核苷酸，和与所述扩增的靶的第二部分互补的核酸探针混合形成 DNA：RNA 杂交寡核苷酸复合体，来检测所述多个扩增的靶而进行所述靶核酸的检测，其中 DNA：RNA 杂交体通过 DNA：RNA 杂交体特异性结合剂检测，并通过所述寡核苷酸复合体分离进行选择，从而检测所述多个靶核酸中的每一个。

2. 如权利要求 1 所述的方法，其中在捕获的所述步骤中，所述固体载体与 DNA：RNA 杂交体特异性结合剂结合，由此将所述靶核酸捕获至所述固体载体上。

3. 如权利要求 1 所述的方法，其中所述 DNA：RNA 杂交体特异性结合剂选自由以下组成的组：DNA：RNA 杂交体特异性抗体、单克隆抗体或多克隆抗体或其片段、蛋白质、催化失活的核糖核酸酶 H，和核酸、核酸适配子或结合并形成三链体结构的寡核苷酸。

4. 如权利要求 2 所述的方法，其中所述 DNA：RNA 杂交体特异性结合剂为 DNA：RNA 杂交体特异性抗体。

5. 如权利要求 2 所述的方法，其中所述 DNA：RNA 杂交体特异性结合剂为与所述靶核酸互补的核酸探针。

6. 如权利要求 3 所述的方法，其中所述 DNA：RNA 杂交体特异性结合剂被可检测地标记。

7. 如权利要求 6 所述的方法，其中所述标记通过比色法、化学发光、

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荧光和光散射方法检测。

8. 如权利要求 1 所述的方法，其中所述捕获的靶核酸或核酸探针通过等温扩增被扩增。

9. 如权利要求 8 所述的方法，其中在所述等温扩增中使用 DNA 聚合酶。

10. 如权利要求 8 所述的方法，其中在所述等温扩增中使用随机引物。

11. 如权利要求 10 所述的方法，其中所述随机引物为五聚体。

12. 如权利要求 10 所述的方法，其中所述随机引物包含疾病特异性引物序列的子集。

13. 如权利要求 1 所述的方法，其中所述选择性寡核苷酸被结合至固体载体。

14. 如权利要求 1 所述的方法，其中所述固体载体选自由以下所组成的组：平板、微平板、微量板、载玻片、皿、微珠、颗粒、微粒、杯子、条状物、芯片、微芯片、带状物、膜、微阵列和试管。

15. 如权利要求 14 所述的方法，其中所述固体载体的制造材料选自由以下所组成的组：玻璃、硅、塑料、聚苯乙烯、聚乙烯、聚丙烯和聚碳酸酯。

16. 如权利要求 14 所述的方法，其中修饰所述固体载体以使其包含：进行修饰以便表面包含羧基、氨基、酰肼、醛基、核酸或核苷酸衍生物、伯氨基和染料。

17. 如权利要求 1 所述的方法，其进一步包括添加阻断剂探针。

18. 一种检测多个靶核酸中的每一个的方法，其包括：

a）将多个靶核酸和与所述靶核酸的至少一部分互补的核酸探针杂交，形成 DNA：RNA 杂交体；

b)用结合至固体载体上的 DNA：RNA 杂交体特异性抗体捕获所述 DNA：RNA 杂交体；

c）将未结合的靶核酸与所述 DNA：RNA 杂交体分离；

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d）使用随机引物和 DNA 聚合酶扩增所述捕获的靶核酸或核酸探针，形成多个扩增的靶；

e）杂交与所述扩增的靶的第一部分互补的核酸探针，形成 DNA：RNA 杂交体；

f）将结合至多个固体载体上的寡核苷酸和所述扩增的靶的第二部分杂交，其中所述固体载体为选择性的；和

g）通过所述选择性固体载体选择所述多个扩增的靶中的每一个；和

h）通过 DNA：RNA 杂交体特异性结合剂与所述 DNA：RNA 杂交体的结合，检测所述多个扩增的靶中的每一个，其中多个扩增子的存在表明存在所述靶核酸。

19. 如权利要求 18 所述的方法，其中与寡核苷酸结合的所述固体载体为可区别的微珠。

20. 如权利要求 19 所述的方法，其中所述可区别的微珠选自由可检测的标记物所组成的组：荧光标记物、金标记物和酶标记物。

21. 一种检测多个靶 DNA 的多重方法，其包括：

a）多个靶 DNA 和与所述靶 DNA 的至少一部分互补的 RNA 探针杂交，形成 DNA：RNA 杂交体；

b）用结合至微珠上的 DNA：RNA 杂交体特异性抗体捕获所述 DNA：RNA 杂交体；

c）将未结合的核酸与所述 DNA：RNA 杂交体分离，形成富集的靶；

d）利用随机引物和 DNA 聚合酶扩增所述富集的靶 DNA，形成多个扩增的靶；

e）杂交与所述扩增的靶 DNA 的第一部分互补的 RNA 探针，形成 DNA：RNA 杂交体；

f）将 DNA 寡核苷酸和所述靶 DNA 的第二部分杂交，其中所述 DNA 寡核苷酸结合至多个选择性微珠上；和

g）通过所述 DNA：RNA 杂交体特异性抗体与所述 DNA：RNA 杂交体的

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结合，检测所述多个扩增的靶 DNA，并根据特定的选择性微珠选择每一个所述扩增的靶。

22. 如权利要求 1 所述的方法，其中与所述靶核酸的至少一部分互补的所述核酸探针和寡核苷酸序列选自由以下所组成的组：

GTACAGATGGTACCGGGGTTGTAGAAGTATCTG [SEQ ID NO: 1];
CTGCAACAAGACATACATCGACCGGTCCACC [SEQ ID NO: 2];
GAAGTAGGTGAGGCTGCATGTGAAGTGGTAG [SEQ ID NO: 3];
CAGCTCTGTGCATAACTGTGGTAACTTTCTGGG [SEQ ID NO: 4];
GAGGTCTTCTCCAACATGCTATGCAACGTCCTG [SEQ ID NO: 5];
GTGTAGGTGCATGCTCTATAGGTACATCAGGCC [SEQ ID NO: 6];
CAATGCCGAGCTTAGTTCATGCAATTTCCGAGG [SEQ ID NO: 7];
GAAGTAGTAGTTGCAGACGCCCCTAAAGGTTGC [SEQ ID NO: 8];
GAACGCGATGGTACAGGCACTGCAGGGTCC [SEQ ID NO: 9];
GAACGCGATGGTACAGGCACTGCA [SEQ ID NO: 10]; ACGCCCACCCAATGGAATGTACCC [SEQ ID NO: 11]; TCTGCGTCGTTGGAGTCGTTCCTGTCGTGCTC [SEQ ID NO: 12];
*(TTATTATTA)CTACATACATTGCCGCCATGTTCGCCA [SEQ ID NO: 13];
(TTATTATTA)TGTTGCCCTCTGTGCCCCCGTTGTCTATAGCCTCCGT [SEQ ID NO: 14];
(TTATTATTA)GGAGCAGTGCCCAAAAGATTAAAGTTTGC [SEQ ID NO: 15];
(TTATTATTA)CACGGTGCTGGAATACGGTGAGGGGGTG [SEQ ID NO: 16];
(TTATTATTA)ACGCCCACCCAATGGAATGTACCC [SEQ ID NO: 17] ;
(TTATTATTA)ATAGTATTGTGGTGTGTTTCTCACAT [SEQ ID NO: 18];
(TTATTATTA)GTTGGAGTCGTTCCTGTCGTG [SEQ ID NO: 19];
(TTATTATTA)CGGAATTTCATTTTGGGGCTCT [SEQ ID NO: 20];
GCTCGAAGGTCGTCTGCTGAGCTTTCTACTACT [SEQ ID NO: 21];
GCGCCATCCTGTAATGCACTTTTCCACAAAGC [SEQ ID NO: 22];
TAGTGCTAGGTGTAGTGGACGCAGGAGGTGG [SEQ ID NO: 23] ;
GGTCACAACATGTATTACACTGCCCTCGGTAC [SEQ ID NO: 24];
CCTACGTCTGCGAAGTCTTTCTTGCCGTGCC [SEQ ID NO: 25];
CTGCATTGTCACTACTATCCCCACCACTACTTTG [SEQ ID NO: 26];

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CCACAAGGCACATTCATACATACACGCACGCA [SEQ ID NO: 27];
GTTCTAAGGTCCTCTGCCGAGCTCTCTACTGTA [SEQ ID NO: 28];
(TTATTATTA)TGCGGTTTTGGGGGTCGACGTGGAGGC [SEQ ID NO: 29];
(TTATTATTA)AGACCTGCCCCCTAAGGGTACATAGCC [SEQ ID NO: 30];
(TTATTATTA)CAGCATTGCAGCCTTTTTGTTACTTGCTTGTAATAGCTCC [SEQ ID NO: 31];
(TTATTATTA)ATCCTGTAATGCACTTTTCCACAAA [SEQ ID NO: 32];
(TTATTATTA)GCCTGGTCACAACATGTATTAC [SEQ ID NO: 33];
(TTATTATTA)CAGGATCTAATTCATTCTGAGGTT [SEQ ID NO: 34];
TGCGGTTTTGGGGGTCGACGTGGAGGC [SEQ ID NO: 35];
GGCGCAACCACATAACACACAGAACCACAAAAC [SEQ ID NO: 36];
GTTCTACACGGGTTTGCAGCACGATCAACAACG [SEQ ID NO: 37];
CGCTGCTTGTGGTGGTCGGTTATCGTTGTCTG [SEQ ID NO: 38];
GACGTAGTGTCGCCTCACATTTACAACAGGAC [SEQ ID NO: 39];
CTCGCTTGGTGGGGTTGTAGGGGAGCTCGG [SEQ ID NO: 40];
GCTGTAGTTGTCGCAGAGTATCCCGTGAGG [SEQ ID NO: 41];
GTGAGCCTGTGTTATATGTAGTGCCCGAATCCC [SEQ ID NO: 42];
CCACCTCCTGCGTCCACTACACCTAGCACTA [SEQ ID NO: 43];
TGCGTGCGTGTATGTATGAATGTGCCTTGTGG [SEQ ID NO: 44];
AATTAGCGCATTGCCCCGTCCAACGTCCCG [SEQ ID NO: 45];
CGCCGTGCACGTGTAGCCACCATATTTAATCAC [SEQ ID NO: 46];
CGAATTGTGTGAGGTGCTGGAAGAATCGGTGC [SEQ ID NO: 47];
GATCGTTCACAACTTTTACCTGCACCGGATCC [SEQ ID NO: 48];
CTAGGTTCTCTAGATGTTTGTCTCCAGCACCCC [SEQ ID NO: 49];
CTGTCGGTATTGTCTGTGTCGCTGATGTGTG [SEQ ID NO: 50];
GATACACACACATTTGCAGCCCGGTCCACACA [SEQ ID NO: 51];
GGTGGCAAAGGACGTATGTGAGTGCAGAGGAC [SEQ ID NO: 52];
GCGTTGCGGAGGGGTATGATAGTTGCTCAGAAG [SEQ ID NO: 53];
GTCTAGGCGTGTAGGAGGAAACAAGATGGGG [SEQ ID NO: 54];
CTGAACACAGCAGTTCTCTATACCAATGGCGCTATTTC [SEQ ID NO: 55];

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TTGGTTGCCCCTGAGCAGTCGGACCCTATGGATA [SEQ ID NO: 56];
GCGCCGCATTGCTGCACCTCGTTTATATAGCAGGGCATTTTC [SEQ ID NO: 57];

CCTGGCGCATGTCATACACACCACATTACTC [SEQ ID NO: 58];
CACGAAGTGTCAGTGCACAGTATGCCTTGC [SEQ ID NO: 59];
GCCATGTACTTCACAAACTGTTAATACTGGTGATTGTCCC [SEQ ID NO: 60];

CCTACACAGTACAAGTGGAGGCCATCACCCG [SEQ ID NO: 61];
GTCTGACACATACTGTTGTAACCCATAGTTAAACACAGG [SEQ ID NO: 62];

GCTGTCTCCCTGTCTTCCTGTGTATTGTTTAT AAGTGTATT [SEQ ID NO: 63]; 和
GTACAGCACTAAAATGGAGTTTGGTGTGTTACTATAGTGCATAC [SEQ ID NO: 64]。

23. 一种在权利要求 17 的方法中使用的组合物，其中与所述靶核酸的至少一部分互补的所述核酸探针、寡核苷酸序列和阻断剂探针选自由以下所组成的组：

GTACAGATGGTACCGGGGTTGTAGAAGTATCTG [SEQ ID NO: 1];
CTGCAACAAGACATACATCGACCGGTCCACC [SEQ ID NO: 2];
GAAGTAGGTGAGGCTGCATGTGAAGTGGTAG [SEQ ID NO: 3];
CAGCTCTGTGCATAACTGTGGTAACTTTCTGGG [SEQ ID NO: 4];
GAGGTCTTCTCCAACATGCTATGCAACGTCCTG [SEQ ID NO: 5];
GTGTAGGTGCATGCTCTATAGGTACATCAGGCC [SEQ ID NO: 6];
CAATGCCGAGCTTAGTTCATGCAATTTCCGAGG [SEQ ID NO: 7];
GAAGTAGTAGTTGCAGACGCCCCTAAAGGTTGC [SEQ ID NO: 8];
GAACGCGATGGTACAGGCACTGCAGGGTCC [SEQ ID NO: 9];
GAACGCGATGGTACAGGCACTGCA [SEQ ID NO: 10]; ACGCCCACCCAATGGAATGTACCC [SEQ ID NO: 11]; TCTGCGTCGTTGGAGTCGTTCCTGTCGTGCTC [SEQ ID NO: 12];
*(TTATTATTA)CTACATACATTGCCGCCATGTTCGCCA [SEQ ID NO: 13];
(TTATTATTA)TGTTGCCCTCTGTGCCCCCGTTGTCTATAGCCTCCGT [SEQ ID NO: 14];
(TTATTATTA)GGAGCAGTGCCCAAAAGATTAAAGTTTGC [SEQ ID NO: 15];

(TTATTATTA)CACGGTGCTGGAATACGGTGAG GGGGTG [SEQ ID NO: 16];

(TTATTATTA)ACGCCCACCCAATGGAATGTACCC [SEQ ID NO: 17];

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(TTATTATTA)ATAGTATTGTGGTGTGTTTCTCACAT [SEQ ID NO: 18];
(TTATTATTA)GTTGGAGTCGTTCCTGTCGTG [SEQ ID NO: 19];
(TTATTATTA)CGGAATTTCATTTTGGGGCTCT [SEQ ID NO: 20];
GCTCGAAGGTCGTCTGCTGAGCTTTCTACTACT [SEQ ID NO: 21];
GCGCCATCCTGTAATGCACTTTTCCACAAAGC [SEQ ID NO: 22];
TAGTGCTAGGTGTAGTGGACGCAGGAGGTGG [SEQ ID NO: 23];
GGTCACAACATGTATTACACTGCCCTCGGTAC [SEQ ID NO: 24];
CCTACGTCTGCGAAGTCTTTCTTGCCGTGCC [SEQ ID NO: 25];
CTGCATTGTCACTACTATCCCCACCACTACTTTG [SEQ ID NO: 26];
CCACAAGGCACATTCATACATACACGCACGCA [SEQ ID NO: 27];
GTTCTAAGGTCCTCTGCCGAGCTCTCTACTGTA [SEQ ID NO: 28];
(TTATTATTA)TGCGGTTTTGGGGGTCGACGTGGAGGC [SEQ ID NO: 29];
(TTATTATTA)AGACCTGCCCCCTAAGGGTACATAGCC [SEQ ID NO: 30];
(TTATTATTA)CAGCATTGCAGCCTTTTTGTTACTTGCTTGTAATAGCTCC [SEQ ID NO: 31];
(TTATTATTA)ATCCTGTAATGCACTTTTCCACAAA [SEQ ID NO: 32];
(TTATTATTA)GCCTGGTCACAACATGTATTAC [SEQ ID NO: 33];
(TTATTATTA)CAGGATCTAATTCATTCTGAGGTT [SEQ ID NO. 34];
TGCGGTTTTGGGGGTCGACGTGGAGGC [SEQ ID NO: 35];
GGCGCAACCACATAACACACAGAACCACAAAAC [SEQ ID NO: 36];
GTTCTACACGGGTTTGCAGCACGATCAACAACG [SEQ ID NO: 37];
CGCTGCTTGTGGTGGTCGGTTATCGTTGTCTG [SEQ ID NO: 38];
GACGTAGTGTCGCCTCACATTTACAACAGGAC [SEQ ID NO: 39];
CTCGCTTGGTGGGGTTGTAGGGGAGCTCGG [SEQ ID NO: 40];
GCTGTAGTTGTCGCAGAGTATCCCGTGAGG [SEQ ID NO: 41];
GTGAGCCTGTGTTATATGTAGTGCCCGAATCCC [SEQ ID NO: 42];
CCACCTCCTGCGTCCACTACACCTAGCACTA [SEQ ID NO: 43];
TGCGTGCGTGTATGTATGAATGTGCCTTGTGG [SEQ ID NO: 44];
AATTAGCGCATTGCCCCGTCCAACGTCCCG [SEQ ID NO: 45];
CGCCGTGCACGTGTAGCCACCATATTTAATCAC [SEQ ID NO: 46];

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CGAATTGTGTGAGGTGCTGGAAGAATCGGTGC [SEQ ID NO: 47];

GATCGTTCACAACTTTTACCTGCACCGGATCC [SEQ ID NO: 48];

CTAGGTTCTCTAGATGTTTGTCTCCAGCACCCC [SEQ ID NO: 49];

CTGTCGGTATTGTCTGTGTCGCTGATGTGTG [SEQ ID NO: 50];

GATACACACACATTTGCAGCCCGGTCCACACA [SEQ ID NO: 51];

GGTGGCAAAGGACGTATGTGAGTGCAGAGGAC [SEQ ID NO: 52];

GCGTTGCGGAGGGGTATGATAGTTGCTCAGAAG [SEQ ID NO: 53];

GTCTAGGCGTGTAGGAGGAAACAAGATGGGG [SEQ ID NO: 54];

CTGAACACAGCAGTTCTCTATACCAATGGCGCTAITTC [SEQ ID NO: 55];

TTGGTTGCCCCTGAGCAGTCGGACCCTATGGATA [SEQ ID NO: 56];

GCGCCGCATTGCTGCACCTCGTTTATATAGCAGGGCATTTTC [SEQ ID NO: 57];

CCTGGCGCATGTCATACACACCACATTACTC [SEQ ID NO: 58];

CACGAAGTGTCAGTGCACAGTATGCCTTGC [SEQ ID NO: 59];

GCCATGTACTTCACAAACTGTTAATACTGGTGATTGTCCC [SEQ ID NO: 60];

CCTACACAGTACAAGTGGAGGCCATCACCCG [SEQ ID NO: 61];

GTCTGACACATACTGTTGTAACCCATAGTTAAACACAGG [SEQ ID NO: 62];

GCTGTCTCCCTGTCTTCCTGTGTATTGTTTATAAGTGTATT [SEQ ID NO: 63];

GTACAGCACTAAAATGGAGTTTGGTGTGTTACTATAGTGCATAC [SEQ ID NO: 64];

和 ACTCCAACGACGCAGA [SEQ ID NO: 65];

TTTTGTGGTTCTGTGTG [SEQ ID NO: 66];

TTATGTGGTTGCGC [SEQ ID NO: 67];

CGTTGTTGATCGTGC [SEQ ID NO: 68];

TGCAAACCCGTGTAG [SEQ ID NO: 69];

CAGACAACGATAACCG [SEQ ID NO: 70];

ACCACCACAAGCAGC [SEQ ID NO: 71];

GTCCTGTTGTAAATGTG [SEQ ID NO: 72];

AGGCGACACTACGTC [SEQ ID NO: 73];

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CGAGCTCCCCTACAA [SEQ ID NO: 74];

CCCCACCAAGCGA [SEQ ID NO: 75];

CCTCACGGGATACTC [SEQ ID NO: 76];

TGCGACAACTACAGC [SEQ ID NO: 77];

GGATTCGGGCACTA [SEQ ID NO: 78];

CATATAACACAGGCTCAC [SEQ ID NO: 79];

TAGTGCTAGGTGTAGTGG [SEQ ID NO: 80];

ACGCAGGAGGTGG [SEQ ID NO: 81];

TACATACACGCACGCA [SEQ ID NO: 82];

CCACAAGGCACATTCA [SEQ ID NO: 83];

GCTACACGTGCACGGCG [SEQ ID NO: 84];

GTGATTAAATATGGTGG [SEQ ID NO: 85];

GGGACGTTGGACG [SEQ ID NO: 86];

GGCAATGCGCTAAT [SEQ ID NO: 87];

CACCGATTCTTCCAG [SEQ ID NO: 88];

CACCTCACACAATTCG [SEQ ID NO: 89];

GGATCCGGTGCAG [SEQ ID NO: 90];

GTAAAAGTTGTGAACGATC [SEQ ID NO: 91];

GGTGCTGGAGACAA [SEQ ID NO: 92];

ACATCTAGAGAACCTAG [SEQ ID NO: 93];

CACACATCAGCGACA [SEQ ID NO: 94];

CAGACAATACCGACAG [SEQ ID NO: 95];

TGTGTGGACCGGG [SEQ ID NO: 96];

CTGCAAATGTGTGTGTAT [SEQ ID NO: 97];

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GTCCTCTGCACTCACAT [SEQ ID NO: 98];

ACGTCCTTTGCCAC [SEQ ID NO: 99];

TTCTGAGCAACTATCATAC [SEQ ID NO: 100];

CCCTCCGCAACG [SEQ ID NO: 101];

CCCCATCTTGTTTCC [SEQ ID NO: 102];

TCCTACACGCCTAGAC [SEQ ID NO: 103]; TATAGAGAACTGCTGTGTTC [SEQ ID NO: 104];

GAAATAGCGCCATTG [SEQ ID NO: 105];

CTCAGGGGCAACCA [SEQ ID NO: 106];

ATCCATAGGGTCCGAC [SEQ ID NO: 107];

CAATGCGGCGC [SEQ ID NO: 108];

TATAAAC GAGGTGCAG [SEQ ID NO: 109];

GAAAATGCCCTGCTA [SEQ ID NO: 110];

ACATGCGCCAGG [SEQ ID NO: 111];

GAGTAATGTGGTGTGTATG [SEQ ID NO: 112];

GCAAGGCATACTGTG [SEQ ID NO: 113];

CACTGACACTTCGTG [SEQ ID NO: 114];

GGACAATCACCAGTATTA [SEQ ID NO: 115]; AGTTTGTGAAGTACATGG [SEQ ID NO: 116];

CGGGTGATGGCC [SEQ ID NO: 117];

CCACTTGTACTGTGTAGG [SEQ ID NO: 118];

CTGTGTTTA ACT ATGGGT [SEQ ID NO: 119];

TACAACAGTATGTGTCAGAC [SEQ ID NO: 120];

AAGACAGGGAGACAGC [SEQ ID NO: 121]; CTTATAAACAATACACAGG [SEQ ID NO: 122]; ATGCACTATAGTAACACACC [SEQ ID NO: 123]; 和 ACTCCATTTTAGTGCTGTA

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[SEQ ID NO: 124]。

24. 一种组合物，其包括选自由 SEQ ID NO: 1-124 所组成的组的核酸序列。

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通过靶特异性杂交捕获方法检测核酸

本申请是按照 35 U.S.C. § 120 在 2004 年 12 月 6 日提交的美国专利申请序号第 11/005,617 号、名称为“通过靶特异性杂交捕获方法检测核酸”的部分继续申请，上述申请为 2004 年 10 月 20 日提交，现在已放弃的美国专利申请序号第 10/971,251 号的部分继续申请，所放弃的申请为 2000 年 6 月 15 日提交的美国专利申请序号第 10/971,251 号的部分继续申请，所有这些申请通过引用结合入本文。

发明领域

本发明涉及核酸检测方法的领域，具体涉及通过靶特异性杂交捕获方法检测核酸。

背景技术

生物样品中存在的特异性核酸序列的检测对鉴定和分类微生物、诊断传染病、检测和表征遗传异常、鉴定癌症相关的遗传改变、研究对疾病的遗传易感性以及测量对各种类型的治疗的响应都是重要的。检测和定量特异核酸序列的常规技术为核酸杂交和靶扩增。

可以获取各种杂交方法用于核酸的检测和研究。在传统的杂交方法中，所鉴定的核酸或者在溶液中或者附于固体载体上。所述核酸使用能够与核酸杂交的标记的核酸探针进行检测。最近，已经开发出新的杂交方法以提高检测的灵敏度和特异性。一个实例是在美国申请序号第 07/792,585 号和美国专利第 6,228,578 号中描述的 Hybrid Capture®方法。虽然这些新杂交方法比传统方法有了显著改善，但它们仍然不具备在高度同源的核酸序列之间进行完全区分的能力。

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聚合酶链式反应（PCR）是最常使用的靶核酸扩增方法。然而，当同时扩增大量的不同靶（即多重反应）时，PCR 在某种程度上受到限制，多重反应可能不仅会引起 PCR 伪影（artifacts）如引物二聚体，还会引起虚假的靶扩增。为了尝试克服这种限制，当多个靶具有同源区域时可以使用通用引物。然而，物种间的同源从来都不是百分之百的，因此引物与不同靶将具有若干错配，从而引起非均匀扩增。当样品中存在不同量的靶时，不同 PCR 的扩增效率也变化，导致不同靶的非均匀和非特异性扩增。

因此提供检测靶核酸序列的方法是本发明的一个目的，该方法不仅提供了提高的速度和灵敏度，而且是高度特异的，并且能够在多个高度同源的核酸靶序列之间进行区分。

发明内容

本发明提供了新的核酸检测方法，这里称为靶特异性**杂交捕获**（“TSHC”）。TSHC 是快速的、高度特异和灵敏的方法，能够区分和检测高度同源的核酸靶序列。

本发明的一个实施例涉及用于快速且灵敏检测靶核酸序列的检测和/或定量一个或多个靶核酸的方法，该方法包括靶向富集、扩增和检测的步骤。

实施例在本发明方法的一个实施例中，一个或多个靶核酸通过以下检测：通过将靶核酸、与所述靶核酸互补的核酸探针（其中一个是 RNA，另一个是 DNA）和固体载体混合来将靶核酸捕获至固体载体上；除去未结合的靶核酸和核酸探针；扩增所捕获的靶核酸或核酸探针，形成多个扩增子，其中所述扩增子的存在表明存在所述靶核酸；以及通过将靶核酸、与所述靶核酸的一部分杂交的选择性且可区别的寡核苷酸（即捕获序列探针，CSP）和与所述靶核酸的其它部分互补的核酸探针（即信号序列探针，SSP）混合来检测所述靶核酸，其中所述探针或靶中任一个为 RNA，另一个为 DNA，通过被直接或间接标记的 DNA：RNA 杂交体特异性结合剂检测 DNA：RNA 杂交体，从而检测靶核酸。所述 SSP 不仅仅限于作为产生检测信号的手段；

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也可以通过与靶核酸杂交，利用 DNA：RNA 杂交体特异性结合剂启动捕获来用于靶富集步骤。

在另一个实施例中，多个靶核酸通过以下检测：将多个靶核酸杂交至与所述靶核酸互补的核酸探针上，形成 DNA：RNA 杂交体；利用结合至固体载体上的 DNA：RNA 杂交体特异性抗体捕获所述 DNA：RNA 杂交体；除去未结合的靶核酸和核酸探针；利用随机引物和 DNA 聚合酶扩增所捕获的靶核酸或核酸探针，形成多个扩增子，其中所述多个扩增子的存在表明存在所述靶核酸；杂交与所述靶核酸序列的一部分互补的核酸探针，从而在靶和探针之间形成 DNA：RNA 杂交体；结合至固体载体上的寡核苷酸与靶核酸其它部分杂交，其中所述固体载体为选择性的；选择所述寡核苷酸复合体；并通过 DNA：RNA 杂交体特异性结合剂与所述 DNA：RNA 杂交体的结合来检测多个靶核酸。

在另一个实施例中，一个或多个靶 DNA 通过多重方法检测，所述多重方法的步骤为：多个靶 DNA 和与所述靶 DNA 互补的 RNA 探针杂交，形成 DNA：RNA 杂交体；利用结合至微珠上的 DNA：RNA 杂交体特异性抗体捕获所述 DNA：RNA 杂交体；通过清洗过量的核酸和探针，除去未结合的核酸和核酸探针；使用随机引物和 DNA 聚合酶等温扩增所述靶 DNA，形成多个扩增子；杂交与所述靶 DNA 的一部分互补的 RNA 探针（即 SSP），形成 DNA：RNA 杂交体；特异性 DNA 寡核苷酸与靶 DNA 的其它部分杂交，其中所述 DNA 寡核苷酸结合至选择性微珠上；并通过可检测地标记的 DNA：RNA 杂交体特异性抗体与所述 DNA：RNA 杂交体的结合以及使用结合选择性寡核苷酸（即 CSP）的微珠选择靶 DNA 来检测所述多个靶 DNA，其中所述 DNA：RNA 杂交体特异性抗体被可检测地且可区别地标记。通过 SSP 利用所述被标记的 DNA：RNA 抗体检测每个靶的存在，所述 SSP 与靶形成 DNA：RNA 杂交体，同时根据结合有寡核苷酸（即 CSP）的微珠分离和选择各种靶。扩增子和 DNA：RNA 杂交体的存在表明存在所述靶 DNA。

具体实施方式

本发明提供了用于富集、扩增和检测试验样品中一个或多个核酸的存

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在的方法。更具体地，本发明提供了高度特异和灵敏的方法，该方法能够区分和检测高度同源的核酸序列。

本发明的一个实施例涉及用于靶核酸序列多重检测的、快速灵敏的方法，其可以同时检测多个不同的靶核酸序列。这种方法可以自动化。所述方法主要包括三个步骤：靶富集，靶扩增和靶检测，它提供了用于核酸诊断的半定量或定性方法。本发明的这个实施例可以以多重的方式来进行纯化或未纯化样品中靶核酸序列的检测，其中靶的浓度可以为大约 50 个拷贝之少或者小于每毫升样品中大约 100 个靶拷贝，或每毫升中 10^8 个拷贝之多。生物样品中单一病原体或靶的检测，例如但又不限于 HSV 和 HPV 类型，可以以多重的方式进行，优选但不限于 50 丛（50-plexes）。

本发明的优点是，这种检测多个靶核酸序列的多重方法是相对快速、灵敏和准确的方法，其具有两个水平的特异性，这对于临床检测是一个有利的特征。通过使用靶特异性序列，所述两个水平的特异性在靶的富集和检测步骤中获得。

靶富集是第一步，它通过将非特异性核酸和污染物同特异性靶分离来制备用于扩增的样品。通过干扰扩增和检测，非特异的或不需要的核酸序列的存在降低了靶扩增的灵敏度。靶富集步骤将样品从可能的抑制剂中纯化出来并清除了非特异性核酸，从而提供特异性的第一个水平。清除非特异性核酸允许等温扩增步骤中的有效扩增，并且由此使同源靶之间的竞争最小化。靶富集还使双链靶变性，产生用于有效扩增的单链 DNA。为了提高靶的捕获，可以在所述靶富集步骤中使用各种试剂。例如，可以使用枯草杆菌蛋白酶以提高靶捕获（尤其在血液临床样品中）。枯草杆菌蛋白酶或羰基水解酶，是由杆菌属成员分泌的碱性蛋白酶。当通过微珠捕获靶核酸时，枯草杆菌蛋白酶的存在使微珠形成紧密的粒状沉淀，从而提高洗脱未结合或不需要的物质的容易程度。另外，靶富集步骤还将靶核酸浓缩成小体积。

通过将靶核酸、DNA：RNA 杂交体特异性结合剂和固体载体混合来实现靶的富集，所述 DNA：RNA 杂交体特异性结合剂例如与所述靶核酸互补的核酸探针，比如信号序列探针，其中所述靶核酸和 DNA：RNA 杂交体特异

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性结合剂杂交以在固体载体上形成 DNA：RNA 杂交体，并除去不形成 DNA：RNA 杂交体的或不被固体载体捕获的任何核酸。

特别地，通过形成 DNA：RNA 杂交体实现所需要的靶核酸和非特异性核酸的分离，其中所述 DNA：RNA 杂交体被捕获在固相或固体载体上，如被 DNA：RNA 杂交体特异性结合剂修饰的顺磁性微珠，该 DNA：RNA 杂交体特异性结合剂例如但不限于 DNA：RNA 杂交体特异性抗体（如**杂交捕获**抗体（HC-Ab））、单克隆抗体或多克隆抗体或其片段、蛋白质、催化失活的核糖核酸酶 H、核酸、核酸适配子或具有结合并形成三链体结构能力的寡核苷酸。Haruki 等人报道了核糖核酸酶 H 在没有 Mg^{2+}和催化剂时通过表面等离子体共振与 DNA：RNA 杂交体的结合亲和性[“Kinetic and stoichiometric analysis for the binding of Escherichia coli ribonuclease HI to RNA-DNA hybrids using surface plasmon resonance”（使用表面等离子体共振的大肠杆菌核糖核酸酶 HI 和 RNA-DNA 杂交体的结合的动力学和化学计量学分析“）*J. Biol. Chem.* 272:22015-22022, 1997, 通过引用并入]。这种靶富集的步骤可以在任何固体载体上进行，如在微量板、微芯片、微珠、顺磁性/非顺磁性微珠或之前提到的任何固相上。例如，将靶 DNA、RNA 探针或不同的 RNA 探针的集合（如果是在多重反应中的话）和与轭合的 DNA：RNA HC-抗体结合的微珠混合。靶 DNA 和 RNA 探针形成 DNA：RNA 杂交体。所述 DNA：RNA HC-抗体结合至固体载体上，如顺磁性微珠。一旦 DNA：RNA 杂交体被捕获至固体载体上，洗脱所有未结合的核酸序列和污染物，优选通过使用不会降低或影响所述杂交的缓冲液的重复洗涤。

作为一种选择或除了使用 DNA：RNA 杂交体特异性抗体外，可以使用任何长度的、与结合至固相上的靶互补的寡核苷酸或多聚核苷酸来捕获靶核酸序列。例如，识别特异性 HPV 类型的寡核苷酸或探针可以结合至固相上，例如平板、芯片或微珠。若干与特异且已知的探针结合的微珠形成微珠集合。例如，每一个微珠集合特异性地针对一种 HPV 类型。在多重方式中，可以使用多个鉴定不同 HPV 类型的微珠集合，每个微珠集合对应一个 HPV 类型。对靶核酸捕获有用的寡核苷酸可以部分或全部为锁核酸（LNA）、

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肽核酸（PNA）或具有其它修饰[*Current Protocols in Nucleic Acid Chemistry*（核酸化学的最新实验规程），Eds. Serge L. Beaucage 等人，John Wiley & Sons © 2004]。核酸探针的长度可以达到靶核酸长度的大约 100%，且长度范围在大约 50 个碱基至大约 10,000 个碱基之间。靶核酸可以为 DNA 或 RNA。如果靶核酸是 RNA，那么可以通过任何已知的常规方法从所述 RNA 靶产生 cDNA，或者所述靶 RNA 可以被 DNA 探针捕获，其中被杂交的 DNA 探针将被扩增并检测。

靶富集步骤不受这里所阐述的实施例的限制。根据这里阐述的原理，本领域技术人员应当理解如何纯化样品中的靶核酸，以便从该样品中除去或清除污染物、抑制剂和其它非特异性核酸。通过商业可得的方法和试剂盒，如质粒小量或大量制备试剂盒、凝胶回收试剂盒以及 DNA 和 RNA 纯化试剂盒，样品制备在本技术领域中是已知的且可理解的。在较低灵敏度可接受的某种情况下，可以使用这些方法和试剂盒以方便靶富集步骤。

靶富集之后，样品进行核酸扩增。使用随机序列的寡核苷酸引物和具有链置换活性的 DNA 聚合酶的核酸等温靶扩增，克服了多种限制，例如但不包括多重 PCR 的受制约的多重能力和不均匀扩增。通过与序列特异性寡核苷酸杂交来鉴定所扩增的序列或扩增子。也可以利用单个靶而不是多重方式进行扩增，并且可以结合扩增子用于杂交试验。

可以使用 DNA 聚合酶延伸引物和激活链置换。可选择地，如果使用 DNA 探针捕获靶 RNA，那么扩增和检测所杂交的 DNA 探针。另一个实施例需要反转录靶 RNA 以产生作为 DNA 靶的 cDNA，该 cDNA 可以如上所阐述被捕获。这个第二步骤优选地使用靶核酸的等温扩增。具体地，随机序列的寡核苷酸引物和具有链置换活性的 DNA 聚合酶可能在等温扩增中有用。在一个实施例中，固相或固体载体例如，但不限于磁性微珠，所述微珠具有的所捕获的靶核酸可以直接用于扩增混合物中。可以使用任何 DNA 聚合酶（包括但不限于 phi29 DNA 聚合酶、Bst DNA 聚合酶、T4 DNA 聚合酶、T7 DNA 聚合酶和 DNA 聚合酶 I）扩增 DNA 靶，所述扩增通过随机引物引导。

为了最佳效率，随机引物可以具有特别比例的 dG、dC、dT 和 dA 单体。为了最佳操作，可以完全省略一个或两个核苷酸碱基。随机引物库不必包

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括所有可能的序列。所述库中所包括的序列也不必为等摩尔浓度。事实上，随机引物库可以包括选择性针对特定疾病的引物序列的子集。长度可以在大约 4 至大约 20 个核苷酸之间变化，且优选在大约 5 至大约 8 个核苷酸之间。长度取决于引物中单体的比例。最佳扩增温度在大约 25℃至大约 70℃之间变化，优选在大约 28℃至大约 40℃之间，该扩增温度可以由本领域的技术人员根据引物的长度确定且不需要过多的试验。所述最佳温度可以通过使用预测寡核苷酸退火温度的商业可得的软件进行估测。这种商业可得的软件的一个实例为 6.0 或 6.41 版的 OLIGO™ (Molecular Biology Insights; Cascade, CO)。例如，可以用五聚体引物结合 phi29 DNA 聚合酶扩增靶 DNA 2 个小时。

例如，通过修饰保护五聚体引物不受外切核酸酶的降解。引物的结构可包括硫代磷酸酯键或 2’-O-甲基。根据所需要的灵敏度，扩增反应优选进行 1-3 小时，它比所发表的用于 phi29 DNA 聚合酶反应的实验方案的持续时间短很多[Gary J. Vora 等，*Applied and Environmental Microbiology*, 70(5): 3047-3054, 2004, 通过引用并入]。独特的试剂配方与所选择的靶富集步骤以及靶检测步骤相结合，能够使扩增时间相对变短。

本发明的靶扩增步骤优选利用等温扩增，所述等温扩增能够实现：数量不受限制的靶的均衡效率的扩增；整个序列的扩增，而不是像聚合酶链式反应（PCR）情况中片段的扩增；并且允许靶检测，即使生物样品中一部分靶序列被除去。例如，HPV 中 L1 区域经常被缺失，然而通过使用针对 L1 区域和 E 区域的杂交探针[MH Einstein 和 GN Goldberg, *Cancer Invest.* 20:1080-1085, 2002]，所述被缺失的序列仍然可以在相同水平的灵敏度下进行检测，因而即使一部分所述序列不存在也能够检测靶。多重等温扩增可以在大约 25℃至大约 40℃的温度范围下进行，反应时间范围为 1 小时到 3 小时。等温扩增的非限制性实例包括滚环扩增[Lizardi P., Huang X., Zhu Z., Bray- Ward P., Thomas D., Ward D. “Mutation detection and single molecule counting using isothermal rolling circle amplification”(“使用等温滚环扩增的突变检测和单分子计数”) *Nat.*

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Genet. 1998, 19:225-232]、多置换扩增[Little M.等，“Strand displacement amplification and homogenous real-time detection incorporated in a second generation probe system”(“引入到第二代探针系统的链置换扩增和同源实时检测”) *Clin. Chem.* 1999, 45:777-784]和蛋白引导的DNA扩增[Blanco M., Lazaro J., de Vega M., Bonnin A., Salas M “Terminal protein-primed DNA amplification”(末端蛋白引导的DNA扩增”) *Proc. Natl. Acad. Sci USA,* 1994, 91:12198-12202]。其它靶扩增实施例包括这样的实施例——在该方案中在将靶加入到扩增混合物之前从固体载体上释放所述靶。用于释放靶核酸的非限制性方法包括碱处理、高温孵育和核糖核酸酶H处理。独特的试剂配方与所选择的靶富集步骤以及靶检测步骤相结合，允许使用相对短的扩增时间。用于等温扩增的试剂（任选包括核糖核酸酶 H）可以与靶扩增同时操作，这将增加靶DNA上的引导位点的数量。这些方法允许变性和固体载体释放，使靶扩增更有效率。

下一步骤包括在所扩增的核酸中单个靶核酸序列的检测。这个步骤的灵敏度和特异性通常取决于扩增效率。通过将所扩增的靶产物或扩增子的不同部分与单个捕获序列探针杂交，来进行靶核酸的检测和说明，所述单个捕获序列探针结合至固体载体上并且特异性探针与靶序列互补形成靶复合体。靶核酸序列的不同部分与两个探针的杂交提供两个水平的特异性，保证了样品中特异性靶的检测和鉴定。

结合至固体载体（优选选择性微珠）的寡核苷酸或捕获序列探针在获取一个水平的特异性中是有用的。CSP 与所扩增的靶核酸的杂交、随后紧接的微珠的选择，能够从未结合或部分结合的实体中分离靶复合体。CSP通常过量加入以保证结合。优选地，CSP 和 SSP 没有重叠序列；但是有可能在一个CSP和SSP具有与靶核酸序列互补的重叠序列的实施例中进行所述方法。

捕获序列探针（CSP）或寡核苷酸可以被结合或连接到固相、固体载体或固体基质上，它们包括例如聚苯乙烯、聚乙烯、聚丙烯、聚碳酸酯或任何固体塑料材料，形状为平板、载玻片、皿、微珠、颗粒、杯子、条状

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物（strands）、芯片、带状物（strips）、微平板和微阵列。固相还包括玻璃微珠、玻璃试管和任何其它适当的玻璃产品。也可以使用功能化的固相如塑料或玻璃，它们已经被修饰成表面包含羧基、氨基、酰肼、醛基、核酸或核苷酸衍生物。可以使用任何固相如塑料、金属、磁性微粒或玻璃微粒、微珠、带状物、试管、载玻片、条状物、芯片、微芯片或微量板。

捕获序列探针或寡核苷酸为核酸探针，它们包含至少 8 个碱基，优选 15 至 100 个碱基，且更优选 20 至 40 个碱基。所述捕获序列探针优选为可鉴定的，或者通过它们在固体载体上（如亚孔平板（sub-well plate））的已知位置，或者例如当结合至微珠上时，通过可区别的有色染料。用于鉴定所述捕获序列探针的方式是本领域已知的，并且在本说明书中举例说明。

与可以连接在固体载体上的序列特异性探针的杂交，不仅允许特异性靶的鉴定，而且还为试验提供了第二个水平的特异性，使它成为更可信赖的临床试验。如果需要较低的灵敏度时，就不需要信号放大，而是使用利用连接在报道基因（生物素、荧光团、酶等）上的（信号）寡核苷酸或多聚核苷酸的检测。每个靶可以有多于 1 个的检测寡核苷酸。捕获寡核苷酸和检测寡核苷酸两者都可以被额外地修饰，如 PNA、LNA 等。寡核苷酸或核酸探针的长度范围可以从大约 15 个碱基到大约 10,000 个碱基，或者达到 100%的靶长度。

信号序列探针(SSP)可以是用以形成 DNA：RNA 杂交体的未标记的 RNA，该杂交体在检测步骤通过例如被标记的杂交体特异性结合剂进行识别。由于 SSP 没有被标记，因此当不与靶结合时它不会产生背景噪声，由此产生更高的特异性。例如，如果所述 RNA 信号探针的大小为 8,000 个碱基（Kb）且每个抗体结合 20 个碱基，那么可以获得被放大大约 400 倍的信号。如之前所阐述，通常信号序列探针包含至少 15 个碱基，但可以达到或多于大约 1000 个碱基，优选在大约 15 个碱基至 100 个碱基之间。信号序列探针的其它非限制性实例包括被标记的 RNA 探针和被标记的 DNA 探针。杂交不限于非重叠区域，而是实际上可以包括重叠区域。结合至固体载体上的 RNA：DNA 杂交复合体可以以多重的方式进行检测，例如使用 PE-标记的抗

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体、羧化的可区别微珠和流式细胞术检测。

靶（或扩增子）检测的一个实施例利用基于液体的阵列。微珠阵列是商业可得的，并且在这个实施例中优选羧化聚苯乙烯微珠阵列。例如，96孔平板的每一个孔都有微珠集合混合物。13 从就有 13 个微珠集合，其中每一个微珠集合都有特异性“签名”（“signature”），并且通过每个微珠内部的染料来提供所述签名。这些染料的比例对于每一个微珠集合来说都是特异的，并且在每个微珠集合之间能够区别。特异针对一个靶核酸的捕获序列探针（CSP）或寡核苷酸被应用或结合至一个特定微珠集合上。当所述靶被杂交至结合有 CSP 或寡核苷酸的微珠上时，使用互补核酸探针和被标记的 DNA：RNA 杂交体特异性结合剂，来选择特定的微珠集合然后进行检测。所述选择或分离可以在流式细胞仪中进行，其中所述微珠一个接一个地前进通过两个激光器：其中一个选择微珠上的所述签名，同时另一个检测由被标记的 DNA：RNA 杂交体特异性结合剂鉴定的靶。在这种方法中，可以区别和检测多个靶。另外，被标记的 DNA：RNA 试剂允许增强信号的检测，由此提高试验的特异性和灵敏度。

本发明的基于液体的微珠阵列的另外一个实施例利用了亚孔平板。亚孔平板为微量板，具有例如 96 个主孔，其中每一个单独的主孔被再分成多个亚孔。以前已经阐述了所述亚孔平板作为平台用于多重化[A. Roda, 等，*Clin. Chem* 46: 1654-1660, 2002, 通过引用并入]。然而，如之前所使用和阐述的，由于不同亚孔间的“串扰”（“cross-talk”）问题，亚孔平台的用途受到限制。然而，通过使用掩蔽物（mask），这里所阐述的被修饰的亚孔平台大体上消除了不同亚孔间的所述串扰问题。每一个亚孔优选具有连接在所述孔上的已知的靶特异性捕获序列探针。可以在所述主孔中加入靶核酸，在此所述靶核酸杂交至与它互补的核酸探针即信号序列探针上。在靶核酸的一部分和信号序列探针之间形成 DNA：RNA 杂交体。捕获序列探针结合靶核酸的不同部分，从而将所述 DNA：RNA 杂交体捕获至固相上。如之前所阐述的，使用与靶核酸杂交的互补核酸探针和被标记的 DNA：RNA 杂交体特异性抗体，并通过例如已知的捕获序列探针进行靶核酸的检测。信号放大和掩蔽物的使用均使具有最小背景噪声的、特异且灵敏

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的靶核酸的检测能够进行，所述掩蔽物作为盖子消除了亚孔间的串扰。

可以获取很多种可用于检测和鉴定靶序列的方法，所述方法包括但不限于：斑点杂交、尼龙膜上的反向斑点杂交、载玻片阵列的杂交、芯片阵列、微珠阵列、多孔平板底部上的阵列等。用于杂交的固体载体的非限制性实例包括微珠阵列（如商业渠道可得的微珠阵列产品）、载玻片阵列、平板阵列（如微量板每一个孔的底部上的阵列）、亚孔平板（在每一个孔中具有 16-64 个小亚孔的微量板）和电极阵列如 GenOHM 系统(GeneOhm Sciences, Inc.)。Drummond 等， *Nature Biotech,* 21: 1192-1199 (2003)。通过例如荧光、化学发光和金纳米颗粒，可以应用不同类型的报道基因以产生检测信号。如之前所讨论的，检测的方式可以包括荧光、任何基于酶的信号、化学发光或比色信号、金颗粒的光散射等。

在一个实施例中，报道基因可以连接在杂交体特异性结合剂上，例如但不限于：RNA：DNA 杂交体特异性抗体（HC-Ab）、蛋白质、催化失活的核糖核酸酶 H、核酸、核酸适配子或具有结合并形成三链体结构能力的寡核苷酸。每个被标记的结合剂结合 DNA：RNA 杂交体的核苷酸区段，例如至少 20 个核苷酸的长度，从而提供信号放大。

本发明的另一个实施例用于未纯化样品中多个靶 DNA 的多重检测，该方案包括以下步骤：1）使靶 DNA 变性以产生单链靶序列；2）加入 RNA 探针和磁性微珠，其中所述微珠之前已经被结合至杂交体特异性抗体上；3）通过洗涤除去任何未结合的靶和污染物；4）在 DNA 聚合酶和其他启动扩增和形成扩增子的试剂存在下，将所捕获的靶进行相对短时间的等温扩增，如 1-3 小时；4）使所述扩增子变性以产生单链序列；5）所扩增的靶与寡核苷酸（捕获探针）和信号序列探针杂交，所述寡核苷酸结合至固体载体上，如微珠或微量板；和 6）根据捕获探针的特征通过区别靶并检测信号序列探针复合体，来检测每一个靶 DNA 的存在或不存在。

在本发明的一个实施例中，靶和探针的杂交可以与利用杂交体结合剂的捕获步骤在同一混合物中且在升高的温度下同时进行。在整个过程中，升高的温度可以允许增加靶捕获的特异性，同时缩短反应时间。应当理解，使用本领域技术人员所熟知且实践的多种成份、缓冲液和温度，可以改变

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低的、适度的且高度严紧的杂交/洗涤条件。对于另外的严紧性条件，参见 T. Maniatis 等，Molecular Cloning: A Laboratory Manual（分子克隆：实验室手册），Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)。根据整体试验条件，杂交和捕获一步化还可以比依序进行杂交和捕获更有效率。

在其它核酸存在下，通过靶富集、靶扩增和靶检测对感兴趣的靶核酸进行检测的方法，提供了在同一反应样品中同时检测多个靶的、快速灵敏的方法。这种方法在临床诊断应用中是有用的，其用于鉴别多种疾病状况，例如患者是否感染了人乳头瘤病毒（HPV）并确定患者患有哪种特异性 HPV 类型，以便更好地对患者进行诊断和治疗。其它与特异性核酸相关的疾病在所属领域是容易得知的且可使用本发明所阐述的检测方法进行鉴定。

任何来源的纯化或未纯化形式的核酸，都可以用作试验样品。例如，试验样品可以为食品或农产品，或者人类或兽医临床样本。典型地，试验样品为生物体液如尿液、血液、血浆、血清、唾液等。可选择地，试验样品可以是怀疑带有感兴趣核酸的组织样本。试验样品中的靶核酸最初可以以离散分子存在，以便待检测的序列组成整个核酸，或者可以仅为较大分子的组分。待检测的核酸序列最初不必以纯的形式存在。试验样品可以包含核酸的复杂混合物，该混合物中的靶核酸可能与整个人类基因组 DNA 或 RNA 或致病生物体的一部分核酸序列中所包含的感兴趣基因相符合，所述生物体是临床样品中较少的组份。

试验样品中的靶核酸可以为任何来源的 DNA 或 RNA，如信使 RNA，该来源包括细菌、酵母、病毒和更高级生物体（如植物或动物）的细胞或组织。靶也可以为由 RNA 通过反转录合成的 cDNA。提取和/或纯化这种核酸的方法为本领域所熟知。靶核酸可以为双链或单链。在本方法的一个实施例中，靶核酸为单链，或在该方法的杂交和扩增步骤之前通过常规变性技术制成单链。在一个实施例中，使用碱基变性技术或高温以使双链靶 DNA 变性。

当术语“寡核苷酸”在这里使用时，它指由两个或多个脱氧核糖核苷酸或核糖核苷酸组成的核酸分子。需要的寡核苷酸可以通过任何适合的方

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法制备，如通过分子生物学的手段从天然存在的核酸中纯化或通过从头合成。在这里阐述了寡核苷酸和核酸探针的非限制性实例。

核酸探针是与试验样品中互补 RNA 或 DNA 序列杂交的核酸序列。所述探针的检测表明试验样品中存在特定的核酸序列。可以直接或间接地检测所述核酸探针。在一个实施例中，靶特异性**杂交捕获**方法应用了两种类型的核酸序列探针：捕获序列探针（CSP）和信号序列探针（SSP）。

捕获序列探针或寡核苷酸包括这样的核酸序列：其能够与靶核酸的独特区域杂交并被捕获到固相上或者其能够捕获靶核酸序列。在所述检测方法中使用的 CSP 可以为 DNA、RNA、肽核酸（PNA）或其它核酸类似物。PNA 为糖-磷酸骨架被聚酰胺或“伪肽”骨架取代的寡核苷酸。在一个优选实施例中，CSP 为 DNA。CSP 的最小长度为至少 8 个碱基，优选在 15 至 100 个碱基之间，并且更优选在 20 至 40 个碱基之间。CSP 与其要杂交的靶核酸序列大体互补。CSP 的序列优选至少 75%与靶杂交区域互补，更优选地，与该序列 100%互补。还优选的是，CSP 包含与试验样品中认为存在的、不需要的非靶序列相比，小于或等于 75%的序列同一性，更优选小于 50%的序列同一性。靶核酸中与 CSP 结合的序列优选为至少大约 12 个碱基，更优选 20-40 个碱基。与 CSP 杂交的靶核酸序列优选为单一序列或簇特异性序列。簇特异性序列为多个相关序列，它们形成离散的簇。例如，CSP 可以包含这样的序列，该序列识别特异的人乳头瘤病毒类型，例如但不限于 HPV-16、HPV-18 和 HPV-31。

在一个实施例中，在所述检测方法中使用的 CSP 可以在核酸中包含一个或多个修饰，该修饰允许将探针特异性地捕获至固相上。例如，可以通过本领域技术人员所熟知的方法将至少一个配体给 CSP 加标签来对 CSP 进行修饰，所述方法包括例如缺口翻译、化学或光化学掺入。另外，可以在多个位置利用一种或多种类型的标记物给 CSP 加标签。例如，可以利用结合链霉亲和素的生物素、结合抗地高辛的地高辛、或结合抗-DNP 的 2,4-二硝基苯酚（DNP），给 CSP 加标签。也可以使用荧光团来修饰探针。荧光团的实例包括荧光素和衍生物、藻红蛋白、别藻蓝蛋白、藻蓝蛋白、罗丹明、德克萨斯红或其它专有的荧光团。所述荧光团通常通过化学修饰连接

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并与荧光团特异性抗体结合，如抗荧光素。本领域技术人员应当理解：也可以通过掺入修饰的碱基给 CSP 加标签，所述修饰的碱基包含由特异性抗体识别的任何化学基团。用于捕获至包被有底物的固相上的其它标签和核苷酸序列加标签的方法是本领域技术人员所熟知的。核酸标记物的综述可以在 Landegren 等的“DNA Diagnostics-Molecular Techniques and Automation”(“DNA 分子诊断技术和自动化”) Science, 242:229-237 (1988)的文章中找到，该文章通过引用结合入本文。在另一个实施例中，利用生物素在核苷酸序列的 5’和 3’末端给 CSP 加标签。在另一个实施例中，CSP 没有被修饰，但是由于 CSP 所包含的序列能够与基质杂交，因而 CSP 被捕获至固相基质上。

在所述检测方法中使用的 SSP 可以是 DNA 或 RNA。信号序列探针包含能够与靶核酸中与 CSP 识别的独特区域相邻的区域杂交的核酸序列。在本发明的一个特定的实施例中，SSP 和靶核酸形成 DNA：RNA 杂交体。因此，在这个实施例中，如果靶核酸为 DNA，那么优选的 SSP 为 RNA。类似地，如果靶核酸为 RNA，那么优选的 SSP 为 DNA。SSP 的长度通常为至少 15 个碱基。然而，SSP 的长度可以达到或大于 1000 个碱基。优选更长的 SSP。SSP 可以包括单个核酸片段，或多个较小的核酸片段，它们每一个的长度都优选在 15 至 100 个碱基之间。

选择 CSP 和 SSP 的序列，以便它们不与靶核酸的同一个区域杂交或者不相互杂交。为了消除 CSP 和 SSP 之间的竞争，可以对与靶核酸序列的区域相对应的 SSP 序列和 CSP 序列进行修饰。例如，SSP 可以具有 CSP 所不包括的缺失。另外，选择 CSP 和 SSP 以便与靶中的区域杂交，例如所述区域彼此相距 50,000 个碱基。靶核酸中与 CSP 杂交的序列和与 SSP 杂交的序列之间的距离优选在大约 1 至 50,000 个碱基之间。更优选地，CSP 和 SSP 在靶核酸上之间的距离小于 3,000 个碱基，并且最优选地，所述距离小于 1,000 个碱基。

在另一个实施例中，在所述检测方法中使用的 SSP 的一部分与单链靶核酸序列形成 DNA：RNA 杂交体，该杂交体通过 DNA：RNA 杂交体特异性结合剂进行检测，并且 SSP 另一部分能够与靶核酸杂交。所述 SSP 可以通过

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如下制备：首先将与靶核酸中的序列互补的单链 DNA 序列克隆至单链 DNA 载体中，然后杂交与 DNA 载体序列互补的 RNA 以便产生 DNA：RNA 杂交体。例如，如果 M13 被用作 DNA 载体，M13 RNA 与载体中的 M13 DNA 序列杂交以产生 DNA：RNA 杂交体。由此产生的 SSP 形成了 DNA：RNA 杂交体部分和能够与靶核酸中的序列杂交的单链部分。单链 DNA 的长度应该为至少 10 个碱基，并且长度可以达到或大于 1000 个碱基。可选择地，SSP 的 DNA：RNA 杂交体部分可以在 SSP 的单链部分与靶核酸杂交的反应中或反应后形成。SSP 可以为线性的、环状的或者这两种或更多形式的组合。SSP 的 DNA：RNA 杂交体部分使用例如 DNA：RNA 杂交体特异性抗体来提供用于被捕获的杂交体检测的放大信号，所述 DNA：RNA 杂交体特异性抗体通过这里所阐述的被标记的实体进行标记或者识别。

在另一个实施例中，所述检测方法中使用的 SSP 为不含能够与靶核酸杂交的序列的分子。在这个实施例中，使用了桥探针（bridge probe），该桥探针包含能够与靶核酸杂交的序列和能够与 SSP 杂交的序列。桥探针可以为 DNA、RNA、肽核酸（PNA）或其它核酸类似物。

在另一个实施例中，SSP 的一部分与互补核酸探针形成 DNA：RNA 杂交体，并且 SSP 的单链部分包含与桥探针中的序列互补的序列。能够与靶核酸和 SSP 两者杂交的桥探针，作为中间体用于将 SSP 连接至靶核酸上。通过可以被标记实体标记或检测的 DNA：RNA 杂交体特异性结合剂来检测 DNA：RNA 杂交体。CSP 与靶核酸序列的不同部分杂交，由此将靶、桥探针和 DNA：RNA 杂交复合体捕获至固相或固体载体上。可以如以上所阐述的制备 SSP。

在另一个实施例中，在一个靶核酸检测方法中所使用的 SSP 包含多组重复序列，所述重复序列与能够与桥探针杂交的单链 RNA 序列互补。含与所述重复序列互补的序列的 DNA 寡核苷酸探针可以被用于与 SSP 杂交，以产生信号放大所需要的 DNA：RNA 双链体。

在另一个实施例中，除了包含能够与靶核酸杂交的序列外，桥探针还包含聚（A）尾。在这个实施例中所使用的 SSP 包含聚（dT）DNA 序列。因此，桥探针能够通过它的聚（A）尾与 SSP 杂交。含聚（A）序列的 RNA 探

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针可以用于与 SSP 中剩余的聚（dT）DNA 序列杂交，以形成 DNA：RNA 杂交体。含聚（dT）序列的 SSP 和含聚（A）序列的 RNA 探针优选长度为 100 至 5,000 个碱基。

如同使用以上所阐述的方法和/或本领域中已知的方法的 CSP 一样，本发明的检测靶核酸序列的方法中所使用的 SSP 可以为未修饰的或修饰的。在优选的实施例中，SSP 为未共价修饰的探针。

应当理解，在本发明的检测方法中可以应用多个 CSP 和/或 SSP。

在另一个实施例中，包含靶核酸的两个或多个不同区域的互补序列的寡核苷酸探针被融合在一起，并被用作本发明的方法中的捕获序列探针。可选择地，可以设计并产生单个探针，它包含与单个或多个靶核酸互补的序列。这里这种类型的探针也被称为“融合”CSP。当以同样的浓度使用时，所述融合捕获序列探针与两个未融合的 CSP 的组合一样有效。

本发明的核酸探针可以通过本领域已知的任何合适的方法生产，包括例如化学合成、从天然存在的来源中分离、重组生产和不对称 PCR (McCabe, 1990 In: *PCR Protocols: A guide to methods and applications*（在 PCR 实验方案：方法和应用指南中），San Diego, CA., Academic Press, 76-83)。如同在微芯片上制备寡核苷酸微阵列的情况一样，可以优选使用一个或多个区段并且随后连接所述区段来化学合成探针。Narang 等(1979 *Meth. Enzymol.* 68:90), Brown 等(1979 *Meth. Enzymol.* 68:109) 和 Caruthers 等(1985 *Meth. Enzymol* 154:287)阐述了几种化学合成方法，所有这些文章通过引用结合入本文。可选择地，克隆方法可以提供方便的核酸片段，可以分离所述片段以作为启动子引物使用。在与靶核酸杂交前，使用例如常规变性方法，首先使双链 DNA 探针提供单链。

杂交在本领域技术人员所熟知的标准杂交条件下进行。探针与核酸序列杂交的反应条件随探针变化而变化，其取决于多种因素，如探针长度、序列中的 G 核苷酸和 C 核苷酸的数量，以及杂交反应所使用的缓冲液的组成。通常本领域技术人员所了解的适度严紧杂交条件为：低于碱基完全配对的双链 DNA 的解链温度的、大约 25℃的条件。通常，通过利用具有更高温度的孵育条件，换句话说更严紧的条件，来获取更高的特异性。熟知的

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Sambrook 等的试验手册，MOLECULAR CLONING: A LABORATORY MANUAL, （分子克隆：实验手册）第二版，Cold Spring Harbor Laboratory Press, 纽约（1990）（通过引用结合入本文）的第 11 章，很详细地阐述了寡核苷酸探针的杂交条件，包括所牵涉的因素和保证特异性杂交所必须的严紧水平的阐述。通常，杂交在缓冲水溶液中进行，选择针对所述杂交的条件如温度、盐浓度和 pH 以提供足够的严紧性，以便探针与它们各自的靶核酸序列而非其它任何序列特异地杂交。

通常，在以下条件下探针和靶之间的杂交效率得到了改善：所加入的探针的量相对模板是摩尔过量的，优选 2 到 10^6 摩尔过量，更优选 10^3 到 10^6 摩尔过量。为了有效的捕获，所提供的每种 CSP 在最终杂交溶液中的浓度为至少 25 fmole/ml (25 pM)，优选在 25 fmole/ml 至 10^4 fmole/ml (10 nM)之间。每种 SSP 在最终杂交溶液中的浓度为至少 15 ng/ml，优选 150 ng/ml。表 A 显示了以 ng/ml 表示的 SSP 浓度至摩尔浓度的转换。

表 A

SSP 浓度从 ng/ml 至 fmole/ml 的转换		
SSP 浓度（ng/ml）	SSP 浓度 fmole/ml （pM）	
	SSP 为 3kb RNA	SSP 为 5kb RNA
15 ng/ml	15.1	9
150 ng/ml	151	90
600 ng/ml	606	364

在检测步骤中，可以同时或以任一顺序连续地进行 CSP 和 SSP 与靶核酸的杂交。在一个实施例中，CSP 与靶核酸的杂交和 SSP 与靶核酸的杂交同时进行。在一个实施例中，所形成的 DNA：RNA 杂交体然后可以被捕获至固相上。可以利用底物包被所述固相，所述底物与连接在 CSP 上的配体特异性结合。靶核酸序列的一部分和 SSP 形成 DNA：RNA 杂交体，同时靶核酸序列的另外部分与连接在固相上的 CSP 杂交。在另一个实施例中，在 CSP 与靶核酸的杂交之后进行 SSP 与靶核酸的杂交。在这种情况下，CSP 可以在杂交之前或杂交之后固定在固相上。CSP 和靶核酸序列两者都可以

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在 SSP 杂交反应的过程中结合至固相上。

本领域的技术人员应当理解，固相、固体载体或固体基质可以交换使用，并且包括例如玻璃、硅、金属、硝化纤维素、聚苯乙烯、聚乙烯、聚丙烯、聚碳酸酯或任何固体塑料材料，形状为平板、载玻片、皿、微珠、超微珠（microbeads）、颗粒、微粒、杯子、试管、载玻片、条状物、芯片、微芯片、带状物、膜、微平板、带有亚孔的微量板和微阵列。固相也包括玻璃微珠、玻璃试管和任何其它适当的玻璃产品。也可以使用功能化的固相如塑料或玻璃，所述塑料或玻璃已经被修饰以便其表面含羧基、氨基、酰肼、醛基、核酸或核苷酸衍生物。

在一个实施例中，利用生物素标记 CSP 并且应用链霉亲和素包被或亲和素包被的固相来捕获杂交体。在另一个实施例中，使用了链霉亲和素包被的微量板或微平板。这些平板可以被被动地或共价地包被。另一个实施例使用了微量板，其中每个单独的孔都有几个亚孔，CSP 单独连接在每个亚孔中。另一个实施例通过任何已知的且常用的结合方法，如使用间隔子或连接体来应用结合至微珠上的 CSP。当 CSP 被结合至固体载体上时，所述 CSP 可以用于捕获和/或选择与其互补的靶核酸。

可以通过本领域熟知的常规方式检测所捕获的 DNA：RNA 杂交体。可以使用被标记的或可区别的 DNA：RNA 杂交体特异性结合剂直接或间接地检测 DNA：RNA 杂交体，所述特异性结合剂如特异于 DNA：RNA 杂交体的被标记的单克隆或多克隆抗体或其片段、特异于一个或多个连接在 SSP 上的配体的抗体、被标记的抗体，或者 SSP 本身可检测的修饰。

一个优选的方法通过使用 DNA：RNA 杂交体特异性抗体检测所捕获的杂交体。在这个实施例中，DNA：RNA 杂交体特异性抗体优选利用酶、荧光分子或生物素-亲和素物进行标记，且优选为非放射活性的。所述标记可以通过本领域已知的常规手段如比色计、光度计或荧光检测器进行直接或间接地检测。一个优选的标记是例如碱性磷酸酶。也可以应用本领域技术人员已知的其它标记作为检测所结合的双链杂交体的手段。通过本领域常规使用的方法进行检测，例如在 Coutlee 等的 *J. Clin. Microbiol.* 27:1002-1007 (1989)中所阐述的比色法或化学发光法，通过引用并入。

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优选地，通过加入可以被碱性磷酸酶激活的底物利用化学发光法，来检测被结合的碱性磷酸酶物。被碱性磷酸酶激活的化学发光底物是本领域所熟知的。

优选通过在孵育步骤的过程中将所结合的 DNA：RNA 杂交体特异性结合剂，例如但不限于 DNA：RNA 杂交体特异性抗体，结合至 DNA：RNA 杂交体上，来实现所捕获的 DNA：RNA 杂交体的检测。在一个实施例中，所述 DNA：RNA 杂交体特异性抗体结合至固相上，如顺磁性微珠。通过将结合至 DNA：RNA 杂交体特异性抗体上的顺磁性微珠放置在磁场力中，从而捕获所述 DNA：RNA 杂交体，然后清洗表面以除去任何过量的抗体和其它未被杂交的物质。这个步骤富集或纯化了靶核酸序列。这些靶富集技术是本技术领域已知的。例如，可以使用重复吸管或注射器、由分解控制器调控的简单的泵或通过带有连接管道的贮器的重力流，进行人工洗涤。也可以使用商业可得的有效的管子洗涤系统。

在本发明中有用的捕获序列探针的非限制性实例包括那些具有针对 HSV-1 的序列和针对 HSV-2 的序列的捕获序列探针。针对 HPV 的 CSP 包括那些具有 SEQ ID NO: 1-64 的 CSP。这些序列所涵盖的 HPV 类型包括：HPV16、HPV18、HPV26、HPV31、HPV33、HPV35、HPV39、HPV45、HPV51、HPV52、HPV56、HPV58、HPV59、HPV66、HPV68、HPV73 和 HPV82，其中几个为高危 HPV。

在另一个实施例中，所述方法除了 CSP 和 SSP 外还应用了阻断剂探针。阻断剂探针包含与 CSP 的序列大体互补的序列。阻断剂探针的序列与 CSP 序列的互补优选为至少大约 75%，更优选与 CSP 100%互补。在杂交反应混合物中加入所述阻断剂探针，抑制了未杂交的 CSP 与靶中存在的交叉反应性核酸序列的杂交，并且因此提高检测的特异性。通常，阻断剂探针为至少大约 5 个碱基至大约 12 个碱基。在本发明的一个实施例中，杂交反应中阻断剂探针的浓度优选过量于 CSP 和 SSP 的浓度。优选地，阻断剂探针以大约 2 倍的摩尔过量存在，虽然它可以以达到 10,000 倍的摩尔过量存在。阻断剂探针可以是 DNA、RNA、肽、非特异性核酸（PNA）或其它核酸类似物。

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在一个实施例中，使用与全长或几乎全长的 CSP 互补的阻断剂探针。在 CSP、SSP 和靶核酸之间形成杂交体的反应之后，可以在反应中加入一个或多个阻断剂探针并使杂交持续需要的时间。然后如以上所阐述的方法检测杂交产物。

在另一个实施例中，使用了仅与 CSP 的一部分互补且比 CSP 短的阻断剂探针。与 CSP 相比，这些阻断剂探针具有更低的解链温度。优选地，所述阻断剂探针的解链温度比 CSP 的解链温度低 10 度。在这个实施例中，阻断剂探针优选地与 CSP 和 SSP 同时加入到靶核酸中。由于阻断剂探针具有比 CSP 更低的解链温度，因而选择杂交的起始温度以便阻断剂探针不干扰 CSP 与其靶序列的杂交。然而，当杂交混合物的温度被调整至低于用于靶杂交的温度时，阻断剂探针与 CSP 杂交并有效地阻止 CSP 与交叉反应性核酸序列的杂交。例如，在杂交捕获过程中，当杂交产物在链霉亲和素包被的微量板上进行室温孵育时，可以加入阻断剂探针。阻断剂探针的非限制性实例见表 2。

以下实施例举例说明本发明的扩增方法和检测试验以及试剂盒的使用。这些实施例仅用于说明和解释本发明，且不应以任何方式限制本发明的范围。这里所阐述的所有参考文献通过引用全部清楚地结合入本说明书。

实施例 1

靶特异性杂交捕获（TSHC）试验方案

使用阴性对照培养基 (Digene Corp., Gaithersburg, MD)或阴性颈部样本 (Digene Corp)对已知浓度的 1-型单纯疱疹病毒（HSV-1）和 2-型单纯疱疹病毒（HSV-2）病毒颗粒(Advanced Biotechnologies, Inc., Columbia, MD)或临床样品进行稀释。制备各种稀释液并等分至单个微量离心管(microfuge tube)中。加入一半体积的变性试剂 5100-0431 (Digene Corp.)。试验样品在 65℃孵育 45 分钟以使样品中的核酸变性。

变性之后，向样品中加入含信号序列探针（SSP）(每份 600 ng/ml)

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和捕获序列探针（CSP）（每份 2.5 pmole/ml)的杂交溶液，并在 74℃孵育 1 小时。然后向杂交混合物中加入阻断剂探针溶液，并在 74℃孵育 15 分钟，所述阻断剂探针溶液含一体积的 4x 探针稀释液(Digene Corp.)、一体积的变性试剂和两体积的阴性对照培养基。

在第二系列试验中，核酸变性后，将含 SSP(每份 600 ng/ml)、CSP（每份 2.5 pmole/ml)和阻断剂探针（每份 250 pmole/ml)的杂交溶液加入到样品中，并在 74℃孵育 1 小时。

含反应混合物的试管被冷却至室温，持续 5 分钟，从每个试管中取出等分试样并转移至 96 孔链霉亲和素捕获平板(Digene Corp.)的单个孔中。将所述平板在室温下以 1100 rpm 摇动 1 小时。然后轻轻倒出上清液，并用 SNM 洗涤缓冲液(Digene Corp.)洗涤该板两次，并且暂时倒置以除去残留的洗涤缓冲液。然后向每孔中加入碱性磷酸酶抗-RNA/DNA 抗体 DR-1 试剂(Digene Corp.)，并在室温下孵育 30 分钟。然后对各孔进行多次洗涤，步骤包括：1)用 Sharp 洗涤缓冲液(Digene Corp.)在室温下洗涤 3 次；2)将所述平板与 Sharp 洗涤缓冲液在加热块（heat block）上 60℃孵育 10 分钟；3）在室温下用 Sharp 洗涤缓冲液洗涤两次；4）在室温下用 SNM 洗涤缓冲液(Digene Corp.)洗涤一次。除去残留液体之后，向每孔中加入化学发光底物 5100-0350 (Digene Corp.)，并在室温下孵育 15 分钟。然后在平板发光计上读取单个孔以获取相对光单位（RLU）信号。

利用含阴性对照培养基或已知的 HSV 阴性颈部样本的溶液作为试验样品的阴性对照。以试验样品中获得的平均 RLU 与阴性对照的平均 RLU 的比来计算信噪比（S/N)。信噪比被用作测定捕获效率和靶核酸检测的基础。任意指定 2 或更大的 S/N 值作为阳性信号，而小于 2 的 S/N 值被认为是阴性。通过获得重复试验的标准偏差，除以平均值并乘以 100 以得到的百分数值来计算变异系数（CV)，变异系数测定的是一个样品组中试验的变异性。

使用 Cook 等 (1988 *Nucl. Acid. Res.*, 16: 4077-95)所阐述的方法合成在试验中使用的捕获序列探针和阻断剂探针。除非另有指出，否则在这里所阐述的实验中所使用的捕获序列探针在 5’和 3’末端被生物素标

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在试验中使用的信号序列探针为 RNA 探针，这些探针使用 Yisraeli 等(1989, *Methods in EnzymoL*, 180: 42-50)所阐述的方法进行制备。

对所有 CSP 进行试验，以确定它们在这里所阐述的**类型特异性杂交捕获**中的类型特异性能力。下列的 CSP，即 SEQ ID NO: 36-56 不显示与其它类型的交叉反应性特征，并且可以在试验中使用。

表 1：HPV 的捕获序列探针

探针	序列	大小（bp）	HPV 类型和序列位置
ZL-1	GTACAGATGGTACCGGGGTTGTAGAAGTATCTG [SEQ ID NO: 1];	33	HPV16 5360-5392
ZL-4	CTGCAACAAGACATACATCGACCGGTCCACC [SEQ ID NO: 2]	31	HPV16 495-525
DP-1	GAAGTAGGTGAGGCTGCATGTGAAGTGGTAG [SEQ ID NO: 3]	31	HPV16 5285-5315
DP-4	CAGCTCTGTGCATAACTGTGGTAACTTTCTGGG [SEQ ID NO: 4]	33	HPV16 128-160
SH-1	GAGGTCTTCTCCAACATGCTATGCAACGTCCTG [SEQ ID NO: 5]	33	HPV31 505-537
SH-4	GTGTAGGTGCATGCTCTATAGGTACATCAGGCC [SEQ ID NO: 6]	33	HPV31 5387-5419
VS-1	CAATGCCGAGCTTAGTTCATGCAATTTCCGAGG [SEQ ID NO: 7]	33	HPV31 132-164
VS-4	GAAGTAGTAGTTGCAGACGCCCCTAAAGGTTGC [SEQ ID NO: 8]	33	HPV31 5175-5207

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AH-1	GAACGCGATGGTACAGGCACTGCAGGGTCC [SEQ ID NO: 9]	30	HPV18 5308-5337
AH-2	GAACGCGATGGTACAGGCACTGCA [SEQ ID NO: 10]	24	HPV18 5314-5337
AL-1	ACGCCCACCCAATGGAATGTACCC [SEQ ID NO: 11]	24	HPV18 4451-4474
PA-4	TCTGCGTCGTTGGAGTCGTTCCTGTCGTGCTC [SEQ ID NO: 12]	32	HPV18 535-566
18-1AB	*(TTATTATTA)CTACATACATTGCCGCCATGTT CGCCA [SEQ ID NO: 13]	36	HPV18 1369-1395
18-2AB	(TTATTATTA)TGTTGCCCTCTGTGCCCCCGTTG TCTATAGCCTCCGT [SEQ ID NO: 14]	46	HPV18 1406-1442
18-3AB	(TTATTATTA)GGAGCAGTGCCCAAAAGATTAA AGTTTGC [SEQ ID NO: 15]	38	HPV18 7524-7552
18-4AB	(TTATTATTA)CACGGTGCTGGAATACGGTGAG GGGG TG [SEQ ID NO: 16]	37	HPV18 3485-3512
18-5AB	(TTATTATTA)ACGCCCACCCAATGGAATGTAC CC [SEQ ID NO: 17]	33	HPV18 4451-4474
18-6AB	(TTATTATTA)ATAGTATTGTGGTGTGTTTCTCA CAT [SEQ ID NO: 18]	35	HPV18 81-106
18-7AB	(TTATTATTA)GTTGGAGTCGTTCCTGTCGTG [SEQ ID NO: 19]	30	HPV18 538-558
18-8AB	(TTATTATTA)CGGAATTTCATTTTGGGGCTCT [SEQ ID NO: 20]	31	HPV18 634-655

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PE-1	GCTCGAAGGTCGTCTGCTGAGCTTTCTACTA CT [SEQ ID NO: 21]	33	HPV18 811-843
PZ-2	GCGCCATCCTGTAATGCACTTTTCCACAAAGC [SEQ ID NO: 22]	32	HPV45 77-108
PZ-5	TAGTGCTAGGTGTAGTGGACGCAGGAGGTGG [SEQ ID NO: 23]	31	HPV45 5295-5325
CS-1	GGTCACAACATGTATTACACTGCCCTCGGTAC [SEQ ID NO: 24]	32	HPV45 500-531
CS-4	CCTACGTCTGCGAAGTCTTTCTTGCCGTGCC [SEQ ID NO: 25]	31	HPV45 533-563
PF-1	CTGCATTGTCACTACTATCCCCACCACTACTTTG [SEQ ID NO: 26]	34	HPV45 1406-1439
PF-4	CCACAAGGCACATTCATACATACACGCACGCA [SEQ ID NO: 27]	32	HPV45 7243-7274
PA-1	GTTCTAAGGTCCTCTGCCGAGCTCTCTACTGTA [SEQ ID NO: 28]	33	HPV45 811-843
45-5AB	(TTATTATTA)TGCGGTTTTGGGGGTCGACGTGG AGGC [SEQ ID NO: 29]	36	HPV45 3444-3470
45-6AB	(TTATTATTA)AGACCTGCCCCCTAAGGGTACATA GCC [SEQ ID NO: 30]	36	HPV45 4443-4469
45-8AB	(TTATTATTA)CAGCATTGCAGCCTTTTTGTTACTT GCTTGTAATAGCTCC [SEQ ID NO: 31]	49	HPV45 1477-1516
45-9AB	(TTATTATTA)ATCCTGTAATGCACTTTTCCACAAA [SEQ ID NO: 32]	34	HPV45 79-103

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45-10AB	(TTATTATTA)GCCTGGTCACAACATGTATTAC [SEQ ID NO: 33]	31	HPV45 514-535
45-11AB	(TTATTATTA)CAGGATCTAATTCATTCTGAGGTT [SEQ ID NO: 34]	33	HPV45 633-656
ON-1	TGCGGTTTTGGGGGTCGACGTGGAGGC [SEQ ID NO: 35]	27	HPV45 3444-3470
ZZ-1	GGCGCAACCACATAACACACAGAACCACAA AAC [SEQ ID NO: 36]	33	HPV18 5285-5315
DXA-1	GTTCTACACGGGTTTGCAGCACGATCAACAA CG [SEQ ID NO: 37]	33	HPV33 1175-1207
PRA-1	CGCTGCTTGTGGTGGTCGGTTATCGTTGTCTG [SEQ ID NO: 38]	32	HPV33 3389-3420
TT-1	GACGTAGTGTCGCCTCACATTTACAACAGGAC [SEQ ID NO: 39]	32	HPV35 732-763
TT-7	CTCGCTTGGTGGGGTTGTAGGGGAGCTCGG [SEQ ID NO: 40]	30	HPV35 3432-3461
NH-1	GCTGTAGTTGTCGCAGAGTATCCCGTGAGG [SEQ ID NO: 41]	30	HPV39 833-862
FT-7	GTGAGCCTGTGTTATATGTAGTGCCCGAATCCC [SEQ ID NO: 42]	33	HPV39 5358-5390
SHA-4	CCACCTCCTGCGTCCACTACACCTAGCACTA [SEQ ID NO: 43]	31	HPV45 5295-5325
SHA-1	TGCGTGCGTGTATGTATGAATGTGCCTTGTGG [SEQ ID NO: 44]	32	HPV45 7243-7274

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OCA-1	AATTAGCGCATTGCCCCGTCCAACGTCCCG [SEQ ID NO: 45]	30	HPV51 482-511
TAA-7	CGCCGTGCACGTGTAGCCACCATATTTAATCAC [SEQ ID NO: 46]	33	HPV51 4124-4156
LFA-1	CGAATTGTGTGAGGTGCTGGAAGAATCGGTGC [SEQ ID NO: 47]	32	HPV52 140-171
PTA-7	GATCGTTCACAACTTTTACCTGCACCGGATCC [SEQ ID NO: 48]	32	HPV52 5066-5097
ZOA-1	CTAGGTTCTCTAGATGTTTGTCTCCAGCACCCC [SEQ ID NO: 49]	33	HPV56 521-553
NAA-4	CTGTCGGTATTGTCTGTGTCGCTGATGTGTG [SEQ ID NO: 50]	31	HPV56 3543-3573
LA-1	GATACACACACATTTGCAGCCCGGTCCACACA [SEQ ID NO: 51]	32	HPV58 1180-1211
LA-7	GGTGGCAAAGGACGTATGTGAGTGCAGAGGAC [SEQ ID NO: 52]	32	HPV58 5376-5407
ZV-4	GCGTTGCGGAGGGGTATGATAGTTGCTCAGAAG [SEQ ID NO: 53]	33	HPV59 3385-3417
ZV-10	GTCTAGGCGTGTAGGAGGAAACAAGATGGGG [SEQ ID NO: 54]	31	HPV59 7543-7573
PNA-1	CTGAACACAGCAGTTCTCTATACCAATGGCGCTATTTC [SEQ ID NO: 55]	38	HPV68 81-118
PNA-4	TTGGTTGCCCCTGAGCAGTCGGACCCTATGGATA [SEQ ID NO: 56]	34	HPV68 5211-5244

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CDA-4	GCGCCGCATTGCTGCACCTCGTTTATATAGCAGGG CATTTTC [SEQ ID NO: 57]	42	HPV82 4827-4868
CDA-11	CCTGGCGCATGTCATACACACCACATTACTC [SEQ ID NO: 58]	31	HPV82 7596-7626
CTA-1	CACGAAGTGTCAGTGCACAGTATGCCTTGC [SEQ ID NO: 59]	30	HPV73 6006-6045
CTA-16	GCCATGTACTTCACAAACTGTTAATACTGGTG ATTGTCCC [SEQ ID NO: 60]	40	HPV73 720-749
DLA-14	CCTACACAGTACAAGTGGAGGCCATCACCCG [SEQ ID NO: 61]	31	HPV26 3566-3596
RBA-16	GTCTGACACATACTGTTGTAACCCATAGTTAAA CACAGG [SEQ ID NO: 62]	39	HPV26 7759-7797
BNA-1	GCTGTCTCCCTGTCTTCCTGTGTATTGTTTAT AA GTGTATT [SEQ ID NO: 63]	41	HPV66 1051-1092
DLA-32	GTACAGCACTAAAATGGAGTTTGGTGTGTTACT ATAGTGCATAC [SEQ ID NO: 64]	44	HPV66 7420-7464

*括号中的序列为 HSV 未指出的“尾”序列

表 2：HPV 的阻断剂探针

探针	序列	大小	与 CSP 结合
PA-6	ACTCCAACGACGCAGA [SEQ ID NO: 65]	16	PA-4
ZZ-2	(TTTTGTGGTTCTGTGTG [SEQ ID NO: 66]	17	ZZ-1
ZZ-3	TTATGTGGTTGCGC [SEQ ID NO: 67]	14	ZZ-1
DXA-2	CGTTGTTGATCGTGC [SEQ ID NO: 68]	15	DXA-1
DXA-3	TGCAAACCCGTGTAG [SEQ ID NO: 69]	15	DXA-1

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PRA-2	CAGACAACGATAACCG [SEQ ID NO: 70]	16	PRA-1
PRA-3	ACCACCACAAGCAGC [SEQ ID NO: 71]	15	PRA-1
TT-2	GTCCTGTTGTAAATGTG [SEQ ID NO: 72]	17	TT-1
TT-3	AGGCGACACTACGTC [SEQ ID NO: 73]	15	TT-1
TT-8	CGAGCTCCCCTACAA [SEQ ID NO: 74]	15	TT-7
TT-9	CCCCACCAAGCGA [SEQ ID NO: 75]	13	TT-7
NH-2	CCTCACGGGATACTC [SEQ ID NO: 76]	15	NH-1
NH-3	TGCGACAACTACAGC [SEQ ID NO: 77]	15	NH-1
FT-8	GGATTCGGGCACTA [SEQ ID NO: 78]	14	FT-7
FT-9	CATATAACACAGGCTCAC [SEQ ID NO: 79]	18	FT-7
SHA-5	TAGTGCTAGGTGTAGTGG [SEQ ID NO: 80]	18	SHA-4
SHA-6	ACGCAGGAGGTGG [SEQ ID NO: 81]	13	SHA-4
SHA-2	TACATACACGCACGCA [SEQ ID NO: 82]	16	SHA-1
SHA-3	CCACAAGGCACATTCA [SEQ ID NO: 83]	16	SHA-1
TAA-8	GCTACACGTGCACGGCG [SEQ ID NO: 84]	17	TAA-7
TAA-9	GTGATTAAATATGGTGG [SEQ ID NO: 85]	17	TAA-7
OCA-2	GGGACGTTGGACG [SEQ ID NO: 86]	13	OCA-1
OCA-3	GGCAATGCGCTAAT [SEQ ID NO: 87]	14	OCA-1
LFA-2	CACCGATTCTTCCAG [SEQ ID NO: 88]	15	LFA-1
LFA-3	CACCTCACACAATTCG [SEQ ID NO: 89]	16	LFA-1
PTA-8	GGATCCGGTGCAG [SEQ ID NO: 90]	13	PTA-7
PTA-9	GTAAAAGTTGTGAACGATC [SEQ ID NO: 91]	19	PTA-7
ZOA-2	GGTGCTGGAGACAA [SEQ ID NO: 92]	14	ZOA-1
ZOA-3	ACATCTAGAGAACCTAG [SEQ ID NO: 93]	17	ZOA-1

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NAA-5	CACACATCAGCGACA [SEQ ID NO: 94]	15	NAA-4
NAA-6	CAGACAATACCGACAG [SEQ ID NO: 95]	16	NAA-4
LA-2	TGTGTGGACCGGG [SEQ ID NO: 96]	13	LA-1
LA-3	CTGCAAATGTGTGTGTAT [SEQ ID NO: 97]	18	LA-1
LA-8	GTCCTCTGCACTCACAT [SEQ ID NO: 98]	17	LA-7
LA-9	ACGTCCTTTGCCAC [SEQ ID NO: 99]	14	LA-7
ZV-5	TTCTGAGCAACTATCATAC [SEQ ID NO: 100]	19	ZV-4
ZV-6	CCCTCCGCAACG [SEQ ID NO: 101]	12	ZV-4
ZV-11	CCCCATCTTGTTTCC [SEQ ID NO: 102]	15	ZV-10
ZV-12	TCCTACACGCCTAGAC [SEQ ID NO: 103]	16	ZV-10
PNA-2	TATAGAGAACTGCTGTGTTC [SEQ ID NO: 104]	20	PNA-1
PNA-3	GAAATAGCGCCATTG [SEQ ID NO: 105]	15	PNA-1
PNA-5	CTCAGGGGCAACCA [SEQ ID NO: 106]	14	PNA-4
PNA-6	ATCCATAGGGTCCGAC [SEQ ID NO: 107]	16	PNA-4
CDA-5	CAATGCGGCGC [SEQ ID NO: 108]	11	CDA-4
CDA-6	TATAAAC GAGGTGCAG [SEQ ID NO: 109]	16	CDA-4
CDA-7	GAAAATGCCCTGCTA [SEQ ID NO: 110]	15	CDA-4
CDA-12	ACATGCGCCAGG [SEQ ID NO: 111]	12	CDA-11
CDA-13	GAGTAATGTGGTGTGTATG [SEQ ID NO: 112]	19	CDA-11
CTA-2	GCAAGGCATACTGTG [SEQ ID NO: 113]	15	CTA-1
CTA-3	CACTGACACTTCGTG [SEQ ID NO: 114]	15	CTA-1
CTA-17	GGACAATCACCAGTATTA [SEQ ID NO: 115]	18	CTA-16
CTA-18	AGTTTGTGAAGTACATGG [SEQ ID NO: 116]	18	CTA-16
DLA-15	CGGGTGATGGCC [SEQ ID NO: 117]	12	DLA-14

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DLA-16	CCACTTGTACTGTGTAGG [SEQ ID NO: 118]	18	DLA-14
RBA-17	CTGTGTTTA ACT ATGGGT [SEQ ID NO: 119]	18	RBA-16
RBA-18	TACAACAGTATGTGTCAGAC [SEQ ID NO: 120]	20	RBA-16
BNA-2	AAGACAGGGAGACAGC [SEQ ID NO: 121]	16	BNA-1
BNA-3	CTTATAAACAATACACAGG [SEQ ID NO: 122]	19	BNA-1
DLA-33	ATGCACTATAGTAACACACC [SEQ ID NO: 123]	20	DLA-32
DLA-34	ACTCCATTTTAGTGCTGTA [SEQ ID NO: 124]	19	DLA-32

实施例 2

CSP 和 SSP 的靶位点之间的距离对捕获效率的影响

评价捕获序列探针（CSP）和信号序列探针（SSP）在 HSV-1 靶核酸上的杂交位点之间的距离对捕获效率的影响。测定与距离 SSP 杂交位点 0.2 kb、3 kb、18 kb、36 kb 和 46 kb 的 HSV-1 核酸序列杂交的 CSP。应用实施例 1 中所阐述的一般的 TSHC 方法。捕获效率分别为 100%、50%、30%、19%和 7%（表 3）。当所述距离从 0.2 kb 增加至 46 kb 时，观察到相对捕获效率的稳定下降。

表 3：靶位点之间的距离对捕获效率的影响

CSP	SSP	靶位点之间的距离	相对捕获效率
BRH 19	H19	0.2 Kb	100%
F15R	H19	3 Kb	50%
F6R	RH5B	18 Kb	30%
F15R	RH5B	36 Kb	19%
F6R	H19	46 Kb	7%

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实施例 3

在 HSV-1 的 TSHC 检测中各种 CSP 和 SSP 的捕获效率

在利用 TSHC 的 HSV-1 检测中，对捕获序列探针（CSP）针对四个 HSV-1 特异性信号序列探针（SSP）：H19、RH5B、RH3 和 R10 中的每一个的捕获效率进行评价。用于设计捕获序列探针的标准为：1）在 HSV-1 核酸序列上，CSP 的杂交位点在 SSP 的杂交位点的 5’ 或 3’ 端的 1 kb 以内，优选在 0.5 kb 以内；并且 2）CSP 包含 HSV-1 独有的序列，该序列没有大于 10 个碱基的 HSV-2 同源序列的延伸片段（stretches）。设计 CSP 以使其靶向与 SSP 杂交位点相邻的 5’ 和 3’ 区域，每个 SSP 优选带有 5’ CSP 和 3’ CSP。商业可得的 OMIGA 软件(Oxford Molecular Group; Campbell, CA)作为鉴定这些位点的手段。设计 CSP 的解链温度（Tm）在 70℃至 85℃之间。应用在实施例 1 中所阐述的一般的 TSHC 方法。对 H19 的 11 个 CSP（结合在 6 个不同的位点上）、RH5B 的 6 个 CSP（结合在 3 个独特的位点上）、RH3 的 6 个 CSP（结合在 6 个独特的位点上）以及 R10 的 2 个 CSP 进行检测。如表 4 所示，找到了信号序列探针 H19、RH5B 和 R10 的有效捕获序列探针。

表 4：HSV-1 的 TSHC 检测的 CSP 和 SSP

SSP	CSP	Cap%	SSP	CSP	Cap%	SSP	CSP	Cap%
R10	ON-3	100%	RH5B	TS-1	50%	H19	HZ-1	50%
R10	ON-3	80%	RH5B	NC-1	75%	H19	HZ-2	20%
			RH5B	VH-4	130%	H19	ZD-1	40%
			RH5B	TS-2	25%	H19	ZD-2	20%
			RH5B	VH-3	50%	H19	BRH19	70%
						H19	VH-2	70%
						H19	F15R	25%

实施例 4

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临床样本检测

使用 TSHC 和杂交捕获 2（hc2™; Digene Corp.）两种方法检测了 64 份临床样本板（clinical specimen panel）的 HSV-1 和 HSV-2。所述板包括 15 份含数量已知的 HSV-1 或 HSV-2 的样品，以及 49 份通过 PCR 检测已知为 HSV-1 和 HSV-2 阴性的样品。因此，“期望”15 份阳性样品在 HC2 和 TSHC 两项试验中为检测阳性，并且“期望”49 份阴性样品在 HC2 和 TSHC 两项检测中为检测阴性。

应用实施例 1 中所阐述的一般 TSHC 方法。表 5 和 6 分别显示了使用 HC2 方法和 TSHC 方法的结果。使用 HC2 方法，在 49 份“期望”产生阴性结果的样品中，5 份样品检测为阳性和 44 份样品检测为阳性。作为比较，使用 TSHC 方法的所有 49 份样品经检测都为阴性。因此，在 HSV-1 和 HSV-2 的检测中，TSHC 方法在特异性方面优于 HC2 方法。

表 5：HSV-1 和 HSV-2 的 HC2 检测的观察结果和期望结果

HC2 结果	期望的结果	
	阳性	阴性
阳性	15	5
阴性	0	44
总数	15	49

表 6：HSV1 和 HSV2 的 TSHC 检测的观察结果和期望结果

TSHC 结果	期望的结果	
	阳性	阴性
阳性	14	0
阴性	1	49
总数	15	49

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实施例 5

在 HSV 的 TSHC 检测中组合探针的效果

评价 HSV-1 特异性信号序列探针和捕获序列探针组的组合对 HSV-1 检测的影响。分别使用两组不同的 RNA 信号序列探针/生物素化捕获序列探针组合(组 1：H19 加 HZ-1；和组 2：RH5b 加 TS-1 和 TS-2)，来进行 HSV-1 和 HSV-2 交叉反应性的 TSHC 检测。还使用 RNA 信号序列探针/生物素化捕获序列探针组的组合进行 TSHC，以评价两个探针组的组合对灵敏度和交叉反应性的影响。应用实施例 1 中所阐述的一般 TSHC 方法。表 7 所显示的结果清楚地表明，组合两个探针组对 HSV-1 检测的累加效应没有 HSV-2 交叉反应性的明显增加。

表 7：通过组合 HSV-1 特异性 CSP 和 SSP 提高灵敏度

捕获序列探针	信号序列探针	VP/ml	RLU	CV	S/N
HZ-1	H19	0	60	3%	1.0
HZ-1	H19	10^5 HSV-1	267	4%	4.5
HZ-1	H19	10^6 HSV-1	2316	6%	38.9
HZ-1	H19	10^7 HSV-2	49	2%	0.8
TS-1，TS-2	RH5B	0	78	6%	1.0
TS-1，TS-2	RH5B	10^5 HSV-1	291	6%	3.8
TS-1，TS-2	RH5B	10^6 HSV-1	2368	11%	30.6
TS-1，TS-2	RH5B	10^7 HSV-2	75	11%	1.0
HZ-1，TS-1，TS-2	H19，RH5B	0	70	12%	1.0
HZ-1，TS-1，TS-2	H19，RH5B	10^5 HSV-1	457	10%	6.5
HZ-1，TS-1，TS-2	H19，RH5B	10^6 HSV-1	4263	1%	60.9
HZ-1，TS-1，TS-2	H19，RH5B	10^7 HSV-2	67	6%	1.0

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实施例 6

以多重的方式检测 HPV 类型

以多重的方式进行代表单个生物样品中不同类型的人乳头瘤病毒（HPV）的 13 种序列的快速、灵敏且特异的检测。这项试验可以以 96 孔微平板的方式在 5 小时时间内分析多达 96 份样品。试验开始后可以在 5 小时内检测一种类型病毒的至少 500 个拷贝。

使用蛋白 G-顺磁性微珠（Dynal Corp.；Brown Deer, WI）和 Luminex® 100™ 羧化微珠（Luminex-Corp., Austin, TX）作为固体载体。通常如本领域所理解的和 Ausubel 等［*Current Protocols in Molecular Biology*, 纽约, Wiley Publishing, 1993, 通过引用结合］所阐述的，通过 HPV 质粒的体外转录制备 HPV RNA。新的试验有三个步骤，包括：1）靶富集；2）靶扩增；和 3）靶检测。

靶富集:

通过将预选的特异性靶和不需要的或非特异性的 DNA 以及污染物分离来制备用于扩增的样品。这个步骤除去不需要的 DNA，该 DNA 的存在大量降低靶扩增的灵敏度。通过将序列特异性 RNA：DNA 杂交体捕获到顺磁性微珠上来进行靶富集。利用 RNA：DNA 杂交体特异性抗体或**杂交捕获**抗体(Digene, Corp.)对所述微珠进行最初的修饰。更具体地，HC-Ab 顺磁性微珠通过以下进行制备:将 1 mL 蛋白 G 顺磁性微珠（2.7×10^{9}微珠/mL; Dynal, Corp.）和 375 μg 的 HC-Ab 混合，并在 PBS 中室温孵育 40 分钟，形成微珠-抗体(微珠-Ab)复合体。通过将平板放置在磁性网格（grid）(Dynal, Corp.)上并用 0.2 M、pH 为 8.2 的三乙醇胺洗涤一次，而使所述微珠-Ab 复合体被聚集在孔的底部。抗体在 20 mM DMP/0.2M 三乙醇胺、pH 8.2 的试剂中与蛋白 G 交联 30 分钟。用 1 mL 的 PBS-0.05% 吐温 20 洗涤所述微珠-Ab 复合体 3 次。然后在 1 mL 的 PBS-吐温中重悬微珠并储存在 4℃。

用各种 HPV 质粒（10 kb）对样品进行系列稀释，所述质粒在含 100 μg/ml 鲱鱼精子 DNA、1 M 胍-HCl、10 mM Tris－HCl (pH 8.0)、10 mM EDTA、0.05%叠氮化钠的去离子水溶液中制备。每份稀释的样品（50 μl）在每孔

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含 25 μl 1.75M NaOH 的 96 孔微平板中混合，并在 50℃下孵育 15 分钟以使样品变性。从表 1 选择的 13 种类型的 HPV RNA(每次试验每种 15 ng)的混合液（cocktail）被加入到变性样品的 25 μL 中和溶液中，该中和溶液为：0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 BES、0.83 M 冰醋酸、3% 聚丙烯酸、5 mM EDTA、0.05% 叠氮化钠和 0.4% Tween-20 (pH 3.6-4.1)。然后立即向被中和的样品中加入与**杂交捕获**抗体（Hc-Ab）结合的顺磁性微珠（每次试验 5.4×10^6 个微珠）。HPV RNA 和顺磁性微珠-抗体物与样品靶序列在 65℃、1100 rpm 摇动下杂交 30 分钟。通过将平板放置在磁性网格(Dynal, Corp.)上，而使磁性微珠-靶复合体聚集在孔的底部。通过移液器吸出上清液并且使用洗涤缓冲液(40 mM Tris pH 8.2、100 mM 氯化钠、0.05%叠氮化钠和 0.05% 吐温-20)洗涤微珠 3 遍，从而除去任何污染物。

靶扩增:

使用随机引物和具有链置换活性的 DNA 聚合酶进行样品靶 DNA 的等温扩增。使用由随机五聚体引导的 phi29 DNA 聚合酶扩增所述 DNA 靶。所合成的五聚体引物带有两个 3’末端硫代磷酸酯键以阻止核酸酶的降解。其它的修饰可以包括，但不限于掺入到所述引物结构的 2’O-甲基。扩增靶 DNA 2 小时。将在靶富集步骤获得的、带有所捕获的靶核酸的顺磁性微珠重悬在 20 μl 反应物中，并在 30℃孵育 2 小时，所述反应物由以下所构成：pH7.5 的 50 mM Tris-HCl、10 mM $MgCl_2$、10 mM $(NH_4)_2SO_4$、4 mM DTT、200 μg/ml 乙酰化的 BSA、每种 dNTP 400 μM、125 μM 随机五聚体和 1U phi29 DNA 聚合酶 (New England Biolabs, Inc)。

下面列出在扩增步骤中可能有用的引物。

探针 HPV16 5’-\FAM\ TCAGGACCCACAGGAGCGACCCAG-3’\BHQ

(SEQ ID NO: 125)

正向引物 5’-GCACCAAAAGAGAACTGCAATGT-3’ (SEQ ID NO: 126)

反向引物 5’-CATATACCTCACGTCGCAGTAACT-3’ (SEQ ID NO: 127)

*双标记（duel-labeled）探针和引物(Integrated DNA Technologies, Inc.；Salt Lake

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City, UT)

*FAM—荧光报道基因；BHQ—黑洞淬灭剂

靶检测

使用基于液体的微珠阵列系统检测所扩增的序列，所述阵列系统整合了光学、流体学和信号处理从而具有多重能力（Luminex 技术）。所述 Luminex 技术使用高度灵敏的试验系统，该系统基于可以在同一个测定孔中检测多个靶的荧光微珠和报道基因分子（参见 5,981,180、6,524,793、5,736,330、6,449,562、6,592,822、6,632,526、6,514,295、6,599,331、6,046,807?和 6,366,354 号美国专利）。Luminex 检测设备使用两个激光器：一个用于检测荧光微珠本身，另一个用于荧光报道基因。二重染色的荧光微珠能够多重地检测达约 100 个不同的靶。在这个实施例中，Luminex 技术被用于 HPV 分型。这种方法提高了平台的灵敏度和稳固性。

使用制造商的方案，将与特异性 HPV 类型的晚期（L）和早期（E）区域互补的两个寡核苷酸序列[MH Einstein 和 GN Goldberg, *Cancer Invest.* 20: 1080-1085, 2002, 通过引用并入]结合至商业可得的、不同染色的 Luminex 羧化微珠上。捕获寡核苷酸的序列在表 1 中显示。将一个特异性 HPV 类型的两个寡核苷酸连接在同一个微珠组上，从而消除由可能发生在癌细胞中的 HPV L-区域的缺失引起的假阴性结果。由于杂交已经在保留第一个靶富集步骤过程中引入的顺磁性微珠的同一个试管中进行，因而在检测步骤之前不需要扩增子的纯化步骤。

试验的检测步骤始于扩增子在 70℃、75 μl 438 mM NaOH 中变性 15 分钟。将 13 种不同类型的 HPV RNA(每次试验每种 15 ng)的混合液加入到变性样品的 25 ul 溶液中，该溶液为：0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 BES、0.83 M 冰醋酸、3% 聚丙烯酸、5 mM EDTA、0.05% 叠氮化钠和 0.4% Tween-20 (pH 3.6-4.1)。然后将 13 种类型的寡核苷酸分别结合至羧化的 Luminex 聚苯乙烯微珠上（每次试验 5×10^3 个微珠），并立即加入到被中和的样品中。RNA 和微珠-寡核苷酸物与靶在 65℃、同时以 1100 rpm 摇动下杂交 30 分钟。然后将样品通过 96 孔滤板(Whatman)过滤，并将微珠重悬在 100 μl 含有以下的 1×磷酸缓冲盐水（PBS）中：

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0.05% 吐温-20、10%山羊血清和 10 ng 藻红蛋白标记的小鼠单克隆 DNA：RNA 杂交体特异性抗体（PE-HC-Ab）。样品在室温（18-25℃）、同时以 1100 rpm 摇动下孵育 30 分钟。通过真空过滤将过量的抗体除去，并将带有靶复合体的微珠重悬在 100 μL 含 0.05%吐温-20 的 PBS 中。然后在将光电倍增管的增益调整至 700 伏后，通过 Luminex 流式细胞仪分析样品。

这个步骤提高的灵敏度归因于允许结合多个藻红蛋白抗体（PE-Ab）的长 RNA 信号序列探针（长度大约 6500 bp），因此，与其它检测技术如使用结合至短的杂交探针上的荧光团的方法相比，其引起大约 500 倍的信号放大。RNA 信号序列探针的长度范围可以从大约 500 个碱基至大约 10,000 个碱基，或达到正好小于靶核酸长度的 100%。

表 8 中所显示的结果示出了在所有 13 种 HPV 类型微珠组存在下，HPV16 检测的灵敏度。如同通过稳健的信噪比所显示的：质粒检测的灵敏度大于每次试验 500 个拷贝。起始样品体积为 50 μl，但可以增加至 500 μl 或者甚至整个样品（用于基于试管的试验，没有显示），这将把试验灵敏度提高到甚至更高的程度。所述结果也示出了 HPV16 检测的特异性。实质上，在微珠组上没有检测到除了 HPV16 以外的其它 HPV 靶特异性信号。表 8 的信号（平均，n=4 个复制，% CV 在 3%至 31%之间）为通过流式细胞仪计数的每份样品的 100 个微珠（事件）的中位荧光强度（MFI）。背景或噪音值为不含靶的样品的 MFI。

表 9 示出所有 13 种 HPV 类型检测的灵敏度和特异性。沿着 X 轴列出了靶 HPV 类型以及沿着 y 轴列出核酸探针。在表的对角突出显示了针对每种类型的相同 HPV 靶和 HPV 探针（阳性结果）。这些方格示出了探针针对其 HPV 靶的高水平特异性。这项试验确定探针对相应的 HPV 类型靶是特异的。表 9 中的结果表明，如 HPV16 靶仅与相应的 HPV16 探针结合（阳性结果），且不与其它 HPV 类型的探针结合（阴性结果）。

表 8：HPV16 检测的灵敏度

靶输入量（拷贝/试验）	特异于单一 HPV 类型的每个微珠组的平均信噪比												
	16	18	31	33	35	39	45	51	52	56	58	59	68

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5×10^2	194	1	1	1	1	2	1	1	1	1	1	1	1
5×10^3	419	1	1	1	1	2	1	1	1	1	1	2	1
5×10^4	553	2	1	1	1	3	1	2	1	1	1	2	1

表 9：HPV 类型的特异性

		每个特异性 HPV 捕获探针/微珠的平均 LMX-MFI（n=2），NB0713，pg：68-69（013004）												
靶	Rxn 拷贝数（双链质粒）	16	18	31	33	35	39	45	51	52	56	58	59	68
16	0.E+00	69	31	22	33	25	91	50	36	22	46	33	66	34
	1.E+02	3945	29	29	39	25	94	53	48	34	46	40	77	38
18	0.E+00	50	16	25	17	25	50	26	31	15	33	18	43	28
	1.E+02	80	3093	18	28	21	90	47	33	26	39	25	69	27
31	0.E+00	62	29	22	35	30	65	43	42	26	42	32	70	35
	1.E+02	76	25	4121	42	26	79	62	42	27	40	34	87	42
33	0.E+00	67	32	26	28	23	71	43	34	24	37	31	65	36
	1.E+02	91	29	23	8389	26	105	54	40	32	56	31	109	38
35	0.E+00	37	20	16	23	15	36	24	25	21	22	20	35	23
	1.E+02	62	31	16	29	3858	51	33	23	21	39	25	57	27
39	0.E+00	63	32	26	24	23	66	44	39	27	47	26	60	38
	1.E+02	104	34	35	39	25	3861	63	52	39	56	40	108	35
45	0.E+00	41	30	21	22	24	48	26	28	16	29	26	43	21
	1.E+02	46	24	23	18	27	53	4859	34	24	35	26	53	18
51	0.E+00	31	17	17	27	26	33	25	24	22	26	20	35	23

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	1.E+02	45	23	24	21	17	46	28	4500	12	26	23	46	18
52	0.E+00	21	25	19	21	16	32	16	19	14	19	19	41	16
	1.E+02	44	36	14	25	23	42	36	24	3500	31	24	51	25
56	0.E+00	44	23	16	25	19	40	27	29	23	24	23	42	29
	1.E+02	52	28	19	39	30	50	37	27	15	5154	26	50	18
58	0.E+00	30	19	16	17	26	34	18	27	17	19	19	36	15
	1.E+02	47	26	11	22	11	30	26	22	14	30	8631	54	22
59	0.E+00	18	22	16	16	19	26	18	17	18	21	14	26	19
	1.E+02	45	24	17	16	22	42	26	28	18	24	19	3045	20
68	0.E+00	22	26	23	13	19	26	12	20	14	20	17	20	15
	1.E+02	37	23	18	19	18	34	30	21	19	22	20	28	5063

实施例 7

HPV16、HPV58 和 HPV33 的检测

通过将每种都插有不同类型的 HPV 序列（10 kb）的 3 种质粒混合在实施例 6 所示的样品缓冲液中，来对感染多种类型 HPV 的颈部样品建模。这个实施例示出了当样品中存在的 HPV 类型多于 1 种时，所述试验检测多个感染的能力。

如实施例 12 所示，进行多个靶样品的试验。样品由 1×10^2 拷贝的 HPV16、1×10^4 拷贝的 HPV58 和 1×10^6 拷贝的 HPV33 组成。图 7 中的结果表明，即使在有高丰度的其它 HPV 类型存在下，也能检测到低丰度的 HPV16 靶。图 7 表明 HPV16 具有低的信噪比，其中信号（平均，n=4 个复制，% CV（变异系数）在 3%至 31%之间）为通过流式细胞仪计数的每份样品的 100 个微珠（事件）的中位荧光强度（MFI）。噪音值为不含靶质粒的样品的 MFI。

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实施例 8

临床样品中 HPV 的检测

在颈部样品中检测了 HPV，其中所述颈部样品被收集在样本转运培养基中(STM; Digene Corp., Gaithersburg, MD)，并且最初使用商业可得的 HC2 High-Risk HPV DNA Test™ assay (Digene Corp.)筛选高危性 HPV 类型。对所述 HC2 试验结果与本发明所阐述的 HPV 分型试验的结果进行比较。

使用刷子收集颈部样品并保存在储存培养基(1M 胍-HCl、10 mM Tris - HCl (pH 8.0)、10 mM EDTA 和 0.05% 叠氮化钠的去离子水)中直至使用。根据生产商的推荐，通过 HC2 (Digene Corp)或通过实施例 6 中所阐述的方法，来分析颈部样品的等分试样（50 μl)。为了比较两项试验的灵敏度，将阳性样品系列稀释至没有 HPV 感染的颈部样品中。

表 10：临床样品检测灵敏度的比较

阳性样品稀释液	HC2，信号/截止值	分型试验，S/N
未稀释	187.2	585.1
1∶10	21.2	310.4
1∶100	2.3	199.2
1∶1000	<1	113.4
1∶10,000	<1	43.1

在表 10 中报告了临床样品的 HC2 的灵敏度，并且显示与大约 10,000 个 HPV 靶/试验相应的信号/截止值为 2.3（100 倍稀释样品)。因此，当与本发明的 HPV 分型试验的灵敏度相比较时，本发明比其高至少 100 倍，且等于 100 个拷贝/试验，信噪比（S/N）为大约 43。

实施例 9

环状、线性和整合形式的 HPV DNA 的检测

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本发明的试验可以检测线性和环状的 DNA 序列。因此，在本发明的试验中进行的扩增不必通过“滚环”现象进行。所以，本发明的试验的靶不必是环状的。它们可以为线性的，因为环状致病的 DNA 因变性而具有切口，或者因为致病的（病毒的）DNA 靶被整合至线性宿主（人类）基因组中，或因为致病的 DNA 为线性基因组。

将样品稀释在 TE 缓冲液中，所述样品包括线性或环状 HPV16 质粒的系列稀释液或者从 CaSki 细胞中纯化的 DNA 的系列稀释液。通过使用标准技术进行环状质粒的限制性内切酶消化，来产生线性质粒。CaSki 细胞 DNA 是人类基因组 DNA，它包含每个人类基因组所整合的 HPV16 病毒基因组的 500 个拷贝。根据人类和 HPV 两个基因组的大小和比例以及所纯化的 CaSki DNA 的光密度测量值，计算每次试验 CaSki 细胞 DNA 的 HPV16 拷贝的浓度。

在扩增之前，靶在随机引物存在下于以下溶液中立即 95℃热变性 3 分钟：50 mM Tris-HCl (pH 8.0)、0.5 mM EDTA 和 50 μM 随机六聚体引物 (3’末端的键被硫代磷酸酯化)。然后在 20 μl 反应物中于 30℃扩增单链靶 120 分钟，该反应物由以下构成：50 mM Tris-HCl (pH 7.5)、10 mM $MgCl_2$、10 mM $(NH_4)_2SO_4$、4 mM DTT、0.2 mg/ml 乙酰化的 BSA、每种 dNTP 400 μM、0.13 mM 随机六聚体和 10 U phi29 DNA 聚合酶 (Epicentre, Inc)。

除了以下之外，按实施例 6 的阐述对所扩增的靶进行检测。首先利用小鼠单克隆抗 RNA：DNA 一抗（10 μg/试验）然后利用结合有藻红蛋白的山羊抗小鼠 IgG 二抗（1.6 μg/试验），给 RNA：DNA 杂交复合体加标签。

表 11 中的结果表明本发明的试验检测环状、线性和整合 DNA 具有稳健的信噪比，从而说明所述试验的有效性不依赖于“滚环”机制。然而，由于扩增环状靶比扩增线性靶的效率更高，因此“滚环”机制可以加速反应。

表 11：利用提议的方法检测环状、线性和整合的靶

靶	信噪比		
（拷贝/试验）	线性 DNA	环状 DNA	整合 DNA
1×10^2	71	123	21

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1×10^4	96	130	83
1×10^6	89	142	77

信号为通过流式细胞仪计数的每份样品的 100 个微珠（事件）的中位荧光强度（MFI）的平均值（n=2）。噪音值为不含靶质粒的样品的平均 MFI。

实施例 10

使用随机寡核苷酸通过 PHI29 DNA 聚合酶催化的等温扩增

靶扩增步骤的另一个实施例使用了对长度进行优化的、修饰的寡核苷酸的随机引物。随机五聚体 5'-NpNpNpsNpsN-3' 由 IDT, Inc 制备。为了获得这个实施例所产生的数据，通过 30℃等温扩增 2 小时，在 20 μl 总反应体积中扩增 HPV16 质粒。DNA 靶在以下 10 μl 溶液中于 95℃加热变性 3 分钟：50 mM Tris-HCl（pH 8.0）、0.5 mM EDTA、50 μM 引物。等温扩增在以下 20 μl 溶液中于 30℃进行 2 小时：50 mM Tris-HCl（pH 7.5）、10 mM $MgCl_2$、10 mM $(NH_4)_2SO_4$、4 mM DTT、200 μg/ml 乙酰化 BSA、每种 dNTP 400 μM、125 μM 随机五聚体和 1 U phi29 DNA 聚合酶（New England Biolabs, Inc）。扩增之后，在 Luminex 流式细胞仪系统中使用实施例 11 所阐述的“一抗/二抗”检测方案，来检测一半体积（10 μl）的扩增子。

表 12：随机 DNA 五聚体为最佳引物长度。所有引物在 3' 末端都具有两个硫代磷酸酯修饰。

拷贝/试验	5-mer	6-mer	7-mer	8-mer	9-mer	10-mer
0	10	9	7	13	9	14
1×10^2	5873	990	16	10	17	12
1×10^4	9864	5766	1469	176	36	26
1×10^6	12257	7567	7187	5444	2421	1621

*值 = Luminex 平均 MFI

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表 13：不同长度和修饰的随机引物的比较

拷贝/试验	1 个硫键		2 个硫键	
	5-mer	6-mer	5-mer	6-mer
0	11	10	12	7
1×10^2	851	4765	6972	922
1×10^4	10972	15250	15793	10758
1×10^6	22420	21097	19734	22313

*值 = Luminex 平均 MFI

表 12 和 13 表明带有一个硫代磷酸酯修饰的随机 DNA 六聚体引物为最佳，并且带有两个硫代磷酸酯修饰的随机 DNA 五聚体引物为最佳。

实施例 11

检测扩增靶的不同方法

比较检测所扩增的靶的三种不同的方案。一种方法利用生物素化探针，该探针是 5’ 末端标记有生物素的脱氧寡核苷酸。通过 Integrated DNA Technology Inc.; Salt Lake City, UT 的化学合成来制备这些寡核苷酸。其它两种方法使用被标记的一抗 HC-Ab (Digene Corp.)，以及依序地使用一抗 HC-Ab 和被标记的二抗 (Digene Corp.)，它们代表本发明的两个实施例。抗体标记或染色微珠的流式细胞仪检测和信号放大的联合，导致增强的信号，并由此把试验的灵敏度提高超过 10 倍。信号放大就如微珠上存在大复合体一样，通常会增强试验的背景，从而降低或消除灵敏度。然而，所阐述的本发明的方法提供了增强的信号和提高的灵敏度。所述微珠上的大复合体可干扰 Luminex 流式细胞仪系统中依赖于微珠的某种尺寸的检测模式。本发明的方法克服了这两个问题。

A. 在一种方法中，与靶核酸互补的生物素化探针被应用于与藻红蛋白(phycoethithrin)结合的链霉亲和素(SA-PE; Pierce)。所扩增的 HPV16 质粒在 75 μl 438mM 的 NaOH 中于 70℃变性 15 分钟。将两种 5’ 生物素化

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探针(5’-生物素-TATTTTATATGACACAATGT-3’ (SEQ ID NO: 128)；5’-生物素-GGTTTGTGCTAACAATAAATGTATCCATAG-3’ (SEQ ID NO: 129))中的每一种（2 纳克）加入到 25 μl 所述变性靶核酸样品的以下溶液中：0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 BES、0.83 M 冰醋酸、3% 聚丙烯酸、5 mM EDTA、0.05%叠氮化钠和 0.4% 吐温-20 (pH 3.6-4.1)。将大约 1000 个聚苯乙烯微珠结合至 HPV16 L 区域和 E 区域特异性的寡核苷酸上（见表 1）并立即加入到被中和的样品中。在 50℃、同时以 1100 rpm 摇动下进行 30 分钟杂交。每次反应加入含 SA-PE（100 ng）的 100 μl 以下溶液：438 mM NaOH、0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 BES、0.83 M 冰醋酸、3% 聚丙烯酸、5 mM EDTA、0.05% 叠氮化钠 和 0.4% Tween-20，并在室温下孵育 5 分钟。然后将样品转移到滤板，在此未被结合的物质通过真空过滤被除去。微珠重悬在 100 μl 的 PBS-0.05%吐温-20 中，并在将光电倍增管的增益调整至 700 伏后，通过 Luminex 流式细胞仪分析。

B. 在第二种方法中，所扩增的 HPV16 质粒在 75 μl 438mM 的 NaOH 中于 70℃变性 15 分钟。将 13 种不同类型的 HPV RNA（每次试验每种 15 ng，如实施例 6 所述获得）的混合物加入到 25 μl 所述变性样品的以下溶液中：0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 BES、0.83 M 冰醋酸、3% 聚丙烯酸、5 mM EDTA、0.05%叠氮化钠和 0.4% 吐温-20 (pH 3.6-4.1)。将 13 组结合有寡核苷酸的聚苯乙烯微珠（每次试验 5×10^3 个微珠）立即加入到被中和的样品中。每组微珠都有两个与特异性 HPV 类型的晚期（L）区域和早期（E）区域互补的寡核苷酸序列。捕获寡核苷酸的序列如表 1 所示。RNA 和微珠-寡核苷酸物与靶核酸在 1100 rpm 摇动、65℃下杂交 30 分钟。然后将样品在 96-孔滤板（Whatman）中过滤，并加入 100 μl 含 1 μg 一抗 HC-Ab 的 PBS-0.05% 吐温-20。室温摇动 5 分钟后，过滤反应物并加入 100 μl 含 8μg PE-标记的二抗（山羊-抗小鼠）的 PBS（0.05% Tween-20 和 10 %山羊血清溶液）。在室温下进行摇动的同时持续孵育 20 分钟。通过真空过滤将过量的抗体除去并将带有靶复合体的微珠重悬在 100 μl 的 PBS(含 0.05% 吐温-20)中。然后在将光电倍增管的增益调整至 700 伏后，通过 Luminex 流式细胞仪分析样品。

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C. 在第三种方法中，所扩增的 HPV16 质粒在 75 μl 438mM 的 NaOH 中于 70℃变性 15 分钟。将 13 种不同类型的 HPV RNA（每次试验每种 15 ng，如实施例 6 所述获得）的混合物加入到 25 μl 所述变性样品的以下溶液中：0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 BES、0.83 M 冰醋酸、3% 聚丙烯酸、5 mM EDTA、0.05%叠氮化钠和 0.4% 吐温-20（pH 3.6-4.1）。将 13 组结合有寡核苷酸的聚苯乙烯微珠（每次试验 5×10^3 个微珠）立即加入到被中和的样品中。每组微珠都有两个与特异性 HPV 类型的晚期（L）区域和早期（E）区域互补的寡核苷酸序列。捕获寡核苷酸的序列如表 1 所示。RNA 和微珠-寡聚体物与靶在 1100 rpm 摇动、65℃下杂交 30 分钟。然后将样品通过 96-孔滤板（Whatman）过滤，并将微珠重悬在 100 μl 含有以下的 PBS 中：0.05% 吐温-20、10%山羊血清和 10 ng 标记有藻红蛋白的小鼠单克隆 RNA：DNA 特异性抗体（PE-HC-Ab）。然后将样品在 1100 rpm 摇动、室温（18℃-25℃）下孵育 30 分钟。通过真空过滤将过量的抗体除去，并将带有靶复合体的微珠重悬在 100 μl 含 0.05% 吐温-20 的 PBS 中。然后在将光电倍增管的增益调整至 700 伏后，通过 Luminex 流式细胞仪分析样品。

如实施例 6 所描述的进行 HPV16 靶的等温扩增。使用在以上方案 A、B 或 C 中所阐述的三个方案中的一个来检测所扩增的靶。结果在表 14 中显示。在所阐述的三种方案中，利用生物素化探针的检测（方案 A）涉及的实施该方法的时间最少。然而，这种检测方法也产生最低的信号强度。使用被标记的一抗的检测方案获得了最大的信号强度和信噪比（表 14），该方案实现了 HC-Ab 信号放大（方案 B 和 C）。方案 C 比方案 B 使用的步骤少一步，并且是优选的。

表 14：等温扩增后 HPV16 检测的比较

靶输入量（拷贝/试验）	3 个中位荧光强度（MFI）的平均值		
	方案 A	方案 B	方案 C
5×10^2	174	4165	2506
5×10^3	517	7466	4715

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5×10^4	776	9310	7001

实施例 12

96 孔 NUCLEOLINK 平板中的 HPV 分型

在一个可选择的实施例中，可以用微量板代替微珠作为用于序列检测的固体载体。单一 HPV 类型特异性寡核苷酸被结合至所述孔的底部。靶核酸与捕获寡核苷酸杂交，然后通过化学发光进行检测。

在 NucleoLink™多孔平板(Nalge Nunc International; 纽约)的制备中，使用以下所阐述的试验方案，将两个与特异性 HPV 类型的晚期（L）区域和早期（E）区域互补的寡核苷酸序列结合至 Nucleolink 平板上。表 1 显示了捕获寡核苷酸的序列。通过将两个寡核苷酸放置在每个孔中，可以消除由常见于癌细胞中的 HPV L 区域的缺失引起的假阴性结果。在新鲜配制的、pH 为 7 的、含 10mM 1-甲基咪唑的 10mM 1-（3-二甲氨基丙基）-3-乙基碳二亚胺溶液中，制备终浓度为 100 nM（0.1 pmole/μL）的、含 5’-C12-氨基连接体的类型特异性捕获寡核苷酸（每个 HPV 类型两个）。将这种溶液以每孔 100 μL（每孔一种类型）加入到 Nucleolink 96 孔平板中，用热稳定条带密封该平板并在 50℃孵育 4 小时。然后轻轻倒出所述溶液并洗涤孔三次，浸湿 5 分钟并全部用含 150 mM 氯化钠和 0.1% 吐温-20 的 pH 7.5 的 100 mM Tris-HCl 在室温下洗涤三次。然后洗涤孔三次，浸湿 5 分钟并用分子生物学级别的水洗涤孔三次。然后允许所述平板在室温下干燥 30 分钟。将所述平板放在带有干燥剂的乙烯袋中于 4℃贮存直至需要。

贮存之后将共价结合有寡核苷酸的 NucleoLink 平板取出至室温。该平板每孔用 290 μl 的 6%酪蛋白的含 0.05%叠氮化钠的 100 mM Tris-HCl 溶液（pH7.2）室温封闭 30 分钟。在由以下构成的去离子水溶液中制备 HPV 质粒（10 kb）稀释液的标准样品（100 pg/mL）：100 μg/mL 鲱鱼精子 DNA、1 M 胍-HCl、10 mM Tris - HCl (pH 8.0)、10 mM EDTA、0.05%叠氮化钠。将样品（50 μl）在 96 孔微平板中与 25 μL 1.75M 的 NaOH 混合，并在

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70℃孵育 30 分钟。将未标记的 RNA 探针（每次试验 15 ng）的以下溶液加入到杂交平板的每个孔中：0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 N,N-双(2-羟乙基)-2-氨基乙烷磺酸、0.83 M 冰醋酸、3%聚丙烯酸、5 mM EDTA、0.05% 叠氮化钠和 0.4% 吐温-20 (pH 3.6-4.1)。所述溶液变澄清或微黄色。将每一个孔中的内容物转移到它们各自在 Nucleolink 平板上的孔中。在 1150 rpm 摇动、65℃下孵育平板 1 小时。将所述平板冷却至室温并轻轻倒出溶液。在每孔中加入结合有小鼠单克隆抗 RNA：DNA 抗体的碱性磷酸酶溶液（100 μL/孔）和 30%封闭溶液（含 6% 酪蛋白的 pH 7.2 的 100 mM Tris-HCl 和 0.05% 叠氮化钠），并在 37℃孵育平板 30 分钟，所述碱性磷酸酶溶液为：0.1M mM Tris-pH 7.4、0.6 M 氯化钠、0.1 mM 氯化锌、1 mM 氯化镁、0.05% 叠氮化钠、0.25% 吐温-20、0.2 mg/mL 核糖核酸酶、4% 羟丙基-β-环糊精、0.05% 山羊 IgG、0.0008% 磺酰罗丹明 B。将所述溶液轻轻倒出，并用含有以下的溶液洗涤平板两次：128 mM Tris pH 7.2、0.6 M 氯化钠、0.05% 叠氮化钠、0.24% 吐温；然后在每孔中加入 250 μL 含有以下的溶液：pH 7.2 的 128 mM Tris、0.6 M 氯化钠、0.05% 叠氮化钠、0.24% 吐温。所述平板用热稳定性条带密封并在 60℃孵育 10 分钟。将所述溶液轻轻倒出并用以下溶液再次洗涤平板两次：pH 7.2 的 128 mM Tris、0.6 M 氯化钠、0.05%叠氮化钠。然后用以下溶液洗涤平板两次：pH 8.2 的 40 mM Tris、100 mM 氯化钠、0.05%叠氮化钠。轻轻倒出溶液并在每孔中加入 100 μL 的 CDP-Star 底物。平板在黑暗中孵育 20 分钟并在 DML 照度计上读数。表 15 显示了通过以上所阐述的方法检测的、100 pg/mL 的 7 种不同的 HPV 克隆靶所产生的信噪比。

表 15：HPV16、18、31、33、35、45 和 52 在 96 孔平板上的分型

捕获探针	HPV 靶，信噪比							
	16	18	31	33	35	45	52	没有靶
16	30.6	1.2	1.1	0.7	0.3	0.6	0.8	1.0
18	0.5	21.9	1.1	0.5	1.1	1.5	0.7	1.0
31	0.6	0.5	264.4	0.6	0.6	1.7	1.8	1.0

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33	0.4	1.2	1.8	91.7	0.5	1.2	0.4	1.0
35	0.4	0.3	1.7	0.4	103.6	0.7	0.4	1.0
45	1.1	1.9	0.8	0.8	1.0	34.3	0.5	1.0
52	0.7	0.5	0.5	0.5	0.7	0.5	44.4	1.0

实施例 13

在与亚孔平板结合中使用掩蔽物的效果

将碱性磷酸酶溶液加入到 CDP-Star 底物中。将这种溶液(20 μL)加入到 384 孔平板(Digene Corp.)上所指明的亚孔中并孵育 20 分钟。然后在加和没有加掩蔽物(Digene Corp.)的情况下，在 384 孔平板照度计上大约 450 nm 处读取平板。表 16 显示了掩盖每一个孔/亚孔的掩蔽物提高 S/N 比例大于 100 倍。带有掩蔽物的亚孔平板具有克服之前已知的亚孔之间的串扰的优势。表 16 的每一个格子代表单个亚孔。只有两个亚孔具有化学发光底物，其用黑体字表示。

表 16：邻近亚孔间的串扰

没有掩蔽物时的相对光单位			
1712	5598	2192	1210
4076	**21986**	5181	1262
1594	5024	2830	6046
1004	1051	4844	**30128**
1712	5598	2192	1210

有掩蔽物时的相对光单位			
15	48	15	8
33	**56548**	40	26

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18	24	12	16
31	16	23	**64942**
15	48	15	8

*添加有化学发光底物的亚孔用黑体表示

实施例 14

384 亚孔平板中的 HPV 分型

以亚孔平板的方式进行单种和多种类型 HPV 的检测。在这个实施例中，将样品加入到每个孔中，并且单个靶迁移并杂交至相应的捕获序列探针上。在所述孔中加入检测试剂，该试剂在此与阳性亚孔中的靶结合。利用化学发光进行检测。

在含 1 mM 乙二胺四乙酸二钠盐的 pH 为 8.5 的 500 mM 磷酸氢二钠溶液中制备终浓度为 5 μM（5 pmole/μL）的、含 5'-C12-氨基连接体的类型特异性捕获寡核苷酸（每种 HPV 类型两个寡核苷酸）。将这种溶液以每孔 20 μL 加入到亚孔平板的每一个亚孔中（每个亚孔一种类型），用热稳定条带密封该平板并在 42℃孵育 4 小时。轻轻倒出所述溶液并用 TBS 洗涤所述孔三次。然后将平板立即用于 HPV 分型试验。

该平板用含 6%酪蛋白的 pH7.2 的 100 mM Tris-HCl、0.05%叠氮化钠（每个大孔 1 mL）室温封闭 30 分钟。在由以下构成的去离子水溶液中制备 HPV 质粒（10 kb）稀释液组成的标准样品（100 pg/mL）：100 μg/mL 鲱鱼精子 DNA、1 M胍-HCl、10 mM Tris - HCl (pH 8.0)、10 mM EDTA、0.05%叠氮化钠。样品（500 μl）在微量离心管中与 250 μl 1.75M 的 NaOH 混合，并在 70℃孵育 30 分钟。接着，将 RNA 探针的以下溶液（每次试验 15 ng）加入到每个试管中：0.125 M 柠檬酸钠、0.125 M 磷酸钠、0.6 M 三乙醇胺、0.6 N,N-双(2-羟乙基)-2-氨基乙烷磺酸、0.83 M 冰醋酸、3%聚丙烯酸、5 mM EDTA、0.05% 叠氮化钠和 0.4% 吐温-20 (pH 3.6-4.1)。所述溶液目前澄清或为微黄色。将每一个试管中的内容物（1 mL）转移到

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亚孔平板上的相应孔中。在 1150 rpm 摇动下于 65℃孵育平板 1 小时。将所述平板冷却至室温并轻轻倒出溶液。在每孔中加入结合有小鼠单克隆抗 RNA：DNA 抗体的碱性磷酸酶溶液（每大孔 1 mL）和 30%封闭溶液（含 6% 酪蛋白的 pH 7.2 的 100 mM Tris-HCl 和 0.05% 叠氮化钠），并在 37℃孵育平板 30 分钟，所述碱性磷酸酶溶液为：100 mM Tris-pH 7.4、0.6 M 氯化钠、0.1 mM 氯化锌、1 mM 氯化镁、0.05% 叠氮化钠、0.25% 吐温-20、0.2 mg/mL 核糖核酸酶、4% 羟丙基-β-环糊精、0.05% 山羊 IgG、0.0008% 磺酰罗丹明 B。将所述溶液轻轻倒出，并用以下溶液洗涤平板两次：128 mM Tris pH 7.2、0.6 M 氯化钠、0.05% 叠氮化钠、0.24% 吐温，然后在每孔中加入 1mL 以下溶液：pH 7.2 的 128 mM Tris、0.6 M 氯化钠、0.05% 叠氮化钠、0.24% Tween。所述平板用热稳定性条带密封并在 60℃孵育 10 分钟。将所述溶液轻轻倒出并用以下溶液再次洗涤平板两次：pH 7.2 的 128 mM Tris、0.6 M 氯化钠、0.05%叠氮化钠、0.24% 吐温。然后用 pH 8.2 的 40 mM Tris、100 mM 氯化钠、0.05%叠氮化钠洗涤平板两次。轻轻倒出溶液并在每孔中加入 20 μL 的 CDP-Star 底物(Applera Corp.)。如实施例 13 所阐述的，将平板在黑暗中孵育 20 分钟，并在带有掩蔽物的 384 照度计上在大约 450 nm 处读数。

表 17 中的结果表明亚孔平板中单种和多种 HPV 类型检测的特异性。以黑体字显示的信号表明检测的特异性。例如，当仅用 HPV16 探针表示时 HPV16 靶显示一个阳性信号，而用其它 HPV 类型探针时不显示信号。但是，多个感染显示多个信号。

表 17：在 384 亚孔平板中利用被动结合的 HPV 分型

靶	捕获探针							
	16	18	31	33	35	45	52	0
HPV 号	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
16	**22.3**	1.7	1.0	0.9	0.7	1.2	1.1	1.1
18	1.7	**4.9**	0.8	1.0	1.0	1.0	0.8	0.9
31	1.5	1.6	**2.6**	0.9	0.9	0.8	0.9	0.9

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33	2.6	2.2	1.2	30.7	0.8	1.1	0.9	1.3
35	2.2	2.1	1.0	1.1	11.0	1.1	0.8	1.1
45	1.7	1.8	0.9	0.9	1.1	8.4	0.7	1.2
52	1.9	2.1	1.1	1.1	1.0	1.1	6.8	1.1
16, 18, 31, 33, 35, 45, 52	35.7	8.2	4.4	28.4	11.1	9.9	5.1	1.2
16, 18, 31, 33, 35, 45	19.9	6.9	5.7	21.0	21.6	9.4	1.0	1.4
16, 18, 31, 33, 35	27.8	5.9	5.4	16.9	18.4	1.4	1.0	1.0
16, 18, 31, 33	25.4	5.7	6.2	17.9	0.9	1.0	1.1	1.5
16, 18, 31	27.5	5.3	4.7	1.3	1.2	1.2	1.1	1.6
16, 18	24.1	5.5	1.1	1.2	1.1	1.2	0.8	1.7
阳性 STM 样品	78.8	11.3	5.3	2.0	2.1	1.4	1.3	1.9

实施例 15

使用微量板和微珠的 HPV 检测方法的比较

比较本发明的两种试验方式，其中检测步骤在微量板（如通过化学发光检测）或 Luminex 羧化微珠（如通过荧光检测）上进行。

按照实施例 6 所阐述的进行靶富集和靶扩增步骤。包括杂交和检测的第三个步骤以以下两种方式进行：一种按照实施例 6 所阐述的在 Luminex 羧化微珠上进行，另一种按照实施例 14 所阐述的在微量板上进行。

表 18 的结果表明平板试验在 30 分钟的扩增中可以检测到 500 个输入拷贝，S/N 比为 9。在扩增 1 小时时，平板试验的灵敏度比 Luminex 方式高出至少一个数量级。

表 18：使用平板和微珠检测方式的 HPV 检测

靶输入量	微珠（荧光）		
拷贝/试验	30 分钟扩增	60 分钟扩增	120 分钟扩增

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	S/N	%CV	S/N	%CV	S/N	%CV
0	1	20%	1	21%	1	16%
5×10^2	1	8%	24	3%	1009	13%
5×10^3	2	8%	281	21%	1548	5%
5×10^4	18	14%	1288	9%	1520	19%
靶输入量 拷贝/试验	平板（化学发光）					
	30 分钟扩增		60 分钟扩增		120 分钟扩增	
	S/N	%CV	S/N	%CV	S/N	%CV
0	1	42%	1	81%	1	57%
5×10^2	9	8%	1046	29%	12320	7%
5×10^3	102	15%	9054	6%	11715	15%
5×10^4	929	8%	13019	5%	12649	2%

实施例 16

随机引物中核苷酸的比例对等温扩增效率的影响

使用在实施例 6 所阐述的方法的靶扩增步骤中核苷酸的不同组合和比例，来化学合成随机引物。表 19 中的结果表明 dG 和 T 在实现有效扩增中起主要作用。省略 dA 的影响较不显著，省略 dC 没有任何影响。利用五聚体引物进行等温扩增 2 个小时。按照实施例 6 的阐述进行整个试验。以信噪比计算相对效率，其中标准的引物组成的效率为(1.0)。

表 19：靶检测的相对效率取决于随机引物的组成

引物组成	靶输入量（拷贝/试验）	相对试验效率
dA:T:dG:dC	100	(1.0)
1: 1: 1: 1	1000	(1.0)
dA:T:dG	100	0.95
1: 1: 1	1000	1.0
T: dG:dC	100	0.4
1: 1: 1	1000	0.6
dA: dG:dC	100	0.10

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1: 1: 1	1000	0.12
dA:T: dC	100	<0.1
1: 1: 1	1000	<0.1
dA:T:dG:dC	100	0.7
1: 1: 3: 1	1000	0.6
dA:T:dG:dC	100	0.95
2.5: 2.5: 4: 1	1000	1.0
dA:T:dG	100	1.5
1: 1:2	1000	1.2

实施例 17

在微芯片阵列上的 HPV 分型

可以在微芯片阵列形式上进行 HPV 类型（单个和多个类型的 HPV）的检测。根据实施例 6 进行靶核酸捕获和扩增。根据用于洗涤载玻片、膜、芯片等的商业可得的产品和说明，实施试剂的配制和洗涤试验方案。根据实施例 6，靶检测步骤包括靶变性和与 RNA 探针或信号序列探针的杂交。

在这个实施例中，按实施例 6 的阐述，在寡核苷酸点阵列而非微珠上，进行靶与序列特异性捕获序列寡核苷酸探针的杂交。术语“点阵列”（spotted array）指固体载体表面上的二维系列元素，在此每个元素为沉积在特定位置的寡核苷酸的等分试样。用于这种方式的固体载体包括：玻璃载玻片、尼龙膜或微芯片（玻璃或硅）。另外的实施例和阐述在“Microarrays And Cancer Research”（“微阵列和癌症研究”） Ed. J. Warrington, Eaton Publishing, 2002 中找到。

把用 5’-C12-氨基连接体修饰的类型特异性捕获序列寡核苷酸探针（每种 HPV 类型两个寡核苷酸）点样在固体载体如微阵列上，每个点对应于一种特异的 HPV 类型。寡核苷酸通过例如伯氨基连接。固体载体在室温下用 2×SSC（氯化钠和柠檬酸钠）缓冲液平衡 10 分钟，然后在 37℃在预杂交缓冲液（2×SSC/0.05%封闭试剂/5% 硫酸葡聚糖/0.1% SDS)中孵育 30 分钟，该预杂交缓冲液含 50 μg/mL 变性的鲱鱼精子 DNA。然后在加入所扩

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增的靶核酸和 RNA 信号序列探针的相同预杂交缓冲液中进行杂交。当在摇动下于 37℃孵育 3 小时时，杂交溶液中的靶核酸、RNA 信号序列探针和类型特异性捕获序列寡核苷酸探针发生了杂交。

含扩增子的点的检测可以用两种方法进行：a)使用被 Cy 荧光染料标记的**杂交捕获**抗体（HC-Ab），随后使用商业可得的扫描仪进行扫描；或 b）使用碱性磷酸酶标记的 HC-Ab，随后应用起沉淀作用的底物（例如 5-溴-4-氯-3-吲哚基-磷酸酯和四唑硝基蓝：NBT/BCIP）。在后一种情况下，信号不用仪器辅助设备就可以检测。

上述各种实施例的阐述目的是为了举例说明和描述。其并不能够穷举或者限制本发明公开的精确形式。根据以上说明，明显的修饰或变更都是可能的。选择和阐述所讨论的实施例以提供例证及其实际应用，由此使本领域普通技术人员能够利用各种实施例和适合所关注的特定用途的各种修饰。如同所附的权利要求所确定的，当根据它们公平、合法、公正地赋予的宽度来解释时，所有这种修饰和变更都包括在所述发明中。

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Lorincz, Attila
Eder, Paul
Lowe, Brian
Mallonee, Richard
Thai, Ha

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<150> US 11/005,617
<151> 2004-12-06

<150> US 10/971,251
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accaccacaa gcagc 15

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Espacenet

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A microarray system and a process for producing microarrays

Inventor(s): DIETER TRAU [SG]; JOHNSON NG KIAN KOK [SG] + (TRAU DIETER, ; NG KIAN KOK JOHNSON, ; LIU WEN-TSO)

Applicant(s): UNIV SINGAPORE [SG] + (UNIV SINGAPORE)

Classification: **- international:** ***G01N21/29***
- cooperative: **B01J19/0046; C12Q1/6834; C12Q1/6883; C40B20/02; C40B30/04; C40B40/04; G01N21/6452; G01N33/54313; G06T5/50; G06T7/0008; G06T7/0028;** B01J2219/00317; B01J2219/00459; B01J2219/00466; B01J2219/0054; B01J2219/00648; B01J2219/00659; B01J2219/00677; B01J2219/00702; B01J2219/00722; B01J2219/0074; C40B50/16; C40B50/18; C40B60/14 more

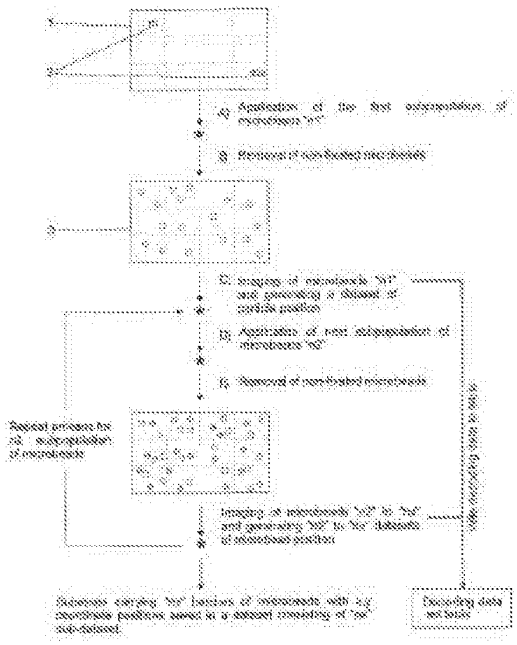
Application number: CN2007828982 20070803

Priority number(s): WO2007SG00232 20070803 ; US20060835648P 20060803

Also published as: CN101529227 (B) WO2008016335 (A1) US2014045721 (A1) US2010075865 (A1) US8927465 (B2) more

Abstract of CN101529227 (A)

The present invention provides a process for making a micro-array. The process comprises the step of depositing a population of microbeads on a substrate having at least one fiducial. The population being comprised of at least two sub-populations, preferably multiple sub-populations, each comprising a known active agent capable of specific binding with at least one target analyte. The said subpopulations are deposited sequentially and at discrete periods of each other. The process also comprises the step of making images of the substrate after deposition of each subpopulation. The images are then compared using the fiducial as a reference to thereby determine the location of each microbead and to identify the subpopulation, and its known active agent, based on differences between each image. Also disclosed in a system for using the microarray.



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[51] Int. Cl.
G01N 21/29 (2006.01)
C12Q 1/68 (2006.01)
C07K 17/14 (2006.01)

[43] 公开日 2009 年 9 月 9 日

[11] 公开号 CN 101529227A

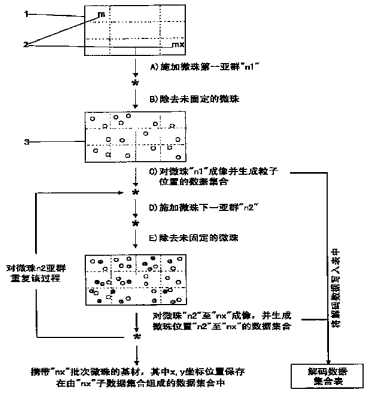
[22] 申请日 2007.8.3
[21] 申请号 200780028982.3
[30] 优先权
[32] 2006. 8. 3 [33] US [31] 60/835,648
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[87] 国际公布 WO2008/016335 英 2008.2.7
[85] 进入国家阶段日期 2009.2.3
[71] 申请人 新加坡国立大学
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[72] 发明人 陶谛德 刘文佐 黄建国
[74] 专利代理机构 永新专利商标代理有限公司
代理人 林晓红
权利要求书 3 页 说明书 26 页 附图 4 页

[54] 发明名称

微阵列系统和用于制备微阵列的方法

[57] 摘要

用于制备微阵列的方法。 该方法包括使微珠的群沉积在具有至少一个基准点的基材上的步骤。 所述群由至少两个亚群、优选多个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂。 将所述亚群依次且以彼此分立的时间段沉积。 该方法也包括在沉积每个亚群后对基材成像的步骤。 随后使用基准点作为参考比较图像从而确定每个微珠的位置，并且旨在基于各图像之间的差异识别所述亚群及其已知活性剂。 还披露了使用所述微阵列的系统。



1. 一种用于制备微阵列的方法，所述方法包括步骤：

使微珠群沉积在具有至少一个基准点的基材上，所述的群由至少两个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂，其中将所述亚群依次且以彼此分立的时间段沉积；

在沉积每个亚群后对所述基材成像；和

使用所述基准点作为参考比较所述图像，以确定每个微珠的位置，并基于各图像之间的差异来识别所述亚群及其已知活性剂。

2. 如权利要求 1 所述的方法，包括步骤：记录指示各所述微珠及其亚群的位置的数据。

3. 如权利要求 1 所述的方法，其中所述的记录步骤包括在计算机存储器中记录所述数据。

4. 如权利要求 1 所述的方法，其中所述的活性剂是生物活性剂。

5. 如权利要求 4 所述的方法，其中所述的生物活性剂是选自蛋白质、核酸、酶、细胞、病毒颗粒和抗体中的至少一种物质。

6. 如权利要求 1 所述的方法，包括以化学反应基使所述微珠官能化的步骤。

7. 如权利要求 1 所述的方法，其中所述微珠的大小是 100 nm-500μm。

8. 如权利要求 1 所述的方法，包括在沉积每种亚群后，除去未与所述基材形成连接的所述微珠。

9. 如权利要求 1 所述的方法，包括在所述基材上形成至少一个基准点的步骤。

10. 如权利要求 1 所述的方法，其中所述的基材选自硅、塑料、玻璃、陶瓷、橡胶、聚合物或其复合材料。

11. 如权利要求 1 所述的方法，形成在所述基材中的三维结构以促进在该基材上容纳所述微珠。

12. 如权利要求 11 所述的方法，其中所述的三维结构是在基本上平坦的基材中形成的凹陷。

13. 如权利要求 1 所述的方法，其中所述亚群依次沉积每个分立时间段的步骤进行 1 次至多达 10,000 次。

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14. 如权利要求 1 所述的方法，包括提供光学标签以指示所述微珠与所述靶分析物结合的步骤。

15. 在用于鉴定样品中存在一种或多种靶分析物的系统内使用的微阵列， 所述微阵列包含沉积在具有至少一个基准点的基材表面上的随机微珠群，所述的群由至少两个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂，其中已经将每个所述亚群依次且以彼此分立的时间段沉积以便能够从每个分立的沉积时间段期间所获取的对比图像中确定每个微珠及其相应亚群相对于所述基准点的位置。

16. 用于鉴定样品中存在一种或多种靶分析物的微阵列系统，所述微阵列系统包含：

沉积在具有至少一个基准点的基材表面上的随机微珠群，所述的群由至少两个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂，其中已经将所述亚群依次且以彼此分立的时间段沉积；

存储器，其记录每个微珠及其相应亚群相对于所述至少一个基准点的位置，每个微珠及其相应亚群的所述位置已经通过比较在所述的依次分立沉积时间段之间的所述微珠的图像进行确定；

检测器，其检测所述微珠在接触所述样品时的变化；和

处理器，其应答于程序指令以询问所述存储器并且比较从所述检测器接收的数据，以便基于每个微珠及其相应亚群的位置而鉴定所述样品中所述一种或多种靶分析物的存在。

17. 如权利要求 16 中所述的微阵列系统，包含用于存储在所述存储器中的解码数据，所述解码数据含有指示每一所述微珠相对于所述基准点的位置和已知活性剂的数据。

18. 如权利要求 17 中所要求保护的微阵列系统，其中所述的解码数据处于用于经计算机网络传输的格式。

19. 如权利要求 17 中所要求保护的微阵列系统，其中需要标识符以获得对所述解码数据的访问权。

20. 用于鉴定样品中靶分析物的方法，所述方法包括以下步骤:

使如权利要求 15 所述的微阵列与所述样品接触；

鉴定已经发生变化的所述微珠亚群，其中所述的变化指示所述活性剂

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与所述样品中存在的靶分析物结合；和

基于显示所述变化的所述微珠的位置和已知活性剂，鉴定所述样品中任何靶分析物的存在。

21. 如权利要求 20 所述的方法，其中所述的变化是光学变化。

22. 如权利要求 21 所述的方法，包括提供光学标签以指示所述微珠与所述靶分析物结合的步骤。

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微阵列系统和用于制备微阵列的方法

技术领域

本发明一般地涉及用于鉴定流体样品中存在靶分析物的微阵列。本发明也涉及用于制备微阵列的方法。

背景技术

微阵列一般通过将生物分子设置在平坦基材上加以制备。已知的微阵列制备技术可以分成两种方法:

1) 生物分子的位置在该生物分子转移至基材之前被指定("有序生物分子沉积法"); 和

2) 生物分子的位置随机地分配在基材上("随机生物分子沉积法")。

有序生物分子沉积法是迄今用于微阵列制备的最常见方式。在 Schena, M.,“DNA Microarrays”, Oxford University Press, 2001 中给出详细的实验描述。也在 Vivian G.等,“Making and reading microarrays”, Nature Genetics 21, 15-19, 1999 中给出技术性综述。在已知的微阵列制备方法中,机器人将靶向分析物的生物分子转移至基材,产生有序阵列。通常,生物分子溶解于缓冲液中。常见的转移体积是 0.1 nl 至 1 ml。生物分子的实例是 DNA、寡核苷酸和蛋白质。

不同技术可用于将生物分子溶液点样在基材上。点样技术可以分成两类:

1) 在打印机与表面之间无物理接触的技术; 和

2) 利用打印头与基材之间物理接触的技术。

属于第一类的技术基于气泡喷墨技术,其中产生小液滴并通过压电诱导或热诱导的压力使小液滴加速,或用马达驱动的微量注射器产生小液滴(Okamoto T.等. “Microarray fabrication with covalent attachment of DNA using Bubble Jet technology”, Nature Biotechnology, 18, 438-441, 2000)。

属于第二类的技术使用可以容纳小体积样品溶液并将该样品溶液的一

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部分通过产生物理接触而转移至基材的打印头。转移的体积依赖于打印头的几何形状、溶剂性质和基材疏水性。

在以上提及的全部技术中，打印头或打印针可以联合成组(通常 8 个至 64 个)以平行地印刷多个样品。平行印刷的点的距离因打印头或打印针的物理大小而是固定的。这限制平行印刷步骤中的最小点距至 2-5 mm。为实现高的点密度，多个打印必须在具有 x 和/或 y 方向上微小偏移的情况下实施。

在全部的上文已知方法中，所印刷点的数目与打印头产品和打印步骤呈线性关系，因此限制产生高质量的阵列。

制备时间随所产生芯片的数目并随所印刷的具体生物识别元件的数目而线性增加。阵列密度因打印设备精度而是有限的。基于机器人的技术的缺点是需要长时间以产生具有高的点密度的大量阵列。

另一种微阵列制备技术基于将聚合物直接合成到微阵列基材上，并且在 PCT 国际专利公开号 WO 9210092 和美国专利号 5,405,783 和 5,770,722 中描述。这些披露的技术限用于寡核苷酸并且使用光刻法。披露的技术仅在需要极高数目(成千)的不同寡核苷酸序列时才是经济的。

在随机生物分子沉积法中，将生物分子点随机地产生在基材上。常见技术使用微珠混合物或由亚群组成的微珠。为确定微珠属于哪种亚群，必须对全部微珠编码。编码微珠的技术基于微珠用不同颜色的荧光团进行标记或用携带不同颜色的微微珠进行标记(WO2004066210 HYBRID RANDOM BEAD/CHIP BASED MICROARRAY)、通过金属标签(Lockhart, D.J.等，“Multiplex Metallica”, Nature Biotechnology, 19, 1122-1123, 2001)、通过射频标签、通过荧光能转移标签(Tong, A.K.等，“Combinatorial fluorescence energy transfer tags for multiplex biological assays”, Nature Biotechnology, 19, 756-759, 2001)、通过寡核苷酸标签或用量子点(Han, M.等，“Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules”, Nature Biotechnology 19, 631-635, 2001)进行标记。光学标签(例如荧光)编码法还在 PCT 国际专利公开号 WO 0016101、WO 0061281 和 WO 9853093 中描述。利用光纤束替代平坦基材的方法在美国专利号 US 5,244, 636 和 US 5,250,264 中描述，其中所述的光纤束具有与其远端连接的染料。使用光纤的好处是避免可能因使用不同染料所致的光干扰问题。

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大量的聚苯乙烯珠(约 3.2 μm)可以通过称作微珠近表面光控电动装配法 (light-controlled electrokinetic assembly of microbeads near surfaces (LEAPS))的方法在蚀刻的硅装置中装配成高密度珠阵列芯片(Li 等. Tissue Antigens 63: 518-528)。在这种方法中，将硅晶片的背侧与金属电极接触。随后使对电极与散布在晶片表面上的悬浮珠溶液接触。使用交流电，可以将珠移动至芯片上指定的低阻抗区域。含有约 4000 个珠子的阵列已经在 300 μm x 300μm 区域内成功排列出来，并且应用于人白细胞抗原基因复合体的单核苷酸多态性(SNP)分析中。虽然没有报道分析次数，不过装配数千个分别可寻址珠的能力使得这种珠阵列芯片成为用于高通量 SNP 基因型分型的引人注目的平台。

在已知方法中，在将全部珠随机布置在基材上后，译解各个点或珠的信息，一般同时使用该阵列用于分析。对微珠解码并且将解码数据与因读取由分析物引起的微珠特性变化而获得数据合并。用于对所编码微珠解码的自动化信息处理法在 PCT 国际专利公开号 WO 0047996 中描述。

与此类方法(如 WO 0047996 中披露的那些方法)相关的解码方法的不足在于解码步骤可能是复杂而耗费时间的。在 WO 0047996 中，解码步骤需要添加第一多种解码性结合配体至包含固定化结合配体的随机微珠阵列并且产生第一数据图像。使用基准点来生成第一寄存数据图像。添加第二多种解码性结合配体至所述随机微珠阵列并且产生第二数据图像。使用所述基准点来生成第二寄存数据图像。随后使用计算机系统来比较第一和第二寄存数据图像以鉴定至少两种生物活性剂的位置。不过，在 WO 0047996 中所教导的使用解码性结合配体具有明显缺点。尤其，需要长时间以读取全部微珠的已编码代码，如 Nolan J. P.等. “Suspension array technology: evolution of the flat-array paradigm”, Trends in Biotechnology, 20, 9-12, 2002 中所讨论。

此外，编码系统的产生是困难的且需要多个加工步骤。需要读出仪器以解码所编码的微珠。例如, 由不同荧光团产生的微珠代码需要多种波长测量法，因而需要多种光源和滤镜。这些仪器是复杂且昂贵的。全部上述因素致使微阵列的制备更复杂，耗费更多时间并且因而执行更昂贵。

需要提供克服或至少改善一种或多种以上所述不足的用于制备微阵列

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的方法。

需要提供克服或至少改善一种或多种以上所述不足的用于鉴定靶分析物存在的系统。

需要提供在制备后无需解读微阵列的用于制备微阵列的方法。

发明简述

根据第一方面，提供用于制备微阵列的方法，所述方法包括步骤：

使微珠群沉积在具有至少一个基准点的基材上，所述的群由至少两个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂，其中将所述亚群依次且以彼此分立的时间段沉积；

在沉积每种亚群后对所述基材成像；和

使用所述基准点作为参考比较所述图像，旨在基于各图像的差异因而确定每个微珠的位置并且旨在鉴定亚群及其已知活性剂。

有利地，能够通过其中确定每个微珠及其相应亚群相对于所述至少一个基准点的位置的比较步骤对微珠解码。更有利地，这是因为成像步骤在所述微珠的连续性沉积步骤之间进行。

有利地，一旦全部微珠已经安置在基材上，则所述方法不包括解读微阵列的步骤。解码在制备微阵列期间于微珠沉积在基材上的同时进行。

有利地，微珠可以随机地沉积在基材上.

有利地，所述方法不需要标识符或标签(如解码性结合配体)来确定每个微珠及其相应群的位置。因此，在一个实施方案中，该方法不包括使用标识符或标签(如解码性结合配体)解读微阵列的步骤。

在一个实施方案中，提供用于制备微阵列的方法，其包括：

使第一亚群的微珠沉积在具有至少一个基准点的基材上，所述亚群包含能够与至少一种靶分析物特异性结合的已知活性剂；

对所述第一亚群的微珠成像；

使第二亚群的微珠沉积在所述基材上，所述的第二亚群包含能够与至少一种靶分析物特异性结合的已知活性剂，其中所述的靶分析物与所述第一群的所述靶分析物不同；

对所述第一和第二亚群的微珠成像；和

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比较所述第一和第二亚群的所述图像以确定每个微珠及其相应亚群相对于所述至少一个基准点的位置。

因此，由于活性剂对于每种亚群是已知的，故无需进行任何其它解码步骤来确定微珠的化学或生物学功能。

该方法可以包括步骤：记录指示每一所述微珠及其亚群的位置的数据。该记录步骤可以包含在计算机存储器中记录所述数据。

所述活性剂可以是生物活性剂。生物活性剂可以是蛋白质或核酸。

该方法可以包括以化学反应基使所述微珠官能化的步骤。

该方法可以包括在沉积每个亚群后除去未与所述基材形成连接的所述微珠。该方法可以包括在所述基材上形成至少一个基准点的步骤。

该方法可以包括在所述基材中形成三维结构以促进在该基材上容纳所述微珠的步骤。所述三维结构可以是在基本上平坦的基材中形成的凹陷。

所述亚群依次沉积每个分立时间段的步骤可以进行 1 次至多达 10,000 次。

根据本发明的第二方面，提供了用于鉴定样品中存在一种或多种靶分析物系统，该系统包括：

沉积在具有至少一个基准点的基材表面上的随机微珠群，所述的群由至少两个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂，其中已经将所述亚群依次且以彼此分立的时间段沉积；

存储器，其记录每个微珠及其相应亚群相对于所述至少一个基准点的位置，每个微珠及其相应亚群的所述位置已经通过比较在所述的依次分立沉积时间段之间所述微珠的图像进行测定；

检测器，其检测所述微珠在接触所述样品时的变化；和

处理器，其应答于程序指令以询问所述存储器并且比较从所述检测器接收的数据，以便基于每个微珠及其相应亚群的位置而鉴定所述样品中所述一种或多种靶分析物的存在。

有利地，对微珠的解码已经在制备所述系统时进行，并且因此解码不在装配微阵列系统时进行。

微阵列系统可以包含用于存储在所述存储器中的解码数据，所述解码数据含有指示每一所述微珠相对于所述基准点的位置和已知活性剂的数

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据。所述解码数据处于用于经计算机网络(如互联网)传输的格式。需要标识符以获得对所述解码数据的访问权。

根据第三方面，提供在用于鉴定样品中存在一种或多种靶分析物的系统内使用的微阵列，所述微阵列包含沉积在具有至少一个基准点的基材表面上的随机微珠群，所述的群由至少两个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂，其中已经将每种所述亚群依次且以彼此分立的时间段沉积以便能够从每个分立的沉积时间段期间所获取的对比图像中确定每个微珠及其相应亚群相对于所述基准点的位置。根据第四方面，提供用于鉴定样品中靶分析物的方法，所述方法包括以下步骤：

使在第三方面中所定义的所述微阵列与所述样品接触；

鉴定已经发生变化的所述微珠亚群，其中所述的变化指示所述活性剂与所述样品中存在的靶分析物结合；和

基于显示所述变化的所述微珠的位置和已知活性剂，鉴定所述样品中任何靶分析物的存在。

所述变化可以是光学变化。用于分析所述样品的方法可以包括提供光学标签以指示所述微珠与所述靶分析物结合的步骤。

定义

本文中所用的以下词汇和术语应当具有所指示的含义：

如本文中所用，术语“微阵列”或“阵列”指微珠在固体支持物上的排列，其中每个微珠具有所选的能够与一种或多种靶分析物结合的生物活性剂。通常根据微珠的大小和基材，微阵列将包括从 2 个至多达十亿个或更多个微珠。

如本文中所用，术语“随机阵列”指这样的阵列，其如此加以制备从而使得每个微珠以通常不可复制的方式随意地沉积在基材上。

术语 “微珠”或“微粒”及其语法变体，指直径 0.1 微米至约 1000 微米的微米大小的分立微粒。

如本文中所用，术语“群”指沉积在微阵列上的微珠总体。如本文中所用，术语“亚群”指在所述群中均包含相同活性剂的微珠集合。

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术语“活性剂”可以指能够与靶分析物反应或与靶分析物间接结合的任何化学制剂或生物学制剂。活性剂可以显示生物学活性并且可以在本说明书中称作“生物活性剂”。示例性生物活性剂包括可以与微珠连接或结合的蛋白质、寡肽、有机小分子、配位络合物、细胞、细胞片段、病毒颗粒、抗原、多糖和多核苷酸。

术语“靶分析物”指能够与所述活性剂结合的待检测物质。示例性靶分析物包括但不限于核酸、多核苷酸、药物、激素、蛋白质、酶、抗体、糖类和抗原。

术语“特异性结合物质”可以指对某种物质具有特异性亲和力的物质。例如，样品中的靶分析物可能能够与活性剂发生特异性结合反应。具体物质与特异性结合物质的组合实例包括：抗原与相应抗体分子、核酸序列与其互补性序列、效应分子与受体分子、酶与抑制剂、含糖链的化合物与凝集素、抗体分子与对前述抗体特异的另一种抗体分子、受体分子与相应抗体分子、以及类似的组合。特异性结合物质的其它实例包括经化学修饰从而其特异结合活性仍保持完整的化合物、以及结合于其它组分的化合物的复合体。此类型的特异性结合物质与特定物质的组合实例包括：生物素蛋白化学修饰的抗体分子或多核苷酸与抗生物素、结合抗生物素的抗体分子与生物素蛋白、以及类似的组合。

如本文中所用，术语“蛋白质”可以定义为两个或多个共价结合的氨基酸，其包括蛋白质、多肽、寡肽和肽。

如本文中所用，术语“氨基酸”和“肽”分别指天然存在的和合成的氨基酸及氨基酸链。

除非另外说明，术语“包含”和“包括”及其语法变体意图代表“开放式”或“包含性”语言，从而它们包括所提及的要素，还允许包括额外未提及的要素。

如本文中所用，术语“约”在配方组分浓度的上下文中，一般意指+/-5%的所述值，更一般地是+/-4%的所述值，更一般地是+/-3%的所述值，更一般地是+/-2%的所述值，甚至更一般地是+/-1%的所述值、并且甚至更一般地是+/-0.5%的所述值。

在本公开的各处，某些实施方案可以以范围的方式披露。应当理解以

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范围的方式进行描述仅出于方便和简明目的，而不应当解释为对所披露范围的不可变化的限制。因此，应当认为对范围的描述具有具体所披露的全部可能子范围，以及在此范围内的各个数值。例如，对范围(如 1-6)的描述应当认为具有具体所披露的子范围，如 1-3、1-4、1-5、2-4、2-6、3-6 等，以及该范围内的各个值，例如 1、2、3、4、5 和 6。无论该范围的宽度，这均适用。

实施方案的详细公开

现在将披露微阵列系统的示例非限制性实施方法、用于制备微阵列的方法和使用所述微阵列鉴定靶分析物的方法。

披露了用于制备微阵列的方法。该方法包括使微珠群沉积在具有至少一个基准点的基材上的步骤。所述群由至少两个亚群、优选多个亚群组成，每个亚群包含能够与至少一种靶分析物特异性结合的已知活性剂。将所述亚群依次且以彼此分立的时间段沉积。该方法也包括在沉积每种亚群后对基材成像的步骤。随后使用基准点作为参考比较图像，旨在基于各各图像的差异因而确定每个微珠的位置、并且旨在鉴定亚群及其已知活性剂。因此，编码/解码步骤在微阵制备过程期间实施而不在制备后实施。这是因为已知活性剂的亚群在分立沉积时间段期间依次地沉积并且在沉积每个亚群后对基材获取图像。在一个实施方案中，记录每个微珠相对于至少一个基准点的位置。因为在每个亚群的每个沉积步骤之间获取图像，因而通过比较连续图像之间的差异，可以不仅知道每个微珠的位置，还知道所述微珠含有的活性剂。因此，在基材上的每个微珠的(化学或生物学)活性剂是已知的，并且因此一旦微珠已经沉积在基材上，则不需要对微阵列进行任何解码。

该方法可以包括提供基材的步骤。基材可以选自：硅、塑料、玻璃、陶瓷、橡胶、聚合物或其复合材料。在一个实施方案中，基材是硅或二氧化硅或氮化硅。

在另一个实施方案中，基材具有疏水性或亲水性表面。有利地，所述的疏水性或亲水性表面可以用来吸引含有所述靶分析物的样品流体，并且可能排斥不想要的流体。例如，若靶分析物处于含水流体中，则基材可以

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是亲水性的以促进将靶分析物吸引至微珠。

示例性基材选自包括：硅、改性硅、玻璃与改性或官能化玻璃、无机玻璃、塑料、丙烯酸类、聚苯乙烯与苯乙烯共聚物、聚丙烯、聚乙烯、聚丁烯、聚氨基甲酸酯、特富龙、多糖、尼龙、硝酸纤维素、树脂、二氧化硅、二氧化硅基材料、碳和金属。有利地，在一个实施方案中，基材不自发荧光。

基材可以在其表面上含有立体特征。立体特征可以选自以下结构以促进微珠停留在基材上：网格、线、凹陷、孔和凝胶垫结构。在一个实施方案中，所述的立体特征可以为微珠的空间位置充当基准点。

沉积步骤可以包括在微珠和基材之间形成连接的步骤。微珠在基材上的连接可以依赖于以下化学相互作用：静电相互作用、离子键、共价键、氢键、疏水性相互作用、亲水性相互作用和偶极-偶极相互作用。

在微珠与基材连接前，可以用化学反应基使基材官能化。有利地，基材的化学官能化可以促进微珠与基材黏附。

示例性化学反应基可以选自：巯醇、羧基、脂族和芳族胺、羧酸、醛、酰胺、氯甲基、酰肼、羟基、磺酸酯和硫酸酯。通过官能化进行化学修饰的位点可以用来共价或非共价地与微珠连接。优选地，官能化不引起任何不想要的基材特性变化。

在微珠与基材之间形成连接的步骤可以包括向基材和微珠中的至少一者施加粘性材料的步骤。在一个实施方案中，将粘性材料施加至基材。可以使用的示例性粘性材料包括基于丙烯酸、基于氨基甲酸酯、基于硅氧烷和基于环氧化物的粘合剂。最优选地，所述粘合剂没有显著的自身荧光。

在一个实施方案中，该方法可以包括向基材施加粘性涂层的步骤。在另一个实施方案中，该方法可以包括在沉积于基材上之前向微珠施加粘性涂层的步骤。

该方法可以包括使预先形成的微珠沉积在基材上的步骤。该方法可以包括从基材表面上除去在所述沉积步骤中与基材未黏附的那些珠的步骤。该方法可以包括在所述除去步骤后对基材表面成像的步骤。

该方法可以包括在基材上形成基准点的步骤。可以在基材上提供多个基准点。有利地，所述的基准点允许均一及稳定地在不同图像之间比较，

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其中所述的不同图像在所述分立时间段期间在依次沉积所述微珠之间获取。优选地，使用多于一个基准点以在比较不同图像时降低不一致性。

基准点可以采取多种形式。在一个实施方案中，基准点可以是具有独特光学标签或其它可检测特征的珠。在另一个实施方案，基准点的形式可以是基材上的一个或多个确定的边缘。在又一个实施方案中，基准点可以是在基材表面上发现的不规则物。

该方法可以包括记录每个所连接微珠相对于基准点的位置的步骤。此记录可以在计算机的存储器中进行。每个微珠相对于基准点的位置可以作为数据以数据表(本文此后称作"编码/解码数据表")的形式记录在计算机存储器中，其中所述的数据表含有每个单一微珠及其对应亚群的空间位置。因此，该编码/解码数据表编码微珠及其相应亚群在基材上的位置并且可以用作解码工具以参考基准点而确定微珠及其相应亚群在基材上的位置。如上所述，每个亚群具有在微珠上的自身独特的已知活性剂并且因而具有检测可能存在于样品中的特定靶分析物的能力。

有利地，与每个微珠相关的位置及标识符数据可以记录在计算机存储器中的编码/解码数据表内。它因此可以容易地由处理器访问，其中所述的处理器在计算机程序的指令下动作以通过确定微珠相对于基准点的位置及其官能性而解读微阵列。计算机可读存储器可以包含采集模块和寄存模块，其中所述的采集模块可以包含可接收来自随机阵列的数据图像的计算机代码，所述的寄存模块包含可使用至少一个基准点来生成寄存数据图像从而记录数据图像的计算机代码。寄存数据图像可以根据需要存储在存储模块中。相同的计算机代码或不同的代码可以用来接收额外的数据图像并生成额外的寄存数据图像，后者也可以被存储。优选地，计算机可读存储器还可以包括包含计算机代码的比较模块，所述的比较模块可以比较寄存数据图像以确定它们之间的差异、允许解读该阵列并检测靶分析物。对至少两种寄存数据图像的比较允许鉴定阵列上至少两种独特的生物活性剂的位置。

例如，比较第一亚群(数据集合“RDI-1”)和第二顺序亚群(数据集合“RDI-2”)之间的寄存数据图像。确定那些存在于 RDI-2 中而不存在于 RDI-1 内的新微珠源自第二顺序亚群，并且那些存在于 RDI-1 中而不存在

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于 RDI-2 内的新微珠源自第一亚群。类似地，比较后续亚群图像并将它们分配至相应亚群组内。指示各个亚群 1、2、……、n 的位置和已知活性剂的数据编译成编码/解码数据表。

可以向想要分析可能含有一种或多种靶分析物的样品的用户提供针对特定基材的编码/解码数据表。在一个实施方案中，该用户可以购买具有位于微阵列上的标识符(即如系列号)的微阵列。该用户可以利用该标识符来访问编码/解码数据表。例如，通过将标识符输入随同微阵列提供的计算机程序中，该标识符可以使编码/解码数据表解锁并使其可用于解读微阵列。或者，该用户可以将标识符输入基于互联网的供应商网址，该网址授予对编码/解码数据表的访问权(即允许下载编码/解码数据表)。这尤其是有利的，因为这允许微阵列供应商控制对微阵列独有的编码/解码数据表的访问。

预期因为对微阵列的编码和解码在制备步骤期间进行，故所述微阵列的发明人可以更严格地控制微阵列的使用并防止未授权的复制。这也可以是发明人借以获得微阵列销售收益的机制，若发明人在向终端用户提供编码/解码数据表时收到资金。

在一个实施方案中，所披露的微珠可以是不规则形状的微珠或规则形状的微珠。微珠也可以具有以下形状：微球、微囊、微杆和微管。最优选地，所述微珠是微球。若需要大的表面积，则微珠也可以是多孔的。示例性微珠由选自形成塑料、陶瓷、玻璃、聚苯乙烯、甲基苯乙烯、丙烯酸聚合物、顺磁性材料、氧化钍溶胶(thoria sol)、石墨碳(carbon graphited)、二氧化钛、乳胶或交联葡聚糖(如 sepharose)、纤维素、尼龙、交联胶束(micelle)和特富龙的材料或在肽、核酸和有机部分合成中所用的相似组成。

微珠的大小范围可以是约 100 nm -约 500μm。更优选地，微珠的大小范围是约 1 μm 微米-约 10μm。每个微珠可以包含连接至或掺入微珠结构中的多种活性剂。在一个实施方案中，每个微珠包含单一类型的生物活性剂。

在一个实施方案中，与所述微珠连接的生物活性剂可以选自无机化合物和有机化合物。优选地，生物活性剂是有机化合物。在另一个实施方案中，生物活性剂是蛋白质。所述蛋白质可以是天然存在的蛋白质或人工合成的蛋白质。

所述生物活性剂可以是天然存在的蛋白质或从天然存在的蛋白质中衍

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生的片段。可以使用含有蛋白质的细胞提取物或者随机或定向消化的蛋白质性质的细胞提取物作为生物活性剂。在另一个实施方案中，生物活性剂是核酸。核酸可以是天然存在的或人工合成的。所述核酸可以是单链或双链的，或者含有双链序列或单链序列部分。核酸可以是 DNA(基因组 DNA 和 cDNA)、RNA 或杂合体。

用作生物活性剂的天然存在的生物分子可以处于细菌、真菌、植物和动物提取物或产物的形式。生物活性剂也可以在连接到微珠上之前通过常规的化学、物理和生物化学手段进行修饰。

可以直接在微珠上合成生物活性剂，或可以产生它们并在合成后进行连接。在一个实施方案中，使用接头来将生物活性剂连接至微珠，旨在提供更好的连接，因灵活性增加而改善与靶分子的相互作用和旨在降低不希望的或非特异的结合作用。生物活性剂在微珠上的连接可以依赖于选自以下各项的化学相互作用：静电相互作用、离子键、共价键、氢键和偶极-偶极相互作用。在生物活性剂与微珠的连接之前，微珠可以用促进结合的化学反应基进行官能化。

在一个实施方案中，靶分析物可以是有机或无机分子。靶分析物可以选自以下物质：核苷酸序列、蛋白质、脂质部分、糖类部分、酶、抗体和抗原。在另一个实施方案中，靶分析物选自以下物质：环境污染物(包括杀虫剂、杀昆虫剂、毒素等)；化学品(包括溶剂、聚合物、有机材料等)；治疗分子(包括治疗药和滥用药物、抗生素等)；生物分子(包括激素、细胞因子、蛋白质、核酸、脂质、糖类、细胞膜抗原和受体(神经受体、激素受体、营养物受体和细胞表面受体)或其配体等)；完整细胞(包括原核生物(如病原菌)和真核细胞(包括哺乳动物细胞))；病毒(包括逆转录病毒、疱疹病毒、腺病毒、慢病毒等)和孢子。靶分析物可以是核酸和蛋白质(包括免疫球蛋白；酶、激素和细胞因子)。靶分析物与生物活性剂的特异性结合作用可以依赖于选自以下各项的化学相互作用：静电相互作用、离子键、共价键、氢键和偶极-偶极相互作用。

当使用微阵列来分析可能含有靶分析物的样品时，可以向微珠和靶分析物中至少之一者添加光学标签。有利地，光学标签可以用来增强识别微珠的位置及随后增强识别在微珠上生物活性剂的位置。光学标签可以显示

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活性剂与靶分析物结合时的光学变化。在一个实施方案中，在含有所述靶分析物的样品已经与微珠的活性剂结合后施加光学标签。

光学标签可以缀合在靶分析物上，后者转而与微珠上相应的生物活性剂结合。光学标签可以直接地与靶分析物缀合或通过接头分子间接地与靶分析物缀合。

在一个实施方案中，光学标签可以是报道染料的混合物，优选地具有荧光性。报道染料的混合物的组成变化可以改变输出光学信号密度，从而提供独特光学标签的巨大可能范围。

该方法可以包括用检测器(如光学检测器)检测光学标签的步骤。光学检测器可以向计算机存储器发出信号，所述信号随后由计算机处理器获得以生成图像文件。与微珠显示光学标签的图像文件相关的数据随后同编码/解码数据表的数据进行比较。显示光学标签的那些微珠是已经与靶分析物结合的那些微珠。因为从编码/解码数据表已知针对微珠亚群的活性剂，故可以鉴定在样品中的靶分析物。

在一个实施方案中，通过使用编码/解码数据表并且因而解码微阵列信息而检测样品中靶分子的方法包括以下步骤：

i) 将含有一种或多种靶分析物的样品与微阵列温育。

ii) 添加处于荧光标记或染料标记或纳米金标记或量子点标记的生物分子形式的光学标签，其中此类生物分子是促进生物测定法性能和在微珠表面上造成光学标签的寡核苷酸或靶分子或 dNTP 或酶底物。

iii) 用检测器从光学标签中光学地对微阵列解读，以通过使用显微镜或者扫描仪或者数字式或视频摄像系统或者通过使用照相胶片而检测并确定在表面上具有光学标签的微珠的位置。可以从含有全部微珠的空间位置的计算机存储器中提取编码/解码数据表，其中所述的微珠具有针对每种亚群的独特活性剂。

iv) 使用源自编码/解码数据表的数据和从微阵列光学读出信息中生成的数据表来通过如此方式解码并且因而检测样品中的靶分析物，即通过合并及比较两份数据表并且鉴定两个数据集合中相应的微珠并因而鉴定样品中的此类微珠及其靶分析物。解码过程可以通过使

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用计算机中的软件加以自动化和/或其中解码数据集合信息的部分可以限定于某些用户和/或其中所述的解码数据集合或该解码数据集合的部分可以经互联网或无线通讯手段传输至用户。

在一个实施方案中，用于基于微珠的随机微阵列和其后续解码过程的产生方法包括步骤：

i) 提供基材材料。该基材材料可以由多个(例如 100 个-10000 个)芯片组成或稍后切割成多个芯片；

ii) 例如通过使用激光雕刻仪，在每个单一芯片的区域上产生基准点，如识别数字。同时，可以在基材上雕刻定义 x 和 y 位置(例如 0，0)的基准点或参考坐标；

iii) 在基材表面上提供粘性层；

iv) 为微珠的每个亚群制备总成分(bulk composition)混悬液，其中所述的微珠携带特定的已知生物活性剂用于开展基于生物识别反应或酶反应的生物测定法；

v) 以如此方式用微珠的第一亚群处理基材，从而将足够数目的微珠固定在每个芯片的区域上；通常，所述数目是 5-20；

vi) 除去松散固定的微珠；

vii) 对那些固定的微珠成像并且将每个单一微珠的相应 x 和 y 位置值和芯片的相应芯片编号存储在存储器内的解码表中；

viii) 以所需数目从 2 至 n 的微珠亚群重复步骤 iv)至 vi)；

ix) 对亚群 1-n 的图像文件进行数据加工。因而生成每个单一芯片的解码数据表，其对于从 1 至 n 的每个微珠亚群具有每个单一微珠的 x、y 位置值；并且

x) 将芯片搁置在芯片架或装置或流动室(flow chamber)或温育室中并组装。

附图简述

以下附图说明所披露的实施方案并且用来解释所披露方案的原理。然而，应当理解附图的设计目的仅在于说明，并且不作为限制本发明的定义。

图 1 是用于根据所披露方案制备微阵列的制备方法的示意说明。

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图 2 是用于施加样品后解读微阵列的流程图的示意说明。

图 3A 是据所披露方案装配在玻璃基材上的微珠的原子力显微照片。该玻璃基材因其含有聚(烯丙基胺盐酸盐(alylamine hydrochlorid))涂层而带正电荷。微珠携带负电荷并且由库仑力固定。

图 3B (i)显示第一亚群的三维微珠结构在形成于表面处的凝胶垫基材上形成；(ii)显示第二亚群的三维微珠结构在形成于表面处的凝胶垫基材上形成；和(iii)显示第一亚组的荧光图像，其中所述的第一亚组因与靶分析物结合而显示荧光；

图 3C 显示固定在基材上的聚苯乙烯微珠的扫描电镜显微照片，其中所述的基材携带微孔的三维结构。

图 4 披露以不连续的时间间隔对两种靶分析物获取的微阵列照片：图 4A 显示来自与抗 IgG-FITC 温育后的 IgG 珠的信号；图 4B 显示来自与 Cy3-标记寡聚物靶标温育后的寡聚物珠的信号；并且图 4C 显示来自 IgG 珠、寡聚物珠和阴性对照珠的信号。使用两种不同滤镜以分别观察如图 4D 和 4E 中所示的 FITC 和 Cy3 信号。在图 4D 中，当使用滤除 Cy3 信号的滤镜时，仅检测到 IgG 珠。在图 4E 中，当使用滤除 FITC 信号的滤镜时，仅检测到寡聚物珠。然而，对于实施该分析而言不需要两种不同染料。

详细描述

本发明非限制性实例将通过参考具体实施例进一步详细描述，其中所述的具体实施无论如何不得解释为限制本发明的范围。

参考图 1，展示了说明制备所披露微阵列的步骤的示意图。提供由次基材(sub-substrate)(芯片 m 至 mx)组成的基材(1)并提供至少一个基准点。

在第一步骤(A)中，基材用第一亚群(n1)(为说明而显示为非填充的圆圈)以及由能够与至少一种靶分析物结合的第一活性剂组成的微珠处理。亚群 n1 的每个微珠由其表面上的相同生物活性剂组成。

在第二步骤(B)中，将未固定的微珠从基材上分离或洗下。

在第三步骤(C)中，对连接的微珠成像，并且所述微珠相对于至少一个基准点的 x、y 坐标位置以编码/解码数据表形式存储在与批次编号(例如 n1)和芯片编号(例如 m26)相关的数据集合中。

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在后续步骤(D)-(x)中，用亚群 n2-nx 依次重复在步骤 (A)-(C)中描述的方法，直至将所需数目的微珠亚群已经施加至基材上。在方法结束时，将产生携带“nx”批次的基材和含有针对每个批次的所有 x,y 粒子位置的编码/解码数据表。

参考图 2，展示了说明解读所披露的微阵列及如何鉴定样品中分析物的步骤的示意图。在一系列步骤中实施解码过程。

在第一步骤(A)中，提供了通过一个实施方案中的披露内容而制备的微阵列及其在制备过程期间所产生的独有解码数据表。

在第二步骤(B)中，将含有潜在靶分析物的样品与微阵列温育。样品中的靶分析物与微珠上相应活性剂的任何结合以 i)杂交及聚合酶延伸作用和/或(ii)抗体抗原相互作用和/或(iii)酶反应为基础。当所述靶分析物与微珠上的活性剂结合及相互作用时，产生光学标签。随后洗涤所述微阵列以除去与微珠的任何非特异性结合并随后除去在微阵列上存在的任何不想要的光学标签。

在下一个步骤(C)中，使用视频或 CCD 摄像机来检测在其表面上携带光学标签的微珠的 x,y 坐标位置(相对于至少一个基准点)并且将读出数据输入计算机以生成读出数据表。

在下一个步骤(D)中，将解码/编码数据表与读出数据表合并和比较，因而解读微阵列以鉴定存在于样品中的靶分析物。

I)计算每块芯片的数据文件大小

此后，词语“芯片”将意指已经被分割的阵列或微阵列的一部分。

每块芯片的数据集合由 n 个子集组成，其中 n 是用来产生芯片的微珠亚群的数目。每个数据子集决定源自亚群的全部微粒的 x,y 位置。该数据集合能够因每个微珠相对于基准点的 x 和 y 位置而以在芯片上的标记结构形式对每个微珠编码。

例如:芯片是具有尺度 2 mm×2 mm 的正方形并且微珠的直径是 5 μm，平均微珠距离是 5 μm，其计算如下：

每 2 mm 的微珠平均数目= 2000 mm/ (5μm + 5 μm) = 200。

因此，每块芯片的平均微珠数目是:

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200×200 = 40000。

对于 n 个亚群，每个亚群(sub-pn)的微珠平均数目是:

sub-pn = 40000/n。

高分辨率视频摄像机提供 2048 像素×2048 像素的分辨率。2 mm×2 mm 视场的光学分辨率是约 1μm。计算机扫描仪的常见分辨率是 4800×4800 dpi (点数每英寸)。光学分辨率是约 5.3μm。该分辨率足以确定直径大于 6μm 的微珠的微珠位置。通过使用计算机扫描仪，微珠应当合适地具有直径 100-500μm。

像素数 2048 等于 2^{12}。为存储多达 2048 个的数字，需要 12 个比特。每个微珠位置可以由针对该微珠位置 x 和 y 值的一个 12 比特数确定。一个微珠需要 24 比特或 3 字节。因此，为存储全部 40000 个微珠的位置，需要 120000 比特或 117 千字节。该数据容量可以存储在任何数据介质上并且可在秒级别上经互联网访问。

数据容量随微珠的数目和微珠大小降低而增加。例如，具有 1 μm 微珠和 1μm 平均微珠距离的 2 mm×2 mm 芯片可以容纳 10^6 个微珠。每个微珠需要约 6 比特。总计约 7.6 兆字节。

II)制备基于珠的阵列

多芯片制备

通过如此方式以微珠粒子混悬液处理大小例如 20 cm×20 cm 的基材，即通过单纯施加微珠粒子混悬液至基材表面上或通过在以上所述的顺序方法中用喷枪喷出微珠混悬液，因而形成微阵列。

在施加微珠的全部亚群并且生成编码/解码数据表后，将基材分割成 2 mm×2 mm 的单个芯片以平行地生成总数至多到 10000 个芯片。每个单一芯片 应当携带标识符以匹配来自芯片的解码数据表的正确数据。

安装基于珠的阵列

阵列可以安装在至支架、流通装置、侧流装置、横流装置中。在任何情况下，使阵列与样品和试剂接触。通过如此方式提供试剂，即通过从例如侧流装置或横流装置内的释放垫中释放试剂，或通过将试剂沉积在阵列的表面上或通过在样品施加至阵列上之前将试剂与样品混合。将一个透明窗安装在阵列上方距离该阵列 10-500μm，形成可以充入分析物/试剂混合物

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的空腔。该空腔可以是流动槽的一部分。

样品施加方法

试剂在施加样品之前或在装置中与样品混合。在常见的基于侧流的装置中，添加样品并将样品借助毛细管力输送穿过该装置。通常从释放垫中释放试剂，其中样品必须移动穿过所述释放垫以抵达微阵列。为使样品经过微阵列连续输送，可以在微阵列后面安装吸收垫。通过基于毛细管力的输送和/或用缓冲液或水洗涤和/或离心力实现从阵列上除去样品和试剂。

读出方法

在与样品温育并分离不反应的组分后，阵列可以通过荧光测量法、反射测光法和透射测光法发光测量法读出。使用本技术领域的扫描仪或可由个人计算机操作的光学驱动器作为光学检测器。通过使用特别适合所用扫描仪的校准(calibration)或软件程序，能够改变或校正读出数据。在读出期间，将获得以下数据:

i) 含有全部微珠的强度或信号的图像；

ii) 图像，其含有相对于全部微珠的处于标记形式的基准点的位置；

iii) 图像，其含有与产生阵列相关的信息，尤其亚群编号、制备日期、阵列编号和/或用该阵列开展分析所需的任何其它信息或编码的信息。

从含有以上信息的图像中，可以生成以下数据：

i) 相对于标记在定义的 x,y 位置处的全部珠的强度/信号；和

ii) 用该阵列开展分析所需的任何其它数据，尤其旨在使正确编码数据集合与阵列关联的数据。

实施例

实施例 1

该实施例说明基于微珠的微阵列用于检测单核苷酸多态性(SNP)的应用。

利用紫外线光聚合作用，在玻片的表面上制备聚丙烯酰胺凝胶垫的阵列。每个分立的凝胶垫进一步由微柱正方形阵列组成。通过分别将珠与靶向特定 SNP 的寡核苷酸探针缀合而制备四组珠。这四种探针含有在靶 SNP

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位点处 ATGC 的全部可能单核苷酸置换。用机器人分配器，使用微靶向法将第一组缀合的珠固定在指定的凝胶垫上。将松散固定的珠洗去，随后使用 CCD 摄像系统对剩余的固定珠成像。全部固定珠的位置从该图像中解码，并且将数据存储在计算机存储器中。随后在相同凝胶垫上对第二组缀合的珠依次重复该过程。

随后将含有靶 SNP 的寡核苷酸的优化杂交缓冲液施加在固定的珠上。随后用 CCD 成像系统检测来自所述珠的阳性杂交信号。由于所述珠已经基于珠的位置进行解码，故识别到这些信号。这使得容易地检测到靶 SNP。可以通过对高通量基因型分型平台的每个凝胶垫上的四组珠重复该方法而实现多个 SNP 的多重检测。将靶向第二 SNP 位点的四个其它组珠以相同方式固定在另一个凝胶垫上，不过在本图中未显示这些珠。

实施例 2

该实施例说明基于微珠的微阵列用于检测环境样品中粪便指示细菌的应用。

设计二十组探针以靶向十种粪便指示物的 16S rRNA 区。每种粪便指示物由一对完美匹配(PM)探针和在中央部分相差一个单核苷酸的错配(MM)探针靶向。使用实施例 1 中所述的相同方法，在不同凝胶垫上固定与 PM 探针缀合的十组珠，并且表征了每个凝胶垫中的珠的位置。对缀合于 MM 探针的珠重复此步骤，从而每个凝胶垫含有与靶向特定粪便指示物的探针对相缀合的珠。这两组珠基于其空间编码进行区分。

制备疑似含有粪便指示物的环境样品并与固定的珠杂交。基于 PM 和 MM 探针之间对 10 个凝胶垫中每一个凝胶垫的相对杂交信号，检测未知环境样品中粪便指示物的存在。该系统可以容易地加以扩展以通过在其它凝胶垫上固定其它组珠而检测多于 10 种的粪便指示物。

实施例 3

该实施例在相同芯片上平行地证实利用抗体抗原相互作用的基于抗体测定法和利用 DNA/DNA 或 DNA/RNA 杂交的基于 DNA 测定法的性能。

通过生物素化准备抗体：

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抗体(多克隆小鼠 IgG 和多克隆小鼠抗 IgG)稀释至大约 2 mg/ml。将生物素蛋白-NHS (10 mg/ml，溶解在 DMSO 中)以 1:1、5:1 和 10:1 的摩尔比(M 生物素蛋白:M 抗体)添加至抗体溶液中，反应管在 25 °C 温育过夜。使用凝胶过滤柱(D-盐葡聚糖脱盐柱，Pierce.)纯化生物素化抗体。生物素化抗体溶解于 PBS 中并在-20 °C 贮藏。

用于抗体的 FITC 缀合的方法：

抗体(多克隆小鼠 IgG 和多克隆小鼠抗 IgG，Arista Biological inc.)稀释至大约 2 mg/ml。将 FITC (10 mg/ml，溶解在 DMSO 中)以 5:1、10:1 和 20:1 的摩尔比(M 生物素蛋白:M 抗体)添加至抗体溶液中。反应在管中 25 °C 温育过夜。使用凝胶过滤柱(D-盐葡聚糖脱盐柱，来自 Pierce Biotechnology Inc.)纯化 FITC-抗体。FITC-抗体溶解于 PBS 中并在-20 °C 贮藏。

制备抗体包被的微珠(亚群 1)：

链霉抗生物素包被的直径 9.95μm 的聚苯乙烯珠(Bangs Laboratories, Fishers, IN)用于固定抗体。将生物素化抗体以 0.5 毫克每 1 微升原始微珠的量(过量 2.5 倍)添加至珠混悬液。温育在 25 °C 过夜进行。未结合的抗体在使用前以 PBST-B (1×PBS，0.05% Tween-20，1%牛血清白蛋白)洗涤 2-3 次。这些珠随后置于如图 4 中所示的基材上。

制备寡核苷酸官能化微珠(亚群 2)：

链霉抗生物素包被的直径 9.95μm 的聚苯乙烯珠(Bangs Laboratories, Fishers, IN)用于固定寡核苷酸。100 μL 贮存(stock)珠(1%固体)在 TTL 缓冲液(100 mM Tris-HCl，pH 8.0，0.1% Tween 20，1 M NaCl)中淋洗三次、沉淀并随后重悬于 20μL TTL 中。此后，以超过微珠结合容量的量(每 mg 微珠约 330 ng DNA)添加 0.5 μL 贮存(stock)探针(0.5μg/μl)。通过在室温下温和混合 12 小时，将 5' 末端处生物素化的捕获探针(5'-GCCCTCACGATCTCTTCC-3')经生物素蛋白-链霉抗生物素结合作用而固定在微珠上。固定后，将微珠淋洗、沉淀并重悬于 80 °C 的 TTL 缓冲液(100 mM Tris-HCl，pH 8.0，0.1% Tween 20，20 mM EDTA)中持续 20 分钟，以除去不稳定的生物素蛋白-链霉抗生物素结合。

这些珠随后添加至如图 4B 中所示的包含抗体包被微珠的微阵列。

制备基于珠的阵列：

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如上所述实施将抗体包被珠的珠的亚群(亚群 1)(图 4A)和寡核苷酸包被珠的亚群(亚群 2)(图 4B)沉积至微阵列基材上。可以将作为阴性对照且不包含任何活性剂的微珠的第三亚群沉积在基材上(图 4C)。该装置由 19×24 聚丙烯酰胺凝胶垫组成的阵列组成，其中在用 Bind Silane(GE Healthcare, Piscataway, NJ)预处理的玻片(Corning, Corning, NY)上制备所述的阵列。所述凝胶垫的水平间距和垂直间距是 300μm，并且每种凝胶垫还包含水平间距和垂直间距 10μm 的 10×10 微柱阵列(10×10×10μm)。

使用光聚合工艺来产生凝胶垫阵列。将光掩模固定在硅烷化玻璃载片上，其中所述的硅烷化玻片用丙烯酰胺溶包被并且在交联仪(UVC500, Hoefer, San Francisco, CA)中暴露于紫外辐射持续 45 分钟。丙烯酰胺溶液的区域暴露于紫外光并发生聚合，导致在玻璃表面上形成凝胶垫。凝胶垫的 10mm 高度由牢固地夹在光掩模与玻片之间的特富龙间隔区决定。聚合后，将玻片在 0.1M $NaBH_4$ 中处理 30 分钟以降低凝胶垫的自身荧光。使用 Biochip Arrayer (PerkinElmer, Boston, MA)来送递 5 nL 不同的珠类型(约 9000 个珠/μL)至凝胶垫上。或者，珠也可以使用移液器手工点样，虽然其精确度较低并且珠同时覆盖几个凝胶垫。

所点样每个单一珠的位置通过相差图像进行记录，用于确定它们的空间地置。随后重复所述步骤直至用于检测特定靶的全部珠亚群固定在相同的凝胶垫上。该装置随后以微流体模块加盖(cap)用于样品流通。或者，缓冲液也可以在无所述模块下施加于点样的珠上。使用常见软刻技术，制备聚二甲基硅氧烷(PDMS)模块。

同时进行 DNA 和免疫测定法：

将含有抗原和互补DNA的样品与阵列温育。该样品含有0.1 ng/μL Cy3-标记的 DNA 靶(5'-AGGAAGAGATCGTGAGGGCA-3')和 FITC 标记的抗 IgG、 500 mM NaCl 和 PBS-T 缓冲液。将 10μL 样品点样在微珠阵列。在温育 30 分钟后，阵列用 20μL 含有 500 mM NaCl 和 PBS-T 的溶液简单淋洗，并且信号随后由荧光显微镜寄存。与微珠结合的 FITC 标记抗 IgG 由图 4D 中照亮的荧光圈显示。与微珠结合的 Cy3 标记 DNA 由图 4 E 中照亮的荧光圈显示。应当指出鉴定样品中靶分析物的方法不要求使用多种光学标签，因为每个微珠的确切位置是已知的。FITC 和 Cy3 标记的使用在本实施例中

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纯粹出于说明目的。

实施例 4

将由已知第一生物活性剂组成的第一亚群微珠添加至基材，随后洗涤，并且将数据图像捕获(图 3Bi)并以编码/解码数据表形式存储。将由已知第二生物活性剂组成的第二亚群微珠添加至基材，随后洗涤，并且将数据图像捕获(图 3Bii)并以编码/解码数据表形式存储。随后合并两个编码/解码数据表以形成如下表 1 中所示的合并编码/解码数据表。每个微珠相对于基准点(X=0，Y=0，如图 3B 中所示)的位置可以通过比较来自如下表 1 中所示合并的编码/解码数据表的数据而确定。将含有与荧光染料缀合的潜在靶分析物的样品添加至微珠的微阵列。将微阵列洗涤并在荧光显微镜下观察(图 3Bii)并且将图像记录并与如下表 1 中所示的合并编码/解码数据表比较以鉴定样品中存在的靶分析物。

生成含有每个微珠的 x，y 位置值的解码数据集合；图 3 B 的实例数据集合在下表 1 中给出。

在图 3B(i)中，在基材上提供来自第一批次的每个珠。在图 3B(ii)中，来自第二批次的每个珠在该图中以“x”显示。因此，计算机程序浏览在图 3B(i)和图 3B(ii)中所示的图像，并且确定额外的珠属于第二亚群。在顶部左边角中显示的大“X”是基准点。

表 1. 图 3 B 中所示微珠的解码数据表。将源自特定亚群的微珠以生物识别分子/生物活性剂包被。如图 3B(iii)中所示，第一批次显示为用荧光染料照亮。应当理解图 3B(i)-(iii)仅在基材上显示两个批次(微珠亚群)用于说明。在实践中，有可能在基材上运行 100 个亚群并且因而能够鉴定更大数目的靶分析物。

阵列识别编号，例如阵列116	
亚群鉴定表	
亚群编号	生物活性剂涂层
1	种类1

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nx		种类x	
微珠位置数据集合			
亚群编号：1		亚群编号：2*	
x-位置	y-位置	x-位置	y-位置
88	22	88	22
		212	22
		242	22
26	51	26	51
57	52	57	52
		88	52
		119	40
118	48	118	48
		119	56
		149	52
		212	53
273	53	273	53
26	82	26	82
119	83	119	83
150	83	150	83
180	83	180	83
		191	83
212	84	212	84

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272	84	272	84
26	114	26	114
		83	113
		89	104
212	115	212	115
		243	115
273	115	273	115
119	134	119	134
118	145	118	145
150	145	150	145
242	147	242	147
56	176	56	176
88	176	88	176
		98	176
		180	176
211	177	211	177
88	207	88	207
118	207	118	207
		118	218
		149	201
		148	212
179	208	179	208

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241	208	241	208
271	207	271	207
25	238	25	238
87	238	87	238
180	239	180	239
236	238	236	238
247	238	247	238
272	227	272	227
		272	237
27	268	27	268
118	268	118	268
178	268	178	268
272	269	272	269
		86	287

*来自第二图像的数据读数含有来自亚群 1 和 2 的微珠的 x,y 位置。该数据用来验证如图中所示的来自亚群 1 的微珠的有效固定作用。

应用

将理解本文中所披露的用于制备微阵列的方法是有成本效率的制备方法并且仅需要简单的读出设备。

有利地在所披露的方法中，微珠微阵列在其制备过程期间解码。这与已知的现有技术微珠微阵列技术相反，其中微阵列在其生成后进行解码。

更有利地，因为将编码/解码步骤执行到制备方法中并且和随阵列提供解码数据，因而更快地进行分析，无需解码过程。

有利地，所披露的微珠微阵列可以伴随更简单的读出设备一起使用，

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无需多波长测量。

有利地，披露的基于微珠的微阵列允许: i)将分析数据访问权限于阵列用户，但是分析性数据可以在特定分析物的解码数据集合对该用户披露后在稍后的任何时间上生成，和 ii)通过经电子邮件、互联网、移动电话、邮件和其它数据传输方式发送编码数据而允许用户访问分析数据。

更有利地，通过提供编码数据控制对分析数据的访问允许在政府、公司或保健组织中更安全的数据交换。例如在地点“A”处实施测量，但是在地点“A”处不能生成分析数据。测量数据传输至地点“B”并且与解码数据集合合并以生成分析数据。在另一个实例中，用个人“P1”提供的样品实施测量。可以从测量中不生成数据，除非个人“P1”或另一个人“P2”出于任何原因提供该数据集合。

有利地，控制对分析数据的访问分析数据可以提供潜在的商业可能性。例如，微阵列读出数据可以含有针对靶全部分析物的全部分析数据，不过仅授权用户访问他人为此付费的特定分析物(解码数据)。

有利地，因为更快地进行分析，无需解码过程，故可以加快检测疾病，如非息肉病性结肠癌、乳腺癌、阿尔茨海默病、囊性纤维化、肺结核、衣原体病和 HIV。更有利地，所披露的微阵列也可以改善法医“DNA 指纹”匹配法的速度。

尽管已经付出合理的努力来描述本发明的等效实施方案，然而本领域技术人员在阅读前述公开后明白可以在其中产生本发明的多种其它修改和改进而不脱离本发明的精神和范围，并且全部此类修改和改变意图处于所附带权利要求书的范围内。

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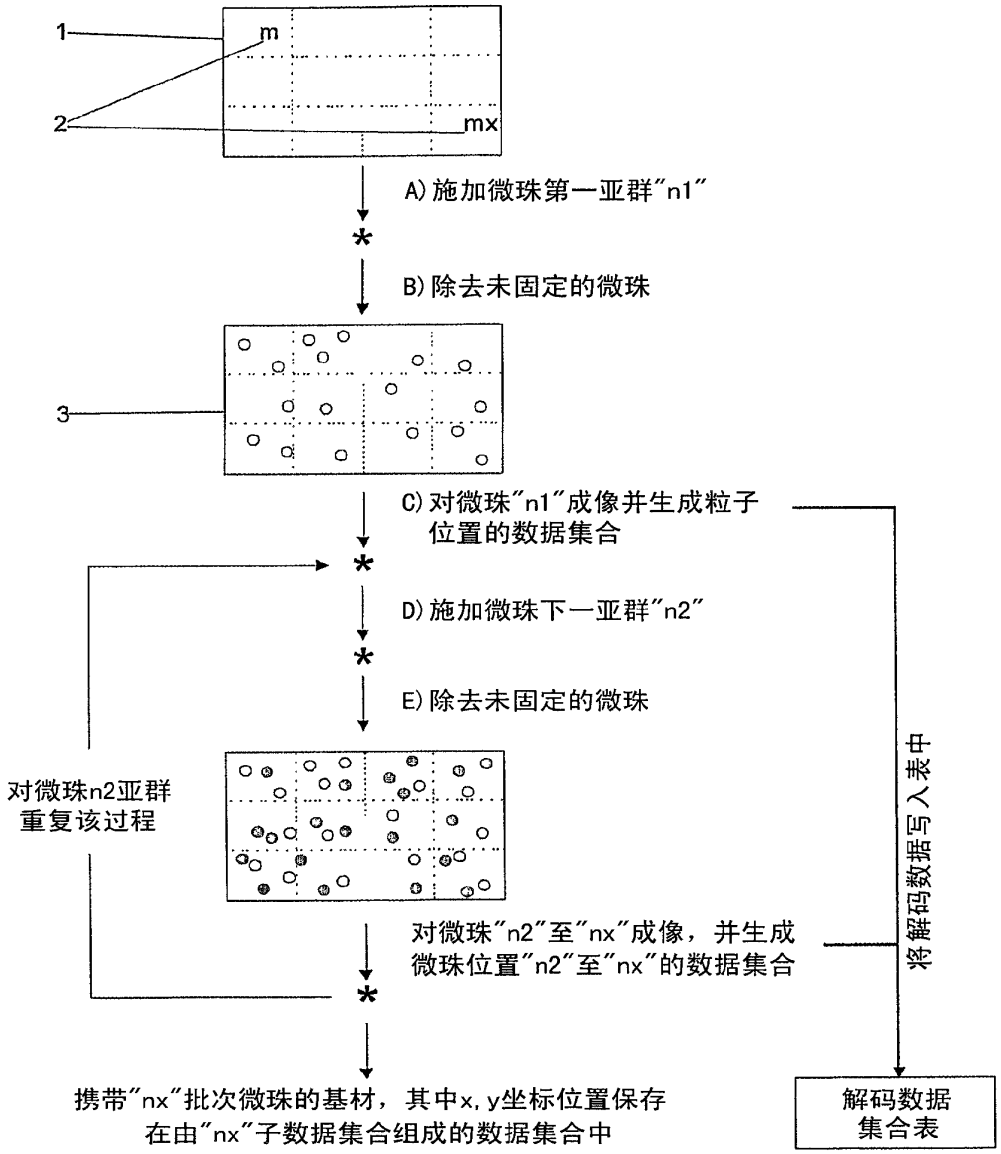


图1

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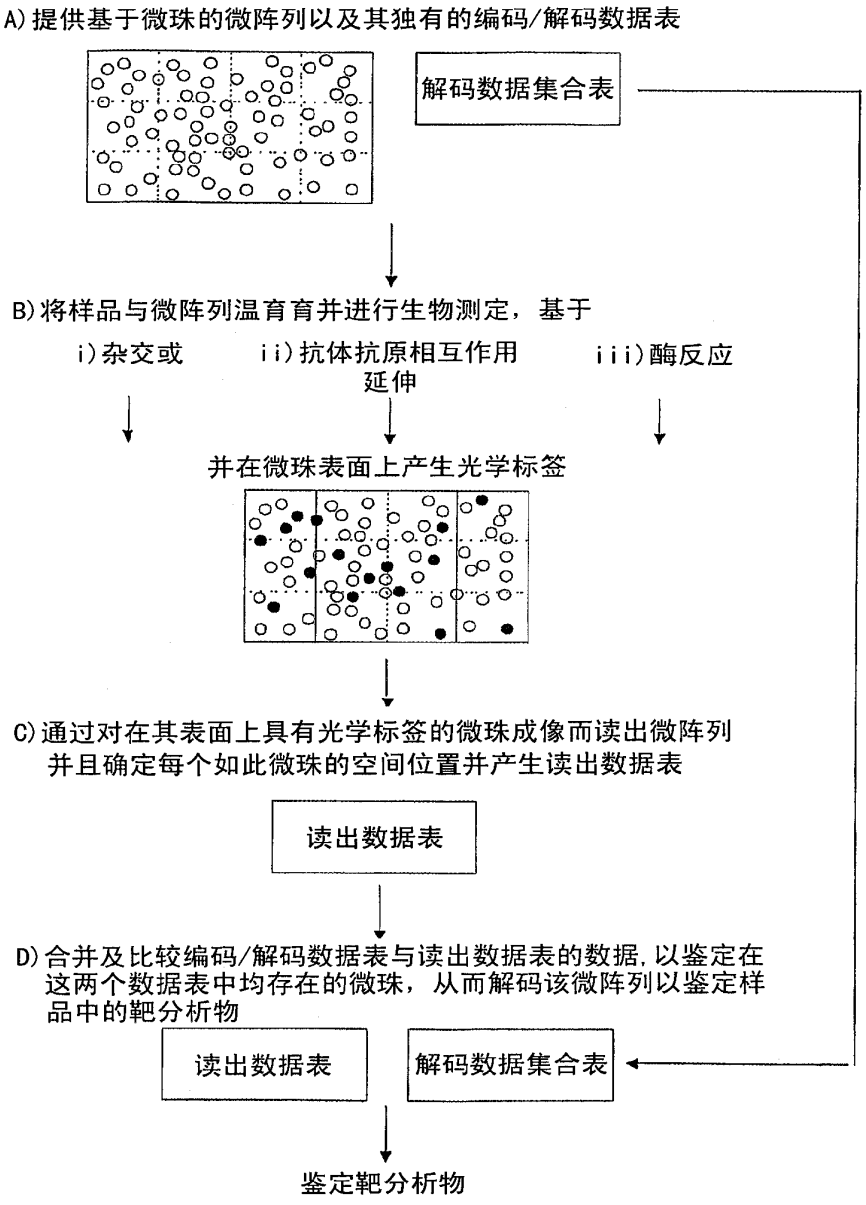


图2

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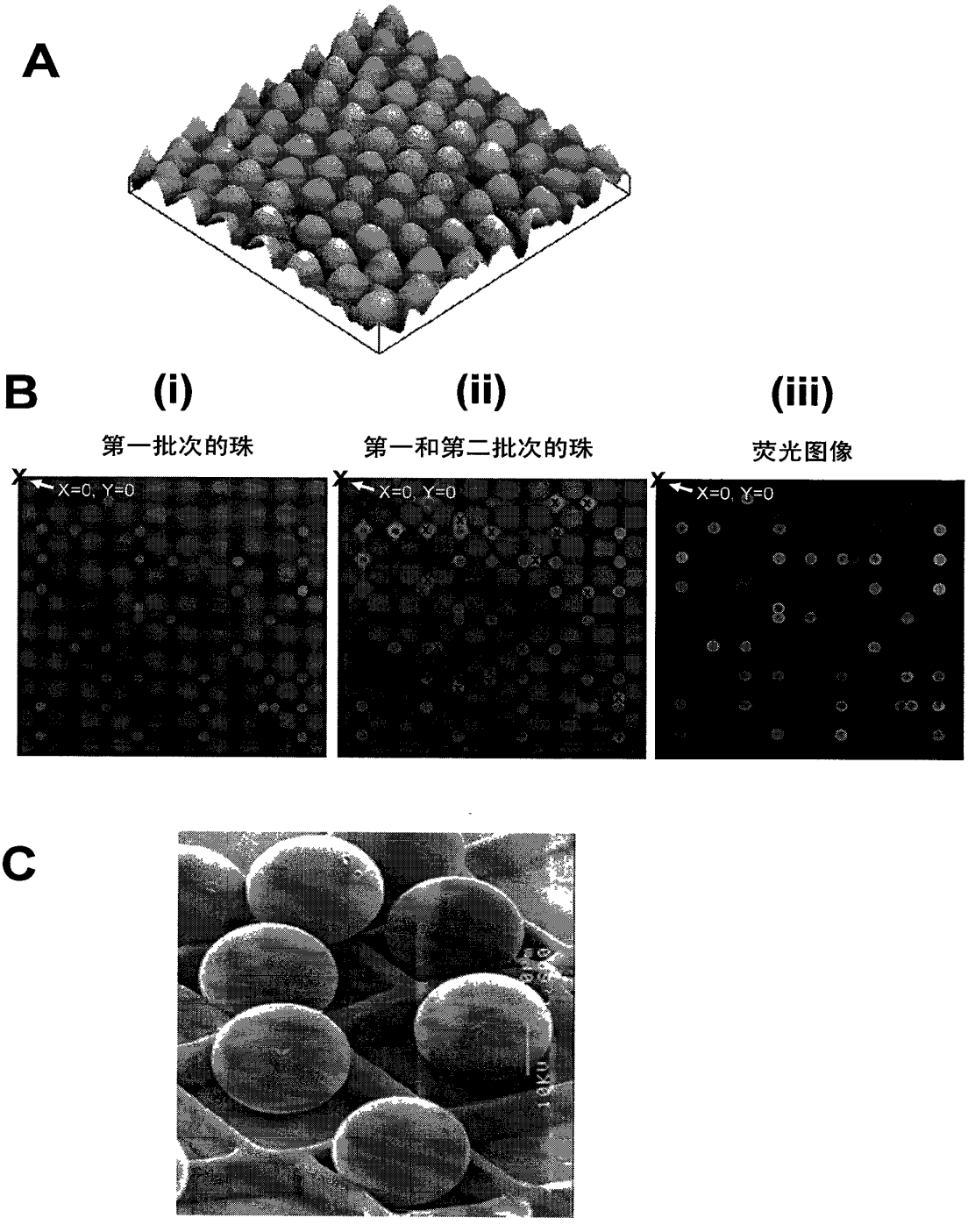
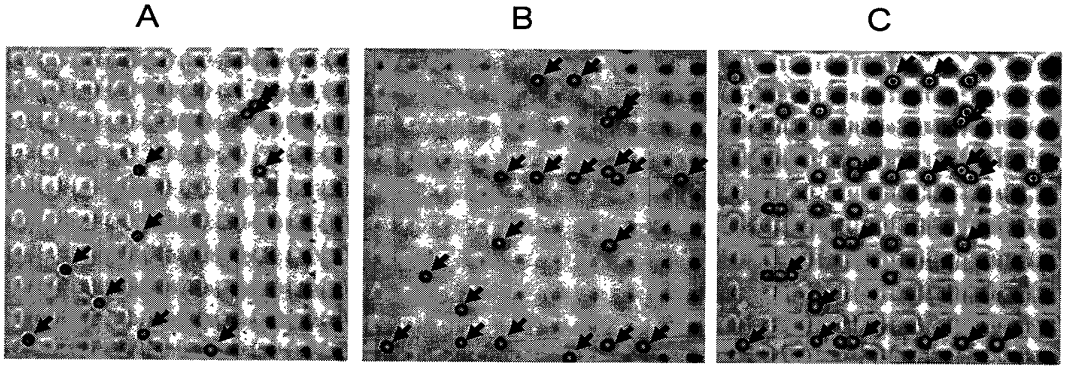
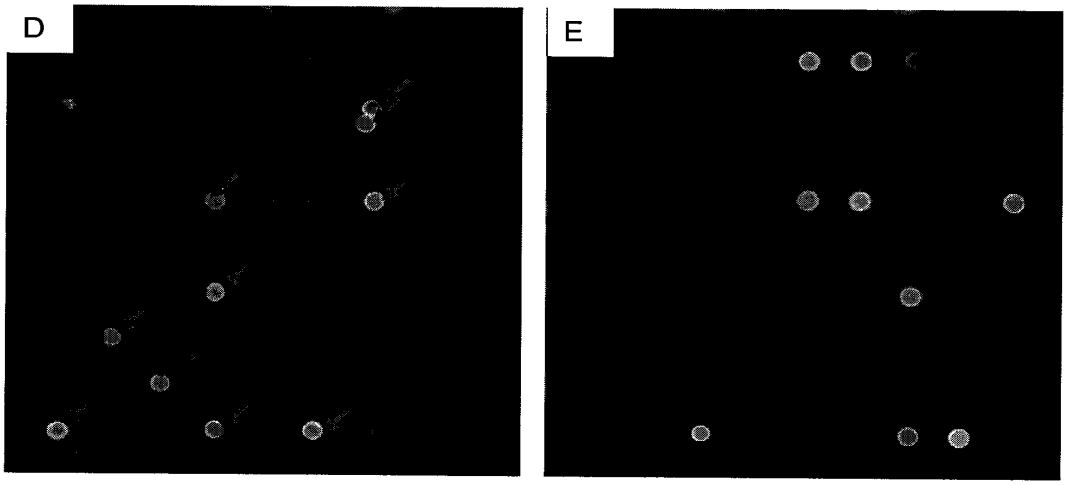


图 3

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IgG 珠　　IgG 珠和寡聚物珠　　IgG 珠、寡聚物珠和阴性对照珠



IgG 信号(FITC)　　寡聚物信号(Cy3)

图 4

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Espacenet

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Hybridization probe assay and array

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Priority number(s): WO2007EP50380 20070116 ; GB20060000927 20060117

Also published as: CN101541974 (B) WO2007082881 (A2) WO2007082881 (A3) WO2007082881 (A8) US2010184022 (A1) more

Abstract of CN101541974 (A)

A probe suitable for coupling with a particulate support such as a microbead, the probe comprising: a) a coupling group which permits coupling of the probe to the surface of the particulate support; b) a spacer; and c) a target-specific oligonucleotide probe sequence, wherein the spacer comprises: i) an oligonucleotide spacer of at least 15 nucleotides between the target specific probe sequence and the support coupling group; and optionally ii) a carbon spacer of between 3 and 50 carbon units between the target specific probe sequence and the support coupling group.

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[51] Int. Cl.
C12Q 1/68 (2006.01)

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[32] 2006.1.17 [33] GB [31] 0600927.8
[86] 国际申请 PCT/EP2007/050380 2007.1.16
[87] 国际公布 WO2007/082881 英 2007.7.26
[85] 进入国家阶段日期 2008.9.17
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权利要求书 4 页 说明书 59 页 附图 4 页

[54] 发明名称

杂交探针测定和阵列

[57] 摘要

适用于与特定支持体例如微珠偶联的探针，所述探针包含：a)允许探针与特定支持体表面偶联的偶联基；b)间隔物；以及 c)目标特异性寡核苷酸探针序列，其中所述间隔物包含：i)在目标特异性探针序列与支持体偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物；以及任选 ii)在目标特异性探针序列与支持体偶联基之间的 3 – 50 个碳单元的碳间隔物。

1. 适合与粒状支持体偶联的探针，所述探针包含:

a) 偶联基，其允许探针与粒状支持体表面偶联;

b) 间隔物；和

c) 目标特异性核苷酸探针序列，

其中所述间隔物包含:

i) 目标特异性探针序列与支持体偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物；和任选的

ii) 目标特异性探针序列与支持体偶联基之间的 3-50 个碳单元的碳间隔物。

2. 权利要求 1 的探针，其中所述间隔物包含碳间隔物和寡核苷酸间隔物两者。

3. 权利要求 1 或 2 的探针，其中所述间隔物是没有碳间隔物的寡核苷酸，而且是 25-150 个核苷酸。

4. 权利要求 1-3 中任一项的探针，其中所述间隔物经过挑选以致于不与目标序列或者目标的侧翼区杂交。

5. 权利要求 1-4 中任一项的探针，其中所述寡核苷酸间隔物包含同聚物或杂聚物。

6. 权利要求 5 的探针，其中所述寡核苷酸间隔物包含 poly T 寡核苷酸或 TTG 重复。

7. 权利要求 1-6 中任一项的探针，其中目标特异性探针序列对人乳头瘤病毒目标序列具有特异性。

8. 与粒状支持体偶联的权利要求 1-7 中任一项的探针。

9. 权利要求 9 的探针，其中所述支持体是珠。

10. 权利要求 8 或 9 的探针，其中所述支持体选自玻璃或聚苯乙烯。

11. 一组权利要求 1-10 中任一项的探针，其包含至少两种目标特

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异性探针序列，所述至少两种目标特异性探针序列与彼此可区别的不同的粒状支持体偶联。

12. 权利要求 11 的一组探针，其中所述不同的粒状支持体被不同的荧光分子标记。

13. 一组 2-1000 种不同的目标特异性探针，每一种探针包含：

a) 允许探针与粒状支持体偶联的偶联基；

b) 间隔物；和

c) 目标特异性寡核苷酸探针序列，

其中所述间隔物包含以下之一或两者：

i) 目标特异性探针序列与支持体偶联基之间的 13-50 个碳单元的碳间隔物；和

ii) 目标特异性探针序列与支持体偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物，所述寡核苷酸间隔物不与目标序列或者目标的侧翼区杂交。

14. 适合与目标特异性探针序列连接的间隔物，所述间隔物包含 13-50 个单元的碳间隔物和至少 15 个核苷酸的寡核苷酸。

15. 试剂盒，其包含探针和粒状支持体例如聚苯乙烯珠，所述探针包含：

a) 允许探针与粒状支持体表面偶联的偶联基；

b) 间隔物；和

c) 目标特异性寡核苷酸探针序列，

其中所述间隔物包含以下之一或两者：

i) 目标特异性探针序列与支持体偶联基之间的 13-50 个碳单元的碳间隔物；和

ii) 目标特异性探针序列与支持体偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物。

16. 试剂盒，其包含探针和关于与粒状支持体例如聚苯乙烯珠偶联的说明书，所述探针包含：

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a) 允许探针与粒状支持体表面偶联的偶联基;

b) 间隔物; 和

c) 目标特异性寡核苷酸探针序列,

其中所述间隔物包含以下之一或两者:

i) 目标特异性探针序列与支持体偶联基之间的13-50个碳单元的碳间隔物; 和

ii) 目标特异性探针序列与支持体偶联基之间的至少15个核苷酸的寡核苷酸间隔物。

17. 权利要求15或权利要求16的试剂盒, 所述试剂盒包含一组两种或更多种不同的目标特异性探针序列, 所述探针序列用于与彼此可区别的不同粒状支持体偶联。

18. 用于检测权利要求1的探针和目标核酸之间的任何相互作用的方法, 所述方法包括以下步骤:

i) 使样品中存在的任何双链目标多核酸变性;

ii) 使所述变性的目标与探针在允许探针与目标之间发生特异性杂交的条件下进行杂交;

iii) (任选进行严格洗涤)

iv) 加入报告分子并与其温育, 以便能够检测探针-目标的结合情况;

v) (任选进行严格洗涤); 和

vi) 检测探针-目标的结合情况

其中所述方法包括从步骤ii)直至步骤iv)结束, 维持杂交温度。

19. 权利要求18的方法, 其中所述探针与粒状支持体例如珠偶联。

20. 权利要求18或19的方法, 其中同时使用2种或更多种不同的探针。

21. 权利要求18-20的方法, 其中不同目标特异性探针的目标特异性探针序列长度不相等。

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22. 权利要求 18-21 的方法，其中探针与目标之间的杂交在离子环境中进行。

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杂交探针测定和阵列

本发明涉及分子之间相互作用的分析。

发明背景

单核苷酸多态性(SNP)被认作是生物功能多样化的重要原因。尽管SNP可以具有重要的效应，但大部分的遗传多样性是在非编码DNA序列中观察到。SNP除了在人类遗传学中的重要性以外，SNP检测在传染病领域也至关重要。基因型分型是人乳头瘤病毒(HPV)感染的重要指标。当今，HPV家族包括超过一百种的基因型，所述基因型可以分成包含重要的人类病原体的不同组(de Villiers等, 2004)。尤其是，已知高危HPV型诱导宫颈癌。因此，识别这些高危型需要强有力的诊断工具，以施行最合适的治疗。

核酸测定基于特异性DNA或RNA序列的检测。通过已标记的检测探针可以识别目标核酸，例如从临床样品中获取的目标核酸。所述测定法的特异性取决于目标与探针之间杂交方法的特异性。然而，SNP的检测需要最高程度的特异性。此外，目前很多技术可用于检测SNP(例如杂交、测序和质谱分析)，但是它们无一能有效地联合高通量和高密度的SNP筛选。然而，对此工具的需求却日渐强烈。

珠(或微珠)(例如在本文中也被称为微珠的球形珠)在多重分析中的使用先前已在例如 Dunbar SA. (Applications of Luminex™(R) xMAPtrade mark technology for rapid, high-throughput multiplexed nucleic acid detection (Luminex™(R) xMAP(商标)技术应用于快速高通量多重核酸检测). Clin Chim Acta. 2005 Aug 15); [Epub ahead of print], Clin Chim Acta. 2006 Jan; 363(1-2):71-82. 中有描述，参见 http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=16102740&query_hl=1)和 Luminex™ 产品

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信息和网站(www.Luminex™corp.com)。Luminex™系统是基于珠的多重(阵列)技术，所述技术已被证明对于分析一个样品中的多种参数或分析物非常有效力(Dunbar 等, 2005)。它将结果呈递到许多生物测定格式上，包括核酸测定、受体-配体测定、免疫测定和酶测定。

液态珠微阵列在 HPV 检测中的应用在 Wallace J 等, (Facile, comprehensive, high-throughput genotyping of human genital papillomaviruses using spectrally addressable liquid bead microarrays." (使用光谱可寻址的液态珠微阵列对人生殖乳头瘤病毒进行方便、广泛、高通量的基因型分型) J Mol Diagn. 2005 Feb; 7(1):72-80.)中描述。

虽然 Luminex™型系统的方案与材料是已知的并被公开，并且在 Luminex 网站上给出，但仍有需要对这类技术和材料予以改进。例如，我们已经发现，使用液态珠微阵列的一些标准方案对于小目标中不同的 SNP，或者对于具有并非总在探针中间的多个点突变的系统并不有效。Luminex 通常所使用的 TMAC 体系也建议在给定的多重反应中固定的探针长度。此外，TMAX 有毒性并且在较高温度下不稳定。

本发明满足了改进适用于基于珠的分析系统(例如 Luminex)的探针和方案设计的要求。

发明概述

本发明涉及用于检测探针与目标核酸之间任何相互作用的方法，所述方法包括以下步骤:

i 使样品中存在的任何双链目标多核酸(polynucleic)变性;

ii 在允许探针与目标之间发生特异性杂交的条件下使变性的目标与探针杂交;

iii 任选进行严格的洗涤;

iv 加入报告分子并与之一起温育，致使能够检测探针-目标的结合情况;

v 任选洗涤; 并且

vi 检测探针-目标的结合情况，

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其中所述方法包括以下额外步骤中的一个或更多个:

a 在步骤(ii)之后的探针与目标之间的杂交步骤之后，维持杂交温度;

b 在杂交步骤(ii)之后立即进行稀释-洗涤步骤;

c 在步骤(iii)的任何严格洗涤期间维持杂交温度;

d 在步骤(ii)中加热下摇动或混合，例如通过在步骤(ii)中使用热混合器;

e 在步骤(iv)的与报告分子温育期间维持杂交温度;

一方面，从步骤(ii)开始维持温度，直到在步骤(iv)中与报告分子的反应完成。

一方面，进行步骤 a 和 c，亦即在探针与目标之间的杂交步骤之后和在步骤(iii)的严格洗涤步骤期间，维持杂交温度。

又一方面，将探针与粒状支持体例如珠偶联。

本发明也涉及适用于与粒状支持体例如珠偶联的探针，所述探针包含:

a) 偶联基(例如 NH_2 基)，其允许探针与粒状支持体表面偶联(例如共价偶联);

b) 间隔物(spacer); 以及

c) 目标特异性寡核苷酸探针序列，

其中所述间隔物包含:

i) 位于目标特异性探针序列与偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物，例如胸腺嘧啶重复间隔物或者包含 TTG 重复单元的间隔物; 和任选的

ii) 位于目标特异性探针序列与偶联基之间的 3-50 个或 13-50 个碳单元的碳间隔物，宜为 C_{18} 间隔物。

一方面，所述间隔物在目标特异性探针序列的 3'末端。

另一方面，所述间隔物在目标特异性探针序列的 5'末端。

本发明也涉及本文中所描述的探针组，其包含与不同粒状支持体

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偶联的至少两种不同的目标特异性探针序列，所述不同粒状支持体相互之间可区别，例如借助不同的标记例如荧光标记或条形码(barcode)来区别。

本发明也涉及一组 2-1000 种(例如 2-50 种)不同的目标特异性探针，每一种探针包含:

a) 允许探针与固相支持体偶联的偶联基;

b) 间隔物; 以及

c) 目标特异性寡核苷酸探针序列，

其中所述间隔物包含以下之一或两者:

i) 位于目标特异性探针序列与支持体偶联基之间的 13-50 个碳单元的碳间隔物; 以及

ii) 位于标特异性探针序列与支持体偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物，所述寡核苷酸间隔物不与目标或目标的邻近区杂交。

本发明也涉及在本文中如本发明的任何方面所定义的间隔物序列本身。

本发明也涉及试剂盒，其包含本发明的间隔物分子和粒状支持体例如珠。

本发明也涉及试剂盒，其包含本发明的间隔物分子和关于与粒状支持体例如珠偶联的说明书。

本发明也涉及与本文中所定义的探针偶联的粒状支持体例如珠。

本发明也涉及试剂盒，其包含与本发明的间隔物分子偶联的粒状支持体例如珠和在检测目标分子中使用的说明书。

本发明也涉及试剂盒，其包含与本发明的探针偶联的粒状支持体例如珠和在检测目标分子中使用的说明书。

本发明也涉及包含探针的试剂盒，所述探针包含:

a) 允许探针与粒状支持体表面偶联的偶联基;

b) 间隔物; 以及

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c) 目标特异性寡核苷酸探针序列，

其中所述间隔物包含以下之一或两者：

i) 位于目标特异性探针序列与支持体偶联基之间的 13-50 个碳单元的碳间隔物；以及

ii) 位于目标特异性探针序列与支持体偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物；

以及粒状支持体例如聚苯乙烯珠。

本发明也涉及包含探针的试剂盒，所述探针包含：

a) 允许探针与粒状支持体表面偶联的偶联基；

b) 间隔物；以及

c) 目标特异性寡核苷酸探针序列，

其中所述间隔物包含以下之一或两者：

i) 位于目标特异性探针序列与支持体偶联基之间的 13-50 个碳单元的碳间隔物；以及

ii) 位于目标特异性探针序列与支持体偶联基之间的至少 15 个核苷酸的寡核苷酸间隔物；

以及关于与粒状支持体例如聚苯乙烯珠偶联的说明书。

附图

图 1a 和 1b 提供了探针与间隔物设计的总体图式概述。

图 1c 作了有关的进一步说明。

图 2 提供了关于基于珠的检测系统的测定方案的概述。

发明详述

在本发明中，已经对检测核酸的标准悬浮珠测定(例如基于 Luminex™的测定法中)的方案与试剂作出了改进。

本发明中使用的粒状支持体具体包括珠，所述珠包括例如球形珠或圆柱形珠。珠也可以称为微珠，或在微阵列中使用的珠。本发明中

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与珠有关的说明也适用于在本发明中所用的其他粒状支持体。

在本发明中使用并且包括本文中所描述的微珠的珠，宜为适合在流式细胞计数分析中使用的珠。珠适宜能够与探针偶联以检测探针与目标之间的相互作用。一方面，用独特的荧光分子或分子组合来标记珠。在珠上或在珠中的标记适宜能够通过使用激光激发珠内的一种或更多种荧光染料来鉴定。一方面，珠是聚苯乙烯珠。另一方面，珠是玻璃珠。

例如，Luminex xMAP 系统引入了用两种光谱不同的荧光染料内部染色的 5.6 μm 的聚苯乙烯微珠(参见上文的 Dunbar 等)。这些珠适合在本发明中使用。

在本发明中使用的其他的珠标记体系包括条形码或数字全息元件(digital holographic element)，例如 Illumina Inc.的经条形码标记的圆柱形珠。条形码或数字全息元件可以用作荧光标记的替代物。

例如 Illumina VeraCode 系统引入了高 240 μm、直径 28 μm 的圆柱形玻璃微珠，所述微珠其中包埋有数字全息元件，以产生独特的珠型。当用激光激发时，每一 VeraCode 珠都发出能被特异性检测到的独特的代码图像。

本发明的一个方面，所述珠也可以具有磁性或顺磁性。

一般来说，所述珠适用于多重系统，以同时检测多种可能的目标与多种探针之间的任何相互作用。

本文中的实施例使用了人乳头瘤病毒(HPV)目标和探针，但所开发的原理原则上可以适用于检测任何来源的多核酸。

标准分子生物学技术在 Sambrook 等(Molecular cloning a laboratory manual, Cold Spring Harbour Press, third edition)中有描述。例如关于探针与目标之间杂交的原理和参数的详细内容，以及目标多核酸扩增的详细内容在 EP1012348 中有描述，上述文献在此引作参考。

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探针

探针通常包含：(1)偶联基(例如 NH_2 基)，其允许探针与珠表面(宜为共价地)偶联，(2) 间隔物，其用来在珠表面与特异性探针序列之间产生间距，以及(3)目标特异性寡核苷酸探针序列(其在本文中也可以称为目标特异性探针序列)。

一方面，探针具有适合与珠或其他支持体上的羧基偶联的伯氨基。

一方面，本发明涉及含有目标特异性 HPV 探针序列的探针，例如已公布的 SPF10 探针组(参见 EP1012348，其在此引作参考)，例如对于 HPV 而言；或者在本文中描述的任何探针或探针组合，特别是实施例 13 中描述的，其任选与多碳(polycarbon)重复连接。

一方面，本发明适用于鉴定在目标核酸的短片段中存在的 SNP，所述短片段通常是从样品扩增的 DNA 片段，例如长度为 20 - 50 个碱基、例如 20 - 30 个碱基、例如 20、21、22、23、24、25、26、27、 28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49 或 50 个碱基的片段。

另一方面，本发明能够辨别处于除探针中间以外的位置的错配。一方面，本发明可以用于辨别与探针中心相距 1、2、3、4、5、6、7、8、9、10 或甚至更远的错配。一方面，本发明可以用于辨别与探针末端之一相距 1、2、3、4、5、6、7、8、9、10 个碱基或甚至更远(宜为 3-10 个碱基)的错配。

本发明的探针允许从在探针末端的邻近位置上的非目标中辨别出目标，这使得能够探测短目标片段中是否存在多种不同的 SNP。

基于碳的间隔物和与寡间隔物联合

本发明的一方面，所述探针包含位于目标特异性探针序列与偶联基之间的 3-50 或 13-50 个碳单元的碳间隔物，一方面包含 C20 – C50 的间隔物，例如 C20 – C40 间隔物，或者例如 C20 – C30 间隔物。可

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以使用任何合适的间隔物，例如C13、14、15、16、17、18、19、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49或C50间隔物。可以根据对结合特异性和信号强度的影响，使用标准技术来选择合适的间隔物，以获得最佳结果。

本发明的一方面，所述探针除了包含碳间隔物以外，还包含寡核苷酸间隔物，所述寡核苷酸间隔物具有至少15个核苷酸或至少20个核苷酸，例如15－150个或20－150个核苷酸，例如25－100个核苷酸、30－75个核苷酸，包括15－20、20－25、25－30、30－35、35－40、40－45和45－50个核苷酸。寡核苷酸间隔物可以是例如同聚物或杂聚物。一方面，寡核苷酸间隔物是聚胸腺嘧啶(poly T)间隔物，或包含其他合适重复核苷酸单元的间隔物例如(TTG)重复单元，或poly A(聚腺嘌呤)间隔物，或poly G间隔物，或poly C间隔物。合适的其他杂聚间隔物包括TTTG、AAG、AAC、AAAG或AAAC的重复。可以使用本领域所熟知的常规方法来测试不同的间隔物，以优化探针-目标的相互作用。

因此，一方面，本发明总的来讲提供包含碳间隔物和寡核苷酸间隔物的探针。

一方面，所述寡核苷酸间隔物位于碳间隔物与特异性探针序列之间。在这种情况下，所述碳间隔物可以是比13个碳单元的长度更短，例如C12或者甚至更短。

一方面，所述寡核苷酸间隔物经过挑选，致使它不与目标序列或目标序列的相邻区杂交。一方面，所述寡核苷酸间隔物经过挑选，致使其在本文中描述的方法中使用时，不与目标序列或目标序列的相邻区域杂交。

一方面，寡核苷酸间隔物经过挑选，致使目标特异性探针侧翼(flanking)的间隔物区不与目标核酸或目标序列的相邻区杂交。这在图1c中阐明。

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多碳间隔物在下述文献中公开：Cowan 等(Transfer of a Mycobacterium tuberculosis genotyping method, Spoligotyping, from a reverse line-blot hybridization, membrane-based assay to the Luminex multianalyte profiling system. J Clin Microbiol (结核分支杆菌基因型分型方法－间隔寡核苷酸基因型分型，从反向线点杂交－基于膜的测定法，转移到 Luminex 多分析物序型分析系统). 2004 Jan;42(1): 474-7.) 和 Taylor 等(Taylor JD, Briley D, Nguyen Q, Long K, Iannone MA, Li MS, Ye F, Afshari A, Lai E, Wagner M, Chen J, Weiner MP. Flow cytometric platform for high-throughput single nucleotide polymorphism analysis. (用于高通量单核苷酸多态性分析的流式细胞计数平台) Biotechniques. 2001 Mar;30(3):661-6, 668-9)。

碳间隔物宜为(CH2)n 间隔物。

寡核苷酸间隔物

因此，一方面，本发明提供了仅包含位于珠偶联基与目标特异性探针序列之间的寡核苷酸间隔物的探针(即不存在碳间隔物)。

一方面，此间隔物具有至少 15 个核苷酸或至少 20 个核苷酸。一方面，此间隔物具有 15－150 或 20－150 个核苷酸，例如 25－100 个核苷酸，30－75 个核苷酸，包括 15－20、20－25、25－30、30－35、35－40、40－45 和 45－50 个核苷酸。

寡核苷酸间隔物可以是例如同聚物或杂聚物。一方面，所述寡核苷酸间隔物是聚胸腺嘧啶(poly T)间隔物，或包含其他合适的重复核苷酸单元的间隔物，例如(TTG)重复单元间隔物，或 poly A (聚腺嘌呤)间隔物，或 poly G 间隔物，或 poly C 间隔物。合适的其他杂聚间隔物包括 TTTG、AAG、AAC、AAAG 或 AAAC 的重复。可以使用本领域所熟知的常规方法来测试不同的间隔物，以优化探针-目标的相互作用。

因此，一方面，本发明提供了包含位于珠偶联基与目标特异性探针序列之间的寡核苷酸间隔物的探针，其中所述寡核苷酸间隔物是聚胸腺嘧啶(poly T)间隔物。

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因此，一方面，本发明提供了包含位于珠偶联基与目标特异性探针序列之间的寡核苷酸间隔物的探针，其中所述寡核苷酸间隔物是TTG 重复间隔物或 polyA 间隔物。

一方面，所述间隔物(或者是碳间隔物 + 寡核苷酸间隔物，或者是单独的寡核苷酸间隔物)位于目标特异性探针序列的 3'末端。另一方面，所述间隔物位于目标特异性探针序列的 5'末端。

一方面，所述寡核苷酸间隔物经过挑选，使它不与目标序列或目标序列的相邻区杂交。一方面，寡核苷酸间隔物经过挑选，使其在本文描述的方法中使用时，不与目标序列或目标序列的相邻区杂交。

一方面，所述寡核苷酸间隔物经过挑选，使目标特异性探针侧翼的间隔物区不与目标序列或目标序列的相邻区杂交。这在图 1c 中阐明。

又一方面，本发明涉及包含至少 2 种探针的探针组，适当地包括本发明的任何探针，其中至少一种探针与珠或间隔物通过探针的 5'末端连接，并且其中至少一种探针与珠或间隔物通过探针的3'末端连接。

间隔物序列

本发明也涉及适合与液态的基于珠的检测系统一起使用的间隔物序列。本发明的间隔物可以是本文所述的任何间隔物。间隔物可以包含例如偶联在一起的多碳重复(例如 $C_{12} - C_{30}$)与寡核苷酸重复(例如长度在 15 – 150 个或 20 – 150 个核苷酸之间的 polyT 或 poly (TTG)或 polyA)，或由其组成，并且适用于与目标特异性探针序列连接。

本发明的间隔物也可以包含总度长为 15 – 150 或 20 – 150 或 25 – 150 个核苷酸的寡核苷酸重复，或者由其组成。

间隔物适宜包含适于与珠连接的偶联基，例如伯氨基。

本发明也涉及与珠偶联的本发明的间隔物分子。

本发明也涉及与目标特异性探针序列偶联、任选地也与珠偶联的本发明的间隔物分子。

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试剂盒

本发明也涉及试剂盒，其包含本发明的间隔物分子和粒状支持体例如珠。

本发明也涉及试剂盒，其包含本发明的间隔物分子和关于与粒状支持体例如珠偶联的说明书。

本发明也涉及试剂盒，其包含与粒状支持体例如珠偶联的本发明的间隔物分子，和关于与目标特异性探针序列偶联的说明书。

本发明也涉及试剂盒，其包含与粒状支持体例如珠偶联的本发明的探针和关于在检测目标分子中使用的说明书。

方法

本发明也涉及对用于通过使用基于珠的技术在核酸水平上检测探针-目标相互作用的已有方案作出的某些方法改进。

基于珠的技术例如 Luminex 技术在本领域及文献中有充分的描述。珠，也称为微珠，宜为在 Dunbar 等以及当中的参考文献中描述的聚苯乙烯珠，以上所有文献在此引作参考。

本发明的总体方法是用于检测探针(宜为本文中所定义的探针)与目标核酸之间的任何相互作用的标准方案。所述方法宜包括以下步骤:

i 使在样品中存在的任何目标多核酸变性;

ii 在允许探针与目标之间发生特异性杂交的条件下，使目标与探针杂交;

iii 任选进行严格的洗涤以基本上除去所有未结合的材料;

iv 加入报告分子并与之一起温育，以便能够检测探针-目标的结合情况;

v 任选进行洗涤; 并且

vi 检测探针-目标的结合情况，

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此方案的详细实例在本文中所包含的实施例中给出。

这些一般步骤宜为连贯的，但一方面，某些步骤可以一起进行，例如，探针与目标和报告分子同时反应。

探针与目标核酸的特异性杂交通常指的是：所使用的试验条件下，所述探针与部分目标区或与整个目标区形成双链体；以及在那些条件下，所述探针不与在所分析样品中存在的多核酸的其他区形成双链体。

严格的洗涤条件为本领域所熟知，包括例如在50℃下3X SSC、0.1% Sarkosyl，以及在本文实施例中描述的那些条件。

步骤(v)的洗涤在本领域所熟知的任何合适的条件下进行，以便例如能够除去多余的报告分子。本发明的一方面，洗涤在比在所述洗涤步骤中所用浓度更低浓度的SSC (例如实质上为2 x SSC、1.5 x SSC，或者实质上为1x SSC)存在下进行。

可以通过任何合适的方法来进行检测，本发明的一方面，使用流式细胞计数分析进行检测，以基于珠的荧光特性(例如在Dunbar (上文中)描述的Luminex珠系统)检测目标-探针的相互作用。尤其是，上述论文表明：例如Luminex xMAP系统引入了用两种光谱不同的荧光染料内部染色的5.6 μm聚苯乙烯微球。使用准确量的这些荧光染料中的每一种，产生出由具有特定光谱地址的不同微球组组成的阵列。每一微球组在其表面可以具有不同的反应物。因为微球组可以通过其光谱地址来辨别，所以可以使它们联合，以允许例如100种或更多种不同的分析物同时在单个反应器中检验。与报告分子偶联的第三荧光染料可定量已在微球表面发生的生物分子相互作用。当微球在Luminex® 100™分析仪中经过两个分开的激光器时，它们在快速流动的液流中单独地被询问。635-nm 10-mW的红光二极管激光器激发包含在微球中的两种荧光染料，而532-nm、13-mW钇铝石榴石(YAG)激光器则激发与微球表面结合的报告荧光染料(R-藻红蛋白、Alex 532或Cy3)。高速数字信号处理基于微珠的光谱地址将其分类，并定量表面上的反

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应。每秒数以千计的微球被询问，从而导致分析系统能够每个样品仅用几秒钟即可分析并报告单个反应器中的例如 100 个或更多个不同的反应。

本发明的一方面，所述珠可以是顺磁珠。一方面，可以基于珠的磁性使用机械混合来使珠与目标和/或报告物混合。

一方面，本发明的方法包括在步骤(ii)之后的探针与目标杂交步骤之后维持杂交温度。一方面，维持杂交温度，至少直到步骤(iii)的严格洗涤。一方面，本发明的方法包括在步骤(iv)的与报告分子温育期间维持杂交温度。

这样，本发明涉及如上文所概括的用于检测探针与目标核酸之间相互作用的方法，其中维持目标和探针之间杂交反应的温度，直到与报告分子的反应基本上完成。

一方面，本发明的方法也包括在杂交步骤(ii)之后立刻使用稀释洗涤的步骤。此步骤增加了目标与探针间反应的体积，似乎降低了非特异性杂交的可能性。使用用于除去本方法步骤 ii 中的任何未结合的材料的洗涤缓冲液来进行稀释。

一方面，本发明的方法包括在加热时摇动或混合，例如通过在步骤(ii)使用热混合器。热混合器通常是在可以预设的温度下提供混合样品的任何装置。在此，混合适宜在杂交温度下，通常为 50℃或更高，例如 50 - 55℃，例如 50℃、52℃、54℃和 55℃

一方面，本发明的方法包括在步骤(vi)之后信号检测之前在 1X SSC 中洗涤最终的探针-目标复合物。此洗涤可以在室温下进行。

一方面，本发明的方法包括在步骤(vi)之后在信号检测之前摇动最终的探针-目标复合物。

又一方面，所述方法在步骤(i)之前包括使珠与探针偶联的又一步骤。这样，目标与探针之间的反应在固相支持体中进行。

又一方面，本发明涉及如上文所概括的方法，其中所述探针与珠(宜为带有荧光染料的聚苯乙烯珠)连接。

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又一方面，本发明涉及如上文所概述的方法，其中同时使用至少2种探针来检测不同的目标。这样的反应通常称为多重反应(multiplex reation)。一方面，本发明的具有不同目标特异性的探针与珠连接，每种珠对每种类型的特异性探针具有特异性。

又一方面，本发明涉及包括2种类型的特异性探针的多重反应，其中所述探针与珠(宜为用不同荧光染料标记的珠)连接，且其中在所述多重反应中不同探针的探针长度不同。例如，当限定的多核苷酸(例如DNA)片段将同时用多种不同类型的特异性探针探测时，则一方面，本发明不需要所有探针的长度都相等，而另一方面，探针的确长度不同。

又一方面，探针与目标之间的杂交在柠檬酸钠(SSC)或等同物(例如2x至4x SSC或3x SSC)存在下进行，适当地为探针-目标相互作用的发生提供离子环境。

本发明在以下的实施例中得以阐明，所述实施例并不限制本发明。

材料与方法:

Wallace等(见上文)的标准杂交方法(逐步)如下:

1. 选择合适的偶联了寡核苷酸的微球组。
2. 通过旋涡和超声处理约20秒以重悬微球。
3. 通过在1.5x TMAC (1x TMAC = 2mol/l TMAC / 0.15% Sarkosyl / 75mmol/l Tris，6mmol/l EDTA)杂交缓冲液中将经偶联的微球储备液稀释至每组 150 个微球/μl，制备工作微球混合物(Working Microsphere Mixture) (注意：每一反应需要33 μl的工作微球混合物)。
4. 通过旋涡和超声作用约20分钟，混合工作微球混合物。
5. 将33μl工作微球混合物加至每个样品或本底孔中。
6. 加入17 μl dH_2O至每一本底孔。

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7. 将扩增的生物素化 DNA 和 dH_2O 加入每个样品孔中至总体积为 17 μl (注意：7 μl PCR 反应液用于检测)。
8. 用移液器上下吹打若干次，以轻柔地混合反应孔。
9. 在 99℃下温育 5 分钟，使扩增的生物素化 DNA 在热循环仪中变性。
10. 在杂交温度(55°C)下温育反应板 15 分钟。
11. 在温育期间，通过用冰冷的 1x TMAC 冲洗两次以准备滤板。随后，将冰冷的 1x TMAC 加入滤板的每个孔。
12. 在温育期间，将链霉素抗生物素-R-藻红蛋白在 1x TMAC 杂交缓冲液中稀释至 2μg/ml，以制备新鲜的报告物混合物(注意：每一反应需要 75 μl 报告物混合物)，并将它放在于杂交温度下的恒温箱(Oven)或水浴中。
13. 通过将整个反应物转移到含有冰冷洗涤缓冲液的滤板中以终止杂交反应。
14. 转移后，通过介入真空过滤，使用冰冷的 1x TMAC 洗涤缓冲液严格地洗涤滤板两次。
15. 每孔加入 75 μl 报告物混合物，并通过用移液器上下吹打若干次以轻柔地混合。
16. 让整个板达到室温约 30 分钟。
17. 在杂交温度下温育反应板 30 分钟。
18. 通过真空过滤终止温育。
19. 通过介入真空过滤，用 1x TMAC 洗涤缓冲液洗涤两次。
20. 通过介入真空过滤，用 1x TMAC 洗涤缓冲液溶解反应物。
21. 于室温下在 Luminex™ 100 分析仪上按照该系统的使用手册进行分析。

[**参见图 2.** 如 Wallace 等(2005)描述的工作流程的总体图示概述]

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检验的灵敏度和特异性基于探针与目标核酸序列之间的特异性杂交。因此，在所述方法中似乎杂交和洗涤以及与 PE 温育是关键性步骤。通过优化不同的参数，例如温度和扩散动力学，对方案进行了修改以优化反应的灵敏度与特异性。这些修改在优化的杂交方案中展示(参见下文)。

材料

A. 缓冲液

0.1 M MES pH 4.5 (偶联缓冲液)

试剂	产品目录编号	终浓度	含量/250 ml
MES (2[N-吗啉代]乙磺酸)	Sigma M-2933	0.1 M	4.88 g
dH_2O	-	-	至 250ml
5 N NaOH	Fisher SS256-500	------	~5 滴

过滤器(45μm)除菌并于 4℃下保存

0.02%吐温 (洗涤缓冲液 I)

试剂	产品目录编号	终浓度	含量/250 ml
吐温 20 (聚氧乙烯山梨坦单月桂酸酯)	Sigma P-9416	0.02%	50 μl
dH_2O	------	------	250 ml

过滤器(45μm)除菌并在室温下保存

20% Sarkosyl

试剂	产品目录编号	终浓度	含量/250 ml
Sarkosyl (N-十二烷酰肌氨酸)	Sigma L-9150	20%	50g
dH_2O	------	------	(调整到) 250 ml

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过滤器(45μm)除菌并在室温下保存

TE pH 8.0 (样品稀释剂)

试剂	产品目录编号	终浓度	含量/250 ml
Tris EDTA 缓冲液 pH 8.0 100X	Sigma T-9285	1 X	2.5 ml
dH_2O	------	------	247.5 ml

过滤器(45μm)除菌并在室温下保存

4.5x SSC / 0.15% Sarkosyl 杂交缓冲液(微球稀释剂)

试剂	产品目录编号	终浓度	含量/50 ml
20x SSC (3M 氯化钠、0.3M 脱水柠檬酸钠，pH 7.0)	Cambrex US51232	4.5x	11.25 ml
20% Sarkosyl	------	0.15%	0.375 ml
dH_2O	------	------	38.375 ml

过滤器(45μm)除菌并在室温下保存

3x SSC / 0.1% Sarkosyl / 1mg/ml 酪蛋白严格洗涤缓冲液

试剂	产品目录编号	终浓度	含量/50 ml
20x SSC	Cambrex US51232	3x	7.5 ml
20% Sarkosyl	------	0.1%	0.250 ml
50mg/ml 酪蛋白 (pH7.2)	VWR BDHA440203H	------	1 ml
dH_2O	------	------	41.25 ml

过滤器(45μm)除菌并在 4℃下保存

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1x SSC / 0.1% Sarkosyl/ 1mg/ml 酪蛋白洗涤缓冲液

试剂	产品目录编号	终浓度	含量/50 ml
20x SSC	Cambrex US51232	1x	2.5 ml
20% Sarkosyl	------	0.1%	0.250 ml
50mg/ml 酪蛋白 (pH7.2)	VWR BDHA440203H	------	1 ml
dH_2O	------	------	46.25 ml

过滤器(45μm)除菌并在 4℃下保存

B. 珠

1. 所使用的珠类型是 L100-C123-01 至 L100-C172-01 (Luminex™ Corp., Austin, TX).

C. 探针(见实施例)

1. 探针由 Eurogentec (Seraing, 比利时)提供

D. 设备

设备	型号
热循环仪(Thermocycler)	ABI GeneAmp PCR system 9700
热混合器(Thermo mixer)	Eppendorf Thermomixer comfort
水浴器(Water bath)	GFL 1001
温育恒温箱(Incubation Oven)	Memmert U25U
Luminex™	Luminex™ X100

方法与方案:

I. 探针偶联

1. 使新鲜等份的-20℃干燥的 Pierce EDC 即盐酸[1-乙基-3-[二甲胺丙基]碳二亚胺粉末温至室温。

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2. 重悬所述胺取代的寡核苷酸(“探针”或“捕获”寡核苷酸(“ “capture” oligo)) 0.2mM (0.2nmol/μl)于 dH_2O 中。

3. 旋涡和超声处理约 20 秒重悬储备的微球。

4. 将 5.0×10^6 的储备微球转移至 USA Scientific 离心管。

5. 微量离心(≥ 8000 x g) 1-2 分钟，使储备微球沉淀。

6. 除去上清液，并通过旋涡和超声处理约 20 秒使沉淀的微球重悬在 50 μl 的 0.1 M MES，pH 4.5 中。

7. 制备 0.2mM 捕获寡核苷酸的 dH_2O 溶液的 1:10 稀释液(0.02nmol/μl)。

8. 将 2 μl (0.04 nmol)的 1:10 稀释的捕获寡核苷酸加至经重悬的微球中，并通过旋涡处理混合。

9. 制备新鲜的 20 mg/ml EDC 的 dH_2O 溶液。在使用前最多 1 分钟，将 10mg EDC 溶解在 500μl dH_2O 中。等份的 10mg EDC(粉末)与硅胶包装在一起于-80℃中干燥保存。

10. 对于每一反应，一个接一个地将 2.5 μl 新鲜制备的 20 mg/ml EDC 加至微球中，并通过旋涡处理混合(注意：等份的 EDC 粉末此时应该丢弃)。

11. 在室温下于黑暗中温育 30 分钟。

12. 制备第二个新鲜的 20 mg/ml EDC 的 dH_2O 溶液。

13. 对于每一反应，一个接一个地将 2.5 μl 新鲜制备的 20 mg/ml EDC 加入到微球中，并通过旋涡混合均匀(注意：等份的 EDC 粉末此时应该丢弃)。

14. 在室温下于黑暗中温育 30 分钟。

15. 将 1.0 ml 的 0.02%吐温-20 加入到已偶联的微球中。

16. 通过微量离心(≥ 8000 x g) 1-2 分钟使已偶联的微球沉淀。

17. 除去上清液，并通过旋涡处理使已偶联的微球重悬在 1.0 ml 的 0.1% SDS 中。

18. 通过微量离心(≥ 8000 x g) 1-2 分钟使已偶联的微球沉淀。

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19. 除去上清液，并通过旋涡和超声处理约 20 秒使已偶联的微球重悬在 100 µl of TE，pH 8.0 中。

20. 通过微量离心(≥ 8000 x g) 1-2 分钟使已偶联的微球沉淀。

21. 除去上清液，并通过旋涡和超声处理约 20 秒使已偶联的微球重悬在 100 µl of TE，pH 8.0 中。

22. 通过血细胞计数器计数已偶联的微球:

a. 用 dH_2O 以 1:100 稀释已重悬的偶联微球。

b. 通过旋涡处理彻底混合。

c. 将 10 µl 转移到血细胞计数器中。

d. 在血细胞计数器网格的 4 个大格中计微球数量。

e. 微球/µl = (4 个大格中微球总数) x 2.5 x 100 (稀释系数)。(注意: 最多 50,000 微球/µl。)

23. 将偶联微球冷藏储存于 2-10℃黑暗中。

II. 优化的杂交和洗涤方案

1. 选择合适的偶联了寡核苷酸的微球组。

2. 通过旋涡和超声处理约 20 秒重悬微球。

3. 通过用 4.5x TMAC / 0.15% Sarkosyl 杂交缓冲液稀释偶联微珠储备液至每组 150 个微珠/µl，制备工作微球混合物(注意: 每一反应需要 33 µl 的工作微珠混合物)。

4. 通过旋涡和超声处理约 20 分钟，混合工作微珠混合物。

5. 将 33µl 工作微珠混合物加入到每个样品或本底孔中。

6. 将 17 µl TE，pH 8 加至每一本底孔。

7. 将扩增的生物素化 DNA 和 TE，pH 8.0 加至每个样品孔，至总体积为 17 µl (注意: 50µl 有效的 PCR 反应液中取 4 µl 通常足够用于检测)。

8. 通过移液器上下吹打若干次，以轻柔地混合反应孔。

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9. 在99-100℃下温育5分钟，使扩增的生物素化DNA在热循环仪中变性。

10. 在热循环仪中于60℃下温育反应板3分钟。

11. 将反应板转移到在杂交温度下预热的热混合器(注意:可以使用8道移液器将反应液同时转移到8个孔中)。

12. 在杂交温度下并以500rpm温育反应板15分钟。

13. 在温育期间，用蒸馏水冲洗以准备Millipore滤板。随后，在杂交温度下将200μl 3xSSC/0.1% Sarkosyl/1mg/ml酪蛋白洗涤缓冲液加至滤板的各孔，并将其放进杂交温度下的恒温箱。

14. 在温育期间，使链霉素抗生物素-R-藻红蛋白在3xSSC/0.1% Sarkosyl/1mg/ml酪蛋白洗涤缓冲液中稀释至2μg/ml，以制备新鲜的报告物混合物(注意：每个反应需要75 μl的报告物混合物)，并将其放在于杂交温度下的恒温器或水浴中。

15. 通过将整个反应物转移到含有杂交温度的洗涤缓冲液的滤板中来终止杂交反应。

16. 转移后，通过介入真空过滤，使用杂交温度的100μl 3xSSC/0.1% Sarkosyl/1mg/ml酪蛋白严格洗涤缓冲液来涤滤板两次。

17. 每孔加入75 μl报告物混合物，并通过用移液器上下吹打若干次以轻柔地混合。

18. 在杂交温度下温育反应板15分钟。

19. 通过真空过滤终止反应。

20. 通过介入真空过滤，使用杂交温度的100μl 1xSSC/0.1% Sarkosyl/1mg/ml酪蛋白洗涤缓冲液来涤滤板两次。

21. 在室温下将反应物溶解在100μl 1xSSC/0.1% Sarkosyl/1mg/ml酪蛋白洗涤缓冲液中。

22. 于室温下在Luminex™ 100分析仪上按照该系统的使用手册取50 μl进行分析。

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III. 读出

1. 使用 Luminex™ 100 IS 版本 2.3 软件读出数据。
2. 在测量期间，使用以下参数:
    a. 样品体积: 50µl
    b. 样品超时(Sample timeout): 60 sec.
    c. XY 加热器温度(℃): 35
    d. 双重鉴别器门控(Doublet Discriminator Gate):
        i. 下限: 8000
        ii. 上限: 18500
    e. 统计值: 中值

IV. 数据处理

1. 将数据保存在含有由 Luminex™100 IS2.3 软件所提供的全部标准输出值的原始 CSV 文件中(逗号隔开的*.csv)。

2. 将获得的中值信号转移到 Excel 文件中，计算出目标与探针比以及信噪比(也参见设计与计算结果)。

本发明提出了 Luminex™方法的不同条目，包括探针设计优化和测试方案优化。

在下文中，将以图 2 所概括的工作流程的顺序来呈现数据。

图 2. 修改过的工作流程的总体图示概括

实施例中的结果表达(设计与计算结果):

有关的实施例与权利要求如以下详述并解释。结果主要以表格形式展现，所述表格含有原始数据(MFI =中值荧光强度)、变量(例如温度)、探针、和被分析的目标、计算结果和注释。计算结果包括目标与探针比(%目标/探针)以及信噪比(信号/噪声)。

目标与探针比以每种探针计算，并将每一信号以阳性对照的百分比来展示，其中阳性对照设定为 100% (也参见实施例表 12)。

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信噪比也以每种探针计算。每一信号值除以所获得的所有信号的中值(也参见实施例表 13)。

目标与探针比和信噪比两者给出了有关信号强度和特异性的良好的总体指示。

某些实施例使用了 EP1012348 中描述的来自 SPF10 引物的探针和探针组的探针，上述文献在此全部引作参考。此专利为本发明专利申请中所用的技术提供了技术背景。

SPF10 引物组产生长度仅 65 bp 且带有 22 个核苷酸的引物间区(interprimer region)的小扩增物。这严重限制了根据所有 HPV 基因型之间的不同错配来定位探针的可能性。

实施例 1

目的:

检验在杂交步骤之后维持杂交温度是否对信号特异性有显著的正面影响。

简介:

在固定于珠上的探针与溶液中变性的目标序列杂交之后，在与报告剂链霉素抗生物素-R-藻红蛋白(PE)温育之前，需要将未结合的材料洗掉。这通过使用滤板(MSBVN12, Millipore)来实现，其中使珠和所有连接分子与溶液中游离的分子分离。因为反应体积小，所以在其环境中易受温度迅速变化的影响。我们检验了在杂交温度之后温度变化的影响。

材料与方法:

使用 SPF_{10} 模型系统，研究在低于杂交的温度下温育对 Luminex™ 信号的影响。

使用了携带 HPV 31 探针(probe 31SLPr31，参见表 1a)的 Luminex™珠。此探针可特异性地鉴定用 SPF_{10} 引物组扩增的 HPV 31

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序列。为了评价交叉反应性，使用了 HPV44 和 HPV16 的扩增物。HPV 31 的目标序列与 HPV 44 的目标序列在 1 个位置上不同，而 HPV 31 的目标序列与 HPV 16 的目标序列在 4 个位置上不同(表 1b)。

于 50℃下进行杂交，而且测定成对地进行。随后，按照标准方案处理其中一组反应物，并立刻于 4℃下在滤板中洗涤珠。在 4℃下开始同样的标准洗涤之前，首先将重复的一组反应物在室温(RT)下温育 1 分钟。与 Wallace 等(2005)相比，在将样品转移到滤板之后，加入洗涤缓冲液(也参见实施例 2)。

结果:

结果在表 1c 中出示。如表所示，在杂交以后且在严格洗涤之前，于室温下仅温育 1 分钟就导致信号增强，但也降低了特异性(观察到的 HPV44 的信号更强表明这一点)。这可以用在杂交后因为短暂的温度下降所导致的严格性降低来解释。

结论

在杂交步骤之后，应该维持反应温度。杂交后，不应该在没有任何延迟以阻止任何温度下降的情况下急于洗涤珠。

实施例 2

目的:

检验在杂交步骤之后立刻稀释洗涤是否对信号特异性有显著的正面影响。

简介:

如果在杂交步骤之后不立刻进行洗涤，则标准 Luminex™测定法有引入非特异性结合的风险(也参见实施例 1)。检验了杂交后立刻稀释样品的情况，以使此风险最小化。

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材料与方法:

为了研究此效果，使用了两种 Luminex™珠的混合物，一种珠携带 HPV 31 的探针(名称: 31SLPr31，参见表 2a)，而另一种珠携带 HPV 51 (名称: 51SLPr2，参见表 2a)。这些探针可分别特异性地鉴定用 SPF_{10} 引物组扩增的 HPV 31 序列和 HPV 51 序列。为了观察与 31SLPr31 的可能的交叉反应性，使用了 HPV44 和 HPV16 的扩增物。HPV 31 的目标序列与 HPV 44 和 16 的的目标序列分别在 1 个和 4 个位置上不同(图 2b)。为了观察与 51SLPr2 的可能的交叉反应性，使用了 HPV33 和 HPV16 的扩增物。HPV 51 的目标序列与 HPV 44 和 16 的的目标序列各自在 4 个位置上不同(图 2c)。

使用标准的方案于 50℃下进行杂交。

随后，在无任何另外的洗涤的情况下，立刻将第一组反应物于 4℃下在滤板中洗涤。与 Wallace 等(2005)相比，在将样品转移到滤板之后，再加入洗涤缓冲液。

如下研究杂交步骤之后立刻进行另外的直接和间接稀释洗涤方法的效果。对于直接和间接的方法，使用了 50℃下的洗涤缓冲液(3x SSC / 0.1% Sarkosyl / 1mg/ml 酪蛋白。此乃严格的洗涤缓冲液)。

通过直接方法来洗涤第二组珠。所述直接方法包括: 在热循环仪中杂交温度下用 200µl 洗涤缓冲液稀释杂交混合物(50µl)，随后将整个稀释后的样品转移至滤板。

通过间接方法来洗涤第三组杂交反应物。所述间接方法包括: 通过将 50µl 杂交混合物转移至已预先装有杂交温度的 200µl 洗涤缓冲液的滤板中，来进行稀释。(也参见 Wallace 等，2005).

结果:

结果在表 2d 中出示。与标准方法相比，两种另外的洗涤方法都引起绝对信号降低，但同时信号的特异性显著提高。直接与间接洗涤方法之间没有显著差异。事实上，在热循环仪中直接稀释洗涤的实践

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性较低，因此优选间接稀释洗涤方法。

结论:

杂交后使用额外的稀释洗涤步骤对信号的特异性有显著的正面影响。出于实践原因，优选间接稀释洗涤方法。

实施例 3

目的:

检验在与链霉素抗生物素-R-藻红蛋白温育之前在严格洗涤期间维持杂交温度是否对信号特异性有显著的正面影响。

简介:

如上文所描述的，在严格杂交之后温度下降的负面作用暗示严格洗涤其本身的温度也可以产生影响。因此，对在 50℃、RT 或 4℃下的严格洗涤温度的影响也作出了研究。

材料与方法:

使用 SPF_{10} 模型系统，对在与链霉素抗生物素-R-藻红蛋白温育之前在杂交步骤之后严格洗涤缓冲液的不同温度的影响进行如下研究。

使用了携带 HPV 31 的探针(名称：31SLPr31，参见图 3a)的 Luminex™珠来研究此影响。此探针可特异性地鉴定用 SPF_{10} 引物组扩增过的 HPV 31 序列。为了观察与 31SLPr31 的可能的交叉反应性，使用了 HPV44 和 HPV16 的扩增物。HPV 31 的目标序列与 HPV 44 和 16 的的目标序列分别在 1 个位置和 4 个位置上不同(表 3b)。

在 50℃下进行杂交。随后，分别将各组反应物分别转移到含有 50℃、RT 或 4℃的洗涤缓冲液的滤板中。

结果:

结果在表 3c 中出示。阳性对照信号的绝对水平在 50℃和 RT 之

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间并无不同，而在4℃洗涤以后则稍微降低。然而，在50℃下洗涤导致信号特异性显著提高，而在RT或4℃下洗涤导致信号特异性降低。因此，优选在50℃的杂交温度下的间接稀释洗涤方法。

结论:

在与链霉素抗生物素-R-藻红蛋白温育之前，在严格洗涤期间维持杂交温度对信号特异性有显著的影响。

实施例4

目的:

检验使用热混合器是否对信号强度有显著的正面影响。

简介:

通过在反应期间混合组分，可以影响杂交反应的动力学。

因此，我们研究了在杂交期间使用热混合器的影响。

材料与方法:

使用 MPF 模型系统，如下对在杂交期间使用热混合器对扩散动力学的影响进行了研究。

使用了携带 HPV18 探针(名称: 18MLPr7，参见表 4a)或 HPV51 探针(名称: 51MLPr2，参见表 4a)的两种 Luminex™珠。这些探针可特异性地鉴定用 MPF 引物组扩增的 HPV18 序列和 HPV 51 序列。使这两种珠与 HPV 18 和 HPV 51 的 MPF 扩增产物混合并进行杂交。HPV 18 的目标序列与 HPV 51 的目标序列在 7 个位置上不同(表 4b 和 c)。以双份对反应物进行检测。

一份反应物在没有振摇的情况下在热循环仪中变性并进行杂交。(也参见 Wallace 等，2005)

副本反应物在用于变性的热循环仪中变性，并立刻转移至用于杂交的热混合器。在50℃下进行杂交。随后，使用优化的杂交和洗涤方

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案，立刻在50℃的滤板中对珠进行洗涤。

结果：结果在表 4d 中出示。使用热混合器显著地增强了阳性对照的绝对信号，而本底仍未受影响。这导致信号特异性的总体提高。

这些结果表明，通过使用热混合器将会提高(改进)信号强度。

结论：

热混合器的使用对信号的强度和特异性产生显著的正面影响。

实施例 5

目标：

检验与链霉素抗生物素-R-藻红蛋白在杂交温度下温育是否对信号强度有显著的正面影响。

简介：

一般来说，温度影响任何反应的动力学，包括用报告剂 PE 检测杂交产物。因此，研究了温度对 PE 温育及随后的洗涤的影响。

材料与方法：

使用了携带 HPV51 探针(名称：51SLPr2，见表 5a)的 Luminex™ 珠。此探针可特异性地鉴定用 SPF_{10} 引物组扩增的 HPV 51 序列。为了观察与此探针的可能的交叉反应性，使用了 HPV33 和 HPV16 的 SPF10 扩增物。HPV 51 的目标序列与 HPV 33 和 HPV 16 的目标序列在 4 个位置不同(表 5b)。

使用本文所概括的优化的杂交和洗涤方案，以两份重复在50℃下进行杂交。经过严格洗涤之后，一组反应物在50℃下与 PE 温育(也参见 Wallace 等，2005)，而另一组在 RT 下与 PE 温育。随后于 50℃下在滤板中洗涤珠。

在另一个实验中，使用优化的杂交与洗涤方案，以两份重复在

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50℃下进行杂交。经过严格洗涤之后，全部反应物都在 50℃下与 PE 温育(也参见 Wallace 等，2005)。经过在 50℃下 PE 温育之后，一组反应物在 50℃下洗涤(也参见 Wallace 等，2005)，而另一组在 RT 下洗涤.

结果:

如表5C中所出示的，在不同的温度下进行PE温育有显著的影响。与在 RT 下进行 PE 温育相比，在 50℃的杂交温度下进行 PE 温育导致绝对信号更强。然而，信号的特异性并无显著不同。

因此，优选在杂交温度下与链霉素抗生物素-R-藻红蛋白进行温育。相比之下，温育之后在 RT 或杂交温度下洗涤并不产生显著影响，尽管这样在一些情形中可能更具实践性。

温度对 PE 温育之后的洗涤步骤的影响并不显著。绝对信号以及特异性两者似乎并无受到洗涤温度的影响。

结论:

在与链霉素抗生物素-R-藻红蛋白温育期间维持杂交温度对信号强度产生显著的影响，但对信号特异性无显著影响。

PE 温育之后的洗涤温度无显著的影响。

实施例 6

目的:

检验通过用 1x SSC 的最后洗涤是否可以防止 Luminex™取样探针的堵塞。

简介:

在我们的优化的杂交和洗涤方案中，杂交在 3x SSC 中进行。在此浓度下，SSC 确实严重地堵塞 Luminex™取样探针，从而阻碍样品处理。因此，研究了最后洗涤时较低 SSC 浓度的影响。

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结果:

起初，我们设法维持杂交的 SSC 浓度。然而，由于用 3xSSC 最后洗涤，导致 Luminex™取样探针严重堵塞，从而致使不能产生有意义的数据。只用 1xSSC 进行此洗涤步骤，即可产生有意义的数据。因此，由于缺乏数据，所以无法出示数据上的比较。未对其他的 SSC 浓度作出研究。

结论:

用 1x SSC 的最后洗涤防止了 Luminex™取样探针的堵塞。

实施例 7

目的:

检验经过最后洗涤之后将可用于测量的样品储存在 4℃下至少 4 天是否对信号产生任何显著的影响。

简介:

为了提高工作地点的灵活性，我们使用 Luminex™系统，分析了关于直接杂交检验方案的若干步骤。特别测试的一个方法是在直接杂交方法的两个步骤之间进行储存。因此，我们研究了在 4℃下储存的影响。

材料与方法:

使用 SPF10 模型系统，对在最后的洗涤步骤之后储存在 4℃下的影响进行如下研究。

为了研究此影响，使用了携带 HPV51 探针(名称: 51SLPr2，参见图 7a)的 Luminex™珠。此探针可特异性地鉴定用 SPF_{10} 引物组扩增的 HPV 51 序列。为了观察与 51SLPr2 的可能的交叉反应性，使用了 HPV31 的扩增物。HPV 51 的目标序列与 HPV 31 的目标序列在 4 个位置上不同(表 7b)。

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在最后的洗涤步骤之后，将各组反应物在4℃中分别储存0、4、24和96小时。随后，在RT下测量这些反应物组。

结果:

结果在表7c中出示。如表所示，在最后的洗涤步骤以后进行储存，并不影响信号的强度或特异性。然而，在此条件下储存似乎导致随时间的过去原始信号强度有非常轻微的增强。因此，如有必要，可以在最后的洗涤步骤之后，储存最多4天以维持原始信号。

结论:

在最后洗涤步骤之后的储存对信号强度和信号特异性无显著的影响，但提高了工作地点的灵活性。

探针(间隔物)设计－介绍

Luminex™系统的关键原理是使特异性寡核苷酸探针固定在微珠的表面上，所述微珠因各个微珠类型的颜色组成而起着独特标记的作用。

在分子大小上，所述珠要比特异性探针大很多。因此，特异性探针序列的定位非常接近Luminex™珠的表面。由于位阻现象和各种珠表面效应例如表面疏水性，对于被固定的探针与溶液中的目标分子之间的杂交动力学来说，此探针位置可能并非最佳。

以下实施例描述了改变将探针定位到珠表面的许多方法，以优化探针与目标之间的杂交动力学。

测试了以下的探针设计的变体:

1. 使用各种长度的碳间隔物
2. 使用各种长度的额外的寡核苷酸间隔物
3. 使用各种组成的寡核苷酸间隔物

所述探针具有三个功能不同的不同区:

1. 偶联基，例如NH2基，其允许探针与珠表面共价偶联;

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2. 间隔物，其可以用来(a)在珠表面与特异性探针序列之间产生间距和/或(b)使特异性探针更多地处于亲水环境；和

3. 真正的目标特异性探针序列。对于探针的此部分，应用了本领域的标准参数，例如探针组成和探针长度。

实施例 8

目的：

检验使用不同长度的碳间隔物的影响。

材料与方法：

使用了携带含 C_{12} 间隔物的 HPV51 探针(名称：51SLPr2，参见表 8a)或含 C_{18} 间隔物的 HPV51 探针(名称：51SLPr2C_{18}，参见表 8a)的 Luminex™珠。这些探针可特异性地鉴定用 SPF_{10} 引物组扩增的 HPV 51 序列。为了观察与这些探针的可能的交叉反应性，使用了 HPV33 的扩增物。HPV 51 的目标序列与 HPV 33 的目标序列在 4 个位置上不同(表 8b)。

结果：结果在表 8c 中出示。与 C12 探针相比，C18 间隔物导致绝对信号减弱，但特异性更强。此现象不仅在 51SLPr2C_{18} 中可以看到，而且在含 C_{18} 间隔物的其他探针(例如 33SLPr21 C_{18}; 表 8a、c 和 d)中也能看到。

结论：

使用不同的碳间隔物长度对信号特异性有显著的影响。就例如 51SLPr2 而论，最佳的探针含有 C_{18} 碳间隔物。

实施例 9

目的：

检验不同长度的寡核苷酸间隔物的影响。

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材料与方法

使用了携带含 0、10、20、30 或 40 个胸腺嘧啶的 HPV51 探针(名称：51SLPr2、51SLPr2T10、51SLPr2T20、51SLPr2T30、51SLPr2T40，参见表 9a)的 Luminex™珠。每种珠类型携带不同的探针变体。这些探针可特异性地鉴定用 SPF_{10} 引物组扩增的 HPV 51 序列。为了观察与这些探针的可能的交叉反应性，使用了 HPV33 的扩增物。HPV 51 的目标序列与 HPV 33 的目标序列在 4 个位置上不同(表 9c)。

除了使用 SPF10 模型系统以外，也使用了 MPF 模型系统来如下研究此影响。使用了携带含 0、20、30 或 40 个胸腺嘧啶的 HPV52 探针(名称：52MLPr2、52MLPr2T20、5MLPr2T30、52MLPr2T40，参见表 9b)的 Luminex™珠。每种珠类型携带不同的探针变体。这些探针可特异性地鉴定用 MPF 引物组扩增的 HPV 52 序列。为了观察与这些探针的可能的交叉反应性，使用了 HPV16 的扩增物。HPV 52 的目标序列与 HPV16 的目标序列在 2 个位置上不同(表 9d)。

结果

结果在表 9e 和 9f 中出示。用胸腺嘧啶序列段延长间隔物增强了绝对信号的水平。与不含添加的胸腺嘧啶间隔物的间隔物相比，特异性也显著地增强。比较了不同长度的间隔物，产生最佳信号需要至少 20 个胸腺嘧啶(例如 51SLPr2)。总的来说，当探针含有 40 个核苷酸的间隔物时(例如 51SLPr2 和 52MLPr2)，其表现最佳。因此优选此间隔物长度。

结论：

使用不同的间隔物不仅对信号强度有显著影响，而且也对特异性产生显著的影响。就 $51SLPr2T_n$ 而言，良好的探针含有至少 20 个胸腺嘧啶的间隔物，这可增强信号强度和特异性两者。一般来说，至少 40 个核苷酸的间隔物长度表现最佳。

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实施例 10

目的:

检验使用修饰型 poly(T)间隔物是否能够防止假阳性的反应性。

简介:

众所周知，许多 Taq DNA 聚合酶会将附加的 A 核苷酸添加到合成链的 3'末端。尚不知晓多个 A 是否也能够添加到 3'末端，而产生在 3'末端带 oligi-A 尾的分子亚群。虽然这些分子将仅代表 PCR 产物总量的极少的一部分，但是出于检测方法的高度灵敏度的原因，这些分子能够导致假阴性结果。这是由于 PCR 产物上的这段 oligi-A 序列与探针的 poly(T)间隔物之间发生杂交这一事实所致。

此 PCR 假象产物(PCR artifact)存在于在某些样品中，并且在难以 PCR 水平上重现。这似乎取决于反应条件中的非常微小的波动。在检测水平上，本底是重现性很高的，即产生本底的 PCR 产物也将是重现性很高的。

此 PCR 假象产物也可以在线性探针测定(line probe assay, LiPA)系统上引起假阳性结果，因为此系统也包含 T 尾探针。在 LiPA 测定中，不论探针的特异性序列如何，这都导致使用所有探针都产生同样微弱的(本底)信号。在 Luminex™系统中也已经观察到此微弱的本底信号读数。因此，对修饰型间隔物的影响作出了研究。

材料与方法:

使用了携带含 T40 间隔物或含修饰型(TTG)13 间隔物的 HPV18 探针(名称: 18MLPr7T40 和 18MLPr7$(TTG)_{13}$，参见表 10a)的 Luminex™ 珠。这些探针可特异性地鉴定用 MPF 引物组扩增的 HPV18 序列。选择了(TTG)三联体作为可供选择的间隔物，因为它对 poly (A)表现出最差的理论结合功效之一。

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为了观察与 18MLPr7T40 和 18MLPr7$(TTG)_{13}$ 的可能的交叉反应性，使用了来自表现出此假阳性本底的样品的扩增物(称为 nc8) 。

结果:

结果在表 10b 中出示。

13 个“TTG”核苷酸三联体的间隔物明显地能够完全消除本底信号，所述本底信号在 T40 间隔物的情况下可观察到。

结论:

使用可供选择的基于 T 的间隔物(例如$(TTG)_{13}$)对信号的特异性产生明显的影响，从而消除了由富 A 的 PCR 假象产物引起的假阳性信号。

实施例 11

目的:

检验将基于胸腺嘧啶的间隔物置于探针的 5’或 3’末端是否抑制与探针-目标结合位点侧翼的富 A 目标区的结合。

简介:

已知探针/目标中间的错配对其结合能的影响最大。接近结合区侧边的错配更难以辨别。与探针/目标结合区侧翼的富 A 序列段位置相结合，这可以对探针的选择性强度造成损害。因此，我们研究了间隔物位置的影响，以便将间隔物与探针-目标结合位点侧翼的富 A 目标区的结合减至最小。

材料与方法:

使用 MPF 模型系统，如下对在探针的 5’或 3’末端的间隔物位置的影响作出了研究，所述间隔物处于 Luminex™珠与特异性探针序列之间。

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为了研究所述影响，使用了携带含基于胸腺嘧啶的间隔物的HPV18 和 HPV45 的探针(名称：18MLPr7T40N5、18MLPr7T40N3、45MLPr8T40N5 和 45MLPr8T40N3，参见表 11a)的 Luminex™珠。这些探针可分别特异性地鉴定用 MPF 引物组扩增的 HPV18 和 HPV45 序列。为了观察可能的与 18MLPr7T40$_n$ 的交叉反应性，使用了 HPV39 的扩增物。HPV 18 的目标序列与 HPV39 的目标序列在 2 个位置上不同(表 11b)。为了观察可能的与 45MLPr8T40$_n$ 的交叉反应性，使用了 HPV13、39 和 40 的扩增物。HPV45 的目标序列与 HPV13、39 和 40 的目标序列分别在 3、2 和 1 个位置上不同(表 11 c)。

结果

结果在表 11d 中出示。如表所示，位于探针 3'末端而非 5'末端的间隔物削弱了其与探针-目标结合位点侧翼的富 A 目标区的结合，并且影响结合能(dG)和解链温度(Tms)。这些非特异性信号的排除可以解释为目标与间隔物和探针结合。这些结果提示，目标与间隔物的结合可以阻碍探针的特异性，应该阻止其发生。从原理上来讲，使用"TTG"核苷酸三联体间隔物也会牵涉同样的机制。因此，当使用基于胸腺嘧啶的间隔物时，可以通过间隔物与特异性目标区侧翼的序列之间的微弱的交叉杂交，来提高探针:目标杂交体的稳定性，所述微弱的交叉杂交可导致假阳性信号，其对于探针设计应该予以考虑。

结论:

基于胸腺嘧啶的间隔物在探针的 5'或 3'末端的位置对于探针-目标结合位点侧翼的富 A 目标区的结合可以产生显著的影响。

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实施例 12

适合于基于珠的方法(例如基于 Luminex 的方法)使用的 HPV 探针:

表 14

名称	探针序列
16MLP4T40N3	GAGCACAGGGCCAC(T)$_{40}$
18MLPr7T40N3	TTACATAAGGCACAGG(T)$_{40}$
26MLP7T40N3	GTTACAACGTGCACAG(T)$_{40}$
31MLPr6T40N3	GGATGCAACGTGCTC(T)$_{40}$
33MLPr4T40N5	(T)$_{40}$CATATTGGCTACAACGT
35MLPr6T40N3	GTGCACAAGGCCATA(T)$_{40}$
39MLPr4T40N5	(T)$_{40}$GCCTTATTGGCTACATAA
45MLPr6T40N5	(T)$_{40}$ggtGTTACATAAGGCCCAG
45MLPr8T40N3	CCAGGGCCATAACAAg(T)$_{40}$
51MLPr2T40N5	(T)$_{40}$TTATTGGCTCCACCGT
52MLPr2T40N5	(T)$_{40}$CCGTACTGGTTACAACGa
53MLPr6T40N5	(T)$_{40}$ATATTGGCTGCAACGT
56MLPr4T40N5	(T)$_{40}$GGCCCAAGGCCATAATAA
58MLPr1T40N5	(T)$_{40}$CTTATTGGCTACAGCGT
58MLPr5T40N3	ACAGCGTGCACAAGG(T)$_{40}$
59MLPr3T40N5	(T)$_{40}$CAAGGCTCAGGGTTTAA
66MLPr6T40N3	TGCACAGGGCCATA(T)$_{40}$
66MLPr7T40N3	TGCAACGTGCACAG(T)$_{40}$
68MLPr8T40N5	(T)$_{40}$CTGCACAAGGCACAG
68MLPr10T40N3	GCACAAGGCACAGG(T)$_{40}$
70MLPr4T40N5	(T)$_{40}$CCTATTGGTTGCATAAGG
82MLPr3T40N3	ATTGGTTGCATCGCG(T)$_{40}$

本发明的一方面，所有以上探针中的任何 1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21 种或全部 22 种可以用于同一条件下的基于珠的多重反应，以同时检测样品中存在的任何 HPV 目标 DNA。这些珠的组别适合在以上概括的优化的反应方案中使用。可以掺入额外的多碳间隔物。因此，本发明涉及包含所有以上探针中的 2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、21 种或全种的任何探针组。

总论

我们推测，空间位阻和疏水排斥导致杂交体末端的次佳(sub-optimal)错配识别。此外，认为此空间位阻会降低灵敏度，因为目

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标次佳地与探针结合。因此，在探针设计中引入间隔物已表明防止了空间位阻，并由此消除了交叉杂交且提高了灵敏度。我们发现，这的确导致特异性和灵敏度更高。

尽管此间隔物显著地增强了探针的性能，但仍然观察到一些交叉反应。我们发现这些交叉反应中大部分在探针(目标结合区)的末端附近具有单个错配。有时候观察到所述交叉反应与目标之探针结合区侧翼的富 A 区相关联。因此，我们推测，此富 A 区可以与间隔物 T 序列段的一部分结合，并因此增强了交叉反应性。于是我们最初设计了在另一端(3'而非 5')含间隔物的探针。然而，考虑到探针结合区侧翼的序列，重新设计间隔物可能是一种替代方案。

我们已经观察到，在 PCR 中有时产生趋于与所有探针结合的假象产物(artificial product)。此产物可能在 3'末端含有许多 A 残基(这是若干 Taq 聚合酶的已知活性)，因此对基于 T 间隔物的亲和力增强。A-T 杂交可以导致交叉反应性增强，引起假阳性杂交结果。

为了进一步了解此现象，我们设计了若干种具有含 TTG 三联体(例如$(TTG)_{13}$)的间隔物的探针。我们估计，特别是此三联体将最具排斥性，并且削弱假象 PCR 产物与探针目标区侧翼的富 A 片段的结合。我们观察到，基于 TTG 的间隔物削弱了此假象 PCR 的总体非特异性结合。基于 TTG 的间隔物也可以削弱与探针侧翼区的结合，并提高了其特异性。

总括:

- 空间位阻和疏水排斥导致杂交体末端的次佳错配辨别
- 在探针设计中引入间隔物防止了空间位阻，并因此消除了交叉杂交，这导致更高的特异性和灵敏度。
- 目标的探针结合区侧翼的富 A 区可以与间隔物的 T 序列段结合，从而增强了交叉反应性。
- 有时在 PCR 中产生在 3'末端含有 A 序列段的假象产物，其趋于与所有探针结合。

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- 此产物富含A，因此对于基于T的间隔物的亲和力增强。
- 此现象可以通过削弱与探针侧翼区的非特异性结合的基于TTG的间隔物来减少，并提高其特异性。

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表实施例 1:

名称	探针组成
31SLPr31	NH_2-C_{12}-GGCAATCAGTTATTTG

表 1a 31SLPr31 = SPF_{10}探针 31 版本 31, C_{12} = 12 个碳原子的片段(stretch)

目标	与探针 31SLPr31 比对	错配数
HPV 31	GGCAATCAGTTATTTG	0
HPV 44	--A----------	1
HPV 16	--T-C-AC---------	4

表 1b "-"指相同氨基酸。

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探针	所杂交的目标	杂交后的温度(℃)	信号(MFI)	目标/探针(%)	信号/噪声	注释	Exp
31SLPr31	SPF_{10} HPV31	50	4457	100	48	特异性	ID28
31SLPr31	SPF_{10} HPV44	50	1279	29	14	交叉反应	ID28
31SLPr31	SPF_{10} HPV16	50	19	<1	<1	阴性	ID28
31SLPr31	SPF_{10} HPV31	RT	7544	100	13	特异性	ID27
31SLPr31	SPF_{10} HPV44	RT	3783	50	6	交叉反应	ID27
31SLPr31	SPF_{10} HPV16	RT	24	1	<1	阴性	ID27

表 1c

表实施例 2:

名称	探针组成
31SLPr31	NH_2-C_{12}-GGCAATCAGTTATTTG
51SLPr2	NH_2-C_{12}-CTATTTGCTGGAACAATC

表 **2a** 31SLPr31 = SPF_{10}探针 31 版本 31, C_{12}= 12 个碳原子的片段

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目标	与探针 31SLPr31 比对	错配数
HPV 31	GGCAATCAGTTATTTG	0
HPV 44	--A----------	1
HPV 16	--T-C-AC-------	4

表 2b “-”指相同氨基酸。

目标	与探针 51SLPr2 比对	错配数
HPV 51	CTATTTGCTGGAACAATC	0
HPV 33	T------T---GG-----	4
HPV 16	-------T---GGT--C-	4

表 2c “-”指相同氨基酸。

探针	所杂交的目标	额外洗涤步骤	信号(MFI)	目标/探针 (%)	信号/噪声	注释	Exp
31SLPr31	SPF_{10} HPV31	无	4457	100	48	特异性	ID28
31SLPr31	SPF_{10} HPV44	无	1279	29	14	交叉反应	ID28
31SLPr31	SPF_{10} HPV16	无	19	<1	<1	阴性	ID28
31SLPr31	SPF_{10} HPV31	直接	2765	100	41	特异性	ID31
31SLPr31	SPF_{10} HPV44	直接	117	4	2	阴性	ID31
31SLPr31	SPF_{10} HPV16	直接	20	1	<1	阴性	ID31

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31SLPr31	SPF_{10} HPV31	间接	3843	100	171	特异性	ID32
31SLPr31	SPF_{10} HPV44	间接	25	1	1	阴性	ID32
31SLPr31	SPF_{10} HPV16	间接	15	<1	1	阴性	ID32
51SLPr2	SPF_{10} HPV51	无	2316	100	201	特异性	ID28
51SLPr2	SPF_{10} HPV33	无	631	27	55	交叉反应	ID28
51SLPr2	SPF_{10} HPV16	无	11	<1	1	阴性	ID28
51SLPr2	SPF_{10} HPV51	直接	2057	100	110	特异性	ID31
51SLPr2	SPF_{10} HPV33	直接	432	21	23	交叉反应	ID31
51SLPr2	SPF_{10} HPV16	直接	18	1	1	阴性	ID31
51SLPr2	SPF_{10} HPV51	间接	1571	100	209	特异性	ID32
51SLPr2	SPF_{10} HPV33	间接	354	23	47	交叉反应	ID32
51SLPr2	SPF_{10} HPV16	间接直接	7	<1	1	阴性	ID32

表 2d

表实施例 3:

名称	探针组成
31SLPr31	NH_2-C_{12}-GGCAATCAGTTATTTG

表 3a 31SLPr31 = SPF_{10}探针 31 版本 31, C12 = 12 个碳原子的片段

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目标	与探针 31SLPr31 比对	错配数
HPV 31	GGCAATCAGTTATTTG	0
HPV 44	--A----------	1
HPV 16	--T-C-AC--------	4

表 3b "-"指相同氨基酸。

探针	所杂交的目标	洗涤温度(℃)	信号(MFI)	目标/探针(%)	信号/噪声	注释	Exp
31SLPr31	SPF_{10} HPV31	50	5747	100	162	特异性	ID90
31SLPr31	SPF_{10} HPV44	50	56	1	2	阴性	ID90
31SLPr31	SPF_{10} HPV16	50	20	<1	<1	阴性	ID90
31SLPr31	SPF_{10} HPV31	RT	5701	100	33	特异性	ID86
31SLPr31	SPF_{10} HPV44	RT	2422	42	14	交叉反应	ID86
31SLPr31	SPF_{10} HPV16	RT	13	<1	<1	阴性	ID86
31SLPr31	SPF_{10} HPV31	4	4889	100	44	特异性	ID34
31SLPr31	SPF_{10} HPV44	4	417	9	4	交叉反应	ID34
31SLPr31	SPF_{10} HPV16	4	33	1	<1	阴性	ID34

表 3c

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表实施例 4:

名称	探针组成
18MLPr7T40	NH_2-C_{12}-$(T)_{40}$-TTACATAAGGCACAGG
51MLPr2T40	NH_2-C_{12}-$(T)_{40}$-TTATTGGCTCCACCGT

表 **4a** 18MLPr7 = MPF 探针 18 版本 7, C_{12} = 12 个碳原子的片段

目标	与探针 **18MLPr7** 比对	错配数
HPV18	TTACATAAGGCACAGG	0
HPV51	C-C--CCGT--G----	7

表 **4b** "-" 指相同氨基酸。

目标	与探针 **51MLPr2** 比对	错配数
HPV51	TTATTGGCTCCACCGT	0
HPV18	A------T-A--TAAG	7

表 **4c** "-"指相同氨基酸。

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探针	所杂交的目标	杂交方法	信号 (MFI)	目标/探针(%)	信号/噪声	注释	Exp
18MLPr7T40	MPF HPV18	热循环仪	1082	100	144	特异性	ID148
18MLPr7T40	MPF HPV51	热循环仪	6	1	1	阴性	ID148
51MLPr2T40	MPF HPV51	热循环仪	1410	100	123	特异性	ID148
51MLPr2T40	MPF HPV18	热循环仪	20	1	1	阴性	ID148
18MLPr7T40	MPF HPV18	热混合器	2154	100	287	特异性	ID148
18MLPr7T40	MPF HPV51	热混合器	6	0	1	阴性	ID148
51MLPr2T40	MPF HPV51	热混合器	2725	100	210	特异性	ID148
51MLPr2T40	MPF HPV18	热混合器	25	1	2	阴性	ID148

表 4d

表实施例 5:

名称	探针组成
51SLPr2	NH_2-C_{12}-CTATTTGCTGGAACAATC

表 5a 51SLPr2 = SPF_{10} 探针 51 版本 2, C_{12} = 12 个碳原子的片段

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目标	与探针 51SLPr2 比对	错配数
HPV 51	CTATTTGCTGGAACAATC	0
HPV 33	T------T---GG-----	4
HPV 16	-------T---GGT----	4

表 5b "-"指相同氨基酸。

探针	所杂交的目标	PE 温育温度 (℃)	信号(MFI)	目标/探针 (%)	信号/噪声	注释	Exp
51SLPr2	SPF_{10} HPV51	50	3681	100	194	特异性	ID44
51SLPr2	SPF_{10} HPV33	50	345	9	18	交叉反应	ID44
51SLPr2	SPF_{10} HPV16	50	30	1	2	阴性	ID44
51SLPr2	SPF_{10} HPV51	RT	3074	100	615	特异性	ID43
51SLPr2	SPF_{10} HPV33	RT	259	8	52	交叉反应	ID43
51SLPr2	SPF_{10} HPV16	RT	5	<1	1	阴性	ID43

表 5c

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探针	所杂交的目标	洗涤温度(℃)	信号(MFI)	目标/探针(%)	信号/噪声	注释	Exp
51SLPr2	SPF_{10} HPV51	50	2433	100	187	特异性	ID90
51SLPr2	SPF_{10} HPV33	50	423	16	33	交叉反应	ID90
51SLPr2	SPF_{10} HPV16	50	8	<1	1	阴性	ID90
51SLPr2	SPF_{10} HPV51	RT	2777	100	179	特异性	ID90
51SLPr2	SPF_{10} HPV33	RT	374	13	24	交叉反应	ID90
51SLPr2	SPF_{10} HPV16	RT	10	<1	1	阴性	ID90

表 5d

表实施例 7:

名称	探针组成
51SLPr2	NH_2-C_{12}-CTATTTGCTGGAACAATC

表 7a 51SLPr2 = SPF_{10} 探针 51 版本 2, C_{12} = 12 个碳原子的片段

目标	与探针 51SLPr2 比对	错配数
HPV 51	CTATTTGCTGGAACAATC	0
HPV 31	T------T---GG-----	4

表 7b "-"指相同氨基酸。

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探针	所杂交的目标	4℃储存 (小时)	信号 (MFI)	目标/探针 (%)	信号/噪声	注释	Exp
51SLPr2	SPF_{10} HPV51	0	1573	100	51	特异性	ID110
51SLPr2	SPF_{10} HPV31	0	30	2	1	阴性	ID110
51SLPr2	SPF_{10} HPV51	4	1611	100	59	特异性	ID111
51SLPr2	SPF_{10} HPV31	4	28	2	1	阴性	ID111
51SLPr2	SPF_{10} HPV51	24	1783	100	60	特异性	ID113
51SLPr2	SPF_{10} HPV31	24	34	2	1	阴性	ID113
51SLPr2	SPF_{10} HPV51	96	1707	100	52	特异性	ID114
51SLPr2	SPF_{10} HPV31	96	33	2	1	阴性	ID114

表 7c

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表实施例 8:

名称	探针组成
51SLPr2	NH_2-C_{12}-CTATTTGCTGGAACAATC
51SLPr2C_{18}	NH_2-C_{18}-CTATTTGCTGGAACAATC
33SLPr21	NH_2-C_{12}-GGGCAATCAGGTATT
33SLPr21C_{18}	NH_2-C_{18}-GGGCAATCAGGTATT

表 8a 51SLPr2 = SPF_{10}探针 51 版本 2, C_{12} = 12 个碳原子的片段, C_{18} = 18 个碳原子的片段

目标	与探针 **51SLPr2** 比对	错配数
HPV 51	CTATTTGCTGGAACAATC	0
HPV 33	T------T---GG-----	4

表 8b "-"指相同氨基酸。

目标	与探针 **33SLPr21** 比对	错配数
HPV 33	GGGCAATCAGGTATT	0
HPV 51	-AA---------C-T--	4

表 8c "-"指相同氨基酸。

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探针	所杂交的目标	信号 (MFI)	目标/探针(%)	信号/噪声	注释	Exp
51SLPr2	SPF_{10} HPV51	4291	100	172	特异性	ID64
51SLPr2	SPF_{10} HPV33	358	8	14	交叉反应	"
51SLPr2C_{18}	SPF_{10} HPV51	3515	100	216	特异性	ID67
51SLPr2C_{18}	SPF_{10} HPV33	16	0	1	阴性	"
33SLPr21	SPF_{10} HPV33	429	100	48	特异性	ID77
33SLPr21	SPF_{10} HPV51	52	12	6	交叉反应	"
33SLPr21C_{18}	SPF_{10} HPV33	429	100	61	特异性	"
33SLPr21C_{18}	SPF_{10} HPV51	4	1	1	阴性	"

表 8d

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表实施例 9:

名称	探针组成
51SLPr2	NH_2-C_{12}-CTATTTGCTGGAACAATC
51SLPr2T10	NH_2-C_{12}-$(T)_{10}$-CTATTTGCTGGAACAATC
51SLPr2T20	NH_2-C_{12}-$(T)_{20}$-CTATTTGCTGGAACAATC
51SLPr2T30	NH_2-C_{12}-$(T)_{30}$-CTATTTGCTGGAACAATC
51SLPr2T40	NH_2-C_{12}-$(T)_{40}$-CTATTTGCTGGAACAATC

表 9a 51SLPr2 = SPF_{10} 探针 51 版本 2, C_{12} = 12 个碳原子的片段, $(T)_{40}$ = 一段 40 个胸腺嘧啶核苷酸的序列

名称	探针组成
52MLPr2	NH_2-C_{12}- CCGTACTGGTTACAACGA
52MLPr2T20	NH_2-C_{12}-$(T)_{20}$- CCGTACTGGTTACAACGA
52MLPr2T30	NH_2-C_{12}-$(T)_{30}$- CCGTACTGGTTACAACGA
52MLPr2T40	NH_2-C_{12}-$(T)_{40}$- CCGTACTGGTTACAACGA

表 9b 52MLPr2 = MPF 探针 52 版本 2, C_{12} = 12 个碳原子的片段, $(T)_{40}$ = 一段 40 个胸腺嘧啶核苷酸的序列

目标	与探针 **51SLPr2** 比对	错配数
HPV 51	CTATTTGCTGGAACAATC	0
HPV 33	T------T---GG-----	4

表 9c "-"指相同氨基酸。

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目标	与探针 52MLPr2 比对	错配数
HPV 52	CCGTACTGGTTACAACGA	0
HPV 16	--T--T----------	2

表 9d "-"指相同氨基酸。

探针	所杂交的目标	信号(MFI)	目标/探针(%)	信号/噪声	注释	Exp
51SLPr2	SPF_{10} HPV51	4291	100	172	特异性	ID64
51SLPr2	SPF_{10} HPV33	358	8	14	交叉反应	ID64
51SLPr2T10	SPF_{10} HPV51	4688	100	122	特异性	ID64
51SLPr2T10	SPF_{10} HPV33	34	1	1	阴性	ID64
51SLPr2T20	SPF_{10} HPV51	8712	100	387	特异性	ID64
51SLPr2T20	SPF_{10} HPV33	32	0	1	阴性	ID64
51SLPr2T30	SPF_{10} HPV51	8077	100	414	特异性	ID64
51SLPr2T30	SPF_{10} HPV33	30	0	1	阴性	ID64
51SLPr2T40	SPF_{10} HPV51	7356	100	320	特异性	ID64
51SLPr2T40	SPF_{10} HPV33	32	0	1	阴性	ID64

表 9e

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探针	所杂交的目标	信号(MFI)	目标/探针(%)	信号/噪声	注释	Exp
51MLPr2	MPF HPV52	423	100	13	特异性	ID69
51MLPr2	MPF HPV16	32	8	1	交叉反应	ID69
51MLPr2T20	MPF HPV52	1233	100	95	特异性	ID69
51MLPr2T20	MPF HPV16	11	1	1	阴性	ID69
51MLPr2T30	MPF HPV52	1250	100	139	特异性	ID69
51MLPr2T30	MPF HPV16	8	1	1	阴性	ID69
51MLPr2T40	MPF HPV52	1510	100	126	特异性	ID69
51MLPr2T40	MPF HPV16	9	1	1	阴性	ID69

表 9f

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表实施例 10:

名称	探针组成
18MLPr7T40	NH_2-C_{12}-$(T)_{40}$-TTACATAAGGCACAGG
18MLPr7$(TTG)_{13}$	NH_2-C_{12}-$(TTG)_{13}$-TTACATAAGGCACAGG

表 10a 18MLPr7 = MPF 探针 18 版本 7, C_{12}= 12 个碳原子的片段, $(T)_{40}$ = 一段 40 个胸腺嘧啶核苷酸的序列, $(TTG)_{13}$ = 一段 13 个胸腺嘧啶-胸腺嘧啶-鸟嘌呤核苷酸三联体的序列(共 39 个核苷酸)

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探针	所杂交的目标	信号(MFI)	目标/探针(%)	信号/噪声	注释	Exp
18MLPr7T40	MPF HPV18	2001	100	13	特异性	ID169
18MLPr7T40	nc8	1104	54	7	交叉反应	ID169
18MLPr7T40	DNA-	2	0	0	阴性	ID169
18MLPr7$(TTG)_{13}$	MPF HPV18	2390	100	199	特异性	ID169
18MLPr7$(TTG)_{13}$	nc8	23	1	2	阴性	ID169
18MLPr7$(TTG)_{13}$	DNA-	2	0	0	阴性	ID169

表 **10b** nc8 =在 LiPA 测定中表现出与所有探针交叉反应的阴性对照 8, DNA- = 阴性对照

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表实施例 11:

名称	探针组成
18MLPr7T40N5	NH_2-C_{12}-$(T)_{40}$-TTACATAAGGCACAGG
18MLPr7T40N3	TTACATAAGGCACAGG-$(T)_{40}$-C_{12}-NH_2
45MLPr8T40N5	NH_2-C_{12}-$(T)_{40}$-CCAGGGCCATAACAAG
45MLPr8T40N3	CCAGGGCCATAACAAG-$(T)_{40}$-C_{12}-NH_2

表 **11a** 18MLPr7 = MPF 探针 18 版本 7, C_{12} = 12 个碳原子的片段, $(T)_{40}$ = 一段 40 个胸腺嘧啶核苷酸的序列, N5 = 5'末端氨基接头, N3 = 3'末端氨基接头

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探针	目标	序列
	MPF HPV18	gactcccagtttgtttaataaaccatatttggTTACATAAGGCACAGGgtcataacaatggtgttttgctggcataatca
18MLPr7T40N5		ttttttttttttttttttttttttttttttttttttttttttttTTACATAAGGCACAGG
18MLPr7T40N3		TTACATAAGGCACAGGttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
	MPF HPV39	gatttcccagtttatttaataaagccttatttggCTTACATAAGGGCCACAGGgccacacaacaatggtatatgtttttggcataatca

表 11b 18MLPr7 = MPF 探针 18 版本 7, N5 = 5'末端氨基接头, N3 = 3'末端氨基接头, 灰框序列= 可以与胸腺嘧啶间隔物(下游例子)和探针序列(上游例子)结合的目标核苷酸, 加粗并下划线的 = 与探针序列错配。

探针	目标	序列
	MPF HPV13	gaggcccagtttgtttaataaaccttattggttacaaaaggcCCAGGGCACATTATggtatatgttggggcaatca
	MPF HPV39	gatttcccagtttatttaataaagccttatttggctacataaggcCCAGGGCACATTATggtatatgttggcataatca
	MPF HPV40	gatttctcagatatttaacaaagccatttgtggatacaaaaggcCCAGGGCCATTACAATggcatatgttttggcaatca
45MLPr8T40N5		ttttttttttttttttttttttttttttttttttttttttttttCCAGGGCCATAACAAg
45MLPr8T40N3		CCAGGGCCATAACAAgttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
	MPF HPV45	gatttctcaatttattaataaagccatatttggttacataaggcCCAGGGCCATACAATggtatattgtttggcataatca

表 11c 45MLPr8 = MPF 探针 45 版本 8, N5 = 5'末端氨基接头, N3 = 3'末端氨基接头, 灰框序列= 可以与胸腺嘧啶间隔物(下游例子)和探针序列(上游例子)结合的目标核苷酸, 加粗并下划线的 = 与探针序列错配。

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探针	所杂交的目标	信号 (MFI)	目标/探针(%)	信号/噪声	注释	Exp
18MLPr7T40N5	MPF HPV18	1146	100	85	特异性	ID141
18MLPr7T40N5	MPF HPV39	518	45	38	交叉反应	ID141
18MLPr7T40N3	MPF HPV18	694	100	139	特异性	ID141
18MLPr7T40N3	MPF HPV39	12	2	2	阴性	ID141
45MLPr8T40N5	MPF HPV13	611	38	51	交叉反应	ID141
45MLPr8T40N5	MPF HPV39	284	18	24	交叉反应	ID141
45MLPr8T40N5	MPF HPV40	1021	64	85	交叉反应	ID141
45MLPr8T40N5	MPF HPV45	1600	100	133	特异性	ID141
45MLPr8T40N3	MPF HPV13	47	8	8	交叉反应	ID141
45MLPr8T40N3	MPF HPV39	17	3	3	阴性	ID141
45MLPr8T40N3	MPF HPV40	116	19	19	交叉反应	ID141
45MLPr8T40N3	MPF HPV45	615	100	103	特异性	ID141

表 11d

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表 12a 和 b:

MFI		
目标	珠/探针 A1	珠/探针 A2
a	988	4399
b	13	14
c	19	19,5
d	5	13
e	3	4
f	11	6
g	14	9
h	3	3

% 目标/探针		
目标	珠/探针 A1	珠/探针 A2
a	100	100
b	1	0
c	2	0
d	1	0
e	0	0
f	1	0
g	1	0
h	0	0

% 目标/探针:

A1, a = 988/988 * 100 = 100%;

A1, c = 19/988* 100 = 2%

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表 13a 和 b:

	MFI	
	球/探针	球/探针
目标	A1	A2
A	988	4399
B	13	14
C	19	19,5
D	5	13
E	3	4
F	11	6
G	14	9
H	3	3
中值	12	11

	信号/噪声	
	球/探针	球/探针
目标	A1	A2
a	82	400
b	1	1
c	2	2
d	0	1
e	0	0
f	1	1
g	1	1
h	0	0

信号/噪声:

A1, a = 988 / 12 (= 中值(988, 13, 19, 5, 3, 11, 14, 3)) = 82;

A1, c = 19 / 12 (中值(988, 13, 19, 5, 3, 11, 14, 3)) = 2.

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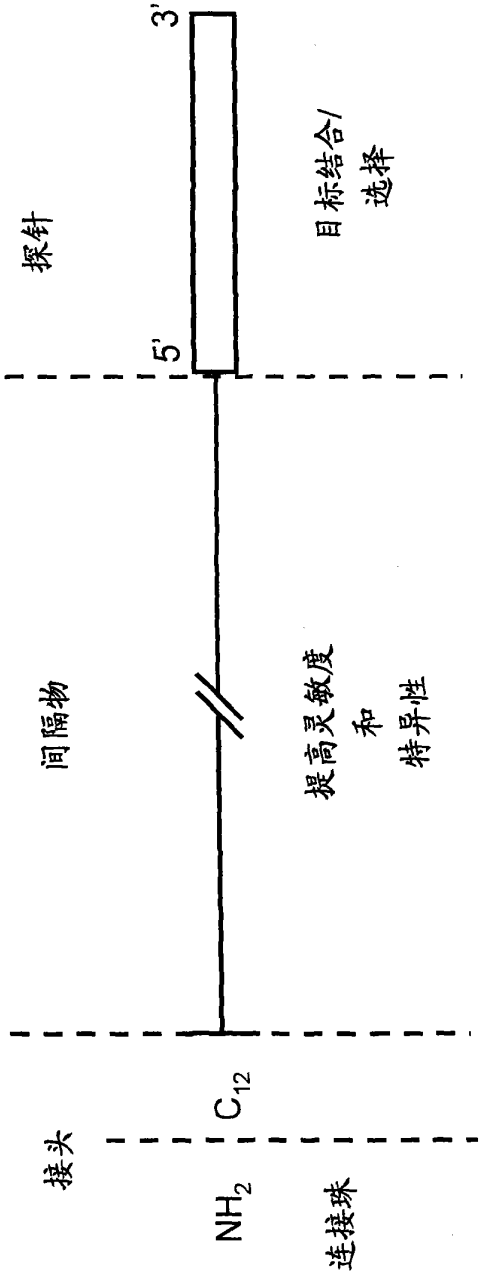


图 1a

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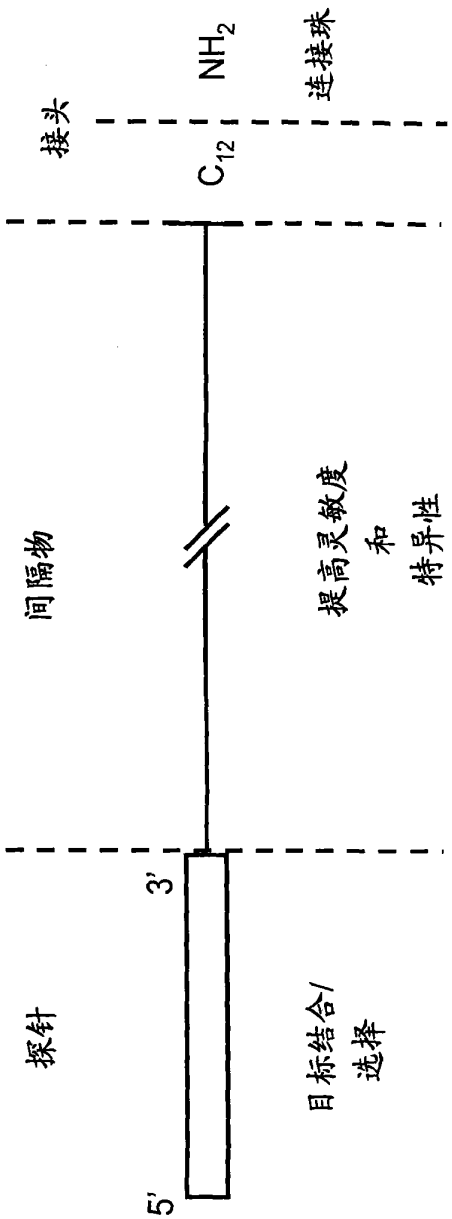


图 1b

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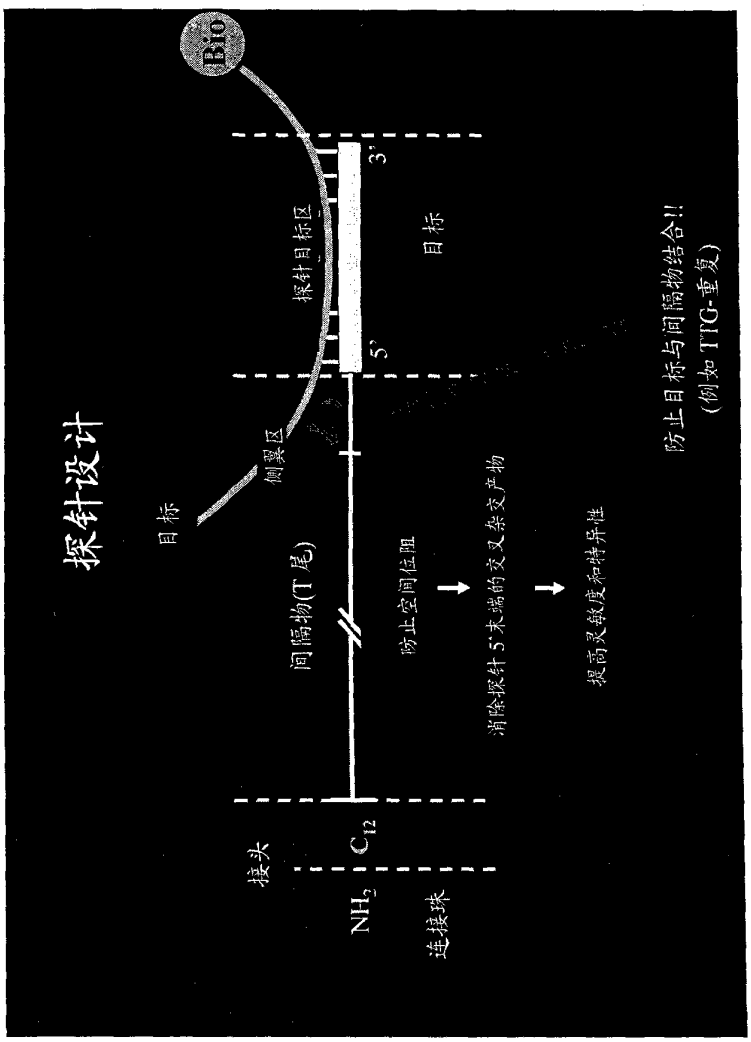


图 1c

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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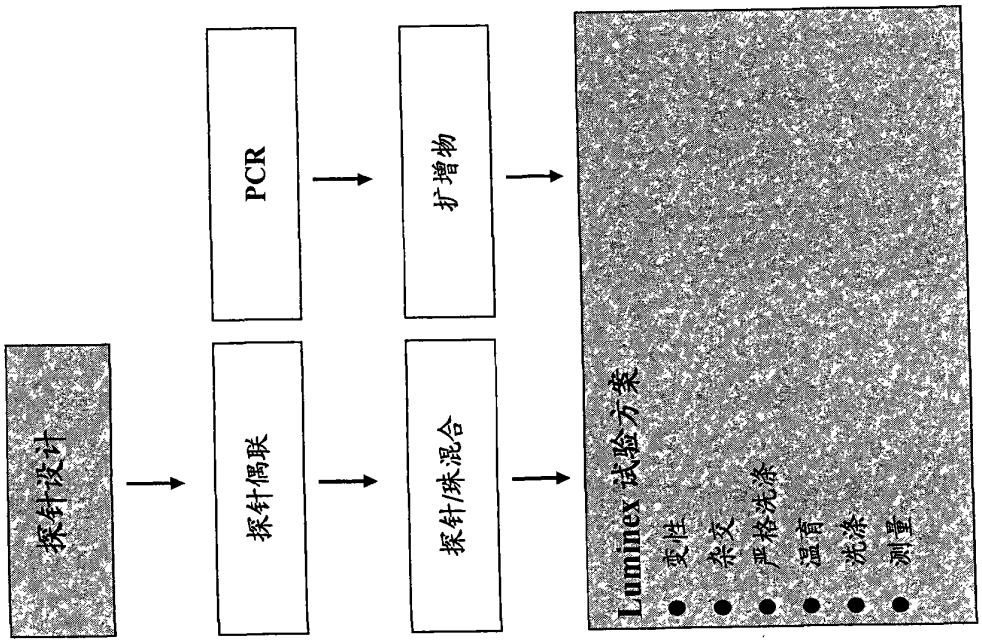


图 2

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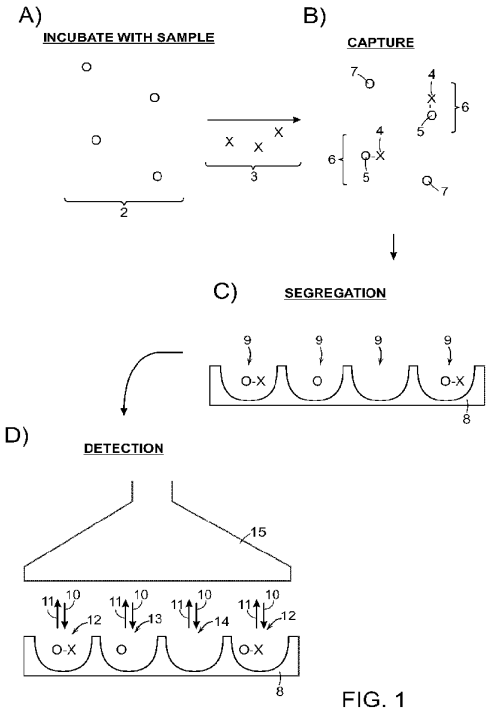


FIG. 1

(57) Abstract: The present invention relates to systems and methods for detecting analyte molecules or particles in a fluid sample and in some cases, determining a measure of the concentration of the molecules or particles in the fluid sample. Methods of the present invention may comprise immobilizing a plurality of analyte molecules or particles with respect to a plurality of capture objects. At least a portion of the plurality of capture objects may be spatially separated into a plurality of locations. A measure of the concentration of analyte molecules in a fluid sample may be determined, at least in part, on the number of reaction vessels comprising an analyte molecule immobilized with respect to a capture object. In some cases, the assay may additionally comprise steps including binding ligands, precursor labeling agents, and/or enzymatic components.

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ULTRA-SENSITIVE DETECTION OF MOLECULES OR PARTICLES USING BEADS OR OTHER CAPTURE OBJECTS

Field of the Invention

Described are systems and methods for detecting analyte molecules or particles in a fluid sample and in some cases, determining a measure of the concentration of the molecules or particles in the fluid sample.

Related Applications

This application is a continuation-in-part of U.S. Patent Application Serial No. 12/731,130, filed March 24, 2010, entitled "Ultra-Sensitive Detection of Molecules or Particles Using Beads or Other Capture Objects," by Duffy et al.., which claims the benefit of U.S. Provisional Patent Application Serial No. 61/309,141, filed March 1, 2010, entitled "Ultra-Sensitive Detection of Molecules or Particles Using Beads or Other Capture Objects," by Duffy et al. Each of the above-indicated applications is incorporated herein by reference.

Background of the Invention

Methods and systems that are able to quickly and accurately detect and, in certain cases, quantify a target analyte molecule in a sample are the cornerstones of modern analytical measurements. Such systems and/or methods are employed in many areas such as academic and industrial research, environmental assessment, food safety, medical diagnosis, and detection of chemical, biological, and/or radiological warfare agents. Advantageous features of such techniques may include specificity, speed, and sensitivity.

Most current techniques for quantifying low levels of analyte molecules in a sample use amplification procedures to increase the number of reporter molecules in order to be able to provide a measurable signal. For example, these known processes include enzyme-linked immunosorbent assays (ELISA) for amplifying the signal in antibody-based assays, as well as the polymerase chain reaction (PCR) for amplifying target DNA strands in DNA-based assays. A more sensitive but indirect protein target amplification technique, called immunoPCR *(see* Sano, T.; Smith, C. L.; Cantor, *C. R. Science* 1992, *258,* 120-122), makes use of oligonucleotide markers, which can subsequently be amplified using PCR and detected using a DNA hybridization assay

(see Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* 2003; *301,* 1884-1886; Niemeyer, C. M.; Adler, M.; Pignataro, B.; Lenhert, S.; Gao, S.; Chi, L. F.; Fuchs, H.; Blohm, *D. Nucleic Acids Research* 1999, *27,*4553-4561; and Zhou, H.; Fisher, R. J.; Papas, T. S. *Nucleic Acids Research* 1993, *21,* 6038-6039). While the immuno-PCR method permits ultra low-level protein detection, it is a complex assay procedure, and can be prone to false-positive signal generation *(see* Niemeyer, C. M.; Adler, M.; Wacker, R. *Trends in Biotechnology* 2005, 23,208-216).

One feature of typical known methods and/or systems for detecting or quantifying low concentrations of a particular analyte in solution is that they are based on ensemble responses in which many analyte molecules give rise to a measured signal. Most detection schemes require that a large number of molecules are present in the ensemble for the aggregate signal to be above the detection threshold. This requirement limits the sensitivity of most detection techniques and the dynamic range (i.e., the range of concentrations that can be detected). Many of the known methods and techniques are further plagued with problems of non-specific binding, which is the binding of analyte molecules or particles to be detected or reporter species non-specifically to sites other than those expected. This leads to an increase in the background signal, and therefore limits the lowest concentration that may be accurately or reproducibly detected.

Accordingly, improved methods for detecting and, optionally, quantifying analyte molecules or particles in a fluid sample are needed, especially in samples where such molecules or particles are present at very low concentration.

Summary of the Invention

Described herein are systems and methods for detecting analyte molecules or particles in a fluid sample and in some cases, determining a measure of the concentration of the molecules or particles in the fluid sample. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises exposing a plurality of capture objects that each include a binding surface having affinity for at least one type of analyte molecule or particle, to a solution containing or suspected of containing the at

least one type of analyte molecules or particles, immobilizing analyte molecules or particles with respect to the plurality of capture objects such that at least some of the capture objects associate with a single analyte molecule or particle and a statistically significant fraction of the capture objects do not associate with any analyte molecule or particle, spatially segregating at least a portion of the capture objects subjected to the immobilizing step into a plurality of separate locations, addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of said locations containing an analyte molecule or particle, and determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations determined to contain an analyte molecule or particle.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises exposing a plurality of capture objects that each include a binding surface having affinity for at least one type of analyte molecule or particle, to a solution containing or suspected of containing the at least one type of analyte molecules or particles to form capture objects comprising at least one immobilized analyte molecule or particle, mixing the capture objects prepared in the exposing step to a plurality of binding ligands such that at least some of the capture objects associate with a single binding ligand and a statistically significant fraction of the capture objects do not associate with any binding ligand, spatially segregating at least a portion of the capture objects subjected to the mixing step into a plurality of locations, addressing at least a portion of the plurality of locations subjected to the spatially segregating step and determining the number of locations containing a binding ligand, and determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of locations determined to contain a binding ligand.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises providing a substrate comprising a plurality of locations, at least a portion of which locations contain a bead, wherein with respect to the total number of beads present on the substrate, the ratio of beads comprising at least one analyte molecule or particle to beads comprising no analyte molecules or particles is between about 8:1 and about 1:10,000,000, addressing at least a portion of the plurality of locations, wherein during the addressing step at least two of the plurality of locations is addressed at least partially concurrently, detecting at

each addressed location the presence or absence of a bead and whether, if present, the bead comprises any analyte molecules or particles, and determining a measure of the concentration of analyte molecules or particles in the fluid sample at least in part by determining the number of locations addressed containing a bead comprising at least one analyte molecule or particle.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises providing a substrate comprising a plurality of locations, at least a portion of which contain a bead, wherein with respect to the total number of beads present on the substrate, the ratio of beads comprising at least one analyte molecule or particle associated with a binding ligand to beads comprising no analyte molecules or particles associated with a binding ligand is between about 8:1 and about 1:10,000,000, addressing at least a portion of the plurality of locations, wherein during the addressing step at least two of the plurality of locations is addressed at least partially concurrently, detecting at each addressed location the presence or absence of a bead and whether, if present, the bead comprises any analyte molecules or particles associated with a binding ligand, and determining a measure of the concentration of analyte molecules or particles in the fluid sample at least in part by determining the number of locations addressed containing a bead comprising at least one analyte molecule or particle associated with a binding ligand.

In some embodiments, an article or kit comprises a plurality of beads having an average diameter between about 0.1 micrometer and about 100 micrometers, and a substrate comprising a plurality of reaction vessels, wherein the average depth of the reaction vessels is between about 1.0 times and about 1.5 times the average diameter of the beads and the average diameter of the reactions vessels is between about 1.0 times and about 1.9 times the average diameter of the beads.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises exposing a plurality of capture objects that each include a binding surface having affinity for at least one type of analyte molecule or particle, to a solution containing or suspected of containing the at least one type of analyte molecules or particles, wherein at least some of the capture objects become associated with at least one analyte molecule or particle, mixing the plurality of capture objects prepared in the exposing step to a plurality of binding ligands comprising an enzymatic component such that a statistically significant fraction of the capture objects associated with at least one analyte molecule or particle associate with a

single binding ligand, spatially segregating at least a portion of the capture objects subjected to the mixing step into a plurality of separate locations, determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part by addressing at least a portion of the plurality of locations subjected to the spatially segregating step to determine the presence of the enzymatic component or a product of a reaction involving the enzymatic component.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises immobilizing a plurality of analyte molecules or particles with respect to a plurality of beads, spatially segregating at least a portion of the plurality of beads into a plurality of separate locations, and addressing at least some of the plurality of locations and determining the number of locations containing a bead, and further determining the number of said locations containing a bead and an analyte molecule or particle, and determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a bead and an analyte molecule and particle, to the number of locations containing a bead.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises immobilizing a plurality of analyte molecules or particles with respect to a plurality of beads, spatially segregating at least a portion of the plurality of beads into a plurality of separate locations, addressing at least some of the plurality of locations and determining the number of locations containing a bead, further determining the number of said locations containing a bead and an analyte molecule or particle, and determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the ratio of the number of locations containing a bead and an analyte molecule and particle, to the number of locations containing a bead but not containing any analyte molecules or particles.

In some embodiments, a method for determining a measure of the concentration of analyte molecules or particles in a fluid sample comprises providing a plurality of capture objects that each are associated with either a single analyte molecule or particle or are free of any analyte molecules or particles, individually addressing at least a portion of the capture objects and determining the number of said capture objects associated with an analyte molecule or particle, and determining a measure of the concentration of analyte molecules or particles in the fluid sample based at least in part on the number of

capture objects subjected to the addressing step determined to be associated with an analyte molecule or particle.

Brief Description of the Drawings

Other aspects, embodiments, and features of the invention will become apparent from the following detailed description when considered in conjunction with the accompanying drawings. The accompanying figures are schematic and are not intended to be drawn to scale. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. All patent applications and patents mentioned in the text are incorporated by reference in their entirety. In case of conflict between the description contained in the present specification and a document incorporated by reference, the present specification, including definitions, will control.

FIG. 1 is a schematic flow diagram depicting one embodiment of steps (A-D) for performing an exemplary method of the present invention;

FIG. 2 is a schematic flow diagram depicting one embodiment of steps (A-D) for performing an exemplary method of the present invention;

FIG. 3 is a schematic diagram depicting one embodiment of a portion of a method of the present invention;

FIG. 4A is a schematic flow diagram depicting one embodiment of steps (A-C) for performing an exemplary method of the present invention;

FIG. 4B is a schematic flow diagram depicting one embodiment of steps (A-D) for performing an exemplary method of the present invention;

FIG. 4C is a schematic diagram depicting one embodiment for performing an exemplary method of the present invention;

FIG. 5 is a schematic flow diagram depicting one embodiment of steps (A-C) for performing an exemplary method of the present invention;

FIG. 6 is a schematic flow diagram depicting an embodiment of a method (steps A-D) for the formation of a plurality of reaction vessels through mating of a substrate and a sealing component and depicting examples of the size (E, F) of a sealing component relative to a substrate;

FIG. 7 depicts an experimental set-up for detection using light, according to one embodiment of the present invention;

FIG. 8 shows a fiber optic array that has been sealed with a sealing component, according to one embodiment;

FIG. 9A shows a schematic diagram depicting a method of indirectly detecting an analyte molecule associated with a capture object, according to some embodiments;

FIG. 9B shows a schematic diagram depicting a method of indirectly detecting an analyte molecule immobilized with respect to a capture object using a binding ligand, according to some embodiments;

FIGS. 10A and 10B show schematic diagrams depicting some embodiments of steps for crosslinking a binding ligand and an analyte molecule, according to some methods of the present invention;

FIGS. 11A and 11B show non-limiting examples of a system employing an optical detection system of the present invention according to some embodiments;

FIG. 12 is a schematic block diagram showing a system employing a fiber optic assembly with an optical detection system according to an embodiment of the invention;

FIG. 13 shows a graph of a schematic calibration curve that may be used to determine the concentration of analyte molecules or particles in a fluid sample, according to some embodiments of the present invention;

FIG. 14 show plots of the fraction of capture objects determined to be associated with an analyte molecule comprising A) PSA, B) TNF-alpha, or C) DNA, versus the concentration of analyte molecules in a fluid sample, according to an exemplary embodiment;

FIG. 15 shows a plot of the log of the fraction of capture objects determined to be associated with an analyte molecule versus the log of the concentration of analyte molecules in a fluid sample, according to an exemplary embodiment;

FIG. 16 shows a graph of the number of reaction vessels comprising a bead versus the total number of beads provided to the reaction vessels, according to a non-limiting embodiment;

FIGS. 17A-17C show non-limiting images of beads contained in arrays comprising a plurality of reaction vessels;

FIG. 18A shows a non-limiting fluorescence image of an array containing beads,

FIG. 18B shows an enlargement of the image from FIG. 18A;

FIGS. 19A and 19B show graphs of the number of reaction vessels determined to contain an analyte molecule versus the concentration of analyte molecules in a fluid sample, according to certain embodiments;

FIG. 19C shows a graph of the total fluorescence read-out versus the concentration of analyte molecules in a fluid sample, according to an exemplary embodiment;

FIG. 20 shows a plot of the %Poisson Noise against the experimental variance over three measurements from the experimental data shown in FIG. 19B.

FIG. 21 shows a plot of the fraction of capture objects determined to be associated with an analyte molecule versus the concentration of binding ligand provided, at two concentrations of analyte molecules, according to an exemplary embodiment;

FIG. 22 shows a plot of the fraction of capture objects determined to be associated with an analyte molecule versus the concentration of binding ligand per capture object provided, at two concentrations of analyte molecules, according to an exemplary embodiment;

FIG. 23 shows a plot of the fraction of capture objects determined to be associated with an analyte molecule versus the concentration of binding ligand provided, at two concentrations of analyte molecules, according to an exemplary embodiment;

FIG. 24 shows a plot of the total chemiluminescence versus the concentration of binding ligand provided, according to an exemplary embodiment;

FIG. 25A and 25B show schematic diagrams depicting one embodiment of steps for performing one method of the present invention;

FIG. 25C shows an image of beads contained in a plurality of reaction vessels, according to an exemplary embodiment;

FIG. 25D shows a fluorescence image of an array comprising a plurality of beads, some of which are associated with an analyte molecule following carrying out a method of the present invention, according to an exemplary embodiment.

FIG. 26 shows a plot of the optical density versus the concentration of TNF-alpha, according to an exemplary embodiment;

FIG. 27 shows a plot of the concentration of PSA determined for a plurality of human subjects;

FIG. 28 shows a histogram of the average fluorescence intensity of reaction vessels in an assay method, according to one embodiment of the present invention;

FIG. 29A shows a fluorescence image taken at a first wavelength of a portion of an array of reaction vessels containing beads;

FIG. 29B is a fluorescence image taken at the first wavelength of a portion of an array of reaction vessels containing beads, wherein the beads are associated with fluorescent entities;

FIG. 29C is a fluorescence image of a portion of the array from FIG. 29B taken at a second wavelength;

FIG. 29D shows a plot of (i) beads and (ii) beads associated with fluorescent entities, at varying concentrations of analyte molecules;

FIGS. 30A and 30B shows plots of dissociation of immunocomplexes over time, according to some embodiments;

FIG. 31 shows plot of average analyte molecules per bead versus concentration of analyte molecules, according to some embodiments.

Detailed Description

Described herein are systems and methods that may in certain embodiments be employed for the detection and/or quantification of analyte molecules, particles (such as, for example, cells, cell organelles and other biological or non-biological particulates), and the like, in a sample. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles. It should be understood, that while much of the discussion below is directed to analyte molecules, this is by way of example only, and other materials may be detected and/or quantified, for example, analytes in particulate form. Some exemplary analyte molecules and particles are described herein.

The systems and methods of the present invention in certain instances may help reduce the negative effects of non-specific binding on detection sensitivity when compared to typical conventional systems and methods for performing similar assays. Non-specific binding is the binding or association in a non-specific fashion of one component of an assay with another component of the assay with which it is not desirable that it interact. For example, association, binding, or immobilization of a binding ligand with a substrate or assay material as opposed to with an analyte molecule or particle to which it has binding specificity. Non-specific binding may lead to false positive signals. Non-specific binding may not only affect the accuracy of the assay measurement, but may also limit the lowest level of detection. Therefore, certain methods and/or systems of the present invention that provide improvements in the level

of non-specific binding, may allow for the detection and/or quantification of analyte molecules in a sample at a lower detection limit as compared to typical conventional technologies. In addition, certain embodiments of the methods and/or systems of the present invention may also allow for the detection and/or quantification of analyte molecules in certain samples in which such analyte molecules have previously been undetected and/or unquantifiable because of the very low concentration in which they are present.

Certain methods of the present invention may be useful for characterizing analyte molecules in a sample. In some cases, the methods may be useful for detecting and/or quantifying analyte molecules in a fluid sample which is suspected of containing at least one type of analyte molecule, since, as explained in more detail below, the inventive assays may be designed such that the number (or equivalently fraction) of interrogated locations (e.g., wells, reaction sites, areas on a surface, etc.) which contain a capture object (e.g., bead, surface, etc. providing a capture surface) comprising an analyte molecule - or, more generally, the number or fraction of interrogated capture objects of a total interrogated population comprising an analyte molecule - can be correlated to the concentration of analyte molecules in the fluid sample. Certain embodiments of present invention thus can provide a measure of the concentration of analyte molecules in a fluid sample based at least in part on the number or fraction of locations, e.g., on a substrate, which contain a capture object associated with an analyte molecule. In some cases, this number/fraction may be related to the total number of locations comprising a capture object (e.g., with or without an associated analyte molecule or labeling agent) and/or to the total number of locations interrogated. Specific methods and calculations of how to quantify analyte molecules in a fluid sample using embodiments of the invention are discussed more below.

In certain embodiments, a method for detection and/or quantifying analyte molecules (or particles) in a sample comprises immobilizing a plurality of analyte molecules with respect to a plurality of capture objects that each include a binding surface having affinity for at least one type of analyte molecule (or particle). For example, the capture objects may comprise a plurality of beads comprising a plurality of capture components (e.g., an antibody having specific affinity for an analyte molecule of interest, etc.). At least some of the capture objects (e.g., at least some associated with at least one analyte molecule) may be spatially separated/segregated into a plurality of locations, and at least some of the locations may be addressed/interrogated. A measure