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UTILITY PATENT APPLICATION TRANSMITTAL

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

Attorney Docket No.	Q0052.70018US03
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
Priority Mail Express® 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*] *(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. [ ] 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*) [ ] **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. [ ] **Other:**

***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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7400515.1

Electronic Acknowledgement Receipt

EFS ID:	36691479
Application Number:	16522237
International Application Number:	
Confirmation Number:	4736
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:	Michael Pomianek/Ellen Paul
Filer Authorized By:	Michael Pomianek
Attorney Docket Number:	Q0052.70018US03
Receipt Date:	25-JUL-2019
Filing Date:	
Time Stamp:	16:43:15
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	Q005270018US03-ADS-MJP.pdf	32300 a94f80d0d3a7e4180e45ea72e96c7a65dc9073172	no	7
Warnings:					

Information:					
This is not an USPTO supplied ADS fillable form					
2	Drawings-other than black and white line drawings	Q005270018US03-DRW-MJP.pdf	59766	no	5
Warnings:					
Information:					
3		Q005270018US03-UTLAPL-MJP.pdf	239627	yes	66

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

Warnings:					
Information:					
4	Transmittal of New Application	Q005270018US03-UTLTRN-MJP.pdf	33088	no	1
Warnings:					
Information:					
		Total Files Size (in bytes):	364781		

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.

Application Data Sheet

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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::	Q0052.70018US03
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	1
Total Drawing Sheets::	5

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Small Entity?::	No
Petition included?::	No
Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2::	No
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::	Prior Appl Status::
This Application	Continuation of	15/269142	09/19/16	Pending
15/269142	Continuation of	14/111326	06/24/14	Abandoned
14/111326	a 371 of international	PCT/US2012/033343	04/12/12	
PCT/US2012/033343	Claims benefit of provisional	61/524693	08/17/11	Expired
PCT/US2012/033343	Claims benefit of provisional	61/474315	04/12/11	Expired

Foreign Priority Information

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Assignee Information Including Non-Applicant Assignee Information

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City of mailing address::	Billerica
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Country of mailing address::	US
Postal or Zip Code of mailing address::	01821

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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.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

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)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ 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.

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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	/Michael J. Pomianek/	Date (YYYY-MM-DD)	2019-07-25
Name	Michael J. Pomianek	Registration Number	46,190

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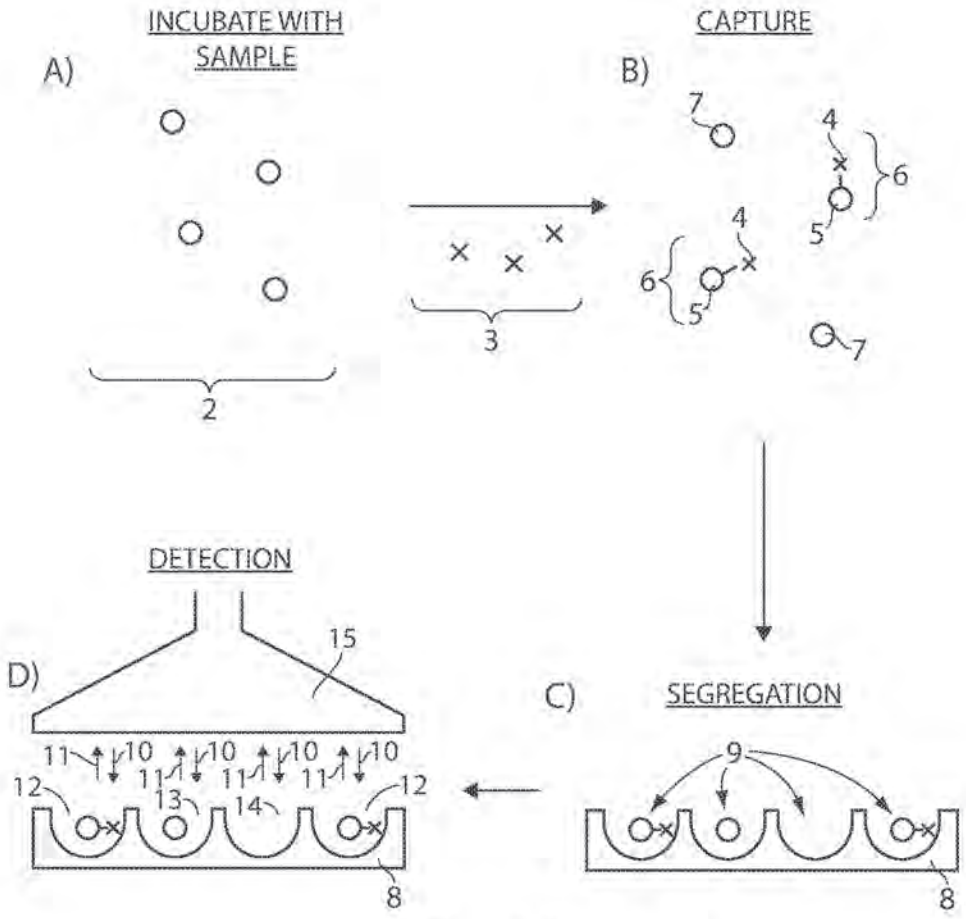

INCUBATE WITH SAMPLE
A)
CAPTURE
B)
2
3
4
5
6
7
SEGREGATION
C)
8
9
DETECTION
D)
10
11
12
13
14
15

Fig. 1A

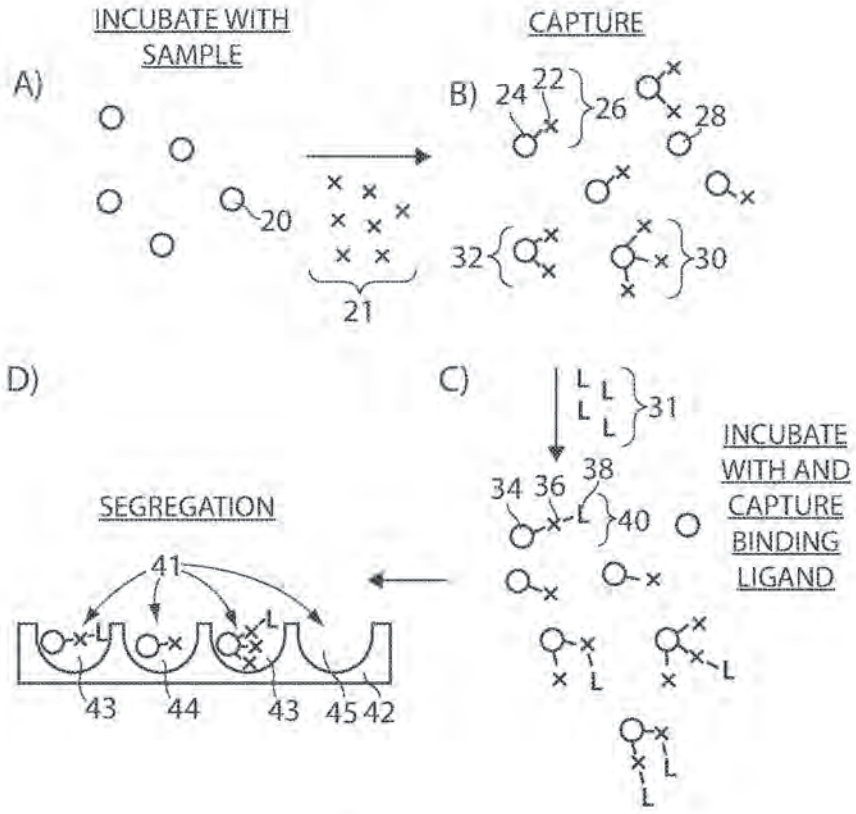

INCUBATE WITH SAMPLE
CAPTURE
A)
B)
20
21
22
24
26
28
30
32
C)
31
INCUBATE WITH AND CAPTURE BINDING LIGAND
34
36
38
40
D)
SEGREGATION
41
43
44
43
45
42

Fig. 1B

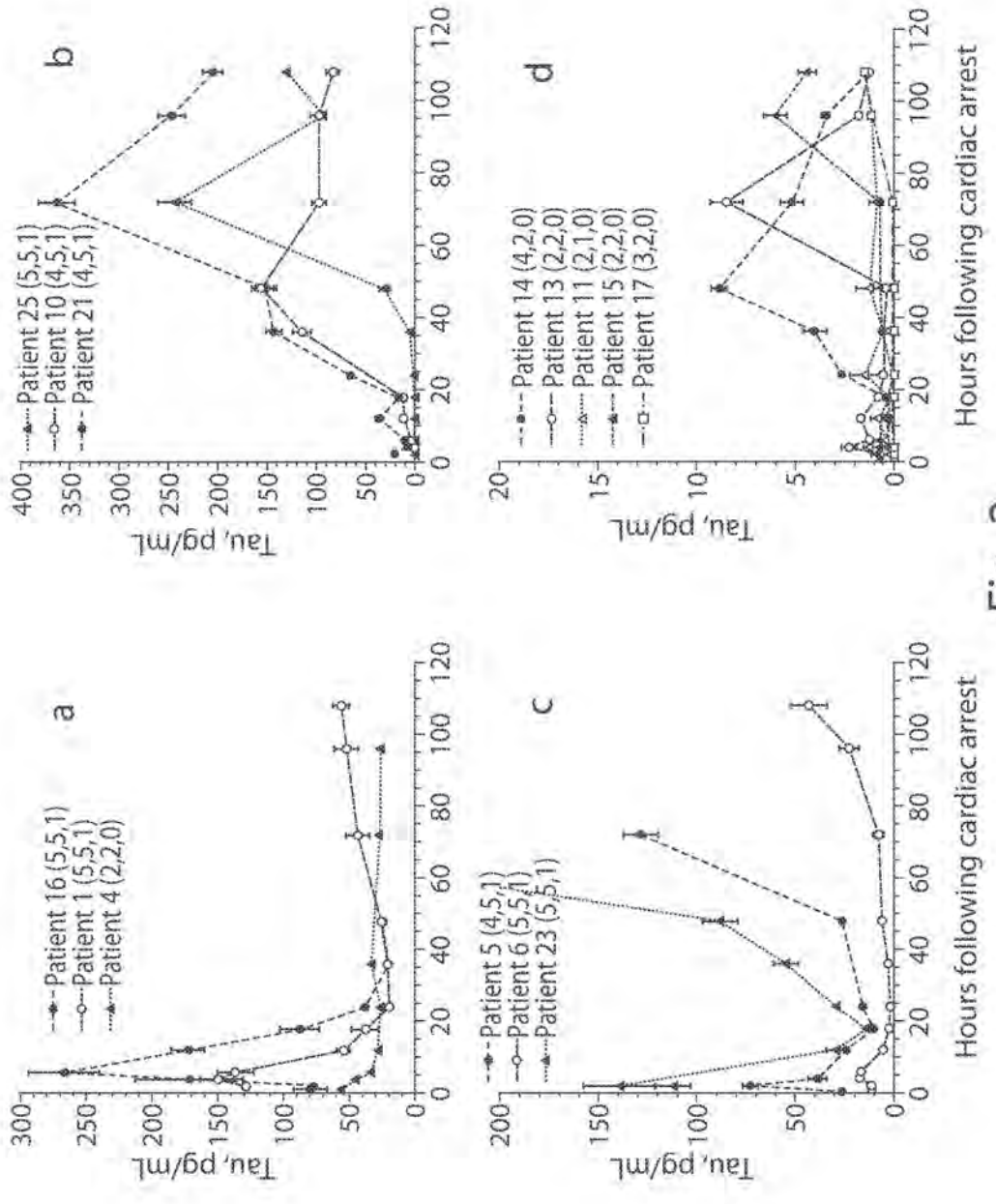

a
Patient 16 (5,5,1)
Patient 1 (5,5,1)
Patient 4 (2,2,0)
Tau, pg/mL
b
Patient 25 (5,5,1)
Patient 10 (4,5,1)
Patient 21 (4,5,1)
Tau, pg/mL
c
Patient 5 (4,5,1)
Patient 6 (5,5,1)
Patient 23 (5,5,1)
Tau, pg/mL
Hours following cardiac arrest
d
Patient 14 (4,2,0)
Patient 13 (2,2,0)
Patient 11 (2,1,0)
Patient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

Fig. 2

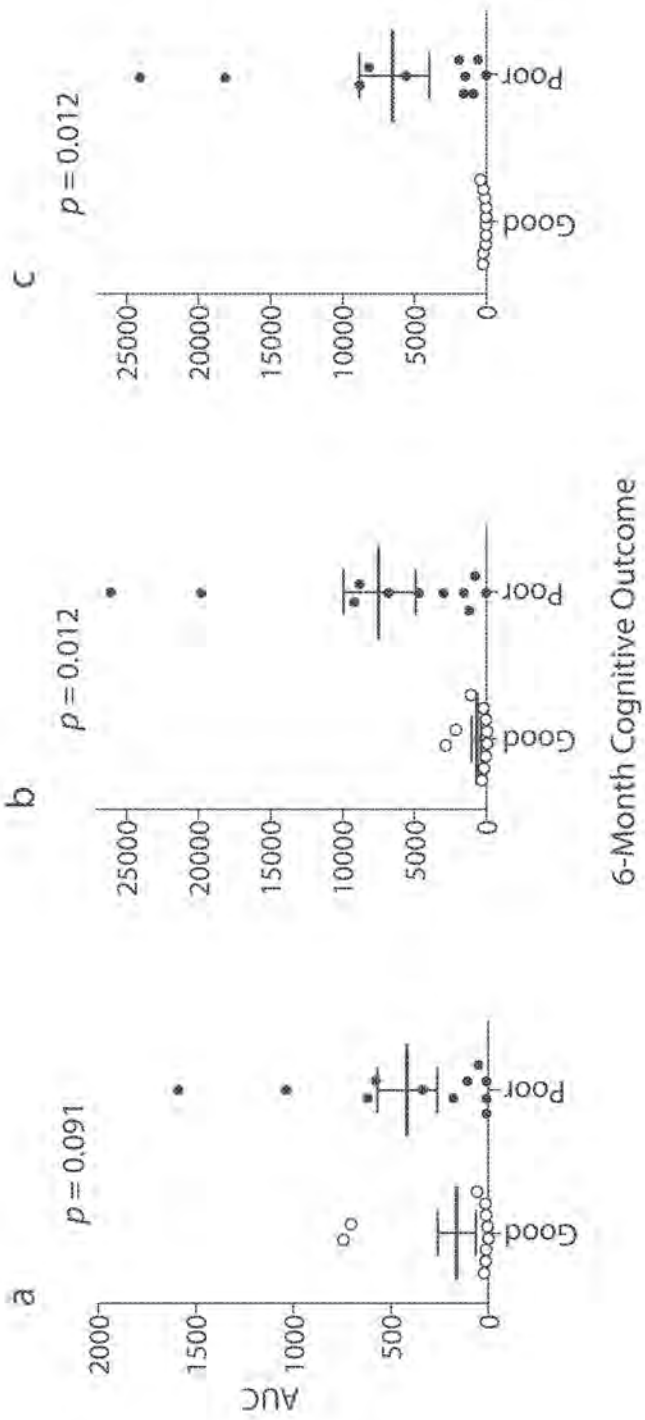


Fig. 3

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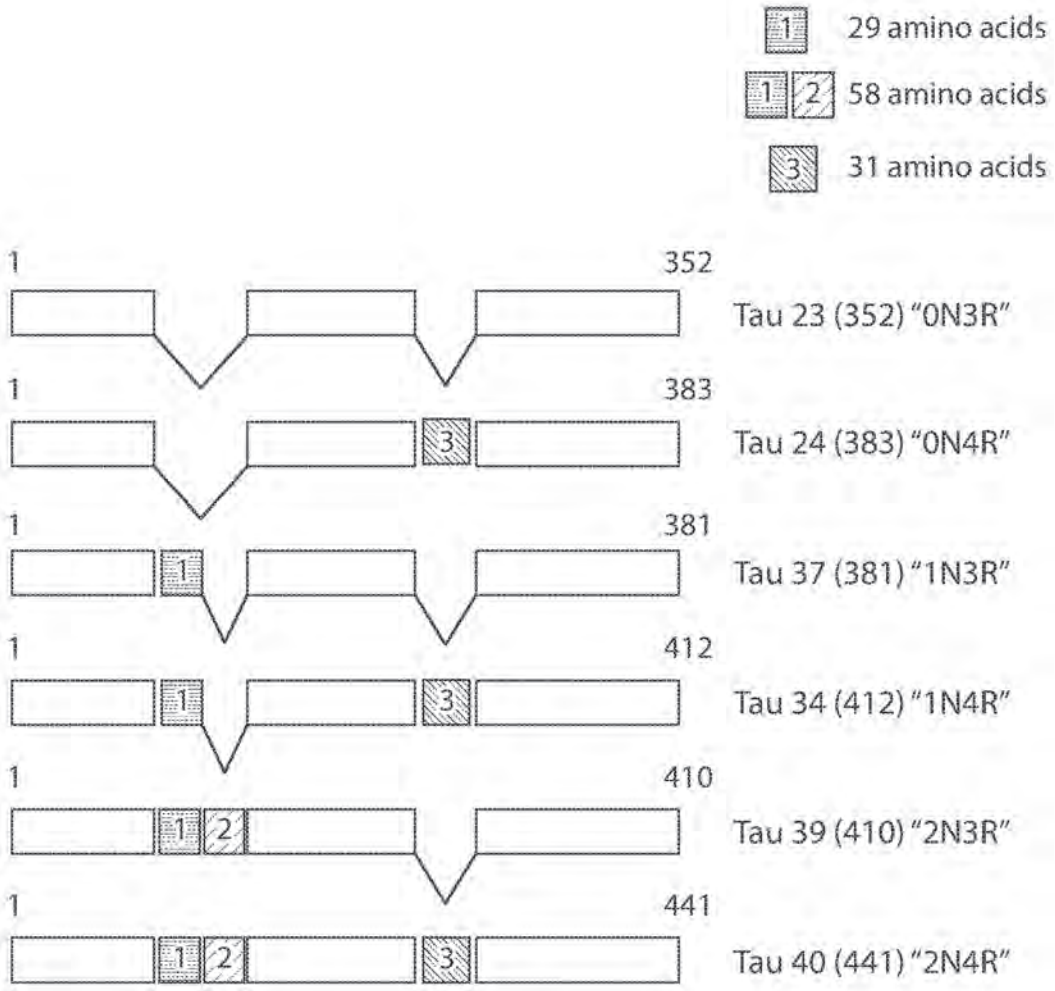

1 29 amino acids
1 2 58 amino acids
3 31 amino acids
1
352
Tau 23 (352) "0N3R"
1
383
3
Tau 24 (383) "0N4R"
1
381
1
Tau 37 (381) "1N3R"
1
412
1
3
Tau 34 (412) "1N4R"
1
410
1 2
Tau 39 (410) "2N3R"
1
441
1 2
3
Tau 40 (441) "2N4R"

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. 15/269,142, filed September 19, 2016, 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 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.

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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 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

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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.

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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

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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

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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.,

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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

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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

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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

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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

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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

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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,

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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.

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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

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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

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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

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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.

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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

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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

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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

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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

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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

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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.,

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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

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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

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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

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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,

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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

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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

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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

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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

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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

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resuscitated with restoration of spontaneous circulation (ROSC). Patients were >18 yrs old, exhibited systolic blood pressure >80mmHg after ROSC, and a Glasgow Coma Scale ≤7.

Upon admission, hypothermia treatment was started immediately after resuscitation. Ventilation was administered during the coma period, with a target PaO_2 of ≥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.

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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).

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Table 2.

Patient	AUC </= 24 hr		AUC all		AUC 2nd peak only	
	Good	Poor	Good	Poor	Good	Poor
2		1590		4759		1440
3	708.9		2145		0	
4	748.3		2861		154.2	
5		625.2		3007		1890
6		179.9		1232		897.5
7	4.72		92.86		54.5	
8		11.19		1646		1545
9	59		1141		0	
10		109.7		9336		8206
11	14.63		84.56		0	
12		52.15		848.2		567.8
13	26.01		288.5		215.8	
14	12.93		431.5		393	
15	12.31		184.4		168.2	
17	0.02		30.68		30.9	
18	15.78		46.62		0	
20		334.6		6827		5511
21		580.8		19875		18051
23		1039		26150		23935
24		12.92		72.9		35.94
25		9.37		8987		8842
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

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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.

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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

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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

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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

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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

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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

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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:

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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.

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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.

35. 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.

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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.

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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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DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 16522237 Document Date: 07/25/2019

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via DAV or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: March 1, 2019

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
16/522,237	07/25/2019	1629	0.00	Q0052.70018US03	91	8

CONFIRMATION NO. 4736

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

FILING RECEIPT

OC000000011022998

Date Mailed: 08/08/2019

Receipt is acknowledged of this non-provisional utility 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 FIRST INVENTOR, 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 corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction 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 provided that the request is grantable.

Inventor(s)

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

Applicant(s)

Quanterix Corporation, Billerica, MA;

Assignment For Published Patent Application

Quanterix Corporation, Billerica, MA

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/269,142 09/19/2016 PAT 10393759
which is a CON of 14/111,326 06/24/2014 ABN
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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Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

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: 08/07/2019

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/522,237**

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 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.

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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).

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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

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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.

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MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

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

Application Number	Filing Date
16522237	

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				
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17		1				
18	1					
19		1				
20		9				
21		(1)				
22		(1)				
23		(1)				
24		(1)				
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26		(1)				
27		(1)				
28		(1)				
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44		(1)				
45		(1)				
46		(1)				
47		(1)				
48		(1)				
49		(1)				
50		(1)				
Total Indep	8		0		0	
Total Depend	134	↵	0	↵	0	↵
Total Claims	142		0		0	

	*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend
51		(1)				
52		(1)				
53		(1)				
54		(1)				
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UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/522,237	07/25/2019	David Wilson	Q0052.70018US03

CONFIRMATION NO. 4736

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

FORMALITIES LETTER

OC000000110229988

Date Mailed: 08/08/2019

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 $ **15320** 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.)

- $ **300** basic filing fee.
- $ **160** surcharge.
- $ **660** search fee.
- $ **760** examination fee.
- $ **2300** for **5** independent claims over 3.
- $ **12200** for **122** total claims over 20.

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- $ **820** for multiple dependent claim surcharge.
- $(**0**) previous unapplied payment amount.
- $ **17200** TOTAL FEE BALANCE DUE.

Items Required To Avoid Processing Delays:

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  David Wilson
  John Henrik Zetterberg
  Kaj Blennow
  Jeffrey D. Randall

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**.

/mhaile/

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/522,237

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	300
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	142 minus 20 =	* 122			OR	x 100 =	12200
INDEPENDENT CLAIMS (37 CFR 1.16(h))	8 minus 3 =	* 5				x 460 =	2300
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))							820
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	17040

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.: Q0052.70018US03
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	16/522,237
Confirmation No.:	4736
Filed:	July 25, 2019
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 C.F.R. § 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 C.F.R. § 1.6(a)(4).

Dated: January 8, 2020 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.

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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
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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	617-301-9821
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

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	16/522,237-Conf. #4736
Filing Date	July 25, 2019
First Named Inventor	David Wilson
Art Unit	1629
Examiner Name	Not Yet Assigned
Attorney Docket Number	Q0052.70018US03

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
- [X] 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):
  -Inventor Declaration

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Michael J. Pomianek/		
Printed name	Michael J. Pomianek		
Date	January 8, 2020	Reg. No.	46,190

Certificate of Electronic Filing under 37 C.F.R. § 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 C.F.R. § 1.6(a)(4).

Dated: January 8, 2020 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

7623479.1

Electronic Patent Application Fee Transmittal

Application Number:	16522237
Filing Date:	25-Jul-2019
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
Filer:	Michael Pomianek/Ellen Paul
Attorney Docket Number:	Q0052.70018US03

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	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
Pages:				
Claims:				
Miscellaneous-Filing:				
LATE FILING FEE FOR OATH OR DECLARATION	1051	1	160	160
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 $0 paid	1253	1	1400	1400
Miscellaneous:				
		Total in USD ($)		**3280**

Electronic Acknowledgement Receipt

EFS ID:	38241742
Application Number:	16522237
International Application Number:	
Confirmation Number:	4736
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:	Michael Pomianek/Ellen Paul
Filer Authorized By:	Michael Pomianek
Attorney Docket Number:	Q0052.70018US03
Receipt Date:	08-JAN-2020
Filing Date:	25-JUL-2019
Time Stamp:	17:00:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$3280
RAM confirmation Number	E202018H01080341
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Extension of Time	Q005270018US03-EXT-MJP.pdf	24813 d81b3191fc556ea0208f8047c54f0d0d9e9f75d	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	Q005270018US03-FEE-MJP.pdf	32909 a6fd2467b43e30f4a1635c3794f4321f4f5 91da	no	1
Warnings:					
Information:					
3	Oath or Declaration filed	Q005270018US03-INVDEC-MJP.pdf	159176 31708083f6da2c5c10fe52d2c34b3dc702a3a2c95	no	2
Warnings:					
Information:					
4	Applicant Response to Pre-Exam Formalities Notice	Q005270018US03-NTMP-MJP.pdf	25752 649ae356e1a8c60d729bfff1568feaaa68ce0f02	no	2
Warnings:					
Information:					
5		Q005270018US03-PAMN-MJP.pdf	35398 3d43dc0c6666ac7277d5dee90d6a6534a3ae6c079	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	Power of Attorney	Q005270018US03-POA-MJP.pdf	102860 b2fbe93649bee24b305a68e6181d2b10f9...9ab11	no	1
Warnings:					
Information:					
7	Miscellaneous Incoming Letter	Q005270018US03-TRN-MJP.pdf	26454 8b5b3647742b0d4255c3b1076ffbaff91e2f23...469d	no	1
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	39671 7506e2aebb71c02e7b5da302ae51867e8b...3e3992	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	447033		

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.

PTO/AIA/22 (03-13)
Approved for use through 11/30/2020. 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) Q0052.70018US03
Application Number 16/522,237-Conf. #4736	Filed July 25, 2019
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 46,190 .

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

/Michael J. Pomianek/ — Signature
January 8, 2020 — Date

Michael J. Pomianek — Typed or printed name
617.646.8000 — 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 C.F.R. § 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 C.F.R. § 1.6(a)(4).

Dated: January 8, 2020 — Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

7839072.1

FEE TRANSMITTAL

Complete if known	
Application Number	16/522,237-Conf. #4736
Filing Date	July 25, 2019
First Named Inventor	David Wilson
Examiner Name	Not Yet Assigned
Art Unit	1629
Practitioner Docket No.	Q0052.70018US03

[ ] 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.

TOTAL AMOUNT OF PAYMENT ($)3,280.00

METHOD OF PAYMENT (check all that apply)

[ ] Check [X] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[X] 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**

[X] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17, **except for any excess claims fees or multiple dependent claim fee**

[ ] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17

[X] 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	300	150*	75	660	330	165	760	380	190	1,720.00
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 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)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

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

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

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

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(i) 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, ...	160.00

SUBMITTED BY

Signature	/Michael J. Pomianek/	Registration No. (Attorney/Agent)	46,190	Telephone	617.646.8000
Name (Print/Type)	Michael J. Pomianek			Date	January 8, 2020

Certificate of Electronic Filing under 37 C.F.R. § 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 C.F.R. § 1.6(a)(4).

Dated: January 8, 2020 Electronic Signature for Ellen J. Paul: /Ellen J. Paul/

7839071.1

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.

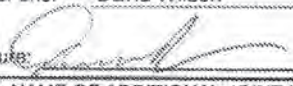
I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. § 1.56.

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: 6/2/14

Signature:  Citizen of: US

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor two: John Henrik Zetterberg Date:

Signature: Citizen of: Sweden

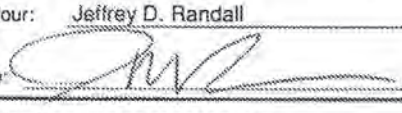
LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

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:  Citizen of: US

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

3566004.1

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000102

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: 01/02/2014

Signature: _____ Citizen of: Sweden

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor three: Kaj Blennow Date: 24/02/2014

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.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000103

Docket No.: Q0052.70018US03
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	16/522,237
Confirmation No.:	4736
Filed:	July 25, 2019
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 C.F.R. § 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 C.F.R. § 1.6(a)(4).

Dated: January 8, 2020 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 August 8, 2019, Applicant respectfully submits a Declaration, a Power of Attorney, a Preliminary Amendment, a Petition for Extension of Time, and the Filing Fee for the Application (as shown on accompanying Fee Transmittal).

Please charge our Credit Card in the amount of $3,280.00 covering the fees set forth in 37 C.F.R. § 1.16(f), 1.16(a), 1.16(k), 1.16(o) and 1.17(a). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed with this Response to Notice to File Missing Parts of Application to our Deposit Account No. 23/2825, under Docket No. Q0052.70018US03.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000104

Dated: January 8, 2020

Respectfully submitted,

Electronic signature: /Michael J. Pomianek/
Michael J. Pomianek
Registration No.: 46,190
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000105

REMARKS

Claims 1-91 were pending in the application. Claims 1-10, 12-19 and 25-91 have been cancelled. Claims 11 and 20-24 have been amended. Claims 92-103 are newly added. 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 11, 20-24 and 92-103 are now pending in this application with claim 92 being the sole independent claim.

In view of the above amendment, applicant believes the pending application is in condition for substantive examination.

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, except for excess claims fees, to our Deposit Account No. 23/2825, under Docket No. Q0052.70018US03.

Dated: January 8, 2020

Respectfully submitted,

Electronic signature: /Michael J. Pomianek/
Michael J. Pomianek
Registration No.: 46,190
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000106

AMENDMENTS TO THE CLAIMS

1. - 10. (Canceled)

11. (Currently Amended) The method of claim 92[[10]], wherein the brain injury results from a hypoxic event.

12. - 19. (Canceled)

20. (Currently Amended) The method of ~~any one of claims 12-19~~claim 92, wherein the limit of detection for the analytical protein concentration measurement 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 92, 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 92, wherein the brain injury comprises a hypoxic event caused by stroke.

23. (Currently Amended) The method of ~~any one of claims 12-20~~claim 92, 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 92, wherein the brain injury comprises a hypoxic event caused by a thrombosis.

25. - 91. (Cancelled)

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000107

92. (New) A method of producing a body fluid sample of a patient recovering from a brain injury containing an analytically quantified amount of endogenous tau protein, said method comprising:

(A) obtaining a volume of body fluid comprising blood, or a blood component selected from plasma and serum,

(B) diluting the volume of body fluid, and

(C) quantifying through the use of an analytical protein concentration measurement assay a concentration of tau protein in the volume of body fluid to produce the body fluid sample containing the analytically quantified amount of endogenous tau protein;

wherein the analytically quantified concentration of endogenous tau protein contained in the body fluid sample is less than about 5 pg/ml, and a limit of quantification of the analytical protein concentration measurement assay used in step (C) for quantifying the concentration of tau protein is less than about 0.2 pg/mL.

93. (New) The method of claim 92, wherein the analytical protein concentration measurement assay used in step (C) comprises:

i. exposing a plurality of capture objects that each include a binding surface having affinity for tau protein molecules to the volume of body fluid;

ii. 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;

iii. determining a proportion of the plurality of capture objects that associated with a tau protein molecule in step ii; and

iv. determining a measure of tau protein molecule concentration in the volume of body fluid based at least in part on the proportion determined in step iii.

94. (New) The method of claim 93, wherein the plurality of capture objects comprises a plurality of beads.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000108

95. (New) The method of claim 92, wherein the analytical protein concentration measurement assay used in step (C) comprises spatially segregated tau protein molecules 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

96. (New) The method of claim 95, wherein the plurality of locations comprises a plurality of reaction vessels.

97. (New) The method of claim 92, wherein each of the tau protein molecules quantified in step (C) 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).

98. (New) The method of claim 97, wherein at least a portion of the tau protein molecules are phosphorylated.

99. (New) The method of claim 92, wherein the volume of bodily fluid comprises blood.

100. (New) The method of claim 92, wherein the volume of bodily fluid comprises plasma.

101. (New) The method of claim 92, wherein the volume of bodily fluid comprises serum.

102. (New) The method of claim 92, wherein the volume of bodily fluid does not comprise cerebrospinal fluid (CSF).

103. (New) The method of claim 92, wherein the has been collected from the patient within 12 hours of the brain injury.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: July 25, 2019	CONFIRMATION NO.: 4736
	FIRST NAMED INVENTOR: David Wilson	
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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
	***	4,631,211		Houghten	12-23-1986
	***	4,780,421		Kameda et al.	10-25-1988
	***	4,883,642		Bisconte	11-28-1989
	***	4,907,037		Boisde et al.	03-06-1990
	***	4,924,870		Wlodarczyk et al.	05-15-1990
	***	4,962,037		Jett et al.	10-09-1990
	***	5,026,159		Allen et al.	06-25-1991
	***	5,028,535		Buechler et al.	07-02-1991
	***	5,089,391		Buechler et al.	02-18-1992
	***	5,091,300		Hurni et al.	02-25-1992
	***	5,108,961		Zhong et al.	04-28-1992
	***	5,152,816		Berkey	10-06-1992
	***	5,190,857		Allen et al.	03-02-1993
	***	5,196,306		Bobrow et al.	03-23-1993
	***	5,213,962		Van Nostrand et al.	05-05-1993
	***	5,234,814		Card et al.	08-10-1993
	***	5,244,636		Walt et al.	09-14-1993
	***	5,244,813		Walt et al.	09-14-1993
	***	5,250,264		Walt et al.	10-05-1993
	***	5,252,494		Walt	10-12-1993
	***	5,262,332		Selkoe et al.	11-16-1993
	***	5,270,165		Van Nostrand et al.	12-14-1993

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	FIRST NAMED INVENTOR: David Wilson	
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	***	5,298,741		Walt et al.	03-29-1994
	***	5,315,375		Allen	05-24-1994
	***	5,320,814		Walt et al.	06-14-1994
	***	5,329,461		Allen et al.	07-12-1994
	***	5,374,395		Robinson et al.	12-20-1994
	***	5,405,783		Pirrung et al.	04-11-1995
	***	5,441,870		Seubert et al.	08-15-1995
	***	5,445,934		Fodor et al.	08-29-1995
	***	5,468,846		Ichikawa et al.	11-21-1995
	***	5,474,990		Olney	12-12-1995
	***	5,488,567		Allen et al.	01-30-1996
	***	5,492,812		Voorheis et al.	02-20-1996
	***	5,512,490		Walt et al.	04-30-1996
	***	5,532,138		Singh et al.	07-02-1996
	***	5,532,379		Fujimoto	07-02-1996
	***	5,545,531		Rava et al.	08-13-1996
	***	5,583,001		Bobrow et al.	12-10-1996
	***	5,593,846		Schenk et al.	01-14-1997
	***	5,620,850		Bamdad et al.	04-15-1997
	***	5,633,972		Walt et al.	05-27-1997
	***	5,690,894		Pinkel et al.	11-25-1997
	***	5,700,637		Southern	12-23-1997
	***	5,705,401		Masters et al.	01-06-1998
	***	5,731,158		Bobrow et al.	03-24-1998
	***	5,770,455		Cargill et al.	06-23-1998
	***	5,807,522		Brown et al.	09-15-1998
	***	5,814,524		Walt et al.	09-29-1998
	***	5,869,266		Wolozin et al.	02-09-1999
	***	5,885,529		Babson et al.	03-23-1999

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	FIRST NAMED INVENTOR: David Wilson	
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	***	5,900,481		Lough et al.	05-04-1999
	***	5,976,817		Davies-Heerema et al.	11-02-1999
	***	5,981,180		Chandler et al.	11-09-1999
	***	5,981,208		Tamburini et al.	11-09-1999
	***	6,001,564		Bergeron et al.	12-14-1999
	***	6,007,690		Nelson et al.	12-28-1999
	***	6,013,445		Albrecht et al.	01-11-2000
	***	6,023,540		Walt et al.	02-08-2000
	***	6,133,436		Koster et al.	10-17-2000
	***	6,143,496		Brown et al.	11-07-2000
	***	6,156,270		Buechler	12-05-2000
	***	6,174,695	B1	Hammock et al.	01-16-2001
	***	6,210,910	B1	Walt et al.	04-03-2001
	***	6,266,459	B1	Walt et al.	07-24-2001
	***	6,285,807	B1	Walt et al.	09-04-2001
	***	6,327,410	B1	Walt et al.	12-04-2001
	***	6,329,139	B1	Nova et al.	12-11-2001
	***	6,368,874	B1	Gallop et al.	04-09-2002
	***	6,377,721	B1	Walt et al.	04-23-2002
	***	6,388,746	B1	Eriksson et al.	05-14-2002
	***	6,396,995	B1	Stuelpnagel et al.	05-28-2002
	***	6,406,845	B1	Walt et al.	06-18-2002
	***	6,432,630	B1	Blankenstein	08-13-2002
	***	6,482,593	B2	Walt et al.	11-19-2002
	***	6,495,335		Chojkier et al.	12-17-2002
	***	6,582,908	B2	Fodor et al.	06-24-2003
		6,599,331	B1	Chandler et al.	07-29-2003
	***	6,602,702	B1	McDevitt et al.	08-05-2003
	***	6,620,584	B1	Chee et al.	09-16-2003

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	***	6,635,452	B1	Monforte et al.	10-21-2003
	***	6,667,159	B1	Walt et al.	12-23-2003
	***	6,670,137		Vanmechelen et al.	12-30-2003
	***	6,680,173		Vanmechelen et al.	01-20-2004
	***	6,713,309	B1	Anderson et al.	03-30-2004
	***	6,714,303	B2	Ivarsson	03-30-2004
	***	6,811,988		Chojkier et al.	11-02-2004
	***	6,821,449	B2	Caplen et al.	11-23-2004
	***	6,838,051	B2	Marquiss et al.	01-04-2005
	***	6,858,394	B1	Chee et al.	02-22-2005
	***	6,859,570	B2	Walt et al.	02-22-2005
	***	6,878,345	B1	Astle	04-12-2005
	***	6,929,924	B2	Bouanani et al.	08-16-2005
	***	6,942,968	B1	Dickinson et al.	09-13-2005
	***	6,943,034	B1	Winkler et al.	09-13-2005
	***	6,991,939	B2	Walt et al.	01-31-2006
	***	6,999,657	B2	Walt	02-14-2006
	***	7,056,746	B2	Seul et al.	06-06-2006
	***	7,060,431	B2	Chee et al.	06-13-2006
	***	7,107,092		Goldstein et al.	09-12-2006
	***	7,115,884	B1	Walt et al.	10-03-2006
	***	7,211,390	B2	Rothberg et al.	05-01-2007
	***	7,244,559	B2	Rothberg et al.	07-17-2007
	***	7,250,267	B2	Walt et al.	07-31-2007
	***	7,264,929	B2	Rothberg et al.	09-04-2007
	***	7,348,181	B2	Walt et al.	03-25-2008
	***	7,442,516		Ohno et al.	10-28-2008
	***	7,480,433	B2	Walt et al.	01-20-2009
	***	7,572,581	B2	Gelfand et al.	08-11-2009

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	FIRST NAMED INVENTOR: David Wilson	
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	***	7,651,841	B2	Song et al.	01-26-2010
	***	7,655,424	B2	Jackowski	02-02-2010
	***	7,749,700	B2	Baird et al.	07-06-2010
	***	7,759,062	B2	Allawi et al.	07-20-2010
	***	7,776,553	B2	Love et al.	08-17-2010
	***	7,838,250	B1	Goix et al.	11-23-2010
	***	8,222,047	B2	Duffy et al.	07-17-2012
	***	8,236,574	B2	Duffy et al.	08-07-2012
	***	8,415,171	B2	Rissin et al.	04-09-2013
	***	8,460,878	B2	Walt et al.	06-11-2013
	***	8,460,879	B2	Walt et al.	06-11-2013
	***	8,492,098	B2	Walt et al.	07-23-2013
	***	8,846,415	B2	Duffy et al.	09-30-2014
	***	9,110,025	B2	Rissin et al.	08-18-2015
	***	9,310,360	B2	Duffy et al.	04-12-2016
	***	9,395,359	B2	Walt et al.	07-19-2016
	***	9,482,622	B2	Sato et al.	11-01-2016
	***	9,482,662	B2	Duffy et al.	11-01-2016
	***	9,551,663	B2	Rissin et al.	01-24-2017
	***	9,678,068	B2	Duffy et al.	06-13-2017
	***	9,809,838		Walt et al.	11-07-2017
	***	9,846,155	B2	Rissin et al.	12-19-2017
	***	9,932,626	B2	Duffy et al.	04-03-2018
	***	9,952,237	B2	Fournier et al.	04-24-2018
		10,261,089	B2	Walt et al.	04-16-2019
		10,393,759	B2	Wilson et al.	08-27-2019
	***	2001-0018191	A1	Merken et al.	08-30-2001
	***	2002-0001857	A1	Vandermeeren et al.	01-03-2002
	***	2002-0009391	A1	Marquiss et al.	01-24-2002

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FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 16/522,237	ATTY. DOCKET NO.: Q0052.70018US03
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	***	2002-0019016	A1	Vanmechelen et al.	02-14-2002
	***	2002-0086009	A1	Ishiguro et al.	07-04-2002
	***	2002-0090650	A1	Empedocles et al.	07-11-2002
	***	2002-0122612	A1	Walt et al.	09-05-2002
	***	2002-0137112	A1	Chojkier et al.	09-26-2002
	***	2002-0157122	A1	Wong et al.	10-24-2002
	***	2002-0188106	A1	Mandelkow et al.	12-12-2002
	***	2003-0027126	A1	Walt et al.	02-06-2003
	***	2003-0091475	A1	Yu et al.	05-15-2003
	***	2003-0096227	A1	Shinitzky et al.	05-22-2003
	***	2003-0096243	A1	Busa	05-22-2003
	***	2003-0104361	A1	Weininger et al.	06-05-2003
	***	2003-0104488	A1	Chojkier et al.	06-05-2003
	***	2003-0138972	A1	Vandermeeren et al.	07-24-2003
	***	2003-0143580	A1	Straus et al.	07-31-2003
	***	2003-0143760	A1	Vandermeeren et al.	07-31-2003
	***	2003-0165575	A1	Iqbal. et al.	09-04-2003
	***	2003-0194742	A1	Vanmechelen et al.	10-16-2003
	***	2003-0198573	A1	Forood et al.	10-23-2003
	***	2004-0013647	A1	Solomon et al.	01-22-2004
	***	2004-0014142	A1	Vanmechelen et al.	01-22-2004
	***	2004-0018561	A1	Decrescenzo et al.	01-29-2004
	***	2004-0038426	A1	Manalis	02-26-2004
	***	2004-0038430	A1	Vandermeeren et al.	02-26-2004
	***	2004-0043502	A1	Song et al.	03-04-2004
	***	2004-0053322	A1	McDevitt et al.	03-18-2004
	***	2004-0071599	A1	Rusch et al.	04-15-2004
	***	2004-0072278	A1	Chou et al.	04-15-2004
	***	2004-0086426	A1	Vann et al.	05-06-2004

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	***	2004-0101918	A1	Cauci	05-27-2004
	***	2004-0132680	A1	Wong et al.	07-08-2004
	***	2004-0142386	A1	Rigler et al.	07-22-2004
	***	2004-0166536	A1	Kerkman et al.	08-26-2004
	***	2004-0219509	A1	Valkirs et al.	11-04-2004
	***	2004-0248325	A1	Bukusoglu	12-09-2004
	***	2004-0253624	A1	Smith et al.	12-16-2004
	***	2004-0253643	A1	Seubert et al.	12-16-2004
	***	2004-0259237	A1	Kellogg et al.	12-23-2004
	***	2005-0042221	A1	Chojkier et al.	02-24-2005
	***	2005-0053996	A1	Tong	03-10-2005
	***	2005-0069968	A1	Seubert et al.	03-31-2005
	***	2005-0112634	A1	Woudenberg et al.	05-26-2005
	***	2005-0112655	A1	Banerjee et al.	05-26-2005
	***	2005-0124016	A1	LaDu et al.	06-09-2005
	***	2005-0129581	A1	McBride et al.	06-16-2005
	***	2005-0130173	A1	Leamon et al.	06-16-2005
	***	2005-0130188	A1	Walt et al.	06-16-2005
	***	2005-0131650	A1	Andersson et al.	06-16-2005
	***	2005-0164289	A1	Quate et al.	07-28-2005
	***	2005-0169925	A1	Bardroff et al.	08-04-2005
	***	2005-0175626	A1	Delacourte et al.	08-11-2005
	***	2005-0181460	A1	Ohno et al.	08-15-2005
	***	2005-0221281	A1	Ho	10-06-2005
	***	2005-0226780	A1	Sandell et al.	10-13-2005
	***	2005-0244308	A1	Tanaami et al.	11-03-2005
	***	2005-0244890	A1	Davies et al.	11-03-2005
	***	2005-0266433	A1	Kapur et al.	12-01-2005
	***	2006-0006067	A1	Unger	01-12-2006

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	***	2006-0013543	A1	Walt et al.	01-19-2006
	***	2006-0024759	A1	Holtzman et al.	02-02-2006
	***	2006-0040297	A1	Leamon et al.	02-23-2006
	***	2006-0068409	A1	Phan et al.	03-30-2006
	***	2006-0078998	A1	Puskas et al.	04-13-2006
	***	2006-0084183	A1	Henricksen	04-20-2006
	***	2006-0139635	A1	Kersey et al.	06-29-2006
	***	2006-0205024	A1	Rogers et al.	09-14-2006
	***	2007-0040095	A1	Walt et al.	02-22-2007
	***	2007-0059754	A1	Kordunsky et al.	03-15-2007
	***	2007-0074972	A1	Nassef et al.	04-05-2007
	***	2007-0116607	A1	Wang et al.	05-24-2007
	***	2007-0154480	A1	Schenk et al.	07-05-2007
	***	2007-0259381	A1	Rissin et al.	11-08-2007
	***	2007-0259385	A1	Rissin et al.	11-08-2007
	***	2007-0259448	A1	Rissin et al.	11-08-2007
	***	2008-0032324	A1	Walt et al.	02-07-2008
	***	2008-0038761	A1	Beernink et al.	02-14-2008
	***	2008-0064113	A1	Goix et al.	03-13-2008
	***	2008-0118924	A1	Buechler	05-22-2008
	***	2008-0131422	A1	Sugimura, et al.	06-05-2008
	***	2008-0131907	A1	Wang et al.	06-05-2008
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				FIRST NAMED INVENTOR: David Wilson	
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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/522,237	ATTY. DOCKET NO.: Q0052.70018US03
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 25, 2019	CONFIRMATION NO.: 4736
				FIRST NAMED INVENTOR: David Wilson	
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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/522,237	ATTY. DOCKET NO.: Q0052.70018US03
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				FIRST NAMED INVENTOR: David Wilson	
Sheet	24	of	28	GROUP ART UNIT: 1649	EXAMINER: Not Yet Assigned

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000137

Electronic Acknowledgement Receipt

EFS ID:	38283196
Application Number:	16522237
International Application Number:	
Confirmation Number:	4736
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:	Michael Pomianek/Nathaniel Taylor
Filer Authorized By:	Michael Pomianek
Attorney Docket Number:	Q0052.70018US03
Receipt Date:	13-JAN-2020
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Application Type:	Utility under 35 USC 111(a)

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	WO2018222585A2.pdf	2415297	no	54
Warnings:					

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2	Foreign Reference	WO2019060607A1.pdf	4074158 d0eaa2a855cf9962f52d3d809456adbd5f7064b1	no	78
Warnings:					
Information:					
3	Other Reference-Patent/App/Search documents	Q005270017US01_OA_mailed_12-12-2019_w_pending_claims.pdf	730352 6f15df04ce9dfd5e0af9000344066d4f2d2e7f7c	no	25
Warnings:					
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(54) Title: SINGLE MOLECULE DETECTION AND QUANTIFICATION OF NUCLEIC ACIDS WITH SINGLE BASE SPECIFICITY

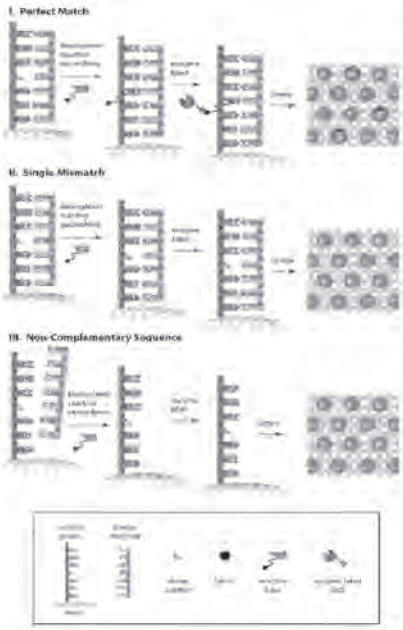


Fig. 1

(57) Abstract: Methods for detecting nucleic acids, particularly small nucleic acids such as microRNAs, involving the use of a peptide nucleic acid (PNA) probe that lacks a base and a labelled modified base corresponding to the omitted base in the PNA probe. A complex containing the target nucleic acid, the PNA probe, and the modified base can be determined using a single molecule array assay.

[Continued on next page]

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Single Molecule Detection and Quantification of Nucleic Acids with Single Base Specificity

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/512,450, filed May 30, 2017, the contents of which are incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

The sensitive detection of specific sequences of nucleic acids (NA) has become an indispensable tool in biological research, drug development and the diagnosis and treatment of diseases. The field of molecular diagnostics - where detection of specific sequences enables diagnosis of cancer, infectious diseases, and hereditary diseases, as well as companion diagnostics for drugs – has emerged from these technologies. Further uses can be imagined in the fields of animal health and plant breeding. Khodakov et al. Adv. Drug Deliv. Rev., 2016, 105, 3-19; and Smith et al., J. Am. Chem. Soc., 2017, 139, 1020–1028. In recent years, the use and measurement of short sequences (<100 bases) of RNA, in particular, has proven useful for greater understanding and control of biological systems. Poliseno et al., Nature, 2010, 465, 1033-1038; and Tay et al., Nature, 2014, 505, 344-352. For example, circulating microRNAs(miRNAs) (~22 bases) regulate gene expression have been described as valuable biomarkers for diagnosing cancer and liver diseases. Moreover, interfering RNA has been used as a therapeutic via gene silencing; and, RNA probes are the basis of gene editing techniques, such as CRISPR/Cas9.

The polymerase chain reaction (PCR) and next generation sequencing (NGS) technologies dominate the detection of NA. Khodakov et al., 2016. These methods have limitations in detecting NA, in particular short strands of RNA. Detection of short sequences is particularly challenging for assays that require multiple binding molecules, *e.g.*, primers of PCR or sandwich binding assays. This requirement often necessitates the elongation of the target molecule using either a ligation step with an extension sequence so that the probes can bind, or

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use of poly(A) polymerase to poly-adenylated target miRNAs, so they can be converted into cDNA. PCR also suffers from bias, sample contamination from production of high concentration of amplicon, and requires laborious sample preparation. Techniques that address the challenges of existing assays, especially for short sequences of RNA, but maintain the high analytical sensitivity and high sequence specificity of PCR are needed, if microRNAs are to fulfill their promise as clinically valuable biomarkers in drug development and detection of cancers and disease.

It is hence of great interest to develop sensitive and specific assay methods for detecting and quantifying short nucleic acid molecules, such as microRNAs.

SUMMARY OF THE INVENTION

The present disclosure is based on the development of an efficient and specific method for detecting nucleic acids having specific sequences such as microRNAs based on the hybridization and labeling of single nucleic acid molecules using a single immobilized probe, without the use of amplification by polymerases. This probe allows the capture of specific target sequences and discrimination of these sequences with single base specificity. This method has been successfully applied to directly detect microRNA miR122 in human serum without the use of polymerase amplification. MiR122 is a regulatory supported biomarker of liver toxicity, in serum.

Accordingly, one aspect of the present disclosure provides a method for detecting a nucleic acid of interest in a sample, comprising: (i) providing a sample suspected of containing a nucleic acid of interest; (ii) providing a plurality of beads, on which a peptide nucleic acid (PNA) probe is immobilized, wherein the PNA probe comprises a nucleotide sequence that is complementary to the nucleic acid of interest or a portion thereof, and wherein the PNA probe lacks a base at a position corresponding to a target nucleotide in the nucleic acid of interest; (iii) incubating the sample with the plurality of beads under conditions allowing for formation of a nucleic acid/PNA complex; (iv) contacting the plurality of beads with a modified base that is complementary to the base of the target nucleotide in the nucleic acid of interest, wherein the

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modified base is conjugated to a first binding moiety and modified with a chemical group capable of reversible covalent reactions that reacts with the PNA probe; (v) contacting the plurality of beads with a second binding moiety that binds the first binding moiety and comprises a detectable label; and (vi) determining the fraction of beads associated with at least one detectable label, which is indicative of the concentration of the nucleic acid of interest in the sample. In some examples, the first binding moiety comprises biotin and the second binding moiety comprises streptavidin. In some examples, the PNA probe has a backbone with glutamic side chains. In some examples, steps (iii) and step (iv) may be performed simultaneously.

In another aspect, the present disclosure provides a method for detecting a nucleic acid of interest in a sample, comprising: (i) providing a sample suspected of containing a nucleic acid of interest; (ii) providing a plurality of beads, on which a peptide nucleic acid (PNA) probe is immobilized, wherein the PNA probe comprises a nucleotide sequence that is complementary to the nucleic acid of interest or a portion thereof, and wherein the PNA probe lacks a base at a position corresponding to a target nucleotide in the nucleic acid of interest; (iii) incubating the sample with the plurality of beads under conditions allowing for formation of a nucleic acid/PNA complex; (iv) contacting the plurality of beads with a modified base that is complementary to the base of the target nucleotide in the nucleic acid of interest, wherein the modified base is conjugated to a first binding moiety and modified with a chemical group capable of reversible covalent reactions that reacts with the PNA probe; (v) contacting the plurality of beads with a second binding moiety that binds the first binding moiety; (v) contacting the plurality of beads with a third binding moiety that binds the second binding moiety and comprises a detectable label; and (vii) determining the fraction of beads associated with at least one detectable label, which is indicative of the level of the nucleic acid of interest in the sample. In some examples, the first binding moiety can be a fluorescein label. In some examples, the second binding moiety comprises an antibody specific to the first binding moiety, for example, an antibody binding to a fluorescein label. In some instances, the antibody can be biotinylated and the third binding moiety comprises streptavidin.

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In some examples, the first binding moiety can be a maleimide group. In some examples, the second binding moiety comprises a biotinylated heterocarbon chain with a thiol group that reacts specifically with the first binding moiety. In some instances, the heterocarbon chain has 1-10 biotin and the third binding moiety comprises streptavidin. In some examples, the second binding moiety comprises nucleic acid with a thiol group that reacts specifically with the first binding moiety. In some instances, the second binding moiety binds to multiple complementary nucleic acids comprising a biotin label that then bind to streptavidin.

In some embodiments, the detectable label used in any of the assay methods described herein may release a signal directly, for example, a dye or a fluorescent agent. In some embodiments, the detectable label may release a signal indirectly, for example, a β-galactosidase, which may convert a fluorogenic substrate to a product that generates a fluorescent signal.

In any of the assay methods described herein, the nucleic acid of interest is a microRNA, *e.g.*, a microRNA associated with a disease. The sample to be analyzed by the assay method may be a biological sample obtained from a subject, for example, a subject suspected of having the disease. In some instances, the chemical group in the modified base can be an aldehyde group, a ketone group, a thiol group, or a diol group capable of reversible covalent reactions. In some examples, the PNA probe has 10-50 monomer units (*e.g.*, 10-20, 10-30, or 10-40 monomer units).

In any of the assay methods, the fraction of beads associated with at least one detectable label may be detected using a single molecule array, for example, a Simoa assay.

The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of an exemplary approach for detecting single molecules of nucleic acid (NA) with single base specificity. Panel I illustrates the reaction when a capture probe has a perfect match with a target NA. Panel II illustrates the reaction when a

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capture probe has one mismatched base with a target NA. Panel III illustrates the reaction when a capture probe does not match a target NA.

Figure 2 is a diagram showing Plot of AEB determined using the assay illustrated in Figure 1 against concentration of calibrators for miR-122 (circles) and miR-122 with a single base mismatch at the 9th position (squares) spiked into buffer. The sequences of these two molecules are shown in Table 4. Error bars (±1 s.d.) are smaller than the size of the data point.

Figure 3 includes diagrams showing measurement of miR122 in clinical samples. A: Scatter plot of AEB for miR-122 measured in the serum of 4 healthy volunteers and 4 individuals after overdosing on acetominophen. The dotted lines represent the mean assay (buffer) background ± 1 s.d.. B: Correlation of concentration of miR-122 in the serum of patients determined using Simoa and PCR (r2 = 0.93; slope = 0.64).

Figure 4 includes diagrams showing structures of compounds used in an exemplary assay. A: structure of an exemplary Amino-PEG linker ("xx" in Table 1) located at the N-terminal end of the abasic PNA probe (1). B: Structure of aldehyde-modified cytosine base presenting biotin.

Figure 5 is a plot showing ES+-TOF MS of base, m/z 1036.5806 $[M+H]^+$; 1054.5632 $[M+H2O+H]^+$;1076.5531 $[M+H2O+Na]^+$; 518.7623 $[M+2H]^{+2}$; 538.7747 $[M+H2O+Na+H]^{+2}$; High Resolution Mass Spectroscopy (HRMS) calculated for C46H82N7O17S ($[M+H]^+$) 1036.5482, found 1036.5806. Hydrated form of aldehyde is detected.

Figure 6 is a plot of Ct values determined using PCR as a function of the concentration of miR-122 (see Table 4). To estimate the limit of detection, the Ct values of the three lowest concentrations were averaged and the standard deviation determined. The Ct value at 3 s.d. below the mean Ct was 34, so the LOD was about 2.6 fM. Error bars are shown as 1 s.d.

Figure 7 is a diagram showing specificity of Simoa assay for miR-122. The chart shows AEB values of the miR-122 specific beads for: 15 nM miR-122 calibrator (1st bar); 15 nM of miR-122 calibrator with a single base mismatch (2nd bar); 15 nM of miR-39 (3rd bar); no nucleic acid (4^{th} bar); no nucleic acid and no nucleobase label (5th bar); no nucleic acid and no reductant (6th bar); and, no nucleic acid, no nucleobase, and no reductant (7th bar). The last 4

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bars in the chart are reagent drop-out experiments that indicate the sources of background in the assay. These data indicate the non-specific binding of the nucleobase label to the beads dominates the background of the Simoa assay. Error bars are s.d. values from 2 replicates. Each bar is labelled with its AEB value.

Figure 8 is a diagram showing Ct values for patients and healthy controls for the same samples measured using Simoa as described in Figure 3, panel A.

Figure 9 is a diagram showing the correlation of concentration of synthetic calibrator for miR-122 spiked into serum determined using Simoa and PCR ($r^2 = 0.998$).

Figure 10 is a diagram showing a Bland-Altman plot for concentrations of miR-122 in serum samples of patients as described herein determined using Simoa and PCR. The dashed line is at the mean of the difference in concentration between the methods; the dotted lines are at ±1.96 s.d. of the mean. Bland et al., Lancet 1986, 1, 307-10.

DETAILED DESCRIPTION OF THE INVENTION

Described herein is a single probe method for detecting single molecules of a nucleic acid of interest (*e.g.*, a small nucleic acid such as a microRNA) from a suitable biological sample (*e.g.*, human serum), with sequence specificity down to a single base. The method described herein may not require amplification of the nucleic acid of interest. The assay methods described herein involve a combination of a single-base label approach and a detection assay capable of counting single molecules/single labels. The single-base label approach has been used to demonstrate gentotyping assays using mass spectrometry, DNA microarrays, and conventional bead-based assays. Bowler et al., *Angew. Chem. Int.* Ed. 2010, 49:1809-1812; Pernagallo et al., *Sensors*, 2012, 12:8100-8111; and Venkateswaran et al., *Talanta*, 2016, 161:489-496. The sensitivity of these analog assays were, however, not sufficient to detect miRNA in clinical samples. Venkateswaran et al., *Talanta*, 2016, 161:489-496. It was demonstrated that single molecule arrays (Simoa) are very sensitive to an enzyme label (limit of detection, LOD = 220 zM), and could be used to develop sensitive assays for proteins and DNA. Rissin et al., Nat.

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Biotechnol. 2010, 28:595-599; and Song et al., *Anal. Chem.* 2013, 85:1932-1939. The Simoa DNA assay was, however, limited to relatively long target sequences (>100 base pairs) because of the requirement for a capture and multiple detection probes, each being 15-20 bases long. Combining these two approaches enables an assay with unexpectedly high sensitivity (*e.g.*, single molecule) and high specificity (*e.g.*, single base) for detecting short RNA target molecules, such as microRNAs.

I. Methods for Detecting Nucleic Acids Involving Single Base Labeling and Single Molecule/Single Label Detection

Figure 1 illustrates an example of the methods described herein for detecting target nucleic acids, particularly small nucleic acids such as microRNAs, with high sensitivity and specificity (*e.g.*, single base specificity and single molecule sensitivity). The exemplary approach combines the specific labeling of an immobilized capture probe with a biotinylated single nucleobase, with detection in arrays of femtoliter wells of single enzymes that bind to the biotin.

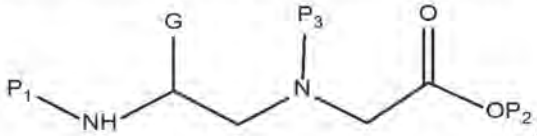
In some embodiments, peptide nucleic acid (PNA) probes are used in the assay methods described herein. PNAs are synthetic polymers mimicking the structures of DNAs or RNAs. As used herein, the term "PNA probe" refers to any synthetic polymer that mimics the structures of DNAs or RNAs and has a peptide backbone, which may include one or more modification moieties. In some instances, the backbone of a PNA molecule is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds, rather than the deoxyribose or ribose sugar backbones in DNAs or RNAs. A PNA molecule contains various purine and/or pyrimidine bases, just like DNAs or RNAs, which are linked to the backbone by a methylene bridge (-CH_2-) and a carbonyl group (-(C=O)-). Such molecules are depicted like peptides, with the N-terminus at the first (left) position and the C-terminus at the last (right) position. The order of the purine and pyrimidine bases from the N-terminus to the C-terminus represents the nucleotide sequence of a PNA molecule, which can form a duplex with a nucleic acid carrying a sequence that is complementary (completely or partially) to the PNA molecule.

Typically, the PNA monomer from which the PNA oligomer is derived, or one or more

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repeat units of the PNA oligomer, is/are derived from a N-(2-aminoethyl)-glycine unit. As stated, the PNA monomer or one or more of the repeat units of the PNA oligomer maycomprise at the/their gamma position(s) a charged moiety, or a moiety capable of carrying a charge at a predetermined pH.

The PNA probes described herein include modified PNA probes, for example, those having a modified backbone. In some examples, the modified PNA probes comprise a N-(2-aminoethyl)-glycine unit. Such modified PNA probes may have the general formula of:



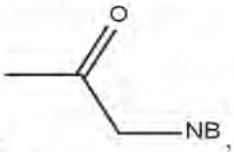
(Formula I), in which:

G is a charged moiety (*e.g.*, a glutamic group), or a moiety capable of carrying a charge at a predetermined pH;

P1 is a protective group P, or is hydrogen;

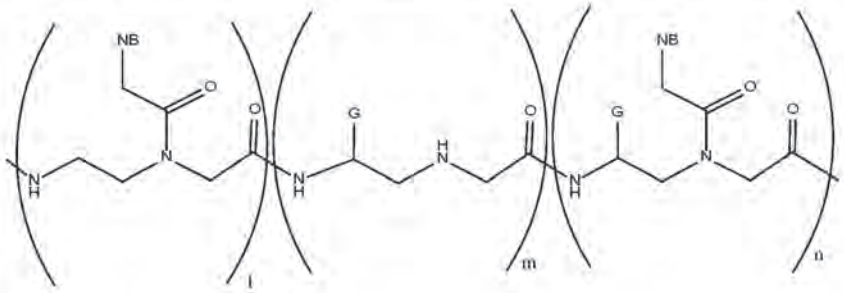
P2 is a protective group P, or is hydrogen, or is a group selected from the list consisting of alkyl, cycloalkyl, aryl, aralkyl, or halogen,

P3 is hydrogen, or is a protective group P, or is a group represented by formula (II) below:



, in which NB is a nucleobase. In some examples, the modified PNA probes have a backbone modified by glutamic side chains.

The PNA probes as described herein may have the general formula:



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Formula (V)

In Formula (V), G is a charged moiety, or a moiety capable of carrying a charge at a predetermined pH; NB is a nucleobase; and $l \geq 0$; $m \geq 0$; $n \geq 0$, with the proviso that $l+m+n \geq 2$ and $n+m \geq 1$. Preferably, $l \geq 1$; $m \geq 1$ (e.g., m=1); and/or $n \geq 1$. In some embodiments, G is a glutamic group.

It will be understood that the repeat units of the PNA oligomer of formula (V) may not necessarily be provided sequentially or as a block polymer of blocks l, m, and n, but that the repeat units may be provided in any order.

In some examples, the total number of PNA units (l+m+n) in the oligomer may be in the range of 5-50, *e.g.*, 7-40, e.g. 10-30. In some examples, the total number of PNA units can be in the range of 12-24.

Other descriptions of PNA probes, including modified PNA probes, can be found in ES201630948 (filed 12th Jul 2016) and GB1616556.5 (filed 29th Sep 2016), the relevant disclosures of which are hereby incorporated by reference for the purposes or subject matter referenced herein.

The PNA probes as described herein may contain up to 50 monomer units (*e.g.*, up to 40, 30, 25, 20, 15, or 10 monomer units). Such probes are complementary to a target nucleic acid or a portion thereof. In some instances, the PNA probes are complementary to a small nucleic acid (*e.g.*, having less than 50 nucleotides, less than 40 nucleotides, less than 30 nucleotides, or less than 20 nucleotides). In some instances, the PNA probe used in an assay method described herein contains less than 20 monomer units. In one specific example, the PNA probe is complementary to a microRNA or a portion thereof, for example, a microRNA that is associated with a disease or disorder. Such target microRNAs are well known in the art.

"Complementary," as used herein, refers to the nucleobase complementarity commonly known in the art. For example, adenine is complementary to thymine (in DNA) or uracil in RNA; and guanine is complementary to cytosine. "Sequence complementarity", or "nucleic acid sequences being complementary to one another", as used herein, means when the two nucleic acid molecules are aligned antiparallel to each other (the 5'-end of a nucleic acid sequence faces

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the C-terminus of a PNA sequence), the nucleotide bases at each position, or at most positions in the sequences are complementary, and that the two nucleic acid molecules can hybridize and form a duplex under suitable conditions, *e.g.*, hybridization temperature. As known in the art, a sequence complementarity needs not be 100% for the two nucleic acid molecules to hybridize and form a duplex. The sequence complementarity between the capture probe (or the detecting probe described herein) and the target nucleic acid may be at least 80% complementary to the corresponding region in the target nucleic acid. In some embodiments, the capture probe contains a fragment that is at least 80% (*e.g.*, 85%, 90%, 95%, 98%, or 100%) complementary to the first segment of the target nucleic acid. In some instances, the capture probe contains a fragment that is completely complementary (100% complementary) to the first segment of the target nucleic acid. Such a capture probe may be used in differentiating the target nucleic acid from substantially similar nucleic acids, for example, nucleic acids having 1, 2, or 3 base differences relative to the target nucleic acid.

As illustrated in Figure 1, the PNA probe described herein is an abasic PNA probe, *i.e.*, lacking one base at a position corresponding to a target nucleotide in the nucleic acid of interest (the nucleic acid to be detected using the PNA probe), for example, a microRNA. The position where the base is lacking can be any position within the PNA probe. In some instances, the base-missing position may be located in the middle of the PNA probe, for example, having at least three bases at the N-terminal, at the C-terminal, or both. The target nucleotide may be at any position inside the nucleic acid of interest. In some instances, the target nucleotide is distinctive to the nucleic acid of interest, *e.g.*, presented in the nucleic acid of interest but not in homologous nucleic acids. For example, if the nucleic acid of interest is a microRNA that belongs to a family containing structurally similar members, the target nucleotide can be located at a position that can be used to distinguish the microRNA of interest from other members of the same family.

The PNA probe as described herein can be immobilized on a support member *via* a conventional method. As used herein, "immobilized" means attached, bound, or affixed, covalently or non-covalently, so as to prevent dissociation or loss of the capture probe, but does

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not require absolute immobility with respect to either the capture probe or the support member. A support member can be a solid or semi-solid member with a surface that can be used to specifically attach, bind or otherwise capture a nucleotide probe (*e.g.*, the capture PNA probe of the present disclosure), such that the PNA probe becomes immobilized with respect to the support member. In some instances, the PNA probe is immobilized onto a support member (*e.g.*, a bead) directly through a covalent bond (*e.g.*, amide, disulfide, hydrazine or thioether). In other instances, the PNA probe may be immobilized onto a support member (e.g., a bead) via linker, such as an oligonucleotide linker (*e.g.*, a polyA or poly T linker) or a peptide linker (*e.g.*, a poly glycine or poly alanine linker).

The support member of the present disclosure may be fabricated from one or more suitable materials, for example, plastics or synthetic polymers (*e.g.*, polyethylene, polypropylene, polystyrene, polyamide, polyurethane, phenolic polymers, or nitrocellulose), naturally derived polymers (*e.g.*, latex rubber, polysaccharides, polypeptides), composite materials, ceramics, silica or silica-based materials, carbon, metals or metal compounds (*e.g.*, comprising gold, silver, steel, aluminum, or copper), inorganic glasses, silica, and a variety of other suitable materials. Non-limiting examples of potentially suitable configurations include beads (*e.g.*, magnetic beads), tubes (*e.g.*, nanotubes), plates, disks, dipsticks, chips, microchips, coverslips, or the like. In one particular example, the support member is a bead.

The surface of the support member of the present disclosure may comprise any molecule, other chemical/biological entity, or solid support modification disposed upon the solid support that can be used to specifically attach, bind or otherwise capture a PNA molecule. Surface compositions that may be used to immobilize a PNA molecule can be readily found in the art. For example, the surface may comprise a complementary nucleic acid or a nucleic acid binding protein, which can be attached to the surface *via* convention methods. Thus, the linkage between the PNA to be immobilized (*e.g.*, the capture probe of the present disclosure) and the surface may comprise one or more chemical or physical (*e.g.*, non-specific attachment via van der Waals forces, hydrogen bonding, electrostatic interactions, hydrophobic/hydrophilic interactions; etc.) bonds and/or chemical linkers providing such bond(s). Alternatively, the surface of the support

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member may comprise reactive functional groups that are capable of forming covalent bonds with the nucleic acid molecules to immobilize. In some embodiments, the functional groups are chemical functionalities. That is, the binding surface may be derivatized such that a chemical functionality is presented at the binding surface, which can react with a chemical functionality on the nucleic acid to be captured, resulting in attachment. Examples of functional groups for attachment that may be useful include, but are not limited to, amino groups, carboxyl groups, epoxide groups, maleimide groups, oxo groups, azides and thiol groups. Functional groups can be attached, either directly or through the use of a linker, the combination of which is sometimes referred to herein as a "crosslinker." Crosslinkers for attaching nucleic acid molecules to a support member are known in the art; for example, homo-or hetero-bifunctional crosslinkers as are well known (*e.g.*, see 1994 Pierce Chemical Company catalog, technical section on crosslinkers, pages 155-200, or "Bioconjugate Techniques" by Greg T. Hermanson, Academic Press, 1996). Non-limiting example of crosslinkers include alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), esters, amide, amine, thiols, azides, triazine, epoxy groups and ethylene glycol and derivatives. A linker may also be a sulfone group, forming a sulfonamide. In some embodiments, the functional group is a light-activated functional group. That is, the functional group can be activated by light to attach the capture component to the capture object surface. One example is PhotoLinkTM technology available from SurModics, Inc. in Eden Prairie, MN.

It is to be understood that the examples provided herein on the support member and the surface composition are not meant to be limiting. Any support members that are known in the art to be suitable for immobilization of nucleic acid molecules may be used in accordance with the present disclosure.

A suitable PNA probe, optionally immobilized on a support member, can be incubated with a sample suspected of having a nucleic acid of interest under suitable conditions to allow hybridization of the PNA probe and the nucleic acid having a complementary sequence to the PNA probe, which lead to formation of PNA/nucleic acid duplexes. See Figure 1. Hybridization refers to the ability of complementary single-stranded DNA/RNA and the PNA probe to form a

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PNA/nucleic acid duplex molecule. The hybridization step of the assay method described herein can be performed under suitable hybridization conditions, which are within the knowledge of those skilled in the art. Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions for attaining particular degrees of stringency are well known in the art, for example, described in Sambrook et al., (1989) Molecular Cloning, second edition, Cold Spring Harbor Laboratory, Plainview, N.Y. (chapters 9 and 11). The hybridization temperatures of the first and second hybridization steps of the assay methods described herein can be determined based on various factors, for example, the length of the complementary regions between the capture/detecting probe and the target nucleic acid, the composition of the complementary regions (*e.g.*, G/C content), and the stringency needed, which are within the knowledge of those skilled in the art.

When needed, the hybridization reaction mixture described above can be washed one or more times to remove unbound nucleic acids and other components so as to enhance sensitivity and specificity of the assay method. Any nucleic acid having a complementary nucleotide sequence of the PNA probe will form a duplex attached to the support member (*e.g.*, the bead). As illustrated in Figure 1, nucleic acids having completely complementary sequences or partially complementary sequences would hybrid with the PNA probe to form a duplex, while nucleic acids having non-complementary sequences would not form duplex with the PNA probe.

During or after hybridization, the mixture containing the PNA/nucleic acid duplex (if any) can be incubated with a modified base, which is complementary to the target nucleotide in the nucleic acid of interest. The modified base contains a chemical group that is capable of reacting with the PNA probe for form a covalent bond. Any modified bases capable of reacting with a PNA molecule can be used in the instant assay methods, for example, those described in US2011/0028337, the relevant disclosures of which are incorporated by reference herein for the purposes or subject matter referenced herein. In some instances, the functional group in the

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modified base can be a group capable of reversible covalent reactions such as an aldehyde group, a ketone group, a thiol group, or a diol group. The modified base is also attached to a first binding moiety, which may be a label capable of releasing a signal directly or indirectly (a detectable label), or a member of a ligand/receptor pair. In one example, the first binding moiety can be biotin. Any methods known in the art involving dynamic labeling chemistry to specifically label the PNA probe bound to the complementary nucleic acid of interest with any of the modified bases described herein can be used in the present disclosure, for example, the method described in Bowler et al., *Angew. Chem. Int.* Ed. 2010, 49:1809-1812, the relevant disclosures of which are incorporated by reference herein for the purposes or subject matter referenced herein.

Afterwards, the reaction mixture may be washed one or more times to remove unbound modified bases. The PNA/nucleic acid duplex labeled with the modified base may optionally be incubated with a second binding moiety which specifically binds the first binding moiety and comprises a detectable label, which may be measured directly.

In some instances, the first binding moiety incorporated into the modified base and the second binding moiety are members of a ligand/receptor pair, for example, biotin and streptavidin. In some instances, the streptavidin is conjugated to a detectable label, *e.g.*, an enzyme, such as beta-galactosidase. In other instances, the second binding moiety may be an antibody or an antigen-binding fragment thereof that specifically binds the first binding moiety.

In some embodiments, the second binding moiety may be an antibody (*e.g.*, an anti-fluorescein antibody) or an antigen-binding fragment thereof that specifically binds the first binding moiety (*e.g.*, a fluorescein molecule). The second binding moiety may be conjugated to an agent (e.g., biotin) that specifically binds a third binding moiety (*e.g.*, streptavidin), which is conjugated to a detectable label.

In some embodiments, the second binding moiety may be a heterocarbon chain containing a thiol groups thereof that specifically binds the first binding moiety (*e.g.*, a maleimide group). The second binding moiety may be conjugated to an agent or multiple agents (*e.g.*, biotin) that specifically binds a third binding moiety (e.g., streptavidin), which is

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conjugated to a detectable label.

In some embodiments, the second binding moiety comprises nucleic acid with a thiol group that reacts specifically with the first binding moiety. In some instances, the second binding moiety binds to multiple complementary nucleic acids comprising a biotin label that then bind to streptavidin. "Conjugated", as used herein, means an agent (*e.g.*, a detectable label) is attached to another molecule (*e.g.*, a binding moiety as described herein) the detecting probe, covalently or non-covalently. The detectable label can be any molecule, particle, or the like, that facilitates detection, directly or indirectly, using a suitable detection technique.

In some embodiments, the detectable label may be a molecule or moiety capable of releasing a signal that can be directly interrogated and/or detected (*e.g.*, a fluorescent label or a dye). In some embodiments, a fluorescent label is used as the detectable agent. Examples include, but are not limited to, fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, fluorescamine and fluorescent metals such as ^{152}Eu or other metals from the lanthanide series, CYE dyes, SETA dyes, and fluorescent proteins such as eGFP, eYFP, eCFP, mKate2, mCherry, mPlum, mGrape2, mRaspberry, mGrape1, mStrawberry, mTangerine, mBanana, and mHoneydrew.

In other embodiments, the detectable agent may be a molecule or moiety (*e.g.*, an enzyme) capable of converting a substrate to a product that is capable of releasing a detectable signal. For example, the detectable label may be a luciferase, which converts luciferin to oxyluciferin to emit detectable lights. Alternatively, the detectable label may be β-D-galactosidase, which can convert its substrate resorufin-β-galactopyranoside (RGP) to a product (resorufin) that has a detectable fluorescent signal.

Other exemplary detectable labels include, but are not limited to, phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, theromatic acridinium ester, imidazole, acridinium salts, oxalate ester, and dioxetane), radio-isotopes (such as ^{3}H, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe, and ^{75}Se), metals, metal chelates or metallic cations (for example metallic cations such as ^{99}mTc, ^{123}I, ^{111}In, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga, and ^{68}Ga. Other examples include chromophores and enzymes (*e.g.*, malate dehydrogenase, staphylococcal

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nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase).

Any of the assay methods described herein employs a step of measuring the intensity of the signal released from the detectable label (directly or indirectly), which can be achieved by a detection assay capable of counting single molecules (*e.g.*, single detectable labels), for example a single molecule array assay (*e.g.*, SiMoA™). In some embodiments, supporting members such as beads to which a complex of PNA probe-nucleic acid of interest-binding moiety-detectable label is attached can be segregated into a plurality of locations (*e.g.*, a plurality of wells of a multi-well plate) to facilitate detection/quantification, such that each location comprises/contains either zero or one or more detectable label molecule. In some instances, each location contains one molecule of the nucleic acid of interest (corresponding to one detectable label molecule).

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 the nucleic acid of interest (*e.g.*, the concentration of a microRNA of interest) in a fluid sample may be determined by detecting the nucleic acids immobilized with respect to a binding surface having affinity for at least one type of nucleic acids. 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 molecule of the nucleic acid of interest may be made. In some cases, based upon the number/percentage/fraction, a measure of the concentration of the nucleic acid of interest in the fluid sample (*e.g.*, the biological sample) may be determined. The measure of the concentration of the nucleic acid of interest 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

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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.

Additional details of exemplary, non-limiting assay methods, which comprise one or more steps of spatially segregating the nucleic acid of interest to be detected by any of the assay methods described herein, will now be described. In certain embodiments, a method for detection and/or quantifying a nucleic acid of interest in a sample comprises immobilizing a plurality of the nucleic acid 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 nucleic acid molecules. For example, the capture objects may comprise a plurality of beads comprising a plurality of capture components (*e.g.*, a PNA probe as described herein). At least some of the capture objects 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 the nucleic acid 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 molecule of the nucleic acid of interest. 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 nucleic acids of interest and/or capture objects associated with a molecule of the nucleic acid of interest 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 molecule of the nucleic acid of interest 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 the nucleic acid of interest 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 molecule of the nucleic acid of interest to the total

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number of locations determined to contain a capture object not associated with any molecule of the nucleic acid of interest, and/or a measure of the concentration of the nucleic acid of interest 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 molecule of the nucleic acid of interest to the number of locations determined to not contain any capture objects, and/or a measure of the concentration of the nucleic acid of interest 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 molecule of the nucleic acid of interest to the number of locations determined to contain a capture object. In yet other embodiments, a measure of the concentration of nucleic acids of interest 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 nucleic acid of interest 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 molecule of the nucleic acid of interest) 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 molecule of the nucleic acid of interest 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 WO 2011/109364, by Duffy et al., the relevant disclosures of which are incorporated by reference herein) of the reaction vessels comprise at least one or, in certain cases, only one capture object associated with at least one molecule of a nucleic acid of interest and at least some (*e.g.*, a statistically significant fraction) of the reaction vessels comprise an capture object not associated with any nucleic acid of interest. The capture objects associated with at least one nucleic acid of interest may be quantified in certain embodiments, thereby allowing for the detection and/or quantification of nucleic acids of interest in the fluid sample by techniques described in more detail herein.

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In some embodiments, the detection step of any of the assay methods described herein involves a single molecule array assay (for example, the SiMoA™ technology) known in the art. Exemplary single molecule array assays have been described previously, for example, U.S. Patent No. 8,460,879, U.S. Patent No. 8,460,878, U.S. Patent No. 8,492,098, U.S. Patent No. 8,222,047, U.S. Patent No. 8,236,574, U.S. Patent No. 8,415,171, US2010-0075862, US2010-0075439, US2010-0075355, US 2011-0212462, US 2012-0196774, US 2011-0245097, WO 2009/029073, WO2010/039179, WO2011/109364, WO2011/109372, WO2011/109379, WO/2014/113502, the relevant disclosures of each of which are incorporated by reference herein for purposes or subject matter referenced herein.

II. Applications

Any of the assay methods described herein can be used to detect the presence and/or measure the level of a nucleic acid of interest (*e.g.*, a microRNA such as one associated with a target disease or disorder) in a suitable sample. In some examples, the sample may be a biological sample obtained from a subject and the results obtained from the assay methods described herein may be used for diagnostic and/or prognostic purposes. In other examples, the assay methods described herein can be used in research settings for detecting presence or measuring the level of a nucleic acid of interest in a sample.

To detect the presence or measure the level (concentration) of a nucleic acid of interest in a sample, a calibration curve may be developed using samples containing known concentrations of the nucleic acid of interest. The concentration of the nucleic acid of interest in a sample may be determined by comparison of a measured parameter to a calibration standard. In some cases, a calibration curve may be prepared, wherein the total measured signal is determined for a plurality of samples comprising the nucleic acid of interest at a known concentration using a substantially similar assay format. For example, the total intensity of the array may be compared to a calibration curve to determine a measure of the concentration of the nucleic acid of interest in the sample. The calibration curve may be produced by completing the assay with a plurality of standardized samples of known concentration under similar conditions used to analyze test

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samples with unknown concentrations. A calibration curve may relate the detected signal of the nucleic acid of interest (and/or detecting probe) with a known concentration of the nucleic acid of interest. The assay may then be completed on a sample containing the nucleic acid of interest or fragment in an unknown concentration, and signals detected from the nucleic acid of interest (and/or detecting probe) may be compared to the calibration curve, (or a mathematical equation fitting same) to determine a measure of the concentration of the nucleic acid of interest in the sample.

The assay methods described herein may be used to detect any nucleic acid molecule and their mimics, including both DNA molecules and RNA molecules. When the target nucleic acid is a DNA molecule, a denaturing step may be performed to produce single-stranded DNA molecules, *e.g.*, by heating the sample to 96°C or added NaOH. In some embodiments, the assay methods are applied to detecting short nucleic acids, for example, nucleic acids having less than 150 nucleotides (nts), e.g., less than 120 nts, less than 100 nts, less than 80 nts, less than 60 nts, less than 50 nts, less than 40 nts, less than 30 nts, less than 25 nts, or less than 20 nts. In a particular example, the assay methods are applied for detecting microRNAs. Given the high sensitivity and specificity of the assay methods described herein, a nucleic acid of interest in a biological sample may not need to be pre-amplified using polymerases or other methods.

In some embodiments, the assay methods are applied to detect a nucleic acid of interest in a biological sample, which may be any sample from a biological source. Exemplary biological samples include tissue samples (such as tissue sections and needle biopsies of a tissue); cell samples (*e.g.*, cytological smears (such as Pap or blood smears) or samples of cells obtained by microdissection); samples of whole organisms (such as samples of yeasts or bacteria); or cell fractions, fragments or organelles (such as obtained by lysing cells and separating the components thereof by centrifugation or otherwise). Other examples of biological samples include blood, blood components (such serum and plasma), urine, semen, fecal matter, cerebrospinal fluid, interstitial fluid, mucous, tears, sweat, pus, biopsied tissue (e.g., obtained by a surgical biopsy or needle biopsy), nipple aspirates, milk, vaginal fluid, saliva, swabs (such as buccal swabs), or any material containing biomolecules that is derived from a first biological

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sample. In some embodiments, the biological sample can be a body fluid, which can be fluid isolated from the body of an individual. For example, "body fluid" may include blood, plasma, serum, bile, saliva, urine, tears, perspiration, and the like. As the method does not require amplification using polymerases that are affected by biological materials found in many samples, it may be used to detect NA in samples without purification of the NA, i.e., the direct detection of NA in samples.

The biological sample may be obtained from a subject in need of the analysis. A "subject" may be a human (*i.e.*, male or female of any age group, for example, pediatric subject (*e.g.*, infant, child, or adolescent) or adult subject (*e.g.*, young adult, middle–aged adult, or senior adult). Alternatively, the subject may be a non–human animal. In certain embodiments, the non–human animal is a mammal (*e.g.*, primate, for example, cynomolgus monkey or rhesus monkey), commercially relevant mammal (*e.g.*, cattle, pig, horse, sheep, goat, cat, or dog), or bird (*e.g.*, commercially relevant bird, such as chicken, duck, goose, or turkey). In other examples, the non-human animal is a fish, reptile, or amphibian. The non-human animal may be a male or female at any stage of development. The non-human animal may be a transgenic animal or genetically engineered animal. In some examples, the subject may also be a plant.

In some embodiments, the sample for analysis may contain one or more nucleic acids that are highly homologous to the nucleic acid of interest, *e.g.*, at least 80%, 90%, 95%, or 98% identical to the target nucleic acid. The "percent identity" of two nucleic acids can be determined using the algorithm of Karlin and Altschul Proc. Natl. Acad. Sci. USA 87:2264-68, 1990, modified as in Karlin and Altschul Proc. Natl. Acad. Sci. USA 90:5873-77, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. J. Mol. Biol. 215:403-10, 1990. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength-12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. Where gaps exist between two sequences, Gapped BLAST can be utilized as described in Altschul et al., Nucleic Acids Res. 25(17):3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. In some examples, the one or

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more homologous nucleic acids may differ from the target nucleic acid by only up to 5 nucleotides (*e.g.*, 4, 3, 2, or 1).

The assay methods may be applied in a diagnostic/prognostic setting to detect the presence or measure the level of a nucleic acid biomarker (*e.g.*, a microRNA) that is associated with a target disease. For example, the methods may be used to detect/measure a specific microRNA, which may be associated with a specific disease, *e.g.*, cancer. The methods can be used in detecting such a nucleic acid biomarker in subjects that are absent of any symptom of the disease for early stage diagnosis. The assay methods can also be used to detect nucleic acids of microorganisms for determining whether a subject has been infected by such microorganisms, for example, viruses (*e.g.*, HBV, HCV, HPV, and HIV). The assay methods can also be applied to monitor an individual drug response. For example, the methods may be used to detect/measure a specific RNA, which may be associated with a specific drug toxicity or drug resistance event.

Those skilled in the art would have known that the application of the ultrasensitive assay methods described herein are not limited to diagnosis/prognosis purposes; such methods can be used to detect nucleic acids of interest for any purposes, for example, for research purposes. In some examples, the assay methods can be applied to detect a nucleic acid such as a microRNA in studies of its biological functions or in studies of biopathways in which the nucleic acid is involved. Alternatively, the assay methods described herein can also be used in development of nucleic acid-based therapeutic agents.

III. Kits for Performing the Ultrasensitive Assay Methods

The present disclosure also provides kits for use in performing any of the assay methods described herein. Such kits may be designed for diagnostic uses or for other purposes, for example, research uses.

The kit described herein may include one or more containers housing components for performing the assay methods described herein and optionally instructions of uses. Specifically, such a kit may include one or more agents described herein (for example, a PNA probe, which

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maybe immobilized on a support member such as a bead, a suitable modified base, and a binding moiety that is conjugated to a detectable label plus ancillary chemicals and buffers), along with instructions describing the intended application and the proper use of these agents. In certain embodiments, the kit may be suitable for a diagnostic purpose. For example, the kit may contain apparatus for sample collection from a patient, and/or reagents for detecting diseases associated nucleic acid molecules. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments.

The kit described herein may contain an abasic PNA probe, which may be immobilized in a support member as described herein. Alternatively, the kit may contain the PNA probe in free form, the support member, and reagents necessary for linking the PNA probe onto the surface of the support member. For example, the support member in the kit may comprise chemical reactive moieties for the covalently linking of the PNA probes. Further, the kit comprises a modified base as described herein. In some embodiments, the modified base is conjugated to a first binding moiety as described herein. The kit may further comprise a second and/or a third binding moiety which is conjugated to a detectable label.

Any of the kits described herein may further comprise components needed for performing the assay methods. For example, it may contain components for use in detecting a signal released from the detectable label, directly or indirectly. In some examples, the detection step of the assay methods involves enzyme reaction, the kit may further contain the enzyme (*e.g.*, β-galactosidase) and a suitable substrate.

Each of the components of the kits, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the components may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (for example, water or certain organic solvents), which may or may not be provided with the kit.

In some embodiments, the kits may optionally include instructions and/or promotion for use of the components provided. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with

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packaging of the disclosure. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, etc.), Internet, and/or web-based communications, *etc*. The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which can also reflects approval by the agency of manufacture, use or sale for animal administration. As used herein, "promoted" includes all methods of doing business including methods of education, hospital and other clinical instruction, scientific inquiry, drug discovery or development, academic research, pharmaceutical industry activity including pharmaceutical sales, and any advertising or other promotional activity including written, oral and electronic communication of any form, associated with the invention. Additionally, the kits may include other components depending on the specific application, as described herein.

The kits may contain any one or more of the components described herein in one or more containers. The components may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other components prepared sterilely. Alternatively the kits may include the active agents premixed and shipped in a vial, tube, or other container.

The kits may have a variety of forms, such as a blister pouch, vials, a shrink wrapped pouch, a vacuum sealable pouch, a sealable thermoformed tray, or a similar pouch or tray form, with the accessories loosely packed within the pouch, one or more tubes, containers, a box or a bag. The kits may be sterilized after the accessories are added, thereby allowing the individual accessories in the container to be otherwise unwrapped. The kits can be sterilized using any appropriate sterilization techniques, such as radiation sterilization, heat sterilization, or other sterilization methods known in the art. The kits may also include other components, depending on the specific application, for example, containers, cell media, salts, buffers, reagents, syringes, needles, pipettes, a fabric, such as gauze, for applying or removing a disinfecting agent, disposable gloves, a support for the agents prior to administration etc.

Without further elaboration, it is believed that one skilled in the art can, based on the

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above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

EXAMPLES

Example 1: Detection and Quantification of microRNA Using Single Nucleobase Labelling In Combination with Simoa assay

An abasic peptide nucleic acid probe (PNA), containing a reactive amine instead of a nucleotide at a specific position in the sequence, for microRNA was conjugated to superparamagnetic beads. These beads were incubated with a sample containing microRNA, a biotinylated reactive nucleobase that was complementary to the missing base in the probe sequence, and a reducing agent. When a target molecule with an exact match in sequence hybridized to the capture probe, the reactive nucleobase was covalently attached to the backbone of the probe by a dynamic covalent chemical reaction. Single molecules of the biotin-labeled probe were then labeled with streptavidin-β-galactosidase (SβG), and the beads were loaded into an array of femtoliter wells, and sealed with oil. The array was imaged fluorescently to determine which beads were associated with single enzymes, and the average number of enzymes per bead was determined. The assay had a limit of detection of 500 fM, approximately 500 times more sensitive than a corresponding analog bead-based assay. This assay was used to measure microRNA-122 (miR-122), a biomarker of liver toxicity, extracted from the serum of patients who had acute liver injury due to acetaminophen, and control healthy patients. All patients with liver injury had higher levels of miR-122 in their serum compared to controls, and the concentrations measured correlated well with those determined using PCR. This approach allows rapid quantification of circulating microRNA with high sensitivity and specificity, and a limit of quantification suitable for clinical use. With further development, this method could facilitate translation of circulating miRNA from research tools into clinical biomarkers.

MATERIALS AND METHODS

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Materials

RNA target molecules were purchased desalted from Integrated DNA Technologies. All chemicals were obtained from SigmaAldrich and used as received. 2.8-μm-diameter superparamagnetic beads presenting carboxylic acid groups (Dynabeads® M-270), and resorufin-β-D-galactopyranoside (RGP) were obtained from ThermoFisher Scientific. Streptavidin-β-galactosidase (SβG) was conjugated using methods previously described. Rissin et al., Nat. Biotechnol. 2010, 28, 595-599. Simoa disks comprised of 24 arrays of 216,000 50-fL-sized microwells molded into cyclic olefin copolymer (COC) and bonded to a microfluidic manifold were obtained from Stratec Consumables. Kan et al., , Lab Chip, 2012, 12, 977–985. Fluorocarbon oil (Krytox®) was obtained from DuPont. Concentrations of stock solutions of RNA and DNA were checked using a ThermoFisher NanoDrop1000 Spectrophotometer.

Synthesis of Capture Probe and Aldehyde-Modified, Biotinylated Nucleobase

A peptide nucleic acid (PNA) probe containing an abasic "blank" position (where the corresponding base is missing) and terminated with an amino-PEG linker was synthesized using standard solid phase chemistry on a MultiPep Synthesiser (Intavis AG GmbH, Germany). The sequence of the probe (1) was designed to allow anti-parallel hybridization with the mature miR-122 target (2). The probe contained 3 thymidine bases modified with propionic acid side chains to increase its negative charge. The sequence of the probe and target are shown in Table 1 below. The structure of an exemplary amino-PEG linker is shown in Fig. 4, panel A. Aldehyde-modified cytosine, tagged with biotin via a 12 ethylene glycol units spacer is shown in Fig. 4, panel B, and was prepared using a synthetic route previously described. Venkateswaran et al., Talanta, 2016, 161, 489–496; and Bowler et al., Chem. Int. Ed. 2010, 49, 1809- 1812.

Table 1. Sequences of capture probe and miR-122 target.

SEQ ID NO:	Name	Peptide with abasic position (N'-C')
1	Capture probe	xx-CACCAT*TGT*_ACACT*CCA
		miRNA sequence (5'-3')

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2	Target miR-122	UGGAGUGUGACAAUGGUGUUUG

Key: xx = amino-PEG linker (Fig. 4, panel A); T* = thymidine containing a propanoic acid side chain at the gamma position; "_" = abasic "blank" monomer presenting a secondary amine; the italicized bases in 2 form a duplex with the capture probe, and G at position 9 (bold) is opposite the "blank" monomer and binds to the aldehyde-modified cytosine. The mature sequence of miRNA-122 is 22 bases long. The probe targets 18 bases out of the 22, leaving out the 4 bases at the 3'-end.

Preparation of Superparamagnetic Beads Presenting Probes

100 µL of superparamagnetic beads (containing 2×10^8 beads) were washed by adding 100 µL 0.01 M NaOH and mixing. The beads were pelleted, supernatant removed, and the beads were washed once in 100 µL 0.01 M NaOH and three times in 100 µL distilled water. The beads were then resuspended in 150 µL of freshly-prepared 50 mg/mL EDC in water, and incubated with slow tilt rotation at 23°C for 30 min. After activation with EDC, the beads were washed once with 100 µL cold water and once with 100 µL cold MES buffer (50 mM, pH 5.0). 100 µL of a solution containing 1400 pmol capture probe (Table 1) and 700 pM of an amino-terminated diluent DNA sequence (5'-H_2N-GAGTGTTAGCTGTGAG-3;' (SEQ ID NO: 6)) in MES buffer (50 mM, pH 5) was added to the activated beads. The mixture of probe and beads was incubated at 4°C for 3 h with slow tilt rotation, and then washed with 50 mM MES buffer (pH 5). The remaining activated carboxyl groups were quenched by incubating the beads with 100 µL of 50 mM ethanolamine in PBS (pH 8) for 1 h, followed by three washes with 100 µL of 10% PEG10K and 0.1% Tween-20 in PBS. The capture beads were stored at 4°C in 100 µL of 10% PEG10K and 0.1% Tween-20 in PBS.

Determination of miR-122 Concentration using Quantitative Real-Time PCR (qRT-PCR)

PCR was performed using a leading commercial kit (Qiagen) and a procedure that has been described in detail elsewhere. Kroh et al., Methods 2010, 50, 298-301. cDNA was generated by reverse transcription using the miScript RT II kit (Qiagen) on 1.5 µL of RNA purified from serum (reaction volume = 20 µL). This step includes the use of a poly(A) polymerase to poly-adenylated the target miRNAs, so that the molecule was sufficiently long to be converted into cDNA. 200 µL of RNase-free water was added to the sample containing

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cDNA, and 1 μL of this solution ([cel-miR-39-3p] = 2.7×10^5 copies/μL) was added to the PCR reaction mixture (reaction volume = 25 μL). Quantitative PCR analysis was performed using the miScript SYBR Green PCR Kit (Qiagen) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, CA), with an initial activation step of 95°C for 15 min followed by 40 cycles of 3-step cycling (Denaturation: 15 s at 94°C; Annealing: 30 s at 55°C; Extension: 30 s at 70°C). Ct values were determined for miR-122 and miR-39 in separate PCR reactions.

A calibration curve of Ct as a function of miRNA was determined by performing PCR on miR-39 at 5×10^5, 5×10^4, 5×10^3, and 5×10^2 copies/μL (independent from serum samples) according to manufacturer's protocol (miRNeasy Serum/Plasma Spiked-In Control, Qiagen). This calibration curve was used to determine the recovery of miR-39 from the samples based on the 10,800 copies/μL spiked control in the final PCR reaction. The calibration curve was also used to determine the concentration of miR-122 in each sample following the protocol reported in the miRNeasy Serum/Plasma Handbook. Concentrations of miR-122 were also corrected for the recovery measured for miR-39.

Determination of miR-122 Concentration using Simoa Assay

Sample hybridization and labeling of the capture probe on the beads was conducted in a 96-well conical bottom microtiter plate (ThermoFisher Scientific; Cat No. 249944). The total volume of the labeling reaction was a 50 μL, containing 7.5 μL of sample or calibrator, 4,000,000 capture probe beads, 5 μM aldehyde-modified biotinylated cytosine (Fig. 4, panel B), 1 mM sodium cyanoborohydride, 0.1% SDS, and 10% w/v PEG-10K, in a buffered solution of 30 mM trisodium citrate and 300 mM sodium chloride, pH adjusted to 6.0 using HCl. The microtiter plate was placed on an incubating shaker (VWR; Cat No. 12620-930), set to 40°C, and mixed at 1000 rpm for 1 h. The beads were then pelleted on a custom-made 96-well plate magnet (VP Scientific), and washed 3 times with 230 μL of PBS and 0.1% Tween 20, followed by resuspension of the beads in 230 μL of PBS and 0.1% Tween 20.

Beads were analyzed on a Simoa HD-1 Analyzer (Quanterix Corporation). Wilson et al.,

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J. Lab. Autom. 2016, 21, 533-47. The microtiter plate containing the labeled beads were loaded onto the instrument, along with enzyme conjugate and enzyme substrate (SβG and RGP, respectively), and consumables (Simoa disks, pipette tips, and reaction cuvettes). The Simoa measurements occurred in two steps on this instrument: liquid handling for enzyme labeling of the biotin-labeled probe beads; and, imaging and image analysis of the beads sealed in the Simoa disk. 100 µL of the solution containing beads in the microtiter plate was pipetted from the well into a reaction cuvette by a disposable tip pipettor. 100 µL of a solution containing 500 pM of SβG was added by a fixed tip pipettor to the reaction cuvette, the cuvette was shaken to disperse the beads, and incubated for 5 min. The beads were then magnetically separated and washed six times in 5× PBS and 0.1% Tween 20, and washed once in PBS. After aspiration of PBS, 25 µL of 100 µM RGP in PBS was added by a disposable tip pipettor to the reaction cuvette, the beads were mixed, and 15 µL of this bead-substrate mixture was transferred into an inlet port of an array on a Simoa disk. The beads were pulled by vacuum over the array of femtoliter wells, and the beads settled into the array of femtoliter wells. The beads were sealed with oil, and the single enzyme signal was generated over 30 s. The arrays were fluorescently imaged at multiple wavelengths at submicron resolution as described previously. Wilson et al., Lab. Autom. 2016, 21, 533-47. These fluorescent images were used to identify the location of beads within the wells, and the enzyme activity associated with each bead. Image analysis software on the instrument determined the average number of enzymes per bead (AEB). Rissin, et al. Anal. Chem. 2011, 83, 2279-2285. From the resulting AEB values of calibrators of known concentration, the concentrations of miR-122 in samples of unknown concentration were determined from interpolation using linear curve fitting in Microsoft Excel. Each sample was analyzed in duplicate to provide a mean AEB and a standard deviation.

RESULTS

Simoa Assay Design

A schematic diagram of an assaying combining the single base labeling and Simoa developed for detecting miR-122 is shown in Fig. 1. The Simoa assay involves a specific 18-mer

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abasic peptide nucleic acid (PNA) probe that was complementary to miR-122. The cytosine base at the 9th position from the C-terminus of the PNA probe was replaced with a secondary amine group to yield a "blank" position in the capture sequence. Thymidine bases (3 total) in the PNA probe were modified with propionic acid at their gamma positions to improve hybridization efficiency (Rissin, et al. Anal. Chem. 2011, 83, 2279-2285), and the N-terminus of the probe was a primary amine.

The probe was covalently attached via its N-terminal amine to superparamagnetic beads presenting surface carboxyl groups. These beads were then incubated with a solution containing the sample, an aldehyde-modified cytosine base that contains biotin (Fig. 4, panel B), and a reducing agent, in reaction buffer with a total volume of 50 μL. If miR-122 was present in the sample then it hybridized to the complementary capture probe on the beads (Fig. 1, panel I). As the target sequence (SEQ ID NO: 2) has a guanine at the 9th position from the 5'-end that lines up with the "blank" position in the capture probe, the cytosine base binds to this guanine and places the aldehyde in close proximity to the secondary amine on the probe backbone. The aldehyde and amine reacted to form a stable iminium complex that was then reduced to a tertiary amine by reductive amination, thereby covalently incorporating biotin into the probe attached to the beads. Molecules with the same sequence but a single base mis-match at the 9th position, hybridize but a stable iminium group did not form and biotin was not incorporated (Fig. 1, panel II). Non-complementary sequences do not hybridize to the capture probe and biotin was not incorporated (Fig. 1, panel III).

The incorporated biotin labels were then labeled with an enzyme, streptavidin-β-galactosidase (SβG). At low concentrations of miR-122, the ratio of enzyme labels to beads was <1, so that the distribution of enzymes on the beads followed a Poisson distribution [8], and single miR-122 molecules were labeled. These enzyme-labeled miR-122 molecules were detected by loading the beads into arrays of 216,000 microwells in the presence of a fluorogenic substrate of β-galactosidase. The wells were sealed with oil, so that the product of the enzyme-substrate reaction was confined to a small volume (~ 50 fL). Single beads and associated enzyme activity in the wells were measured using a fluorescent imager with sub-micron

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resolution. The fraction of active beads and average number of enzymes per bead (AEB) were then determined as previously described. Bland et al., Lancet 1986, 1, 307-10.

Sensitivity and Specificity of the Single Base Labeling-Simoa Assay

The analytical sensitivity of the Simoa assay was measured as the limit of detection (LOD) of miR-122. AEB values for buffered solutions spiked with known concentrations of a synthetic calibrator for miR-122 ranging from 0 to 1500 pM were determined using the Simoa assay (Fig. 2 and Table 2). The LOD of miR-122 using the Simoa assay was 500 fM.

The specificity of the Simoa assay was determined using two nucleic acid molecules (Table 2). First, a random, off-target miRNA molecule (miR-39) was tested. AEB values for the off-target miRNA were at background (Fig. 7). Second, a molecule that differed in sequence from miR-122 by a single base at the complement to the labeling position was tested. AEB values for the single base mismatch miR-122 did not increase significantly over background (Fig. 2). Concentrations up to 15 μM of the single based mismatched molecule were also tested with no increase in signal above background (Table 3). Based on these data, the analytical specificity of the assay to a single-base mismatch was $>3 \times 10^7$-fold.

Table 2. Nucleic acid sequences of synthetic calibrators for miR-122.

SEQ ID NO:	Name	Sequence (5'-3')
3	Calibrator for miR-122*	TGGAGTGTGACAATGGTGTTTG
4	Calibrator for single base mismatch miR-122*	TGGAGTGTTACAATGGTGTTTG
5	Off-target miR-39†	UCACCGGGUGUAAAUCAGCUUG

*DNA analogs were used as calibrators for measuring miRNA in the clinical samples. DNA enabled long term storage of calibrators and more reliable determination of miR-122 concentration in samples. It was previously shown that uracil and thymidine have similar efficiencies for specifically templating the dynamic covalent chemical reaction between aldehyde-modified cytosine and secondary amine of the abasic PNA probe upon duplex formation (Bowler Ph.D. Dissertation, University of Edinburgh, UK, 2011). Equivalent performance of RNA and DNA calibrators for miR-122 in the assay presented here was determined (data not shown).

†miRNeasy Serum/Plasma Spike-In Control (Cat No./ID:219610, Qiagen):10 pmol lyophilized *C.elegans* miR-39 miRNA mimic [5].

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Table 3. AEB values for single base mismatch miR-122 from 0 pm to 15 mM.

Concentration of mismatch miR-122	Mean AEB	SD	CV	Signal-to-background ratio
15 μM	0.086	0.002	2%	0.92
1.5 μM	0.071	0.004	6%	0.76
150 nM	0.079	0.016	20%	0.84
15 nM	0.083	0.014	17%	0.89
1.5 nM	0.091	0.009	10%	0.98
150 pM	0.093	0.007	7%	1.00
15 pM	0.089	0.004	4%	0.96
0 pM (background)	0.093	0.009	9%	1.00

These results demonstrate that the single-base labeling/Simoa assay provided herein is both sensitive and specific for miR-122. The LOD of miR-122 obtained using the Simoa assay was compared to the LOD of miR-122 determined using a PCR kit that is widely used to measure microRNA. Kroh et al., Methods, 2010, 50, 298-301. The LOD of the PCR assay was determined by measuring the Ct value of buffer solutions spiked with known concentrations of the synthetic calibrator for miR-122 ranging from 0 to 10,000 fM (Table 4). The LOD of miR-122 using the PCR assay was 2.6 fM as calculated from a plot of Ct values as a function of miR-122 concentration (Fig. 6). Previous reports provided that the LOD of miR-122 using a conventional, analog bead-based assay was 300 pM. Rissin, et al. Anal. Chem. 2011, 83, 2279-2285.

Table 4. Ct values determined using PCR as a function of the concentration of miR-122.

Concentration (fM)	Ct values	Mean Ct	SD	CV

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0	37.10841	n.d.	n.d.	37.108	n.d	n.d
0.1	35.75835	n.d.	n.d.	35.75835	n.d.	n.d.
1	35.65074	36.61235	36.83607	36.366	0.630	1.7%
10	31.58425	31.55038	31.44276	31.526	0.074	0.2%
100	28.31135	27.97488	28.05834	28.115	0.175	0.6%
1,000	24.08295	24.15437	24.16391	24.134	0.044	0.2%
10,000	19.94744	19.8787	20.23545	20.021	0.189	0.9%

The concentration is the concentration of miR-122 in the reverse transcription (RT) reaction. One RT reaction was carried out, and PCR performed on three aliquots from the RT reaction. n.d. = not determined.

Taken together, results from this study show that the single base labeling-Simoa assay provides fM range detection of miR-122, which is similar to miR-122 concentrations that can be detected using RT-PCR. The fM range limit of detection is approximately 500 times more sensitive than a corresponding analog bead-based assay.

Example 2: Clinical Validation of Single Base Labelling-Simoa Assay for Ultrasensitive Detection and Quantification of MiR-122 in Serum

The Simoa assay provided herein allows detection of short nucleic acids with high specificity using just a single probe, rather than multiple probes and primers used in most approaches for measuring miRNA. This specificity resulted from specific hybridization between the target and probe sequences, and incorporation of a single label. The use of a single probe greatly simplifies the measurement of short sequences of nucleic acids by simplifying probe design, and reducing the number of interactions that need to be screened for cross-reactivity in multiplex assays.

The Simoa assay provided herein is a sensitive and specific assay for detection of nucleic acids (*e.g.*, miR-122). The Simoa assay may be used for detection of any nucleic acid biomarkers, such as nucleic acid biomarkers of liver toxicity, cancer, and sepsis. Alternatively, or in addition to, the Simoa assay provided herein may be used for detection of interfering

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nucleic acid therapies, and measurement of guide RNA used for gene editing systems, such as CRISPR/Cas9.

MATERIALS AND METHODS

Patients with Liver Injury

Samples from 4 patients (Patient Identifiers 3-4, 6, and 9) with acetaminophen (APAP) induced liver injury were recruited as part of the Markers and Paracetamol Poisoning Study (MAPP). Adult patients (16 years old and over) were recruited to the MAPP study if they fulfilled the study inclusion and exclusion criteria. Full informed consent was obtained from every participant and ethical approval for this study was from the South-East Scotland Research Ethics Committee. The inclusion criteria were: a history of APAP overdose that the treating clinician judged to warrant treatment with intravenous acetylcysteine as per the contemporaneous UK guidelines; the first blood sample collected within 24 hours of last APAP ingestion; and, the patient has capacity to consent. Patients were excluded if any of the following applied: patient detained under the Mental Health Act (UK); patient has known cognitive impairment; inability to provide informed consent for any reason; or an unreliable history of overdose. Patients having taken a single acute APAP overdose were recruited at the Royal Infirmary of Edinburgh, UK. The demographic details and clinical chemistry measurements performed on the patients are shown in Table 5.

Table 5. Demographics and clinical chemistry results for patients with liver injury.

Patient	Age (yrs)	Sex	ALT activity (U/L) (ULN 50)	INR	Serum Creatinine (µmol/L)	ALP activity (U/L)	Bilirubin (µmol/dL)
3	56	M	2150	1.3	56	141	23
4	23	F	487	1.5	60	64	14
6	46	M	2963	1.4	62	139	23

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9	63	F	726	1.2	68	169	14

Healthy Volunteers

The study was approved by the local research ethics committee (East Midlands – Nottingham 1 Research Ethics Committee), and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants. A total of 4 adults (23-42 years old) were recruited to this study (Patient identifiers HV1-4). Healthy volunteers were eligible if they had no history of liver disease, they were taking no medications, and they were willing to give blood samples by venepuncture.

Collection and Preparation of Clinical Samples

For both healthy volunteers and patients with liver injury, blood samples were immediately centrifuged at 11,000 × g for 15 min at 4°C, after which serum was separated into aliquots and frozen at −80°C.

Control Sample Preparation

Serum from a healthy donor, who was negative for acute liver failure, was used for spike-in experiments. The blood from the donor was centrifuged at 11,000 × g for 15 min at 4°C after which serum was separated into 6 aliquots. A synthetic calibrator for miR-122 (SEQ ID NO: 3; Table 2) at different concentrations were added to each aliquot to yield 6 control samples (Control samples 1 to 6 containing: 10 nM, 10 nM, 1 nM, 1 nM, 100 pM, and 10 pM, respectively). These samples were frozen at −80°C and subsequently processed in an identical fashion as the clinical samples.

RNA Isolation from Serum Samples

RNA was isolated from serum using the miRNeasy Serum/Plasma kit (Qiagen) according to manufacturer's protocol. Briefly, 500 µL QIAzol lysis reagent was added to 100 µL thawed serum, mixed, and incubated at 23°C for 5 min. To calibrate the concentration of miR-122 and as a control to determine the efficiency of recovery and reverse transcription of miRNA, 3.5 µL

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of cel-miR-39-3p (1.6×10^8 copies/μL) was added to each sample, in addition to 100 μL chloroform. Following shaking, incubation, and centrifugation, the upper aqueous phase was transferred, and 450 μL of ethanol was added and transferred to the RNeasy MinElute column. The column was washed with buffers RWT and RPE from the miRNeasy kit, and 80% ethanol, followed by drying and elution in 14 μL RNasefree water; total miRNA was recovered in ~12 μL of water ([cel-miR-39-3p] = 4×10^7 copies/μL). Quantification and quality assessment of small RNA, including the miRNA fraction, were performed using the Small RNA Assay kit (Agilent Technologies). Purified RNA was stored at <−80°C before analysis with Simoa and RT-PCR.

RESULTS

Clinical Validation

Clinical validation of the single base labeling-Simoa assay for detecting miR-122 in patient serum was performed using the serum of patients who had overdosed on acetaminophen and sustained clinically significant liver injury, and in the serum of healthy individuals.

Concentrations of miR-122 in clinical samples and serum control samples spiked with synthetic miR-122 were determined using the Simoa assay (Table 6) and RT-PCR (Table 7). AEB values for samples from healthy volunteers were close together and slightly offset from the background AEB values, and AEB values for samples from patients having liver toxicity were significantly above background AEB values (Fig. 3, panels A and B). Ct values for samples from healthy volunteers were near background, and Ct values for samples from patients having liver toxicity were significantly above background (Fig. 8).

Table 6. Concentration of miR-122 in clinical samples determined using the Simoa assay.

Patient ID	Sample number*	Sample volume (μL)	Concentration in 50 μL reaction (pM)	Recovery (%)†	Concentration in sample (pM)	Spiked concentration
HV1	1	90	1.51	51	*2.65‡*	
	2	90	3.91	81	*4.27‡*	
HV2	1	95	1.51	84	*1.51‡*	
	2	95	3.81	94	*3.41‡*	

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HV3	1	95	1.34	83	*1.37‡*	
	2	95	2.95	84	*2.97‡*	
HV4	1	100	1.13	78	*1.17‡*	
	2	100	3.32	70	*3.81‡*	
3	1	105	2.85	41	5.28	
	2	105	6.46	58	8.54	
4	1	95	11.57	73	13.34	
	2	95	13.66	79	14.56	
6	1	95	19.67	80	20.71	
	2	95	31.43	64	41.35	
9	1	90	3.21	75	3.81	
	2	90	6.25	98	5.67	
Controls						
1	1	100	n.d.	49	n.d.	10000
2	2	100	n.d.	67	n.d.	10000
3	3	100	162.66	56	232.13	1000
4	4	100	126.47	43	233.96	1000
7	5	100	13.78	45	24.31	100
8	6	100	3.69	44	6.70	10

n.d. = not determined

*For each patient and healthy volunteer, miRNA was extracted from two samples and its concentration was determined.

† Recovery of miRNA from the sample was determined by PCR using miR-39 control spikes. The concentration in samples were corrected for this recovery.

‡ The AEB values determined for these samples were close together and slightly offset from the background AEB (Fig. 3, panels A and B). This observation could be explained by a systematic difference in the background signal of the sample matrix compared to the calibration buffer, rather than by specific measurement of miR-122. Without further experimental confirmation, we regard these samples as not quantified for miR-122.

Table 7. Concentration of miR-122 in clinical samples determined using the PCR assay.

Patient ID	Sample number*	Volume sample (µL)	Ct	Copies/µL in PCR reaction	Copies in sample	Recovery (%)	Concentration in sample (pM)	Spiked concentration
HV1	1	90	34.4	76	3,345,710	51	*0.12†*	
	2	90	34.1	92	4,050,941	81	*0.09†*	
HV2	1	95	32.4	281	12,355,108	84	*0.26†*	
	2	95	32.9	200	8,783,626	94	*0.16†*	
HV3	1	95	33.3	156	6,875,551	83	*0.15†*	
	2	95	33.4	147	6,461,253	84	*0.13†*	
HV4	1	100	33.2	169	7,419,621	78	*0.16†*	
	2	100	33.4	152	6,705,783	70	*0.16†*	
3	1	105	29.1	2,295	100,969,205	41	3.88	
	2	105	27.9	4,942	217,428,017	58	5.96	

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4	1	95	25.6	21,300	937,196,939	73	22.41	
	2	95	25.1	29,113	1,280,986,047	79	28.31	
6	1	95	24.8	35,139	1,546,107,754	80	33.74	
	2	95	24.3	47,006	2,068,243,613	64	56.41	
9	1	90	27.4	6,702	294,868,894	75	7.24	
	2	90	27.8	5,120	225,300,348	98	4.24	
Controls								
1	1	100	14.5	23,245,908	1,022,819,938,047	49	34380.66	10000
2	2	100	14.5	23,790,920	1,046,800,483,612	67	25999.14	10000
3	3	100	18.7	1,653,095	72,736,170,424	56	2151.74	1000
4	4	100	19.0	1,363,925	60,012,704,821	43	2301.44	1000
7	5	100	22.7	130,673	5,749,590,955	45	210.31	100
8	6	100	26.4	12,485	549,318,183	44	20.67	10

*For each patient and healthy volunteer, miRNA was extracted from two samples and its concentration was determined.

† Recovery of miRNA from the sample was determined by PCR using miR-39 control spikes. The concentration in samples were corrected for this recovery.

‡ The Ct values of samples from healthy volunteers were above or slightly below those of background samples (Table 3), so the detection of miR-122 in these samples is ambiguous.

The concentrations of miR-122 in serum from patients determined using the Simoa assay were well correlated with those obtained using the PCR assay (Fig. 9). The Bland-Altman plot for concentrations of miR-122 in serum samples determined using the Simoa assay and the PCR assay showed that the miR-122 concentration as measured by the Simoa assay is lower than those obtained using the PCR assay with an average bias of 6.1 pM (95% limit of agreement = -8.5–20.8 pM) (Fig. 10).

In sum, these data indicate that the sensitivity and specificity of the Simoa assay was sufficient to measure miR-122 in patients, and showed a clear distinction between healthy controls and those with clinical liver toxicity after drug overdose. The sensitivity and specificity of the Simoa assay was also comparable to that of the RT-PCR assay.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential

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characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

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What Is Claimed Is:

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

(i) providing a sample suspected of containing a nucleic acid of interest;

(ii) providing a plurality of beads, on which a peptide nucleic acid (PNA) probe is immobilized, wherein the PNA probe comprises a nucleotide sequence that is complementary to the nucleic acid of interest or a portion thereof, and wherein the PNA probe lacks a base at a position corresponding to a target nucleotide in the nucleic acid of interest;

(iii) incubating the sample with the plurality of beads under conditions allowing for formation of a nucleic acid/PNA complex;

(iv) contacting the plurality of beads with a modified base that is complementary to the base of the target nucleotide in the nucleic acid of interest, wherein the modified base is conjugated to a first binding moiety and modified with a chemical group that reacts with the PNA probe;

(v) contacting the plurality of beads with a second binding moiety that binds the first binding moiety and comprises a detectable label; and

(vi) determining the fraction of beads associated with at least one detectable label, which is indicative of the level of the nucleic acid of interest in the sample.

2. The method of claim 1, wherein the first binding moiety comprises biotin and the second binding moiety comprises streptavidin.

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

(i) providing a sample suspected of containing a nucleic acid of interest;

(ii) providing a plurality of beads, on which a peptide nucleic acid (PNA) probe is immobilized, wherein the PNA probe comprises a nucleotide sequence that is complementary to the nucleic acid of interest or a portion thereof, and wherein the PNA probe lacks a base at a position corresponding to a target nucleotide in the nucleic acid of interest;

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(iii) incubating the sample with the plurality of beads under conditions allowing for formation of a nucleic acid/PNA complex;

(iv) contacting the plurality of beads after with a modified base that is complementary to the base of the target nucleotide in the nucleic acid of interest, wherein the modified base is conjugated to a first binding moiety and modified with a chemical group that reacts with the PNA probe;

(v) contacting the plurality of beads with a second binding moiety that binds the first binding moiety;

(vi) contacting the plurality of beads with a third binding moiety that binds the second binding moiety and comprises a detectable label; and

(vii) determining the fraction of beads associated with at least one detectable label, which is indicative of the level of the nucleic acid of interest in the sample.

4. The method of claim 3, wherein the second binding moiety comprises an antibody specific to the first binding moiety.

5. The method of claim 4, wherein the antibody is biotinylated.

6. The method of claim 5, wherein the third binding moiety comprises streptavidin.

7. The method of any one of claims 3-6, wherein the first binding moiety is a fluorescein label.

8. The method of any one of claims 3-7, wherein the second binding moiety comprises a thiolated heterocabon chain which react specifically with first binding moiety.

9. The method of claim 8, wherein the thiolated heterocarbon moiety is biotinylated.

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10. The method of claim 9, wherein the third binding moiety comprises streptavidin.

11. The method of any one of claims 8-10, wherein the first binding moiety is a maleimide group.

12. The method of any one of claims 1-11, wherein the detectable label releases a signal directly.

13. The method of claim 12, wherein the detectable label is a dye or a fluorescent agent.

14. The method of any one of claims 1-11, wherein the detectable label releases a signal indirectly.

15. The method of claim 14, wherein the detectable label is a β-galactosidase.

16. The method of any one of claims 1-15, wherein the nucleic acid of interest is a microRNA.

17. The method of any one of claims 1-16, wherein the chemical group in the modified base is an aldehyde group, a ketone group, a thiol group, or a diol group.

18. The method of claim 1, wherein step (vi) is performed using single molecule arrays.

19. The method of claim 3, wherein step (vii) is performed using single molecule arrays.

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20. The method of claim 8, wherein step (vii) is performed using single molecule arrays.

21. The method of any one of claims 1-20, wherein the sample is a biological sample obtained from a subject.

22. The method of claim 20, wherein the nucleic acid of interest is a microRNA associated with a target disease.

23. The method of claim 21, wherein the subject is suspected of having the target disease.

24. The method of any one of claims 1-22, wherein the PNA probe has 10-30 monomer units.

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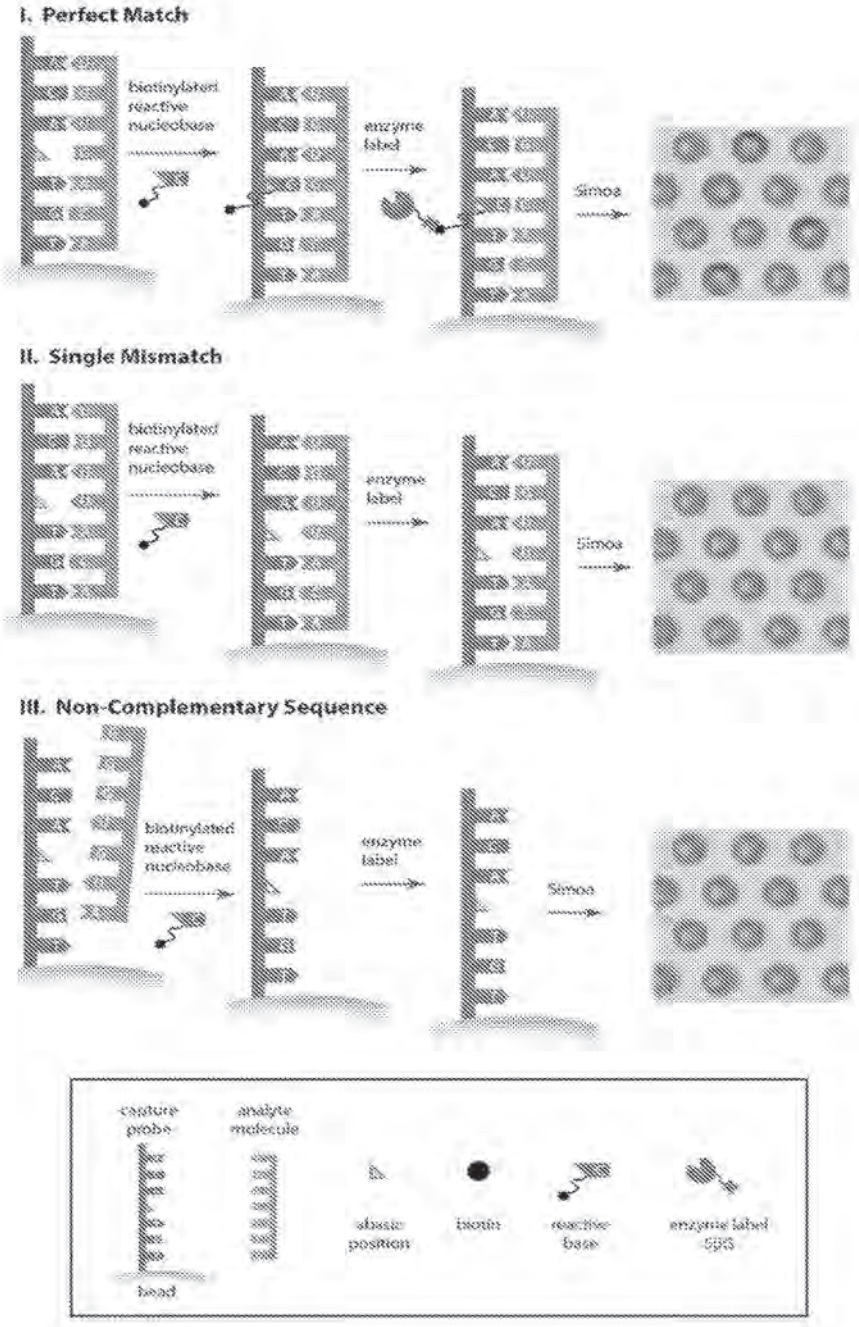

I. Perfect Match
biotinylated reactive nucleobase
enzyme label
Simoa
II. Single Mismatch
biotinylated reactive nucleobase
enzyme label
Simoa
III. Non-Complementary Sequence
biotinylated reactive nucleobase
enzyme label
Simoa
capture probe
analyte molecule
abasic position
biotin
reactive base
enzyme label (SBG)
bead

Fig. 1

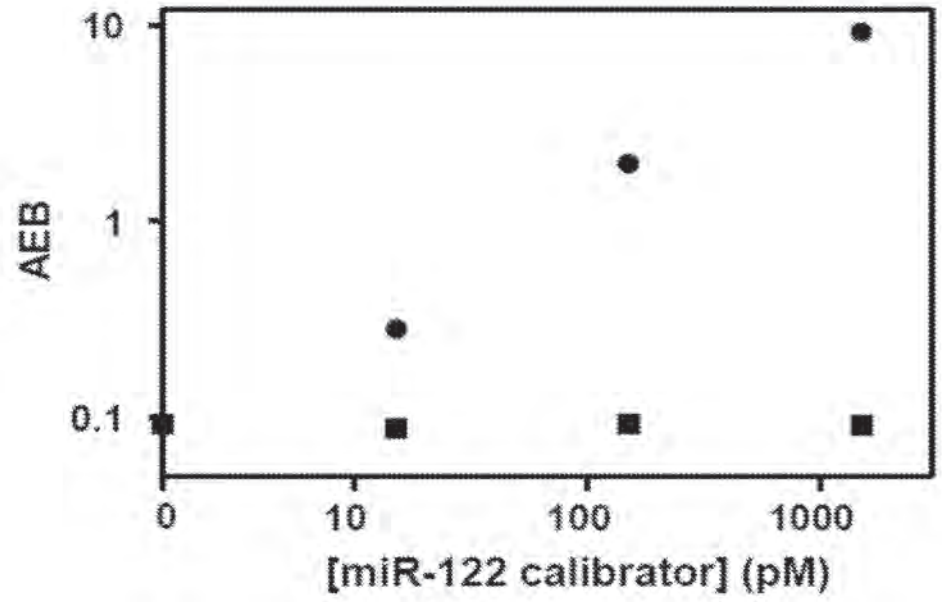


Fig. 2

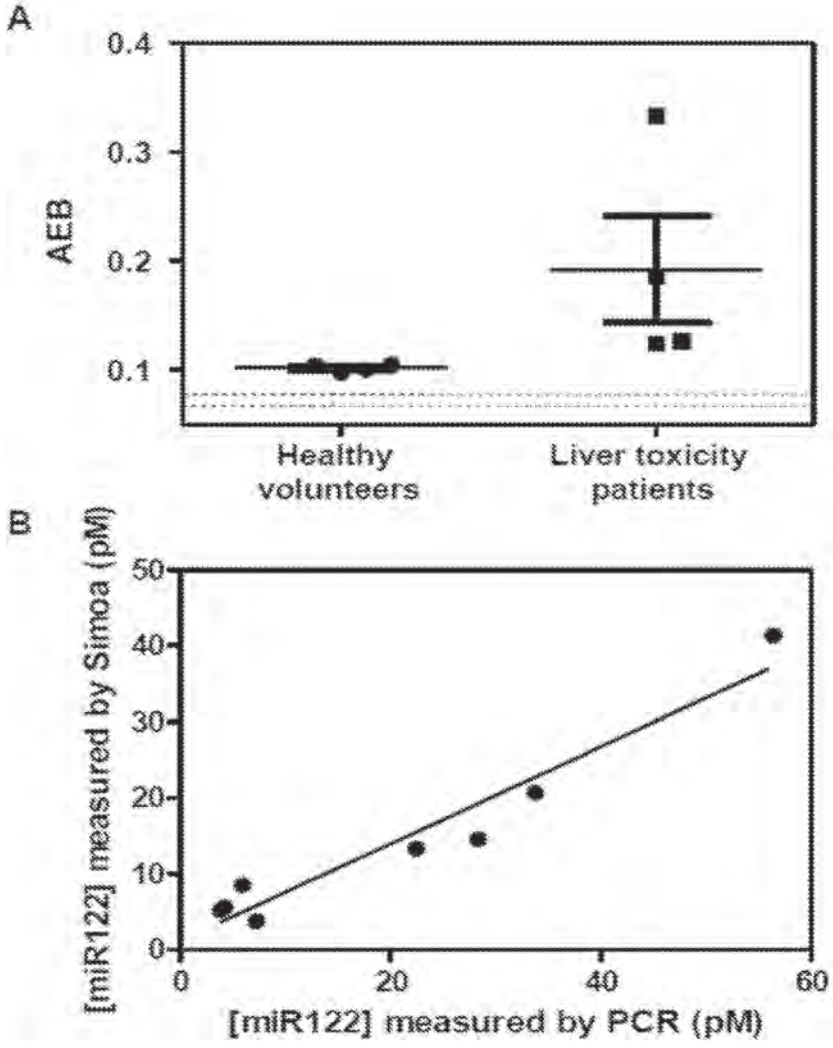

A
0.4
0.3
0.2
0.1
AEB
Healthy volunteers
Liver toxicity patients
B
[miR122] measured by Simoa (pM)
50
40
30
20
10
0
0
20
40
60
[miR122] measured by PCR (pM)

Fig. 3

A.


H2N
O
O
H
N
O
O
O
N
H
Capture sequence

B.

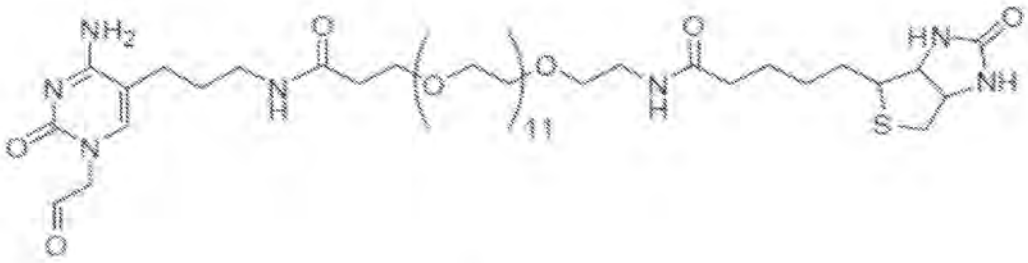

NH2
N
O
N
O
H
N
O
O
O
11
O
H
N
O
HN
O
NH
S

Fig. 4

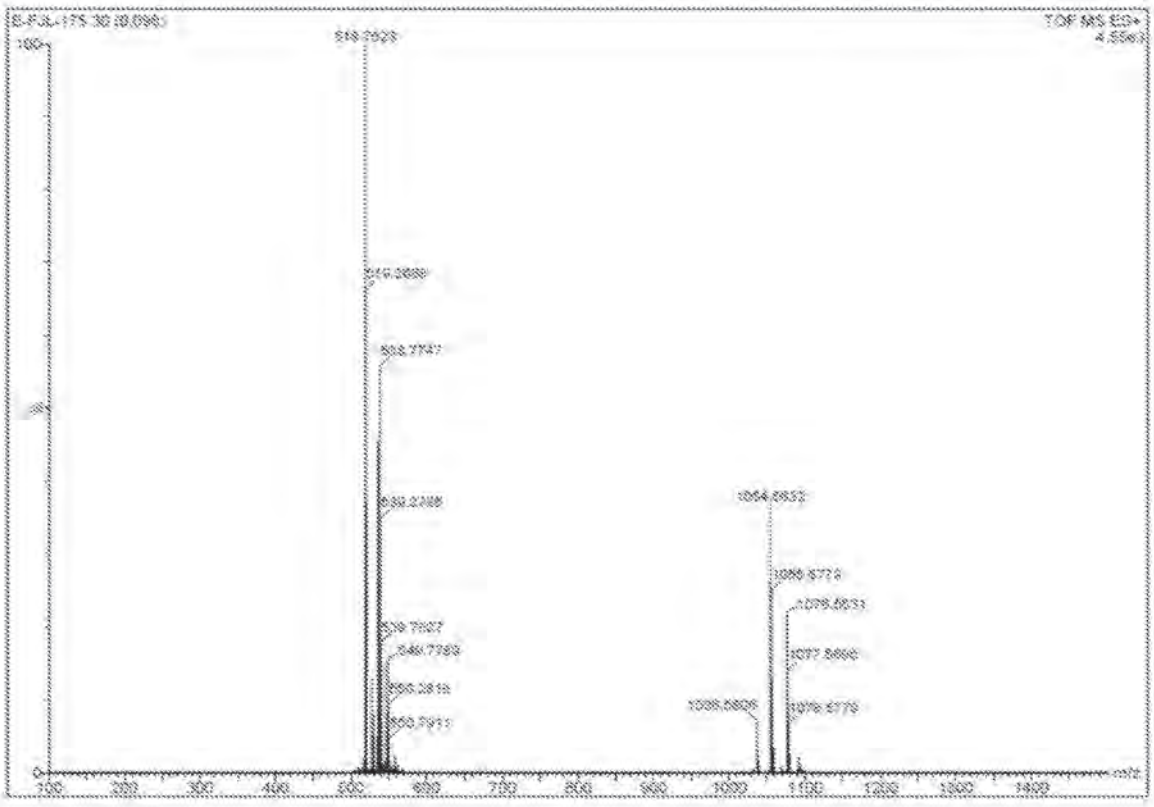


Fig. 5

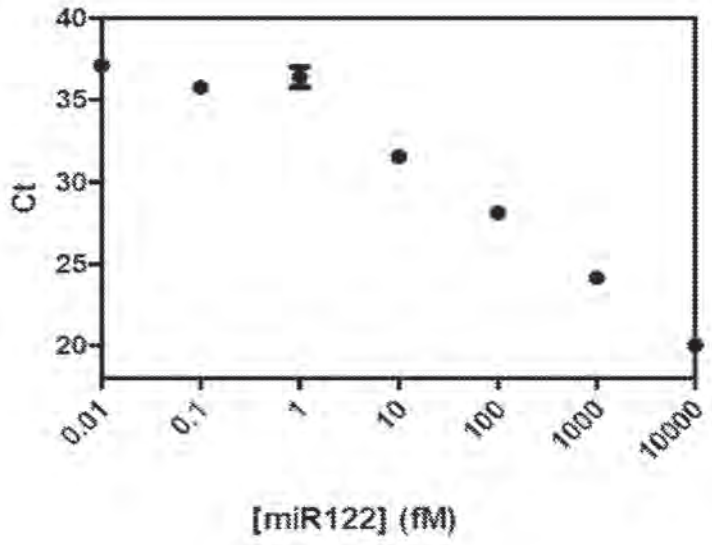


Fig. 6

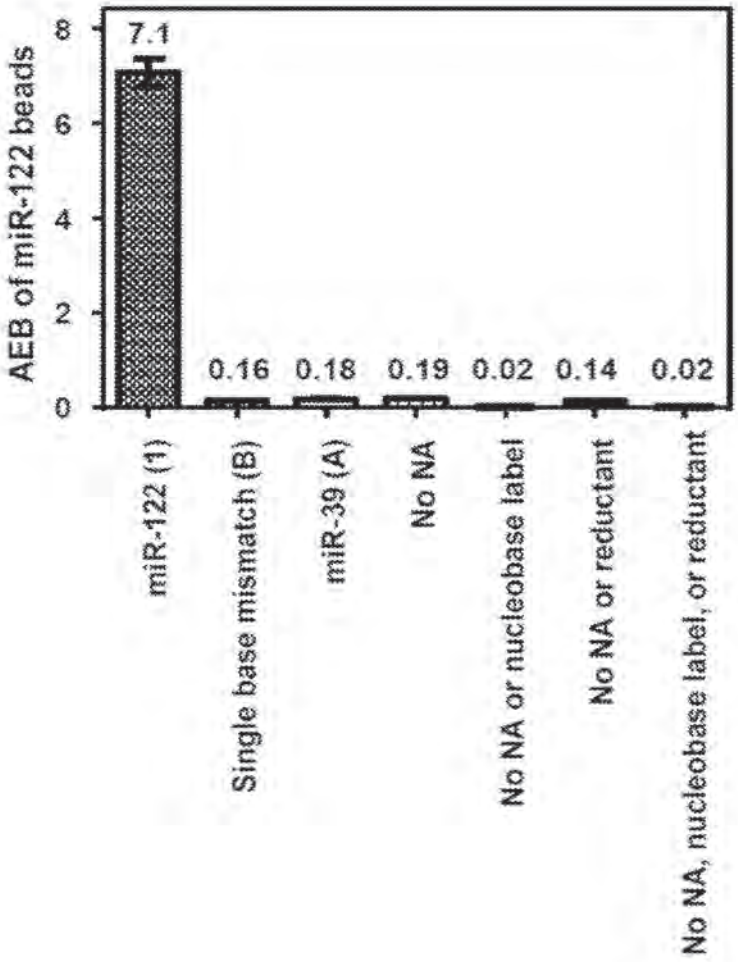

AEB of miR-122 beads
0
2
4
6
8
7.1
0.16
0.18
0.19
0.02
0.14
0.02
miR-122 (1)
Single base mismatch (B)
miR-39 (A)
No NA
No NA or nucleobase label
No NA or reductant
No NA, nucleobase label, or reductant

Fig. 7

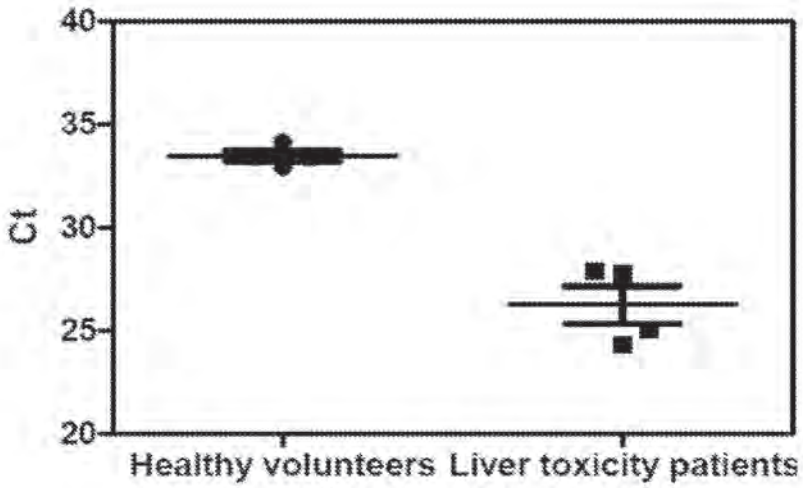

40
35
30
25
20
Ct
Healthy volunteers
Liver toxicity patients

Fig. 8

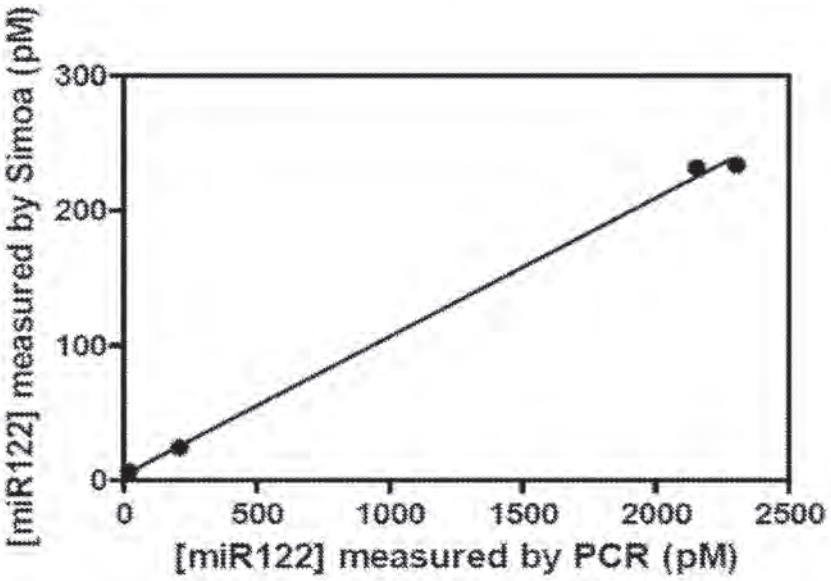

300
200
100
0
[miR122] measured by Simoa (pM)
0
500
1000
1500
2000
2500
[miR122] measured by PCR (pM)

Fig. 9

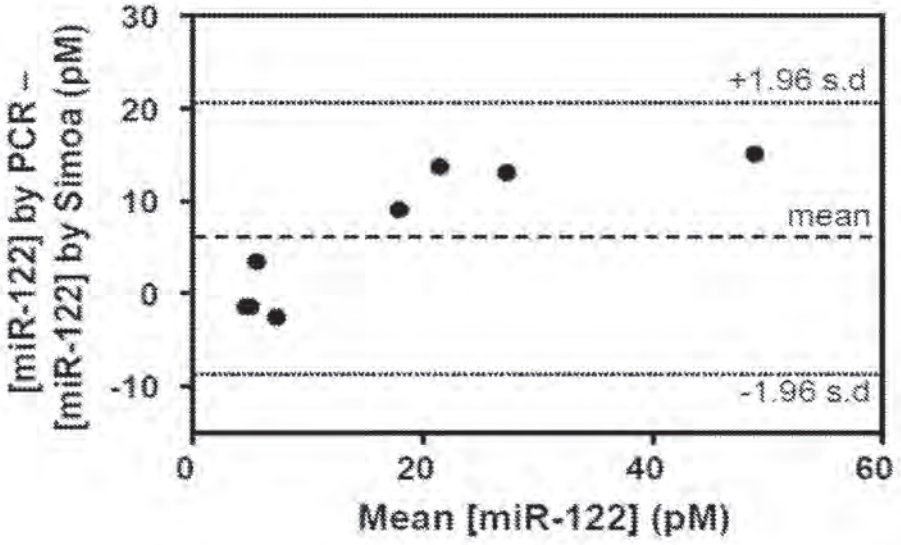

30
20
10
0
-10
+1.96 s.d
mean
-1.96 s.d
0
20
40
60
[miR-122] by PCR – [miR-122] by Simoa (pM)
Mean [miR-122] (pM)

Fig. 10

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(54) **Title:** METHODS AND SYSTEMS FOR THE DETECTION OF ANALYTE MOLECULES

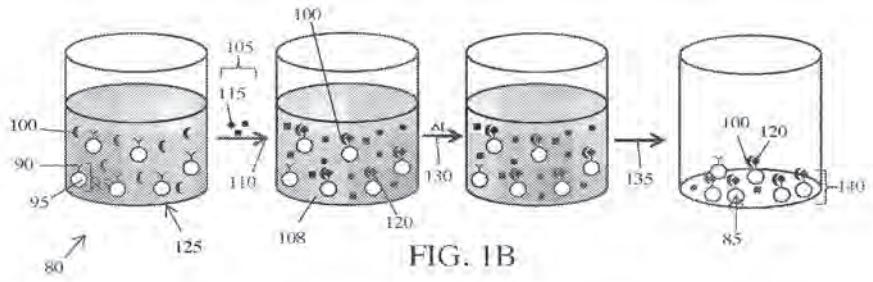


FIG. 1B

(57) **Abstract:** Methods and systems for the detection of analyte molecules are generally described. In some embodiments, a method may comprise using a stabilizing agent prior to, during, and/or after one or more steps of a detection assay. The stabilizing agent may serve to maintain and/or increase the detectable signal indicative of the analyte during one or more assay steps and/or until the signal is measured. In such cases, the stabilizing agent may reduce and/or prevent one or more phenomena associated with signal decay, such as, e.g., dissociation between, aggregation of, and/or denaturation of analyte molecules and/or detection molecules. In some embodiments, the stabilizing agent can be used to improve the sensitivity of new and existing assays with little or no adverse effect on specificity. The methods and systems, described herein, may be used for a plethora of applications, including the detection or quantification of low levels of analyte molecules.

[Continued on next page]

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METHODS AND SYSTEMS FOR THE DETECTION OF ANALYTE MOLECULES

Field of the Invention

Methods and systems for the detection of analyte molecules are generally described.

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. One feature of typical known methods and/or systems for detecting or quantifying low concentrations of a particular analyte molecule 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 are needed, especially in samples where such molecules are present at very low concentration

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Summary of the Invention

Methods and systems for the detection of analyte molecules are generally described. 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 one set of embodiments, methods are provided. In one embodiment a method for perform an assay comprises combining a stabilizing agent and an assay composition to form a first liquid mixture comprising a liquid, wherein the assay composition comprises detection molecules associated with analyte molecules. The method further comprises separating at least a portion of a liquid from at least a portion of the first liquid mixture to form a pellet and a separated liquid, wherein the pellet comprises at least a portion of the assay composition and at least a portion of the stabilizing agent. The method further comprises removing greater than or equal to about 95% of the separated liquid from the pellet, wherein the stabilizing agent comprises a molecule having greater than four hydroxyl groups.

In another embodiment, a method for preventing or reducing signal decay in an assay comprises combining a stabilizing agent with an assay composition to form a first liquid mixture, wherein the assay composition comprises detection molecules associated with analyte molecules; removing greater than or equal to about 95% of a liquid from the first liquid mixture to form a concentrated composition comprising the assay composition and the stabilizing agent; and storing the concentrated composition for at least 1 minute. The method further comprises reconstituting the concentrated composition to form a second liquid mixture and measuring a signal used to determine a concentration of the analyte molecules in the second liquid mixture, wherein a magnitude of the signal measured in the measuring step is greater than or equal to about 10% of the magnitude of a signal used to determine a concentration of the analyte molecules of the analyte molecules in an otherwise identically prepared second liquid mixture except not subjected to the storage step.

Other advantages and novel features of the present invention will become apparent from the following detailed description of various non-limiting embodiments of the invention when considered in conjunction with the accompanying figures. In cases where the present specification and a document incorporated by reference include conflicting and/or inconsistent disclosure, the present specification shall control.

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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. 1A is a schematic flow diagram depicting a detection method without a stabilizing agent, according to certain embodiments;

FIG. 1B is a schematic flow diagram depicting a detection method with a stabilizing agent, according to certain embodiments;

FIG. 2A is a schematic flow diagram depicting a detection method without a stabilizing agent, according to certain embodiments;

FIG. 2B is a schematic flow diagram depicting a detection method with a stabilizing agent, according to certain embodiments;

FIG. 2C is a schematic flow diagram depicting a detection method with a stabilizing agent, according to certain embodiments;

FIG. 3A is a schematic flow diagram depicting a detection method without a stabilizing agent, according to certain embodiments;

FIG. 3B is a schematic flow diagram depicting a detection method with a stabilizing agent, according to certain embodiments;

FIG. 3C is a schematic flow diagram depicting a detection method with a stabilizing agent, according to certain embodiments; and

FIG. 4 is a graph of average intensity versus time for a detection method, according to certain embodiments;

FIG. 5 is a graph of average enzyme per bead (AEB) versus time for a detection method, according to certain embodiments;

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FIG. 6 is a graph of average intensity versus time for a detection method, according to certain embodiments;

FIG. 7 is a histogram of average enzyme per bead (AEB) for various assay mixtures, according to certain embodiments;

FIG. 8 is a histogram of average enzyme per bead (AEB) for various assay mixtures, according to certain embodiments;

FIG. 9 is a graph of average enzyme per bead (AEB) versus percent sucrose, according to certain embodiments;

FIG. 10 is a graph of percent bead fill versus percent sucrose, according to certain embodiments;

FIG. 11 is a graph of normalized average enzyme per bead (AEB) versus time, according to certain embodiments;

FIG. 12A is a schematic flow diagram depicting a detection method without a stabilizing agent, according to certain embodiments; and

FIG. 12B is a schematic flow diagram depicting a detection method with a stabilizing agent, according to certain embodiments.

Detailed Description

Methods and systems for the detection of analyte molecules are generally described. In some embodiments, a method may comprise using a stabilizing agent (e.g., polyol, carbohydrate, disaccharide) prior to, during, and/or after one or more steps of an assay. The stabilizing agent may serve to maintain and/or increase a detectable signal indicative of (e.g., presence of, absence of, quantity of) an analyte molecule during one or more assay steps and/or until the signal is measured. In such cases, the stabilizing agent may reduce and/or prevent one or more phenomena associated with signal decay, such as, e.g., dissociation between, aggregation of, and/or denaturation of analyte molecules and/or detection molecules. For example, the stabilizing agent may prevent dissociation between analyte molecules and detection molecules during storage and/or desolvation. In some embodiments, the stabilizing agent can be used to improve the sensitivity (e.g., detection limit) of new and existing assays with little or no adverse effect on specificity. The methods and systems, described herein, may be used for a plethora of applications, including the detection or quantification of low levels of analyte molecules.

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In some embodiments, the assay is a detection assay. Detection assays often rely on the measurement of a signal indicative of an analyte molecule to determine the presence, absence, and/or quantity of the analyte. However, many analyte molecules do not produce such a signal and/or do not produce a detectable signal at certain concentrations. Accordingly, many detection assays utilize detection molecules that specifically (e.g., exclusively) associate with the analyte molecules and can be used to produce and/or amplify a signal indicative of the analyte molecules. Many such detection assays require steps, such as manipulation of the analyte molecule after association with the detection molecules (e.g., drying, suspension, washing, transfer), which results in a delay before measurements that can result in a change in the signal. This change in the signal may reduce the sensitivity and/or dynamic range of the assay.
The delay between exposure of the analyte molecule to detection molecules and signal measurement (e.g., the time in which a signal of the detection molecules is determined) often results in a signal reduction. For example, in embodiments in which the detection molecule is an antibody and the analyte molecule is an antigen, a time delay between exposure and measurement may allow dissociation to occur between the detection molecule and analyte molecule. The amount of dissociation between the detection molecule and analyte molecule may increase with time, such that the signal measured after a delay is significantly lower than the signal measured immediately after exposure to the detection molecules. This reduction in the signal can reduce the sensitivity and/or accuracy of the assay. Therefore, it is desirable to reduce and/or eliminate any change in the signal.

The time delay between exposure and measurement may also adversely affect calibration curves and comparative analysis of samples. In some embodiments, the time delay between exposure and determining the signal often has greater impact when the signal measured is in or being compared to a calibration curve. For instance, a calibration curve produced from samples having different time delays between exposure and measurement may, in certain instances, be inaccurate. Moreover, even if the calibration curve was produced from samples having the same time delay, use of the calibration curve for samples measured with a different time delay may result in inaccuracies. In some assays, analysis requires comparison of multiple samples. In such assays, multiple samples are prepared at a single time point (e.g., a plurality of samples are exposed to detection molecules at the same time), but only a single sample can be analyzed at a time. Thus, the signal of the first sample would be determined at a much earlier time point

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following exposure to the detection molecule compared to another sample. Variation in time delay between replicates of the same sample can result in imprecision and high coefficient of variation of the measurement, i.e., there will be variability in the signal from test-to-test. This variation can lead to imprecision within a run, run-to-run, day-to-day, instrument-to-instrument, site-to-site, and user-to-user, for examples.

Existing methods have tried to address this problem by ensuring that every sample experiences the same time delay (e.g., the same timing of incubation, washing, and detection during an experiment). In this way, the time between exposure and measurement is fixed. Thus, any change in the signal due to the delay should be similar for all samples and is not expected to significantly affect the precision of the assay. However, in practice, this approach means that each sample has to be processed individually with the same delays, and/or all processed simultaneously. Thus, while the difference in time between exposure and determination could be minimized by keeping the time delay constant for each sample (e.g., sample 1 is exposed to detection molecules at time point X1 and the signal for sample 1 is determined Y minutes after time point X1, and sample 2 is exposed to detection molecules at time point X2 and the signal for sample 2 is determined Y minutes after time point X2, wherein time point X1 and X2 are different), control of the exposure and determination for any significant number of samples would be time consuming and is not possible with many automated assay systems and methods. Existing methods have also tried to address the problem of differences in time delay by applying a mathematical correction to the data to correct for differences in the time between exposure and measurement. While this approach is theoretically feasible, it is dependent on the change in the signal over time being stable and predictable, which is often not true in practice. Experimental variation in the change in the signal over time occur limiting the utility of the mathematical approach. Accordingly, improved methods for reducing and/or minimizing the change in signal due to time delays are needed.

The inventors have developed in the context of the present invention techniques and compositions that employ certain stabilizing agents to reduce and/or prevent signal decay when used prior to, during, and/or after certain assay steps. In some embodiments, polyols, such as sucrose and trehalose, may be used to reduce and/or prevent one or more phenomena associated with signal decay, such as dissociation, reduction of a measurable property of the detection molecules (e.g., enzymatic activity), aggregation, and/or denaturation associated with certain

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assay steps (e.g., desolvation, suspension, storage, prolonged time until measurement). Without being bound by theory, it is believed that the stabilizing agents, described herein, may maintain the structure that proteins and protein complexes form in aqueous solutions without water being necessarily present. In some embodiments, this stabilization of structure is achieved by stabilizing agents that maintain the hydrogen bond network between the surface of proteins and water so that they do not denature. For example, the stabilizing agent may be a non-ionic kosmotrope (e.g., trehalose and sucrose). Non-ionic kosmotropes have no net charge and strongly hydrogen bonding to water. The non-ionic kosmotropes may be used as a stabilizing agent to stabilize the structure of proteins and other biological molecules in solution. Without being bound by theory, it is believed that kosmotropes reduce the volume of water available to hydrate the larger surface exposed by denatured proteins, which tends to prevent the denaturation process. In some embodiments, the stabilizing agents, described herein, may have two or more (e.g., three or more, four or more, five or more, six or more) hydrogen bond donors and/or hydrogen bond acceptors. For example, the stabilizing agent may be a polyol or a sugar. In certain embodiments, the stabilizing agent may be a zwitterion kosmotrope. The zwitterion kosmotrope may have a similar stabilizing effects as the non-ionic kosmotrope. The methods and systems, described herein, may be used to improve the sensitivity and/or dynamic range of a wide variety of new and existing assays.

In some embodiments, a method for performing an assay is provided comprising combining a stabilizing agent and an assay composition (e.g., comprising analyte molecules associated with detection molecules) to form a first liquid mixture comprising a liquid. The assay composition may additionally comprise capture objects (e.g., beads) associated with the analyte molecules. At least a portion of a liquid from at least a portion of the first liquid mixture may be separated to form a pellet and a separated liquid, wherein the pellet comprises at least a portion of the assay composition and at least a portion of the stabilizing agent. Greater than or equal to about 95% of the separated liquid may then be removed from the pellet, wherein the stabilizing agent comprises a molecule having greater than four hydroxyl groups.

In other embodiments, a method for preventing or reducing signal decay in an assay is provided. In some embodiments, a stabilizing agent is combined with an assay composition to form a first liquid mixture, wherein the assay composition comprises detection molecules associated with analyte molecules. Greater than or equal to about 95% of a liquid from the first

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liquid mixture may be removed to form a concentrated composition (e.g., a pellet) comprising the assay composition and the stabilizing agent. The concentrated composition (e.g. pellet) may be stored for any desired period of time (e.g., for at least 5 seconds, for at least 30 seconds, for at least 1 minute, for at least 10 minutes, for at least 30 minutes, for at least 60 minutes, etc.). The concentrated composition (e.g., pellet) may be reconstituted to form a second liquid mixture. For example, a liquid may be added to the concentrated composition. The amount of liquid added may be the same amount of liquid that was removed during the removing step. A signal used to determine a concentration of the analyte molecules in the second liquid mixture may then be determined. In some cases, a magnitude of the signal measured in the measuring step is greater than or equal to about 10% (e.g., greater than or equal to about 20%, greater than or equal to about 30%, greater than or equal to about 40%, greater than or equal to about 50%, greater than or equal to about 60%, greater than or equal to about 70%, greater than or equal to about 80%, greater than or equal to about 85%, greater than or equal to about 90%, greater than or equal to about 95%) of a magnitude of a signal used to determine a concentration of the analyte molecules of the analyte molecules in an otherwise identically prepared second liquid mixture except not subjected to the storage step. In certain embodiments, the magnitude of the signal measured in the measuring step is greater than or equal to about 80% (greater than or equal to about 85%, greater than or equal to about 90%, greater than or equal to about 95%) of a magnitude of a signal used to determine a concentration of the analyte molecules of the analyte molecules in an otherwise identically prepared second liquid mixture except not subjected to the storage step.

In some embodiments wherein capture objects are employed, the pellet or concentrated composition comprises greater than about 50%, or greater than about 60%, or greater than about 70%, or greater than about 80%, or greater than about 90%, or greater than about 95%, or more, of the capture objects initially provided. In some embodiments, the pellet or concentrated composition comprises at least 1000 capture objects, or at least 2000 capture objects, or at least 3000 capture objects, or at least 5000 capture objects, or at least 10,000 capture objects, or at least 20,000 capture objects, or at least 30,000 capture objects, or at least 50,000 capture objects, or at least 100,000 capture objects.

Additional details of the methods will now be described in connection with the figures. As described herein, a stabilizing agent may be used in one or more assay steps to reduce and/or

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prevent signal decay. In some embodiments, as illustrated in FIG. 1 – FIG. 3, the stabilizing agent may be used to reduce and/or prevent dissociation between components (e.g., analyte molecules, detection molecules, capture components, etc.) during one or more assay steps. For instance, the stabilizing agent may be used to reduce and/or prevent dissociation between components that are reversible associated, e.g., via an affinity interaction. As will be known to those of ordinary skill in the art, some reversible affinity interactions, such as those between certain analyte molecules (e.g., antigen) and detection molecules (e.g., capture object, antibody), are governed by thermodynamics. Accordingly, at some point during an assay step, some dissociation may occur. This may result in a reduced number of analyte molecules being detected than are actually present as illustrated in FIG. 1A, FIG. 2A, and FIG. 3A, as described in more detail herein.

In general, the dissociation constant of and/or dissociation rate between a pair of reversibly associated molecules (e.g., antibody-antigen pair), may be altered (e.g., adversely) by certain assay conditions (e.g., ion concentration, pH, temperature steps, solvent) and/or assay steps, such as chemical and/or physical manipulation of the molecules after association (e.g., washing, desolvation, pelleting, drying, exposure to certain fluids, exposure to reagents). Moreover, for certain molecules and/or under certain assay conditions, the amount of dissociation may increase and/or the total number of reversibly associated molecules may decrease as a function of time. Thus, in some existing detection assays, assay conditions, assay steps, and the time between initial association and detection, amongst other factors, may affect the degree to which dissociation alters detection and quantification of analyte molecules. Stabilizing agents may be used, as described herein, to reduce the effects of dissociation processes.

A non-limiting example of dissociation in a detection assay is shown in FIG. 1A. In some embodiments, an existing detection assay 10 may utilize detection molecules, such as capture objects 15, that reversibly associate with analyte molecules, for example, to aid in the detection of the analyte molecules. In certain embodiments, capture objects 15 may comprise a least one capture component 20 and a support 25. Capture component 20 may be selected to reversibly associate with the analyte molecule via an affinity interaction. In some cases, capture component 20 may be selected to undergo a specific affinity interaction (e.g., specific binding) with the analyte molecule. In certain embodiments, support 25 may serve to immobilize, allow for facile

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isolation, and/or allow for facile manipulation of capture component 20 and associated molecules. For instance, support 25 may be a solid capture object, such as in the form of a bead (e.g., magnetic bead) as illustrated that is attached (e.g., covalently, noncovalently) to capture component 20. The bead may be easily separated from other assay component using simple separation techniques based on, e.g., magnetic force, gravitational force, density, centrifugal force, filtration, and/or evaporation. Other types of supports may be used, for example, a planar surface.

In some embodiments, capture objects 15 may be exposed to a sample 30 (e.g., fluid sample) comprising analyte molecules 35 as indicated by arrow 40. In certain embodiments, sample 30 may comprise other molecules 45 in addition to the analyte molecules 35 as illustrated in FIG. 1A. In certain embodiments, sample 30 may be combined with a solution 55 comprising capture objects 15 and a fluid (e.g., liquid) 32 as illustrated in FIG. 1A. In other embodiments, capture objects 15 may be added to sample 30. Upon exposure of the analyte molecules to the detection molecules, at least some of analyte molecules 35 may reversibly associate with capture components 20 to form associations 50. For example, analyte molecules 35 and capture component 20 may associated via an affinity interaction, such as an antibody-antigen interaction. In some such cases, capture component 20 may be an antigen and analyte molecule 35 may be an antibody or capture component 20 may be an antibody and analyte molecule 35 may be an antigen. In some embodiments, after association of at least some of analyte molecules 35 with capture objects 15, solution 55 may be allowed to remain for a period of time prior to the next assay step and/or signal detection as indicated by arrow 60. During this interim time period, at least some of associations 50 may dissociate. For instance, as illustrated in FIG. 1A, association 50A may dissociate.

In certain embodiments, after dissociation of at least a portion of associations 50, the next assay step and/or detection may occur as indicated by arrow 65. For example, the next assay step may comprise isolation and/or desolvation of (e.g., removal of liquid from) associations 50. In some such embodiments, at least a portion of (e.g., greater than or equal to 95%, greater than or equal to 98%, substantially all of) liquid 32 may be separated from associations 50 and optionally removed. For instance, liquid 32 may be separated from capture objects 15 by application of force (e.g., centrifugal force, gravitational force, magnetic force). In some such cases, the application of force (e.g., centrifugal force) may result in the formation of a

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composition comprising separated liquid and a separated assay mixture (e.g., pellet) 70, and the liquid may be removed (e.g., by filtration, decanting, and aspiration). Assay mixture 70 may comprise capture objects 15, associations 50, and optionally at least some molecules 45. In other cases, at least a portion of liquid 32 may be separated from capture objects 15 and/or associations 50 without the application of force and/or the formation of a pellet.

In some embodiments, the separated liquid may comprise at least some (e.g., substantially all) of the free analyte molecules that dissociated from capture objects 15. Removal of the separated liquid may result in the removal of at least some free analyte molecules thereby preventing re-association of the capture objects and the free analyte molecules. Regardless of the next assay step, the dissociation that occurred in the interim period between assay steps may result in a reduced signal and/or a reduced number of analyte molecules being detected.

In some embodiments, the stabilizing agents, described herein, may be used to reduce and/or prevent signal loss due to dissociation, such as that illustrated in FIG. 1A. A non-limiting example of a detection assay using a stabilizing agents to reduce and/or prevent dissociation is shown in FIG. 1B. Detection assay 80 may be substantially the same as detection assay 10, except for the use of a stabilizing agent. For example, capture objects 85 may comprise a capture component 90 and a support 95 (e.g., bead). Capture component 90 may also be selected to reversibly associate with the analyte molecule via an affinity interaction. Support 95 may also serve to immobilize, allow for facile isolation of, and/or allow for facile manipulation of capture component 90 and associated molecules. In some embodiments, in the presence of stabilizing agent 100, capture objects 85 may be exposed to a sample 105 (e.g., fluid sample) comprising analyte molecules 115 as indicated by arrow 110. In certain embodiments, sample 105 may be combined with a solution 125 comprising capture objects 85, stabilizing agent 100, and a fluid (e.g., liquid) 108 as illustrated in FIG. 1B. In other embodiments, the stabilizing agent may be added after (e.g., immediately after, within 30 seconds) exposure of the capture objects to the analyte molecules. Upon exposure of the capture objects to the analyte molecules, at least some of analyte molecules 115 may reversibly associate with capture components 90 to form associations 120. Stabilizing agent 100 may associate with associations 120 and alter (e.g., reduce) the rate of dissociation between and/or dissociation constant of the capture object 85 and analyte molecule 115. After association of at least some of analyte molecules 115 with capture objects 85, solution 125 may be allowed to remain for a period of time prior to the next assay

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step and/or signal detection as indicated by arrow 130. During this interim time period, stabilizing agent 100 may reduce and/or prevent at least some (e.g., substantially all) of associations 120 from dissociating as illustrated by arrow 135. In certain embodiments, the stabilizing agent may serve to reduce and/or prevent dissociation until the next assay step and/or detection.

For instance, in some embodiments, the next assay step may comprise isolation and/or desolvation of associations 120. In some such embodiments, at least a portion of (e.g., greater than or equal to 95%, substantially all of) liquid 108 may be separated from associations 120 and optionally removed as described above with respect to FIG. 1A. For example, separation of the liquid may result in the formation of a composition comprising the separated liquid and an assay mixture (e.g., pellet) 140. Assay mixture 140 may comprise capture objects 85 and stabilizing agents 100 associated with associations 120. In certain embodiments, the separated liquid from FIG. 1B may comprise substantially less free analyte molecules than the separated liquid from FIG. 1A. For instance, separated liquid from FIG. 1B may comprise little or no free analyte molecules due to dissociation. In such cases, removal of the liquid may result in minimal or no signal decay unlike existing assay 10. In some embodiments, the reduced dissociation associated with assay 80 may result in more accurate determination and quantification of analyte molecules.

As described above, existing detection assay 10 may comprise separating and/or removing at least a portion of a liquid from assay mixture 70. In some embodiments, as illustrated in FIG. 2A, a relatively large percentage of the liquid may be removed from the assay mixture, such that at least a portion of associations 50 are desolvated. It should be understood that the last container in FIG. 1A is the first container in FIG. 2A. In some such cases, after removal of the liquid, assay mixture 70 may be allowed to remain for a period of time prior to the next assay step and/or signal detection as indicated by arrow 150. For instance, assay mixture 70 may be stored for a period of time (e.g., at least 1 minute). During storage, at least some of associations 50 may dissociate. For instance, as illustrated in 2A, association 50B may dissociate. In some instances, the dissociations may occur in the time period between formation of the desolvated assay mixture and the next assay step (e.g., washing) and/or signal measurement. In some embodiments, the next assay step may comprise suspending assay mixture 70 in a fluid to form a solution 155 as indicated by arrow 158. Suspension of assay mixture 70 in the fluid may allow analyte molecule 35B to freely diffuse or otherwise move

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away from capture object 15B greatly reducing the likelihood of re-association between the capture object and the analyte molecule. In certain embodiments, analyte molecule 35B may not re-associate with a capture object prior to subsequent removal of the fluid and signal measurement. In such cases, the dissociation that occurred in the desolvated assay mixture may result in a reduced signal and/or a reduced number of analyte molecules being detected.

In some embodiments, the stabilizing agents, described herein, can be used to prevent dissociation in desolvated assay mixtures. For example, as described above, detection assay 80 may comprise separating and/or removing liquid from assay mixture 140. As illustrated in FIG. 1B and FIG. 2B, a relatively large percentage of the liquid may be removed from the assay mixture, such that at least a portion of associations 120 are desolvated. Stabilizing agents 100 may be associated with associations 120 in the desolvated state. In some such cases, after removal of the liquid, assay mixture 140 may be allowed to remain for a period of time prior to the next assay step and/or signal detection as indicated by arrow 160. The stabilizing agent may serve to prevent dissociation between capture objects 85 and analyte molecules 115 by maintaining a similar hydrogen bond network as the hydrogen bond network that was provided by the water prior to desolvation. In such cases, the stabilizing agent may allow a capture object and analyte molecule to maintain a similar structure and association while in the desolvated state as was present in solution. It should be understood that the last container in FIG. 1B is the first container in FIG. 2B. Assay mixture 140 may be suspended in a fluid to form a solution 165, as described above with respect to FIG. 2A, as indicated by arrow 170. Optionally, the fluid used for suspension may comprise additional stabilizing agent 102. In some embodiments, stabilizing agents 100 and/or optionally stabilizing agents 102 may reduce and/or prevent dissociation of at least some (e.g., substantially all) of associations 120. The reduced dissociation may result in more accurate determination and quantification of analyte molecules.

In some embodiments, the stabilizing agents, described herein, can be used to prevent dissociation during suspension of a desolvated assay mixtures that does not comprise a stabilizing agent as illustrated in FIG. 2C. FIG. 2C shows an assay mixture 180 substantially identical to assay mixture 70 of FIG. 2A. At least some of associations 185 in assay mixture 180 may also dissociate in the time period prior to the next assay step as illustrated by the dissociation between capture object 190A and analyte molecule 195A. In some embodiments, assay mixture 150 may be suspended in a solution 200 comprising a stabilizing agent 205, as

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indicated by arrow 210. The stabilizing agent may serve to promote association, such that that dissociated analyte molecules may readily re-associate with capture objects prior to the next assay step and/or signal measurement. For example, analyte molecule 195A that dissociated from capture object 190A in the time period prior to suspension may re-associate after suspension in solution 200.

In some embodiments, as illustrated in FIG. 3, the stabilizing agent may be used to reduce and/or prevent reduction of a measurable property of the detection molecules (e.g., enzymatic activity) and/or denaturation during one or more assay steps. As will be known to one of ordinary skill in the art, a measurable property of the detection molecules, such as enzyme activity, and the structural configuration of certain molecules (e.g., proteins) may be altered (e.g., adversely) by certain assay conditions and/or assay steps. For instance, certain ion concentrations, pHs, temperatures, and/or solvents may reduce the activity of an enzyme and result in denaturation of a protein. As another example, physical manipulation (e.g., washing, desolvation, agitation, pelleting) of certain detection molecules (e.g., enzymes, proteins) can negatively affect the ability of the detection molecules to association with analyte molecules (e.g., due to denaturation) and/or other detection molecules and/or the measurable property of the detection molecule. Thus, in some existing detection assays, certain assay conditions and assay steps, amongst other factors, may affect the degree to which reduction a measurable property of the detection molecules and/or denaturation alters detection and quantification of analyte molecules. Stabilizing agents may be used, as described herein, to reduce the effects of these processes.

A non-limiting example of reduction of a measurable property of the detection molecules and denaturation in a detection assay is shown in FIG. 3A. As described above, existing detection assay 10 may comprise suspension of assay mixture 70 in a fluid to form a solution 155. In some embodiments, production of a signal indicative of the presence, absence, and/or quantity of the analyte molecule may comprise exposing associations 50 to a solution 215 comprising binding ligand 220 have a measurable property (e.g., conductivity, spectroscopic emission, spectroscopic absorbance, fragmentation pattern, electrical potential, nucleic acid tags that can be amplified) as illustrated in FIG. 3A. In certain embodiments, binding ligand 220 comprises a component 225 that facilitates indirect detection of the analyte molecule, for example, by converting a precursor labeling agent into a labeling agent (e.g., an agent that is

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detected in an assay). For instance, binding ligand may comprise a component that reversibly and specifically associates with the analyte molecule (e.g., antibody) and an enzyme that converts precursor labeling agent (e.g., enzymatic substrate) into a labeling agent (e.g., enzymatic product). Upon exposure of analyte molecule 35 with binding ligand 220 as indicated by arrow 30, at least some analyte molecules 35 may associate with binding ligand 220. For instance, analyte molecule 35C in association 50C may reversibly associate with the binding ligand 220C to form a complex 235C. In some such embodiments, analyte molecules 35 and binding ligand 220 may associated via an affinity interaction, such as an antibody-antigen interaction. In some embodiments, after association between analyte molecules 35 and binding ligand 220, solution 215 may be allowed to remain for a period of time prior to the next assay step (e.g., detection). In some embodiments, the next assay step may involve separating and/or removing at least a portion of the fluid in solution 215. For instance, as indicated by arrow 240, detection assay 10 may comprise removing fluid from assay mixture 245 comprising complexes 235. In some embodiments, as illustrated in FIG. 3A, a relatively large percentage of the fluid may be removed from the assay mixture, such that at least a portion of complex 235 are desolvated.

In some embodiments, after removal of the liquid, the desolvated assay mixture may be allowed to remain for a period of time prior to the next assay step and/or signal detection. For instance, assay mixture 245 may be stored for a period of time (e.g., at least 1 minute). During storage, at least a portion of binding ligands 220 may denature and/or a measurable property (e.g., enzymatic activity) of binding ligand 220 may be altered, such that upon rehydration (e.g., suspension in a fluid) the structural configuration of the binding ligand is adversely altered and/or the measurable property is reduced. In certain embodiments, at least some of associations between the binding ligand and the analyte molecule and/or the analyte molecule and the capture object may dissociate. In some embodiments, the next assay step may comprise suspending assay mixture 235 in a fluid to form a solution 255 as indicated by arrow 250. At least a portion of the binding ligands in solution 255 may be denatured. For example, binding ligand 220A may be denatured in the solution. In certain embodiments, the measurable property of binding ligand 220C may be reduced. In embodiments in which dissociation occurs, the suspension of assay mixture may allow binding ligand to freely diffuse or otherwise move away from analyte molecules greatly reducing the likelihood of re-association between the binding ligand and the

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analyte molecule. In certain cases, re-association may not occur prior to subsequent removal of the fluid and/or signal measurement.

In some embodiments, the reduction of a measurable property, denaturation, and/or optional dissociation that occurred in the desolvated assay mixture may result in a reduced signal and/or a reduced number of analyte molecules being detected than are actually present. As illustrated in FIG. 3A, solution 255 may comprise three analyte molecules (e.g., 35C, 35D, 35E). Analyte molecule 35C may be associated with a denatured binding ligand 220A that cannot produce a signal. Analyte molecule 35D may be associated with a binding ligand 220B that is not denatured. The measurable property of binding ligand 220B may not be altered, such that the magnitude of the measurable property is the substantially the same as the magnitude of the measurable property prior to utilization in the assay and/or prior to the removal step. For instance, in embodiments in which the measurable property is enzymatic activity of an enzyme component, the activity of the enzyme is the substantially the same as the activity of the enzyme prior to utilization in the assay and/or prior to the removal step. In some such embodiments, the enzyme may convert precursor labeling agent into labeling agent 260 to produce a signal indicative of the analyte molecule. Conversely, analyte molecule 35E may be associated with binding ligand 220C that has measurable property, which is reduced. In some such cases, the magnitude of the measurable property is less than the magnitude of the measurable property prior to utilization in the assay and/or prior to the removal step. For instance, in embodiments in which the measurable property is enzymatic activity of an enzyme component, the activity of the enzyme is the substantially less than the activity of the enzyme prior to utilization in the assay and/or prior to the removal step. In some such embodiments, the rate of conversion of precursor labeling agent into labeling agent 260 may be reduced relative the rate of conversion of the enzyme in binding ligand 220B. For example, during the same time interval, binding ligand 220C may produce one labeling agent and binding ligand 220B may produce two labeling agents as illustrated in FIG. 3A. In some embodiments, the signal indicative of the analyte molecules may be reduced by at least about 10% (e.g., at least about 15%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%) due to the denaturation and/or reduction of the measurable property of the binding ligand. In some embodiments, the absence of stabilizing agent in detection assay 10 may result in an overall signal decay of at least about 50% (e.g., at least about

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60%, at least about 70%, at least about 80%, at least about 90%, 100%) as illustrated by comparison of labeling agent in FIG. 3A and labeling agent in FIG. 3B.

In some embodiments, the stabilizing agents, described herein, can be used to prevent reduction of a measurable property of the detection molecules and/or denaturation. For example, as described above, detection assay 80 may comprise suspension of assay mixture 140 in a fluid to form a solution 165 containing stabilization agents 100. In some embodiments, production of a signal indicative of the presence, absence, and/or quantity of the analyte molecule may comprise exposing associations 120 to binding ligand 270, thereby forming solution 265. Binding ligand 270 may have a measurable property (e.g., conductivity, spectroscopic emission, spectroscopic absorbance, fragmentation pattern, electrical potential, nucleic acid tags that can be amplified) as described above with respect to FIG. 3A and illustrated in FIG. 3B. Upon exposure of the binding ligands to the analyte molecules, at least some of analyte molecules 115 may associate with binding ligands 270. For instance, analyte molecule 115A in an association may reversibly associate with the binding ligand 270A to form a complex 275A, as described herein. In some embodiments, at least a portion of the fluid in solution 265 may be removed to form a desolvated assay mixture as described above with respect to FIG. 3A. In some embodiments, after removal of the liquid, the desolvated assay mixture may be allowed to remain for a period of time prior to the next assay step and/or signal detection. For instance, assay mixture 280 may be stored for a period of time (e.g., at least 1 minute). Stabilizing agents 100 may be associated with the binding ligand and/or associations. The stabilizing agent may serve to prevent denaturation, dissociation from the analyte, and/or reduction of a measurable property of the binding ligands. In certain embodiments, the stabilizing agent may serve to prevent dissociation between binding ligands (e.g., antibody) and analyte molecules (e.g., protein) as well as capture objects and analyte molecules. Assay mixture 280 may be suspended in a fluid, as described above with respect to FIG. 3A, as indicated by arrow 285 as shown in FIG. 3B. Optionally, the fluid may comprise additional stabilizing agent 282. In some embodiments, stabilizing agents 100 and/or optional stabilizing agents 282 may reduce denaturation, reduction of a measurable property, and/or optionally dissociation.

In some embodiments, utilization of a stabilizing agents in one or more assay steps may result in an increased signal. In certain embodiments, the increase in signal may be due to an increase in the capture and/or retention of analyte molecules due to a reduction in dissociation.

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For instance, as illustrated in FIG. 3B, solution 290 has more associations between capture objects and analyte molecules, as well as between analyte molecules, and binding ligands than solution 255 in detection assay 10 of FIG. 3A. Accordingly, solution 290 in FIG. 3B has more labeling agents 295 than solution 255 in FIG. 3A. In some embodiments, the increase in signal may be due to the reduction in dissociation of the associations necessary for detection.

In certain embodiments, the increase in signal may be due to preservation of a measurable property of the detection molecules as illustrated in FIG. 3C. In some embodiments, the signal of a measurable property may be maintained by exposing the assay mixture to a stabilizing agent 205 during the interim period between an assay step and detection. FIG. 3C depicts the same assay as FIG. 3A, except the assay mixture is exposed to a stabilizing agent prior to liquid removal as indicated by arrow 300 and optionally during suspension as indicated by arrow 305. The stabilizing agent may serve to prevent alteration of the measurable property (e.g., enzymatic activity) that may result in signal loss. Though solution 310 in FIG. 3C has the same number of analyte molecules 195 and complexes 315 as solution 255 in FIG. 3A, solution 310 produces a greater signal than solution 255 due to the maintenance of the measurable property (e.g., enzyme activity) as well as the lack of denaturation.

It should be understood that FIG. 1 – FIG. 3 are for illustrative purposes and that a suitable detection assay may comprise some, all, or none of the assay steps and/or components (e.g., binding ligands, capture objects) illustrated in the figures. Moreover, it should be understood that a stabilizing agent need not be used in every assay step, as illustrated in certain figures, to achieve desirable results (e.g., increase signal, reduction in dissociation, reduction in denaturation, maintenance of a measurable property).

As described herein, a stabilizing agent can be used to reduce and/or prevent signal decay. In some embodiments, the stabilizing agent may be a polyol. In some such embodiments, the stabilizing agent may comprise greater than or equal to about 2, greater than or equal to about 3, greater than or equal to about 4, greater than or equal to about 5, greater than or equal to about 6, greater than or equal to about 7, greater than or equal to about 8, greater than or equal to about 9, greater than or equal to about 10, greater than or equal to about 11, greater than or equal to about 12, or greater than or equal to about 13 hydroxyl groups. In some instances, the stabilizing agent may comprise less than or equal to about 14, less than or equal to about 13, less than or equal to about 12, less than or equal to about 11, less than or equal to about 10, less than or equal

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to about 9, less than or equal to about 8, less than or equal to about 7, less than or equal to about 6, less than or equal to about 5, or less than or equal to about 4 hydroxyl groups. All combination of the above-referenced ranges are possible (e.g., greater than or equal to about 2 and less than or equal to about 14 hydroxyl groups, greater than or equal to about 4 and less than or equal to about 12 hydroxyl groups). In some embodiments, the stabilizing agent may comprise a polyol as a repeat unit with the above-referenced range of hydroxyl groups. For example, the stabilizing agent may be a polysaccharide.

In some embodiments, the stabilizing agent may be a carbohydrate. In certain embodiments, the stabilizing agent may be a small molecule carbohydrate, such as sucrose, trehalose, and combinations thereof. In some embodiments, the stabilizing agent may be a small molecule polyol. In some embodiments, the stabilizing agent may be a kosmotrope (e.g., non-ionic kosmotrope, zwitterionic kosmotrope) and/or an osmolyte. Non-limiting example of suitable stabilizing agents include glycerol, ethylene glycol, propylene glycol, sorbitol, mannitol, ectoine ((S)-2-methyl-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid), proline, Glycine betaine, glucose, trimethylamine-N-oxide, glycine, N-methylglycine (sarcosine), N,N-dimethylglycine, N,N,N-trimethylglycine (betaine), dimethylsulfoniopropionate, glycerophosphorylcholine, myo-inositol, and taurine.

In some embodiments, the beneficial properties of the stabilizing agent may depend, at least in part, on the weight percentage of the stabilizing agent in the assay composition (e.g., assay mixture, solution). For instance, in some embodiments, the weight percentage of the stabilizing agent in the assay composition (e.g., solution, fluid, mixture, pellet) may be greater than or equal to about 0.5 wt.%, greater than or equal to about 1 wt.%, greater than or equal to about 2 wt.%, greater than or equal to about 4 wt.%, greater than or equal to about 5 wt.%, greater than or equal to about 6 wt.%, greater than or equal to about 8 wt.%, greater than or equal to about 10 wt.%, greater than or equal to about 12 wt.%, greater than or equal to about 14 wt.%, greater than or equal to about 15 wt.%, greater than or equal to about 16 wt.%, greater than or equal to about 18 wt.%, greater than or equal to about 20 wt.%, greater than or equal to about 22 wt.%, greater than or equal to about 24 wt.%, greater than or equal to about 25 wt.%, greater than or equal to about 26 wt.%, or greater than or equal to about 28 wt.%. In some instances, the weight percentage of the stabilizing agent in the assay composition (e.g., solution, fluid, mixture, pellet) may be less than or equal to about 30 wt.%, less than or equal to about 28 wt.%, less than

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or equal to about 26 wt.%, less than or equal to about 25 wt.%, less than or equal to about 24 wt.%, less than or equal to about 22 wt.%, less than or equal to about 20 wt.%, less than or equal to about 18 wt.%, less than or equal to about 16 wt.%, less than or equal to about 15 wt.%, less than or equal to about 14 wt.%, less than or equal to about 12 wt.%, less than or equal to about 10 wt.%, less than or equal to about 8 wt.%, less than or equal to about 6 wt.%, less than or equal to about 5 wt.%, or less than or equal to about 3 wt.%. All combinations of the above-referenced ranges are possible (e.g., greater than or equal to about 1 wt.% and less than or equal to about 30 wt.%, greater than or equal to about 10 wt.% and less than or equal to about 30 wt.%).

As noted above, a method may comprise using a stabilizing agent prior to, during, and/or after one or more steps of a detection assay. In general, the stabilizing agent may be used prior to, during, and/or after any suitable assay step. For instance, the stabilizing agent may be used prior to removal of at least a portion (e.g., substantially all) of a fluid from an assay mixture comprising associated molecules (e.g., capture objects, binding ligands, analyte molecules). In some instances, the stabilizing agent may be used prior to and/or during storage of an assay mixture comprising associated molecules (e.g., capture objects, binding ligands, analyte molecules). In certain embodiments, the stabilizing agent may be used prior to (e.g., immediately prior to), during, and/or after (e.g., immediately after) addition of a binding ligand. In certain cases, the stabilizing agent may be used prior to and/or during signal measurement. In some embodiments, the stabilizing agent may be used in substantially all assay steps. Non-limiting examples of assay steps in which a stabilizing agent may be used prior to, during, and/or after include washing, desolvation, agitation, suspension, isolation, incubation, storage, measurement, dilution, mixing, and drying steps.

In some embodiments, the stabilizing agent may be used in a relatively large percentage (e.g., greater than or equal to about 50%, substantially all) of the steps in a detection assay. For instance, the stabilizing agent may be a component in an assay buffer that is used in at least some (e.g., substantially all) of the assay steps that utilize a buffer. As another example, the stabilizing agent may be a component of the wash solution (e.g., wash buffer) for the assay. In some such cases, the stabilizing agent may be used prior to and/or after a relatively large percentage (e.g., greater than or equal to about 50%, substantially all) of steps in a detection assay. In other embodiments, the stabilizing agent may be used in relatively small percentage of assay steps. For instance, the stabilizing agent may be use in less than or equal to about 4 assay steps (e.g., less

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than or equal to about 2 assay steps, a single assay step). Regardless of the number of steps that utilize the stabilizing agent, the stabilizing agent may prevent and/or reduce signal decay.

In some embodiments, the stabilizing agent may be used to reduce and/or prevent signal loss during storage of an assay mixture that comprises, for example, associations (e.g., capture objects, binding ligands, analyte molecules). In general, the assay mixture may be stored for any suitable amount of time with minimal or no loss in signal due to, e.g., dissociation, denaturation, and/or reduction in a measurable property. For instance, an assay mixture may be stored for greater than or equal to about 5 seconds, greater than or equal to about 15 seconds, greater than or equal to about 30 seconds, greater than or equal to about 45 seconds, greater than or equal to about 1 minute, greater than or equal to about 2 minutes, greater than or equal to about 4 minutes, greater than or equal to about 5 minutes, greater than or equal to about 8 minutes, greater than or equal to about 10 minutes, greater than or equal to about 30 minutes, greater than or equal to about 1 hour, greater than or equal to about 2 hours, greater than or equal to about 6 hours, greater than or equal to about 12 hours, greater than or equal to about 18 hours, greater than or equal to about 1 day, greater than or equal to about 2 days, greater than or equal to about 3 days, greater than or equal to about 5 days minutes, greater than or equal to about 1 week, greater than or equal to about 2 weeks, greater than or equal to about 3 weeks, or greater than or equal to about 1 month with minimal or no loss in signal due to, e.g., dissociation, denaturation, and/or reduction in a measurable property. In some instances, an assay mixture may be stored for less than or equal to about 3 months, less than or equal to about 2 months, less than or equal to about 1 month, less than or equal to about 3 weeks, less than or equal to about 2 weeks, less than or equal to about 7 days, less than or equal to about 5 days, less than or equal to about 3 days, less than or equal to about 2 days, less than or equal to about 24 hours, less than or equal to about 18 hours, less than or equal to about 12 hours, or less than or equal to about 6 hours with minimal or no loss in signal due to, e.g., dissociation, denaturation, and/or reduction in a measurable property. All combination of the above-referenced ranges are possible (e.g., greater than or equal to about 5 seconds and less than or equal to about 3 months, greater than or equal to about 12 hours and less than or equal to about 3 months). In general, any suitable storage conditions may be used. In some embodiments, the storage condition may be an assay condition. In certain embodiments, storage may occur at a certain temperature (e.g., between about -85 ^{0}C and about 25 ^{0}C, between about -22 ^{0}C and about 25 ^{0}C, between about -6 ^{0}C and about 25 ^{0}C,

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between about 2 ^{0}C and about 25 ^{0}C, between about 20 ^{0}C and about 25 ^{0}C, between about 2 ^{0}C and about 8 ^{0}C, between about -85 ^{0}C and about 20 ^{0}C, between about -85 ^{0}C and about 8 ^{0}C, between about -85 ^{0}C and about -2 ^{0}C, between about -85 ^{0}C and about -18 ^{0}C, between about -6 ^{0}C and about -2 ^{0}C, between about -18 ^{0}C and about -22 ^{0}C, and between about -75 ^{0}C and about -85 ^{0}C), at a certain pressure (e.g., about atmospheric pressure, less than atmospheric pressure, including vacuum), in a solvated state (e.g., in solution) and/or in a desolvated state (e.g., substantially dry).

As used herein, the terms "storage" and "stored" as well as other grammatical variations have their ordinary meaning in the art and may refer to the interim period between the end of one assay step and the beginning of the next assay step. In some embodiments, storage may occur during the operation of an assay due to, e.g., the time required for the set-up of an assay step and/or the concurrent analysis of multiple replicates and/or samples. For instance, in embodiments in which multiple samples are assayed concurrently, the individual detection of one sample may result in storage of another sample. In some embodiments, storage may occur due to, e.g., temporary cessation of the assay. For instance, an assay may be temporarily suspended after formation of a certain assay mixture to allow for analysis at a different time and/or location. For instance, an assay may be manufactured at a manufacturing facility and stored before shipping to a customer and its ultimate use.

In some embodiments, the stabilizing agent may be used to reduce and/or prevent signal loss during removal of a fluid from an assay mixture comprising associations (e.g., capture objects, binding ligands, analyte molecules). In general, any suitable method may be used to remove at least a portion (e.g., substantially all) of the fluid from the assay mixture. Non-limiting examples include aspiration, vacuum filtration, exposure to reduced pressure below the vapor pressure of the fluid in the presence or absence of a desiccant, evaporation, drying in a stream of air, and/or heating. In some embodiments, removal of the fluid may be result in desolvation of the assay mixture. In some such cases, the assay mixture may be substantially dry. For instance, the desolvated assay mixture may comprise less than or equal to about 5 wt.%, less than or equal to about 4 wt.%, less than or equal to about 3 wt.%, less than or equal to about 2 wt.%, less than or equal to about 1 wt.%, less than or equal to about 0.5 wt.%, less than or equal to about 0.1 wt.%, less than or equal to about 0.05 wt.%, or less than or equal to about 0.001 wt.% of a fluid.

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In some embodiments, the assay mixture may be desolvated prior to certain assay steps (e.g., washing, storage).

In some embodiments, the stabilizing agent may reduce and/or prevent dissociation. For instance, less than or equal to about 20% (e.g., less than or equal to about 18%, less than or equal to about 15%, less than or equal to about 12%, less than or equal to about 10%, less than or equal to about 8%, less than or equal to about 5%, less than or equal to about 3%, less than or equal to about 2%, less than or equal to about 1%, less than or equal to about 0.8%, less than or equal to about 0.5%, less than or equal to about 0.3%, less than or equal to about 0.1%, less than or equal to about 0.05%, less than or equal to about 0.01%) of analyte molecules and/or detection molecules (e.g., binding ligands, capture objects) may dissociate during one or more assay steps and/or the entire assay. In some embodiments, the percent dissociation may be determined using surface plasmon resonance and/or interferometrometric techniques.

In some embodiments, the stabilizing agent may reduce the dissociation constant and/or dissociation rate. For instance, the stabilizing agent may reduce the dissociation constant and/or rate by greater than or equal to about 0.5% (e.g., greater than or equal to about 1%, greater than or equal to about 2%, greater than or equal to about 3%, greater than or equal to about 5%, greater than or equal to about 8%, greater than or equal to about 10%, greater than or equal to about 15%, greater than or equal to about 20%). The dissociation rate and/or constant may be determined using surface plasmon resonance and/or interferometrometric techniques.

In some embodiments, the stabilizing agent may reduce and/or prevent alterations in the structural configuration of a molecule (e.g., denaturation). For instance, less than or equal to about 20% (e.g., less than or equal to about 18%, less than or equal to about 15%, less than or equal to about 12%, less than or equal to about 10%, less than or equal to about 8%, less than or equal to about 5%, less than or equal to about 3%, less than or equal to about 2%, less than or equal to about 1%, less than or equal to about 0.8%, less than or equal to about 0.5%, less than or equal to about 0.3%, less than or equal to about 0.1%, less than or equal to about 0.05%, less than or equal to about 0.01%) of analyte molecules and/or detection molecules (e.g., binding ligands, capture objects) undergo a structural alteration (e.g., denaturation) during one or more assay steps and/or the entire assay. In some embodiments, the structural alteration of a molecule may be determined using NMR, circular dichroism, and/or light scattering.

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In some embodiments, the stabilizing agent may reduce and/or prevent reduction in a measurable property (e.g., enzyme activity) of a binding ligand. For instance, a measurable property of a binding ligand may be reduced by less than or equal to about 20% (e.g., less than or equal to about 18%, less than or equal to about 15%, less than or equal to about 12%, less than or equal to about 10%, less than or equal to about 8%, less than or equal to about 5%, less than or equal to about 3%, less than or equal to about 2%, less than or equal to about 1%, less than or equal to about 0.8%, less than or equal to about 0.5%, less than or equal to about 0.3%, less than or equal to about 0.1%, less than or equal to about 0.05%, less than or equal to about 0.01%) during one or more assay steps and/or the entire assay. The reduction may be measured by determining the measurable property before and after the assay step(s) and/or the entire assay. For example, enzyme activity may be determined using ELISA or Simoa techniques.

In some embodiments, the stabilizing agent may be used to maintain and/or improve a signal indicative of the analyte molecule during one or more assay steps and/or the entire assay. For instance, the stabilizing agent may be used to maintain the signal during storage. In some embodiments, the signal after storage for the times and under the conditions described herein may be greater than or equal to about 10%, may be greater than or equal to about 20%, may be greater than or equal to about 30%, may be greater than or equal to about 40%, may be greater than or equal to about 50%, may be greater than or equal to about 60%, may be greater than or equal to about 70%, greater than or equal to about 72%, greater than or equal to about 75%, greater than or equal to about 78%, greater than or equal to about 80%, greater than or equal to about 82%, greater than or equal to about 85%, greater than or equal to about 88%, greater than or equal to about 90%, greater than or equal to about 92%, greater than or equal to about 95%, greater than or equal to about 98%, greater than or equal to about 99%, or greater than or equal to about 100% of the signal prior to storage.

In some embodiments, the stabilizing agent may be used to reduce and/or prevent signal decay due to desolvation. In such cases, the signal after desolvation (e.g., in the solution after resuspension of the desolvated mixture) may be greater than or equal to about 10%, may be greater than or equal to about 20%, may be greater than or equal to about 30%, may be greater than or equal to about 40%, may be greater than or equal to about 50%, may be greater than or equal to about 60%, may be greater than or equal to about 70%, greater than or equal to about 72%, greater than or equal to about 75%, greater than or equal to about 78%, greater than or

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equal to about 80%, greater than or equal to about 82%, greater than or equal to about 85%, greater than or equal to about 88%, greater than or equal to about 90%, greater than or equal to about 92%, greater than or equal to about 95%, greater than or equal to about 98%, greater than or equal to about 99%, or greater than or equal to about 100% of the signal prior to desolvation.

In some embodiments, the stabilizing agent may be used to maintain and/or improve the biological activity of the analyte and/or detection molecule during one or more assay steps and/or the entire assay. For instance, the stabilizing agent may be used to maintain the biological activity of the detection molecule during storage. In some embodiments, the biological activity after storage for the times and under the conditions described herein may be greater than or equal to about 10%, may be greater than or equal to about 20%, may be greater than or equal to about 30%, may be greater than or equal to about 40%, may be greater than or equal to about 50%, may be greater than or equal to about 60%, may be greater than or equal to about 70%, greater than or equal to about 72%, greater than or equal to about 75%, greater than or equal to about 78%, greater than or equal to about 80%, greater than or equal to about 82%, greater than or equal to about 85%, greater than or equal to about 88%, greater than or equal to about 90%, greater than or equal to about 92%, greater than or equal to about 95%, greater than or equal to about 98%, greater than or equal to about 99%, or greater than or equal to about 100% of the biological activity prior to storage.

In some embodiments, the stabilizing agent may be used to maintain and/or improve the binding affinity (e.g., equilibrium dissociation constant, K_D) of the analyte for the detection molecule(s) during one or more assay steps and/or the entire assay. For instance, the stabilizing agent may be used to maintain the binding affinity of the detection molecule during storage. In some embodiments, the binding affinity after storage for the times and under the conditions described herein may be greater than or equal to about 10%, may be greater than or equal to about 20%, may be greater than or equal to about 30%, may be greater than or equal to about 40%, may be greater than or equal to about 50%, may be greater than or equal to about 60%, may be greater than or equal to about 70%, greater than or equal to about 72%, greater than or equal to about 75%, greater than or equal to about 78%, greater than or equal to about 80%, greater than or equal to about 82%, greater than or equal to about 85%, greater than or equal to about 88%, greater than or equal to about 90%, greater than or equal to about 92%, greater than

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or equal to about 95%, greater than or equal to about 98%, greater than or equal to about 99%, or greater than or equal to about 100% of the binding affinity prior to storage.

As used herein, the term "detection molecule" has its ordinary meaning in the art and may refer to a molecule that directly or indirectly associates with an analyte molecule and is used in the determination of the presence, absence, and/or quantity of the analyte molecule.

As used herein, the phrase "associated with" when used with respect to two or molecules (e.g., a capture object and an analyte molecule) refers to a direct or indirect link between the molecules formed via a chemical and/or biological interaction. In some embodiments, an association between molecules may occur via a biological binding event (i.e., between complementary pairs of biological molecules). For example, a molecule may include an entity such as biotin that specifically binds to a complementary entity, such as avidin or streptavidin, on another molecule. Other examples of biological molecules that may form biological bonds between pairs of biological molecules include, but are not limited to, proteins, nucleic acids, glycoproteins, carbohydrates, hormones, and the like. Specific examples include, but are not limited to, an antibody/peptide pair, an antibody/antigen pair, an antibody fragment/antigen pair, an antibody/antigen fragment pair, an antibody fragment/antigen fragment pair, an antibody/hapten pair, an enzyme/substrate pair, an enzyme/inhibitor pair, an enzyme/cofactor pair, a protein/substrate pair, a nucleic acid/nucleic acid pair, a protein/nucleic acid pair, a peptide/peptide pair, a protein/protein pair, a small molecule/protein pair, a glutathione/GST pair, an anti-GFP/GFP fusion protein pair, a Myc/Max pair, a maltose/maltose binding protein pair, a carbohydrate/protein pair, a carbohydrate derivative/protein pair, a metal binding tag/metal/chelate, a peptide tag/metal ion-metal chelate pair, a peptide/NTA pair, a lectin/carbohydrate pair, a receptor/hormone pair, a receptor/effector pair, a complementary nucleic acid/nucleic acid pair, a ligand/cell surface receptor pair, a virus/ligand pair, a Protein A/antibody pair, a Protein G/antibody pair, a Protein L/antibody pair, an Fc receptor/antibody pair, a biotin/avidin pair, a biotin/streptavidin pair, a drug/target pair, a zinc finger/nucleic acid pair, a small molecule/peptide pair, a small molecule/protein pair, a small molecule/target pair, a carbohydrate/protein pair such as maltose/MBP (maltose binding protein), a small molecule/target pair, or a metal ion/chelating agent pair. Biological interactions between a molecule and another molecule (e.g., binding partner) suitable for use in the embodiments described herein can be selected readily, by those of ordinary skill in the art, based upon the

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description herein as their function, examples of such biological interactions, and knowledge herein and in the art as to simple techniques for identifying suitable chemical interactions.

In some embodiments, molecule may associate via a chemical interaction, such as a chemical bond. In some embodiments, the association is formed via a non-covalent bond (e.g., hydrogen bonding, van der Waals interactions, hydrophobic interactions, magnetic interactions, electrostatic interactions, pi stacking, dipole-dipole interactions, ligand-receptor interaction). In some embodiments, the molecule and binding partner may comprise functional groups capable of forming such bonds. For example, a molecule may include at least one hydrogen atom capable of interacting with a pair of electrons on a hydrogen-bond acceptor of a binding partner to form the hydrogen bond. In some embodiments, a molecule and/or a binding partner may include an electron-rich or electron-poor moiety, such that it may form an electrostatic interaction with another of a binding partner and/or molecule, respectively. It should be understood that non-covalent bonds between components may be formed by any type of reactions, as known to those of ordinary skill in the art, using the appropriate functional groups to undergo such reactions. Chemical interactions suitable for use with various embodiments described herein can be selected readily by those of ordinary skill in the art, based upon the description herein.

As used herein, the term "small molecule" refers to molecules, whether naturally occurring or artificially created (e.g., via chemical synthesis) that have a relatively low molecular weight. Typically, a small molecule is an organic compound (i.e., it contains carbon). The small molecule may contain multiple carbon-carbon bonds, stereocenters, and other functional groups (e.g., amines, hydroxyl, carbonyls, and heterocyclic rings, etc.). In certain embodiments, the molecular weight of a small molecule is at most about 1,000 g/mol, at most about 900 g/mol, at most about 800 g/mol, at most about 700 g/mol, at most about 600 g/mol, at most about 500 g/mol, at most about 400 g/mol, at most about 300 g/mol, at most about 200 g/mol, or at most about 100 g/mol. In certain embodiments, the molecular weight of a small molecule is at least about 100 g/mol, at least about 200 g/mol, at least about 300 g/mol, at least about 400 g/mol, at least about 500 g/mol, at least about 600 g/mol, at least about 700 g/mol, at least about 800 g/mol, or at least about 900 g/mol, or at least about 1,000 g/mol. Combinations of the above ranges (e.g., at least about 200 g/mol and at most about 500 g/mol) are also possible.

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As used herein, the term "mixture" has its ordinary meaning in the art and typically refers to a system that contains two or more chemical substances (e.g., detection molecules, analyte molecules) that are combined such that each substance retains its own chemical identity. The mixture may be homogenous (e.g., a solution) or heterogeneous (e.g., a suspension). In some embodiments, the mixture is homogenous (e.g., a solution). In certain embodiments, the mixture is heterogeneous (e.g., a suspension).

The methods and system, described herein, may be used to extend the dynamic range of a detection assay. The term, "dynamic range" is given its ordinary meaning in the art and refers to the range of the concentration of analyte molecules in a fluid sample that may be quantitated by a system or method without dilution or concentration of the sample or change in the assay conditions producing a similar result (e.g., concentration of reagents employed, etc.), and wherein the measured concentration of the analyte molecules may be substantially accurately determined. The concentration of analyte molecules in a fluid sample may be considered to be substantially accurately determined if the measured concentration of the analyte molecules in the fluid sample is within about 10% of the actual (e.g., true) concentration of the analyte molecules in the fluid sample. In certain embodiments, the measured concentration of the analyte molecules in the fluid sample is substantially accurately determined in embodiments where the measured concentration is 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 analyte 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 about 10%, no greater than about 5%, no greater than about 4%, no greater than about 3%, no greater than about 2%, no greater than about 1%, or no greater than about 0.5%. The accuracy of the assay method may be determined, in some embodiments, by determining the concentration of analyte molecules in a fluid sample of a known concentration using the selected assay method and comparing the measured concentration with the actual concentration.

In some embodiments, the concentration (e.g., unknown concentration) of analyte molecules in the fluid sample that may be substantially accurately determined is less than about 5000 fM (femtomolar), less than about 3000 fM, less than about 2000 fM, less than about 1000 fM, less than about 500 fM, less than about 300 fM, less than about 200 fM, less than about 100

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fM, less than about 50 fM, less than about 25 fM, less than about 10 fM, less than about 5 fM, less than about 2 fM, less than about 1 fM, less than about 500 aM (attomolar), less than about 100 aM, less than about 10 aM, less than about 5 aM, less than about 1 aM, less than about 0.1 aM, less than about 500 zM (zeptomolar), less than about 100 zM, less than about 10 zM, less than about 5 zM, less than about 1 zM, less than about 0.1 zM, or less. In some cases, the limit of detection (e.g., the lowest concentration of an analyte molecule which may be determined in solution) is about 100 fM, about 50 fM, about 25 fM, about 10 fM, about 5 fM, about 2 fM, about 1 fM, about 500 aM (attomolar), about 100 aM, about 50 aM, about 10 aM, about 5 aM, about 1 aM, about 0.1 aM, about 500 zM (zeptomolar), about 100 zM, about 50 zM, about 10 zM, about 5 zM, about 1 zM, about 0.1 zM, or less. In some embodiments, the concentration of analyte molecules or particles 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 0.1 zM, between about 100 fM and about 1 zM, between about 100 aM and about 0.1 zM, or less. The upper limit of detection (e.g., the upper concentration of an analyte molecule which may be determined in solution) is at least about 100 fM, at least about 1000 fM, at least about 10 pM (picomolar), at least about 100 pM, at least about 100 pM, at least about 10 nM (nanomolar), at least about 100 nM, at least about 1000 nM, at least about 10 uM, at least about 100 uM, at least about 1000 uM. at least about 10 mM, at least about 100 mM, at least about 1000 mM, or greater. In some embodiments, the concentration of analyte molecules or particles in the fluid sample determined is less than about 50×10^{-15} M, or less than about 40×10^{-15} M, or less than about 30×10^{-15} M, or less than about 20×10^{-15} M, or less than about 10×10^{-15} M, or less than about, or less than about 1×10^{-15} M.

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

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various parameters discussed above and a method of treatment and/or diagnostic indication that may otherwise not be determinable and/or apparent.

In some embodiments, the analyte molecule may be an enzyme. Non-limiting examples of enzymes include, an oxidoreductase, transferase, kinase, hydrolase, lyase, isomerase, ligase, and the like. Additional examples of enzymes include, but are not limited to, polymerases, cathepsins, calpains, amino-transferases such as, for example, AST and ALT, proteases such as, for example, caspases, nucleotide cyclases, transferases, lipases, enzymes associated with heart attacks, and the like. When a system/method of the present invention is used to detect the presence of viral or bacterial agents, appropriate target enzymes include viral or bacterial polymerases and other such enzymes, including viral or bacterial proteases, or the like.

In other embodiments, the analyte molecule may comprise an enzymatic component. For example, the analyte particle can be a cell having an enzyme or enzymatic component present on its extracellular surface. Alternatively, the analyte particle is a cell having no enzymatic component on its surface. Such a cell is typically identified using an indirect assaying method described below. Non-limiting example of enzymatic components are horseradish peroxidase, beta-galactosidase, and alkaline phosphatase.

In yet other embodiments, the analyte molecule may be a biomolecule. Non-limiting examples of biomolecules include hormones, antibodies, cytokines, proteins, nucleic acids, lipids, carbohydrates, lipids cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, or combinations thereof. Non-limiting embodiments of proteins include peptides, polypeptides, protein fragments, protein complexes, fusion proteins, recombinant proteins, phosphoproteins, glycoproteins, lipoproteins, or the like. As will be appreciated by those in the art, there are a large number of possible proteinaceous analyte molecules that may be detected or evaluated for binding partners using the present invention. In addition to enzymes as discussed above, suitable protein analyte molecules include, but are not limited to, immunoglobulins, hormones, growth factors, cytokines (many of which serve as ligands for cellular receptors), cancer markers, neurological markers, inflammatory markers, markers of cardiovascular disease, etc. Non-limiting examples of biomolecules include PSA, beta-amyloid protein, tau protein, TNF-alpha, *toxins from C. difficile*, Aβ40, Aβ42, C-Peptide, Eotaxin, G-CSF, GM-CSF, IFNα, IFNγ, MCP-3, MIP-1β, pNF-heavy, NSE, PD-L1, PIGF, P-Tau 181, P-Tau 231, TDP43, TNFβ, UCH-L1, VEGF, GM-CSF, IL-1α, IL-1β, IL-2, IL-

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4, IL-3, IL-5, IL-6, IL-8, IL-10, IL-12p40/23, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-17A/F, IL-17C, IL-17F, IL-18, IL-22, IL-23, IL-28A, IL-33, IL-36β, IP-10, MCP-1, NF-light (NFL), HIV p24, TRAIL, troponin-I, α-Synuclein, BDNF, CA 19-9,CA-125, cathepsin S, CEA, c-MET, CRP, CXCL13, GFAP, leptin, and LIF.

In certain embodiments, the analyte molecule may be a host-translationally modified protein (e.g., phosphorylation, methylation, glycosylation) and the capture component may be an antibody specific to a post-translational modification. Modified proteins may be captured with capture components comprising a multiplicity of specific antibodies and then the captured proteins may be further bound to a binding ligand comprising a secondary antibody with specificity to a post-translational modification. Alternatively, modified proteins may be captured with capture components comprising an antibody specific for a post-translational modification and then the captured proteins may be further bound to binding ligands comprising antibodies specific to each modified protein.

In another embodiment, the analyte molecule is a nucleic acid. A nucleic acid may be captured with a complementary nucleic acid fragment (e.g., an oligonucleotide) and then optionally subsequently labeled with a binding ligand comprising a different complementary oligonucleotide.

Suitable analyte molecules and particles include, but are not limited to small molecules (including organic compounds and inorganic compounds), environmental pollutants (including pesticides, insecticides, toxins, etc.), therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.), biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc.), whole cells (including prokaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells), viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.), spores, etc.

The fluid sample containing or suspected of containing an analyte molecule may be derived from any suitable source. In some cases, the sample may comprise a liquid, fluent particulate solid, fluid suspension of solid particles, supercritical fluid, and/or gas. In some cases, the analyte molecule may be separated or purified from its source prior to determination; however, in certain embodiments, an untreated sample containing the analyte molecule may be tested directly. The source of the analyte molecule may be synthetic (e.g., produced in a

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laboratory), the environment (e.g., air, soil, etc.), a mammal, an animal, a plant, or any combination thereof. In a particular example, the source of an analyte molecule is a human bodily substance (e.g., blood, serum, plasma, urine, saliva, tissue, organ, stool, cerebral spinal fluid, or the like). The volume of the fluid sample analyzed may potentially be any amount within a wide range of volumes, depending on a number of factors such as, for example, the number of capture objects used/available, the number of locations us/available, etc.

In some cases, the fluid sample may be diluted prior to use in an assay. For example, in embodiments where the source of an analyte molecule is a human body fluid (e.g., blood, serum), the fluid may be diluted with an appropriate solvent (e.g., a buffer such as PBS buffer). A fluid sample may be diluted about 1-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 10-fold, or greater, prior to use. The sample may be added to a solution comprising the plurality of capture objects, or the plurality of capture objects may be added directly to or as a solution to the sample.

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 molecule 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 molecule 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 500 pg/mL, 250 pg/mL, 100 pg/mL, 50 pg/mL, 40 pg/mL, 30 pg/mL, 20 pg/mL, 10 pg/mL 5 pg/mL, 4 pg/mL, 3 pg/mL, 2 pg/mL, 1 pg/mL, 0.8 pg/mL, 0.7 pg/mL, 0.6 pg/mL, 0.5 pg/mL, 0.4 pg/mL, 0.3 pg/mL, 0.2 pg/mL, 0.1 pg/mL, 0.05 pg/mL, 0.04 pg/mL, 0.02 pg/mL, 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, between about 5 pg/mL and about 0.02 pg/mL, or between about 1 pg/mL and about 0.02 pg/mL. As will be understood by those of ordinary

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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 concentration of analyte molecules (e.g., biomarker molecules) in the fluid sample that may be substantially accurately determined is less than about 5000 fM, less than about 3000 fM, less than about 2000 fM, less than about 1000 fM, less than about 500 fM, less than about 300 fM, less than about 200 fM, less than about 100 fM, less than about 50 fM, less than about 25 fM, less than about 10 fM, less than about 5 fM, less than about 2 fM, less than about 1 fM, less than about 0.5 fM, less than about 0.1 fM, or less. In some embodiments, the concentration of analyte molecules (e.g., 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 analyte molecule (e.g., biomarker molecules) in the fluid sample. In certain embodiments, the measured concentration of the analyte molecules (e.g., 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 analyte molecules (e.g., 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 analyte (e.g., biomarker) molecules into a plurality of locations to facilitate detection/quantification, such that each location comprises/contains either zero or one or more analyte (e.g., biomarker) molecules. Additionally, in some embodiments, the locations may be configured in a manner

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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 analyte molecule (e.g., biomarker) molecule may be made. In some cases, based upon the number/percentage/fraction, a measure of the concentration of analyte molecule (e.g., average enzyme per bead) in the fluid sample may be determined. The measure of the concentration of analyte molecule (e.g., average enzyme per bead) 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, for example, US8460879 (by Walt et al.); US8460878 (by Walt et al.); US8492098 (by Walt et al.); WO2009029073 (by Walt et al.); US20110195852 (by Walt et al.); US20100075862 (by Duffy et al.); US201000754072 (by Duffy et al.); US20100075439 (by Duffy et al.); WO2010/039179 (by Duffy et al.); US20100075355 (by Duffy et al.); US20110212848 (by Duffy et al.); WO 2011/109364 (by Duffy et al.); WO 2011/109372 (by Duffy et al.); US20110212462 (by Duffy et al.); WO 2011/109379 (by Rissin et al.); US20110212537 (by Duffy et al.); US20120196774 (by Fournier et al.); US 20110245097 (by Rissin et al.), US8236574 (by Duffy et al.); US8415171 (by Duffy et al.); WO2012/170776 (by Wilson et al.); WO2012/142300 (by Wilson et al.); WO2012/142301 (by Wilson et al.,); WO2014/113502 (by Duffy et al.); WO2014/183096 (by Rissin et al.); WO2016/115256 (by Duffy et al.); WO2016/130923 (by Pollock et al.), each of which are incorporated herein by reference in their entirety. These assay may also be referred to herein as digital ELISA assay, e.g., based on single molecule array (Simoa) technology. E.g., see

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also Rissin, et al., Nat Biotechnol, 28(6): p. 595-9 (2010); Rissin, et al., Anal Chem, 83(6): p. 2279-85 (2011); Kan, et al., Lab Chip, 2012. 12(5): p. 977-85.

Additional details of exemplary, non-limiting assay methods which comprise one or more steps of spatially segregating analyte (e.g., biomarker) molecules will now be described. In certain embodiments, a method for detection and/or quantifying analyte (e.g., biomarker) molecules in a sample comprises immobilizing a plurality of analyte (e.g., 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 analyte (e.g., 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 analyte 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 analyte 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 an analyte 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 analyte 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 at least one analyte molecule to the total number of locations determined to contain a capture object not associated with an analyte molecule. In yet other embodiments, a measure of the concentration of analyte 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

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object and at least one analyte 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 analyte 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 analyte 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 Publication No. WO2011/109364, filed March 1, 2011, by Duffy et al.; U.S. Patent No. 8,236,574, issued Aug. 7, 2012, by Duffy et al.; U.S. Patent No. 8,415,171, issued April 9, 2013, by Duffy et al.; International Patent Publication No. WO2011/109379, filed March 1, 2011, by Rissin et al.) of the reaction vessels comprise at least one or, in certain cases, only one capture object associated with at least one analyte molecule and at least some (e.g., a statistically significant fraction) of the reaction vessels comprise an capture object not associated with any analyte molecules. The capture objects associated with at least one analyte molecule may be quantified in certain embodiments, thereby allowing for the detection and/or quantification of analyte 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 analyte molecules is provided. An assay consumable comprising a plurality of assay sites is exposed to the sample fluid. In some cases, the analyte molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the assay sites contain a single analyte molecule and a statistically significant fraction of the assay sites do not contain any analyte 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

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measure of the concentration of the analyte 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 analyte molecule and/or the number/fraction/percentage of sites which do not contain any analyte molecules. In some cases, the analyte molecules are provided in a manner (e.g., at a concentration) such that at least some assay sites comprise more than one analyte molecule. In such embodiments, a measure of the concentration of analyte 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 analyte 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 analyte molecules are immobilized with respect to a bead. In some cases, the analyte molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the beads associate with a single analyte molecule and a statistically significant fraction of the beads do not associate with any analyte molecules. At least some of the plurality of beads (e.g., those associated with a single analyte molecule or not associated with any analyte 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 analyte molecule. In some cases, the number of assay sites containing a bead not associated with a analyte 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 analyte molecules in the fluid sample. In some cases, more than one analyte molecule may associate with a bead and/or more than one bead may be present in an assay site. In some cases, the plurality analyte 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 analyte molecules into a plurality of locations.

The analyte molecules may be directly detected or indirectly detected. In the case of direct detection, a analyte 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 analyte molecule. For example, the analyte molecules (e.g., optionally associated with a bead) may be exposed to at least one

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type of binding ligand. A "binding ligand," is any molecule, particle, or the like which specifically binds to or otherwise specifically associates with a analyte molecule to aid in the detection of the analyte 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 analyte 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 analyte 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 analyte 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 analyte 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 analyte molecule (or number of capture objects associated with at least one analyte molecule to total number of capture objects)). Non-limiting examples of enzymes or enzymatic components

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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 analyte 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 analyte 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 analyte 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.

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 FIG. 12A. A plurality of capture objects 402, are provided (step

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(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 analyte molecules 403 (e.g., beads 402 are incubated with analyte molecules 403). At least some of the analyte molecules are immobilized with respect to a bead. In this example, the analyte molecules are provided in a manner (e.g., at a concentration) such that a statistically significant fraction of the beads associate with a single analyte molecule and a statistically significant fraction of the beads do not associate with any analyte molecules. For example, as shown in step (B), analyte molecule 404 is immobilized with respect to bead 405, thereby forming complex 406, whereas some beads 407 are not associated with any analyte molecules. It should be understood, in some embodiments, more than one analyte 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 analyte molecule or not associated with any analyte 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 409. 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 analyte molecule. For example, as shown in step (D), the plurality of reaction vessels are interrogated optically using light source 415, wherein each reaction vessel is exposed to electromagnetic radiation (represented by arrows 410) from light source 415. The light emitted (represented by arrows 411) from each reaction vessel is determined (and/or recorded) by detector 415 (in this example, housed in the same system as light source 415). The number of reaction vessels containing a analyte molecule (e.g., reaction vessels 412) 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 analyte molecule (e.g., reaction vessel 413), the number of wells not containing a bead (e.g., reaction vessel 414) 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 analyte molecules in the fluid sample.

A non-limiting example of an embodiment where a capture object is associated with more than one analyte molecule is illustrated in FIG. 12B. A plurality of capture objects 420 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 analyte

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molecules 421 (e.g., beads 420 are incubated with analyte molecules 421). At least some of the analyte molecules are immobilized with respect to a bead. For example, as shown in step (B), analyte molecule 422 is immobilized with respect to bead 424, thereby forming complex 426. Also illustrated is complex 430 comprising a bead immobilized with respect to three analyte molecules and complex 432 comprising a bead immobilized with respect to two analyte molecules. Additionally, in some cases, some of the beads may not associate with any analyte molecules (e.g., bead 428). The plurality of beads from step (B) is exposed to a plurality of binding ligands 431. As shown in step (C), a binding ligand associates with some of the analyte molecules immobilized with respect to a bead. For example, complex 440 comprises bead 434, analyte molecule 436, and binding ligand 438. The binding ligands are provided in a manner such that a statistically significant fraction of the beads comprising at least one analyte 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 analyte 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 441 on a substrate 442. 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 443), wherein in the number may be related to a measure of the concentration of analyte 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 444), the number of reaction vessels not containing a bead (e.g., reaction vessel 445), 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 analyte 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,

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as opposed to being sequentially addressed/detected, where they would not. Simultaneous addressing/detection can be accomplished by using various techniques, including optical imaging techniques (e.g., CCD or CMOS detectors). 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

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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 or CMOS camera.

In some embodiments of the present invention, the plurality of reaction vessels may be sealed (e.g., after the introduction of the analyte 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 analyte molecules and, optionally, at least one type of precursor labeling agent to facilitate detection of the analyte 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 analyte capture contained in each vessel to a small number, the volume

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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 analyte capture can be fully contained within the reaction vessel (see, for example, U.S. Patent Application No. US 2011-0212848 (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.; International Patent Application Publication No. WO2011/109364 (International Patent Application No. PCT/US2011/026645), filed March 1, 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 analyte 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

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multiple types of analyte 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

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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.

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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 analyte 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 analyte 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 analyte molecules may be spatially segregated into a plurality of droplets. That is, single analyte 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.

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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 analyte 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 analyte 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 analyte molecules, binding ligands, precursor labeling agents, or the like. As another example, following immobilization of the analyte molecules with respect to a plurality of capture objects, the plurality of capture objects may be subjected to a washing step thereby removing any analyte 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 analyte. The assay methods employed should meet the algorithm requirements for LOD and LOQ.

The following examples are intended to illustrate certain embodiments of the present invention, but do not exemplify the full scope of the invention.

EXAMPLES

Example 1

This example describes the effect of 16.7 wt.% of sucrose on signal loss due to storage of an assay mixture. Assay mixtures that were exposed to 16.7 wt.% of sucrose prior to and after desolvation had less signal loss than assay mixtures that were not exposed to sucrose.

A PSA detection assay was used to investigate the effect on sucrose on signal loss. Briefly, paramagnetic beads conjugated with anti-PSA capture antibodies were exposed to solutions containing 30 pg/mL PSA. The captured PSA molecules were then labeled with anti-PSA detection antibody ("DetAb") in the presence or absence of 16.7 wt.% of sucrose. The beads were pelleted and the solution aspirated and the beads were stored in pellet form for various times. Then, the pellets were exposed to a solution containing enzyme conjugate ("SbG") and 16.7 wt.% sucrose. Bead pellets were resuspended in enzyme substrate and each were

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analyzed using single molecule arrays (Simoa). The average fluorescence intensity of beads (I_{bead}) in each pellet was then determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285.

FIG. 4 shows a graph of the I_{bead} signal derived from individual pellets as a function of the storage time for the assay mixtures in the presence or absence of sucrose. As shown in FIG. 4, the DetAb assay mixture including sucrose did not exhibit a loss of signal. The assay mixture not including sucrose did exhibit a loss of signal, and an exponential fit to these data (solid line) showed a rate equal to the previously determined dissociation rate of this detection antibody. These data indicate that the 16.7 wt.% of sucrose reduces the drop of signal due to the dissociation of the detection antibody from the captured PSA molecules.

Example 2

This example describes the effect of 20 wt.% of sucrose on signal loss due to storage of an assay mixture. Assay mixtures that were exposed to 20 wt.% of sucrose prior to storage had less signal loss as a function of time than assay mixtures that were not exposed to sucrose.

A prostate specific antigen (PSA) detection assay was used to investigate the effect of sucrose on signal loss as a function of time. Briefly, paramagnetic beads conjugated with anti-PSA capture antibodies were exposed to solutions containing approximately 10 pg/mL PSA. The captured PSA molecules were then labeled with anti-PSA detection antibody ("DetAb"). Then, the pellets were exposed to a solution containing enzyme conjugate ("SbG") and washed in buffer. After the final wash, the beads were resuspended in a buffer that contained 20% sucrose, pelleted on a magnet, and the supernatant buffer was removed and the pellets dried. The bead pellets were stored for various times and then re-suspended in enzyme substrate. The signal was measured immediately after resuspension in enzyme substrate using single molecule arrays (Simoa). The average number of enzymes per bead (AEB) of each pellet was then determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285. The AEB signal was measured for the different drying times over about a 24 hour time period as shown in Table 1.

FIG. 5 shows a graph of the signal versus the storage time for assay mixtures that were desolvated and stored with sucrose and assay mixtures that were desolvated and stored without sucrose. As shown in Table 1 and FIG. 5, the assay mixture including sucrose showed significantly reduced signal loss during storage.

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Table 1. Signal as a function of storage time (t) for 0 wt.% and 20 wt.% of sucrose

0% Sucrose		20% Sucrose	
t, min	% AEB Maintained	t, min	% AEB Maintained
0	100%	17	94%
24	79%	41	100%
47	77%	61	98%
149	53%	168	94%
174	46%	191	---
196	45%	214	98%
1337	0%	1354	91%
1359	0%	1377	87%

Example 3

This example describes the effect of 16.7 wt.% of sucrose on enzymatic activity after storage of an assay mixture. Assay mixtures that were exposed to 16.7 wt.% of sucrose prior to and after desolvation had little or no reduction in enzymatic activity, whereas assay mixtures that were not exposed to sucrose had a significant reduction in enzymatic activity during storage.

An enzyme detection assay was used to investigation of the effect on sucrose on signal loss. Briefly, paramagnetic beads presenting biotin groups were incubated with solutions containing streptavidin-beta-galactosidase ("SbG"), washed, and then pelleted in the presence or absence of 16.7 wt.% of sucrose, and stored for various periods of time. After storage, the pellets were re-suspended in enzyme substrate and the enzymatic activity was immediately measured by single molecule arrays (Simoa). The average fluorescence intensity of beads (I_{bead}) in each pellet was determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285.

FIG. 6 shows a graph of the average intensity for the active beads in each pellet versus the storage time for the pelleted assay mixtures with and without sucrose. As shown in FIG. 6, the enzymatic activity of SbG when stored in the presence of sucrose was relatively constant over several hours, whereas the enzymatic activity of SbG when stored in the absence of sucrose decreased dramatically after less than about 30 minutes. Since the dissociation rate of biotin and streptavidin is very low, the difference in signal between sucrose and non-sucrose sample was likely due to differences in enzymatic activity (i.e., sucrose reduced denaturation of the enzyme in the dried state) and not dissociation.

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Example 4

This example describes the use of wash buffer containing 16.7 wt.% of sucrose on signal loss when the assay mixture is left dry between wash steps. Sucrose in the wash buffer reduced signal loss due to dissociation and denaturation when the assay mixture was left dry between wash steps. Assays that did not use sucrose in the wash buffer had a significant signal loss.

A PSA detection assay was used to investigate the effect on sucrose on signal loss. Briefly, paramagnetic beads conjugated with anti-PSA capture antibodies were exposed to solutions containing 15 pg/mL (as shown in FIG. 7) or 0.7 pg/mL (as shown in FIG. 8) PSA. The captured PSA molecules were then labeled sequentially with anti-PSA detection antibody ("DetAb") and enzyme conjugate ("SbG") both in the presence of 16.7 wt.% of sucrose. After each incubation with PSA solution, DetAb, or SbG, the beads were washed under four different conditions: 1) DetAb and SbG reagents added immediately after removal of wash buffer, i.e., no drying time, with no sucrose in the wash buffer; 2) DetAb and SbG reagents added 5 min after removal of wash buffer, i.e., 5 min drying time, with no sucrose in the wash buffer; 3) DetAb and SbG reagents added immediately after removal of wash buffer, i.e., no drying time, with 16.7% wt. sucrose in the wash buffer; 4) DetAb and SbG reagents added 5 min after removal of wash buffer, i.e., 5 min drying time, with 16.7% wt. sucrose in the wash buffer. The beads were resuspended in enzyme substrate at the end of the experiment and the signal was measured using single molecule arrays (Simoa). The average number of enzymes per bead (AEB) of each pellet was then determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285.

FIG. 7 and FIG. 8 show bar charts of AEB for the four drying conditions for a sample containing relatively high analyte concentration (15 pg/mL) and a relatively low analyte concentration (0.7 pg/mL), respectively. The data are averages of 4 replicates of the same condition, and the error bars represent one standard deviation of the mean. Regardless of the analyte concentration, the signal loss when the beads were dried for 5 min was lower for assays that used sucrose in the wash buffer. The signal dropped by 37% and 40% compared to the control for low analyte concentration and high analyte concentrations, respectively, when sucrose was not included in the wash buffer.

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Example 5

This example describes the effect of the weight percentage of sucrose on the signal produced without a drying step, e.g., the minimum time required to process bead pellets. The signal increased with the weight percentage of sucrose used during the final pelleting step before the signal was determined. The signal produced using 30 wt.% of sucrose was about 5 times the signal produced when no sucrose was used.

A PSA detection assay was used to investigate the effect of the weight percentage of sucrose on the signal produced after the assay beads were pelleted before the signal was measured. Briefly, paramagnetic beads conjugated with anti-PSA capture antibodies were exposed to solutions containing approximately 30 pg/mL PSA. The captured PSA molecules were then labeled with anti-PSA detection antibody ("DetAb"). Then, the pellets were exposed to a solution containing enzyme conjugate ("SbG") and washed in buffer. After the final wash, the beads were resuspended in a buffer that contained varying concentrations (0-30%) of sucrose, pelleted on a magnet, and the supernatant buffer was removed. The bead pellets were immediately re-suspended in enzyme substrate. The signal was measured immediately after resuspension in enzyme substrate using single molecule arrays (Simoa). The average number of enzymes per bead (AEB) of each pellet was then determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285. FIG. 9 shows a graph of the AEB versus the weight percentage of sucrose. As shown in FIG. 9, the signal increased with increasing sucrose concentration even with the minimum drying time possible.

Example 6

This example describes the effect of the weight percentage of sucrose on aggregation during suspension of dried beads. Sucrose solution containing at least 10 wt.% significantly reduced aggregation of the beads.

A PSA detection assay was used to investigate the effect of the weight percentage of sucrose on aggregation of the beads. Briefly, paramagnetic beads conjugated with anti-PSA capture antibodies were exposed to solutions containing approximately 30 pg/mL PSA. The captured PSA molecules were then labeled with anti-PSA detection antibody ("DetAb"). Then, the pellets were exposed to a solution containing enzyme conjugate ("SbG") and washed in buffer. After the final wash, the beads were resuspended in a buffer that contained varying

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concentrations (0-30%) of sucrose, pelleted on a magnet, and the supernatant buffer was removed. The bead pellets were then dried in air for times varying from 0 to 200 min, re-suspended in enzyme substrate, and the signal was measured using single molecule arrays (Simoa). The bead fill of arrays (i.e., %Fill, equal to the fraction of the wells in the array containing a bead) from each pellet was then determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285. FIG. 10 shows a graph of bead fill percentage versus the weight percentage of sucrose used in the re-suspension step for all drying times. Bead fill is reduced if beads become aggregated during drying as fewer single beads will be available to fit into the wells. As shown in FIG. 10, bead fill was significantly lower at 5% sucrose and below, suggesting that aggregation was significantly decreased when at least 10 wt.% of sucrose was used to suspend the beads.

Example 7

This example describes the effect of various weight percentages of sucrose on signal loss as a function of storage time. The use of sucrose in the assay at all tested weight percentages significantly reduced signal loss over time compared to the absence of sucrose. Sucrose solutions containing between about 10 wt.% and about 30 wt.% had the least signal loss.

A PSA detection assay was used to investigation of the effect of the weight percentage of sucrose on signal loss as a function of time. Briefly, paramagnetic beads conjugated with anti-PSA capture antibodies were exposed to solutions containing approximately 30 pg/mL PSA. The captured PSA molecules were then labeled with anti-PSA detection antibody ("DetAb"). Then, the pellets were exposed to a solution containing enzyme conjugate ("SbG") and washed in buffer. After the final wash, the beads were resuspended in a buffer that contained varying concentrations (0-30%) of sucrose, pelleted on a magnet, and the supernatant buffer was removed. The bead pellets were then dried in air for times varying from 0 to 200 min, re-suspended in enzyme substrate, and the signal was measured using single molecule arrays (Simoa). The average number of enzymes per bead (AEB) of each pellet was then determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285. The normalized signal after 200 minutes of storage for various weight percentages of sucrose is shown in Table 2.

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Table 2. Normalized signal after 200 minutes for various weight percentages of sucrose

%Sucrose	@t=200 min Normalized AEB loss
0	0.238
1	0.749
3	0.713
5	0.765
10	0.804
16.7	1.040
20	0.932
30	0.860

FIG. 11 shows a graph of normalized AEB versus the storage time for various weight percentages of sucrose. As shown in FIG. 11, signal loss was reduced at all weight percentages of sucrose compared to no sucrose. Sucrose solutions containing between about 10 wt.% and about 30 wt.% had the least signal loss.

Example 8

This example describes the effect of various weight percentages of sucrose and various weight percentages of trehalose on signal loss after 200 minutes of storage. Trehalose and sucrose both significantly reduced signal loss compared to the absence of sucrose. Trehalose and sucrose produced a similar reduction in signal loss.

A PSA detection assay was used to investigate the effect of the weight percentage of trehalose and sucrose on signal loss after 200 minutes of storage. Briefly, paramagnetic beads conjugated with anti-PSA capture antibodies were exposed to solutions containing approximately 30 pg/mL PSA. The captured PSA molecules were then labeled with anti-PSA detection antibody ("DetAb"). Then, the pellets were exposed to a solution containing enzyme conjugate ("SbG") and washed in buffer. After the final wash, the beads were resuspended in a buffer that contained varying concentrations of either sucrose or trehalose, pelleted on a magnet, and the supernatant buffer was removed. The bead pellets were then dried in air and stored for 200 min, re-suspended in enzyme substrate, and the signal was measured using single molecule arrays (Simoa). The average number of enzymes per bead (AEB) of each pellet was then determined as described in Rissin et al., Anal. Chem., 2011 83(6), 2279-2285. The normalized AEB signal for

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various sucrose and trehalose concentrations after 200 minutes of storage compared to 0 min of storage time is shown in Table 3.

Table 3. Signal after 200 minutes for various weight percentages of sucrose and trehalose

Sugar	%(Wsugar/Wtotal)	Ratio of AEB @ 200 min compared to 0 min
None	0	0.32
Sucrose	1	0.74
Sucrose	3	0.68
Sucrose	5	0.78
Sucrose	10	0.88
Sucrose	16.7 (3-31-17)	1.06
Sucrose	20	0.86
Sucrose	30	0.96
Trehalose	5	0.80
Trehalose	10	0.80
Trehalose	15	1.16
Trehalose	16.7	0.84
Trehalose	20	0.98
Sucrose	16.7 (4-14-17)	0.94

As shown in Table 3, sucrose and trehalose reduced signal loss at all concentrations. Trehalose and sucrose had a similar reduction in signal loss.

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

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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."

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.

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Claims

What is claimed is:

1. A method for perform an assay, comprising:

combining a stabilizing agent and an assay composition to form a first liquid mixture comprising a liquid, wherein the assay composition comprises detection molecules associated with analyte molecules;

separating at least a portion of a liquid from at least a portion of the first liquid mixture to form a pellet and a separated liquid, wherein the pellet comprises at least a portion of the assay composition and at least a portion of the stabilizing agent; and

removing greater than or equal to about 95% of the separated liquid from the pellet, wherein the stabilizing agent comprises a molecule having greater than four hydroxyl groups.

2. A method for preventing or reducing signal decay in an assay, comprising:

combining a stabilizing agent with an assay composition to form a first liquid mixture, wherein the assay composition comprises detection molecules associated with analyte molecules;

removing greater than or equal to about 95% of a liquid from the first liquid mixture to form a concentrated composition comprising the assay composition and the stabilizing agent;

storing the concentrated composition for at least 1 minute;

reconstituting the concentrated composition to form a second liquid mixture; and

measuring a signal used to determine a concentration of the analyte molecules in the second liquid mixture, wherein a magnitude of the signal measured in the measuring step is greater than or equal to about 10% of the magnitude of a signal used to determine a concentration of the analyte molecules of the analyte molecules in an otherwise identically prepared second liquid mixture except not subjected to the storage step.

3. A method of any preceding claim, wherein the assay composition further comprises a plurality of capture objects associated with a plurality of analyte molecules.

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4. A method of any preceding claim, wherein the pellet or concentrated composition comprises at least 1000 capture objects, or at least 2000 capture objects, or at least 3000 capture objects, or at least 5000 capture objects, or at least 10,000 capture objects.

5. A method of claim 3 or 4, wherein the plurality of capture objects comprise a plurality of beads.

6. The method of any preceding claim, wherein the stabilizing agent is a disaccharide.

7. The method of any preceding claim, wherein the stabilizing agent has a molecular weight of less than or equal to about 500 g/mol.

8. The method of any preceding claim, wherein the stabilizing agent is a sucrose, trehalose, or combinations thereof.

9. The method of any preceding claim, wherein the stabilizing agent comprises a molecule having greater than six hydroxyl groups.

10. The method of any preceding claim, wherein the stabilizing agent alters a dissociation rate between the detection molecules and the analyte molecules in the pellet.

11. The method of any preceding claim, wherein a weight percentage of the stabilizing agent in the first liquid mixture is greater than or equal to about 1 wt.% and less than or equal to about 30 wt%.

12. The method of any preceding claim, wherein a weight percentage of the stabilizing agent in the first liquid mixture is greater than or equal to about 10 wt.% and less than or equal to about 30 wt%.

13. The method of any preceding claim, wherein the concentration of analyte molecules in the assay composition is less than about 50×10^{-15} M.

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14. The method of any preceding claim, wherein the removing step comprises removing greater than or equal to about 98% of the separated liquid from the pellet.

15. The method of any preceding claim, wherein the removing step comprises aspirating greater than or equal to about 95% of the separated liquid from the pellet.

16. The method of any one of claims 1 and 3-15, wherein the removing step comprises exposing the pellet to reduced pressure.

17. The method of any one of claims 1 and 3-16, wherein the removing step comprises exposing the pellet to reduced pressure in the presence of a desiccant.

18. The method of claim 16 or 17, wherein the reduced pressure is less than or equal to about a vapor pressure of the separated liquid.

19. The method of any one of claims 1 and 3-18, wherein the separating step comprises separating the liquid from the first liquid mixture to form the pellet.

20. The method of any one of claims 1 and 3-19, comprising storing the pellet for at least 5 minutes.

21. The method of any preceding one of claims 1 and 3-20, comprising storing the pellet for at least 12 hours.

22. The method of any preceding claim, wherein less than about 5% of the analyte molecules in the composition have dissociated from the detection molecules after the storing step.

23. The method of any one of claims 1 and 3-22, comprising suspending the pellet in a second liquid mixture comprising the stabilizing agent to form a third liquid mixture .

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24. The method of any one of claims 2-15 and 22, wherein a weight percentage of the stabilizing agent in the second liquid mixture is greater than or equal to about 1 wt.% and less than or equal to about 30 wt%.

25. The method of any one of claims 2-15 and 22-24, wherein a weight percentage of the stabilizing agent in the second liquid mixture is greater than or equal to about 10 wt.% and less than or equal to about 30 wt%.

26. The method of any one of claims 23-25, comprising measuring a signal indicative of a concentration of the analyte molecules in the third liquid mixture.

27. The method of any preceding claim, wherein the detection molecules comprises a protein.

28. The method of claim 27, wherein the protein is an enzyme.

29. The method of any preceding claim, wherein the analyte molecule is a biomarker.

30. A method of any one of claims 2-15, 22, and 24-29, wherein the concentrated composition is stored for at least 5 minutes, at least 10 minutes, at least 15 minutes, at least 30 minutes, at least 60 minutes, at least 2 hours, at least 4 hours.

31. The method any one of claims 2-15, 22, and 24-30, wherein the signal used to determine the concentration of the analyte molecules in the second liquid mixture is determined using a digital ELISA assay.

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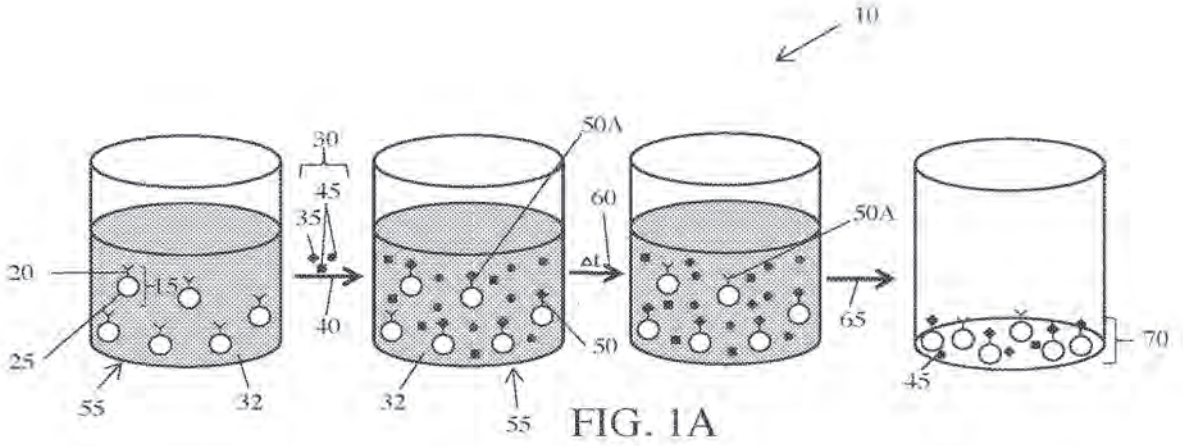


FIG. 1A

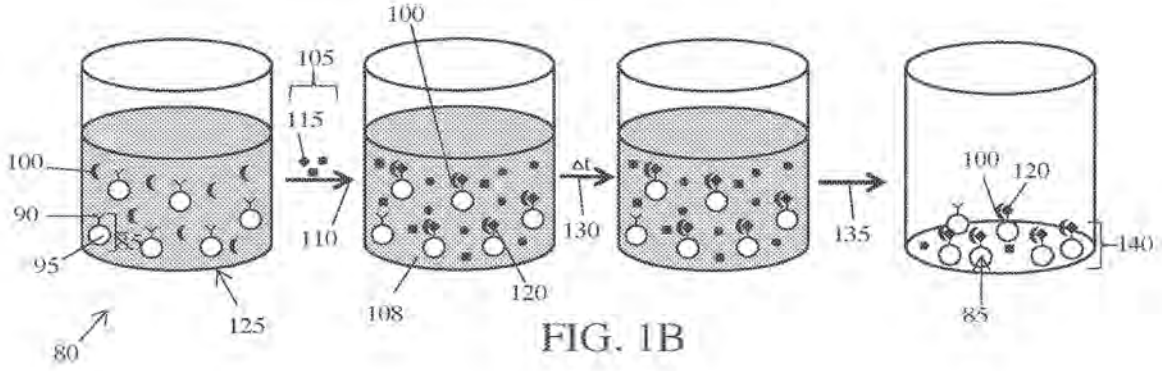


FIG. 1B

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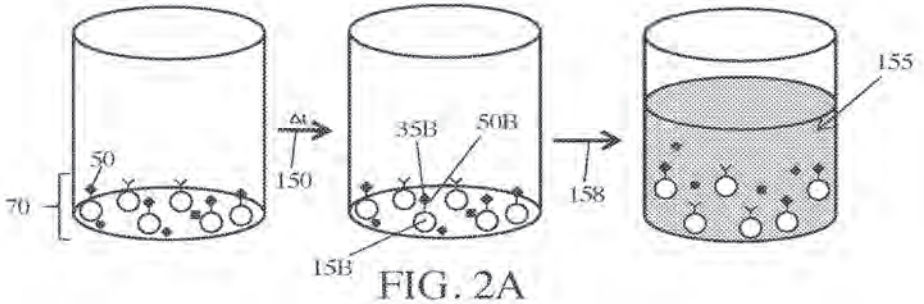


FIG. 2A

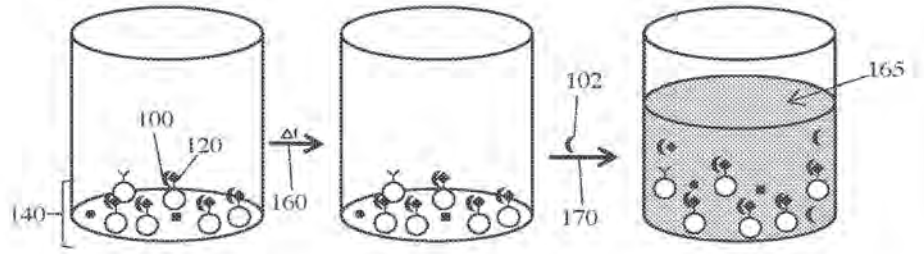


FIG. 2B

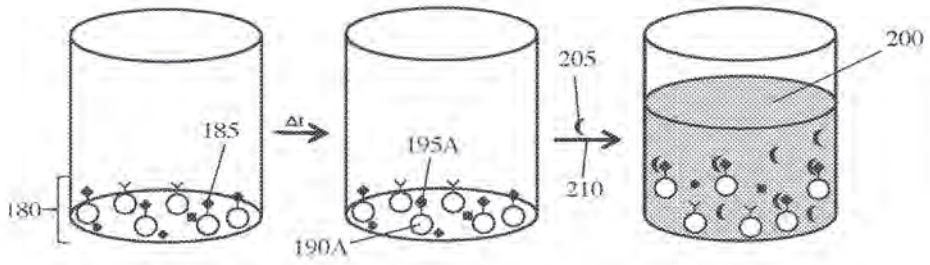


FIG. 2C

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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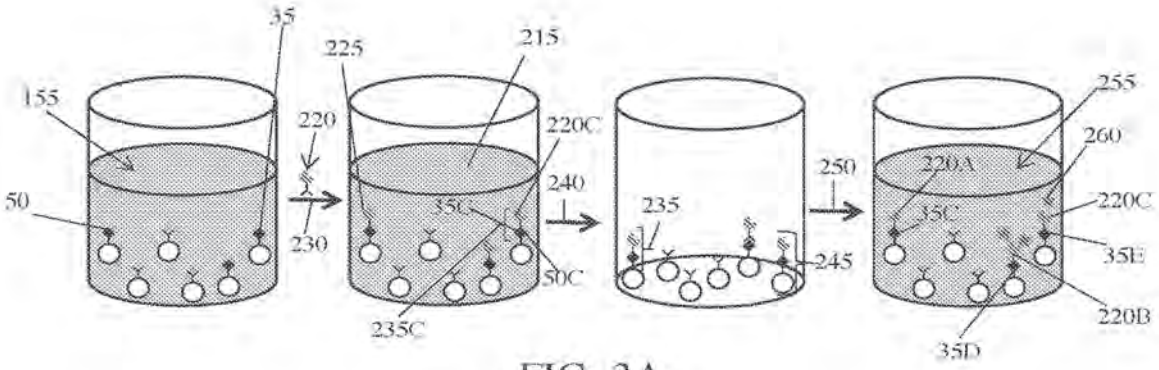


FIG. 3A

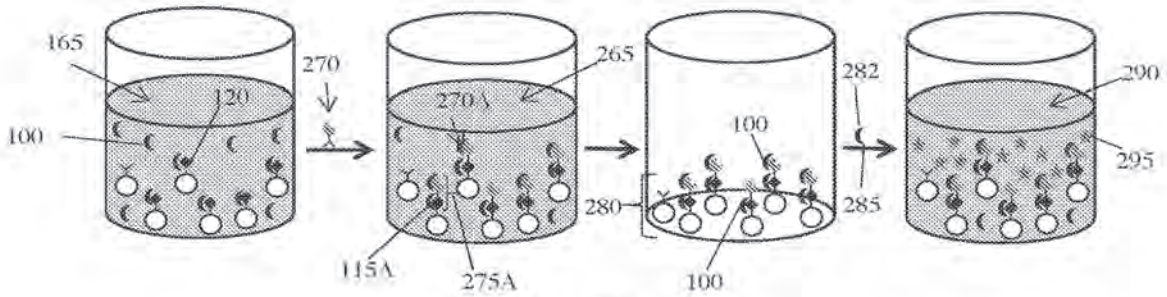


FIG. 3B

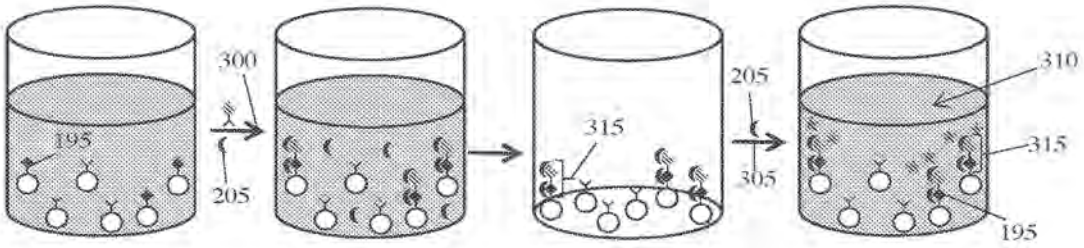


FIG. 3C

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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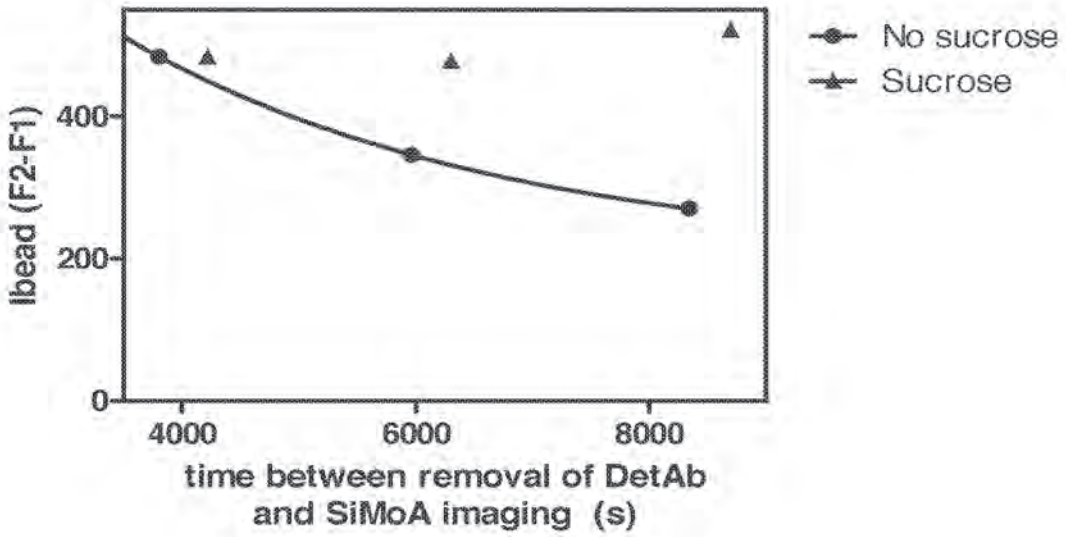


FIG. 4

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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000260

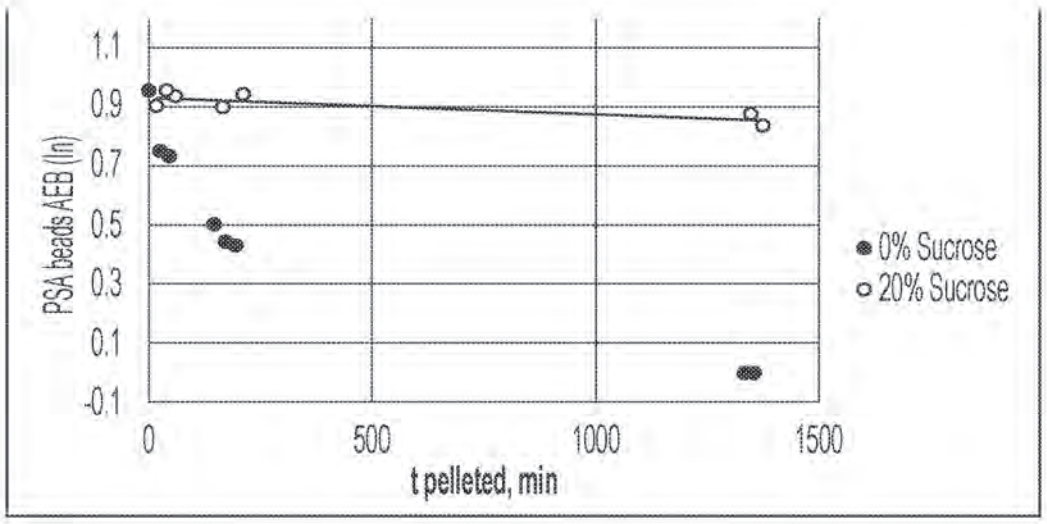


FIG. 5

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000261

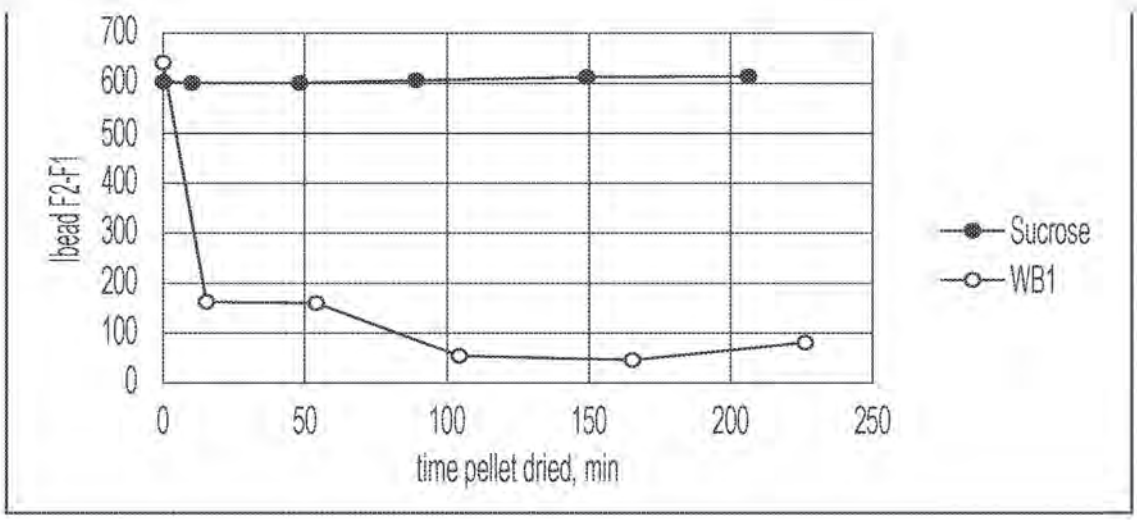


FIG. 6

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000262

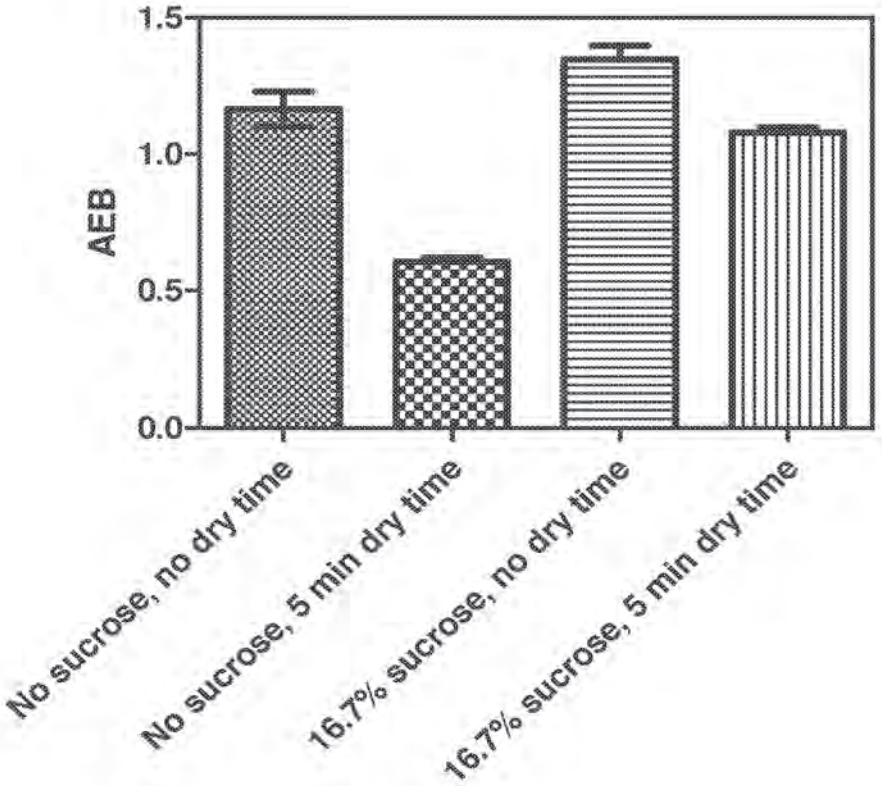


FIG. 7

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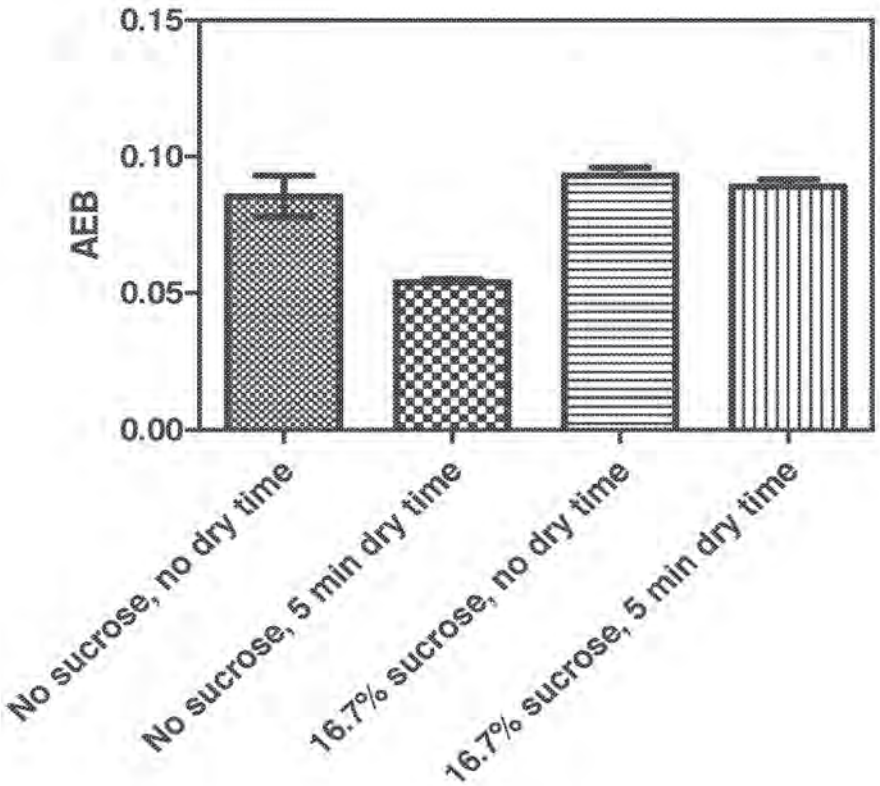


FIG. 8

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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000264

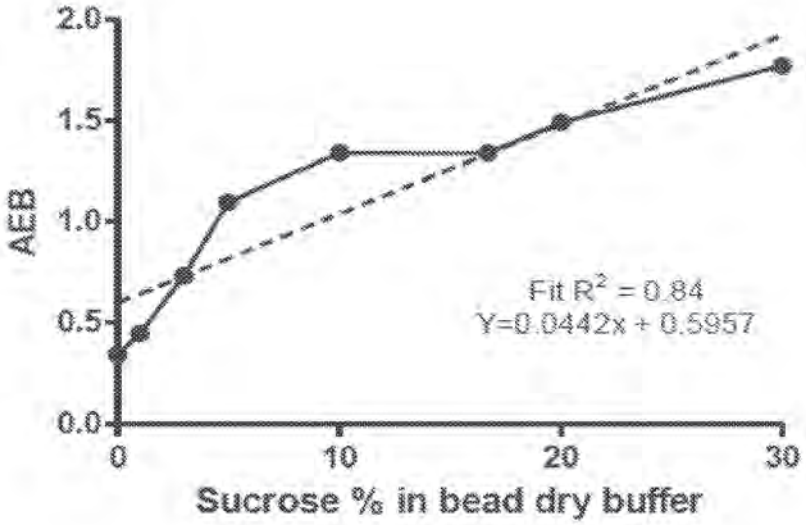


FIG. 9

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000265

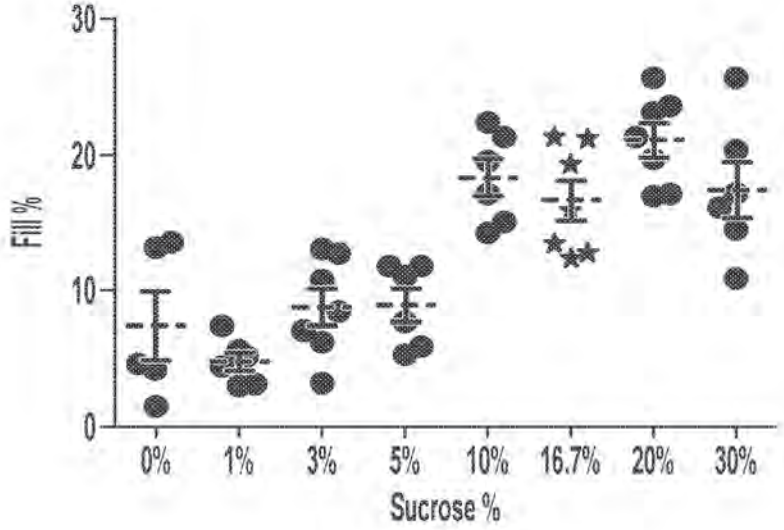


FIG. 10

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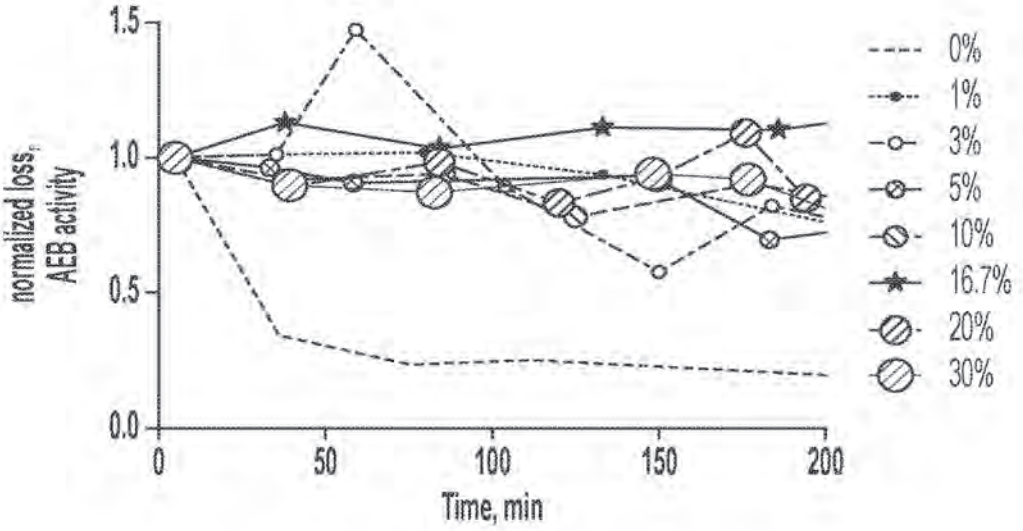


FIG. 11

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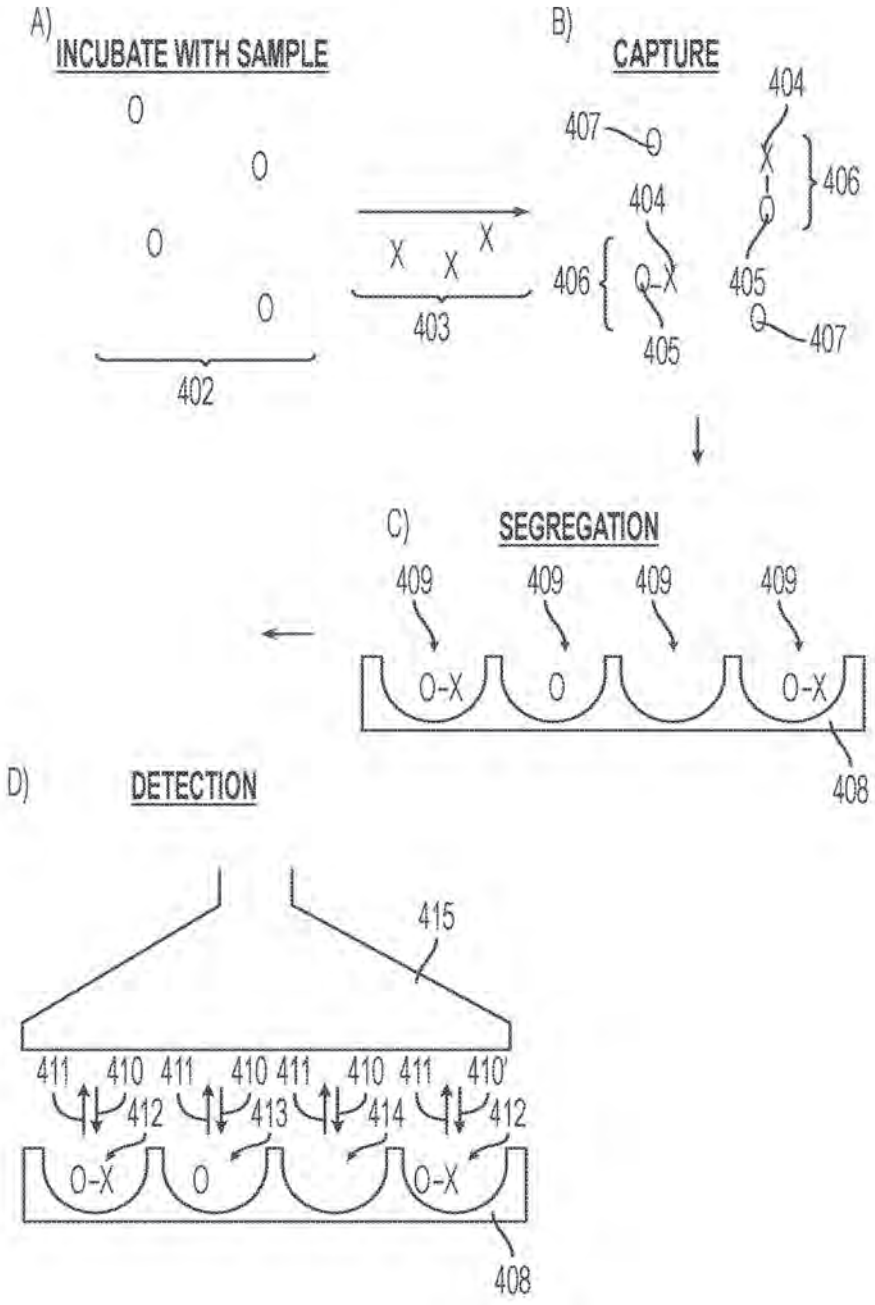


FIG. 12A

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000268

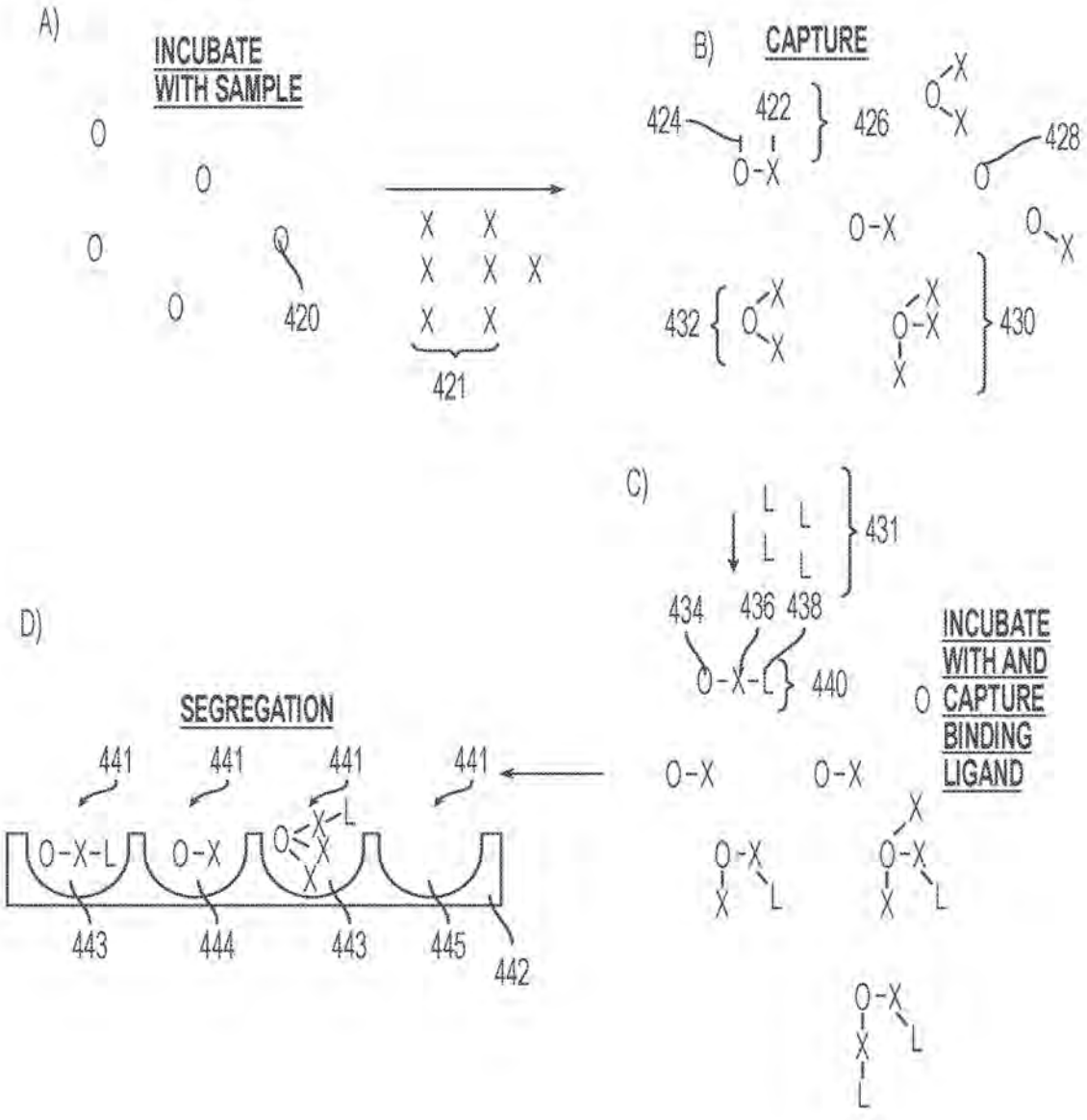


FIG. 12B

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000269

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/052040

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/53 G01N33/542 G01N33/543 G01N33/58
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/091909 A1 (LU WENYUAN [US] ET AL) 21 April 2011 (2011-04-21) abstract paragraph [0022]; examples 1-4 -----	1-31
X	TOSS ET AL: "An evaluation of stabilizing agents in competitive protein-binding assay for 25-hydroxyvitamin D", CLINICA CHIMICA ACTA, ELSEVIER BV, AMSTERDAM, NL, vol. 117, no. 3, 24 December 1981 (1981-12-24), pages 361-364, XP023399630, ISSN: 0009-8981, DOI: 10.1016/0009-8981(81)90124-8 [retrieved on 1981-12-24] page 362, section "Methods".; figure 1 -----	1-31
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 2019	01/02/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jacques, Patrice

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Form PCT/ISA/210 (second sheet) (April 2005)

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/052040

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/58259 A1 (SAVYON DIAGNOSTICS LTD [IL]; OHANA BELLA [IL]) 23 December 1998 (1998-12-23) abstract examples 1-8 -----	1-31
A	JAI K. KAUSHIK ET AL: "Why Is Trehalose an Exceptional Protein Stabilizer? : AN ANALYSIS OF THE THERMAL STABILITY OF PROTEINS IN THE PRESENCE OF THE COMPATIBLE OSMOLYTE TREHALOSE", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 29, 18 July 2003 (2003-07-18), pages 26458-26465, XP055547280, US ISSN: 0021-9258, DOI: 10.1074/jbc.M300815200 the whole document -----	1-31

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2018/052040

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011091909 A1	21-04-2011	CA 2659387 A1	03-04-2008
		CN 101512342 A	19-08-2009
		EP 2047270 A2	15-04-2009
		ES 2427997 T3	05-11-2013
		HK 1127642 A1	07-02-2014
		KR 20090035025 A	08-04-2009
		PT 2047270 E	17-10-2013
		US 2008182341 A1	31-07-2008
		US 2011091909 A1	21-04-2011
		WO 2008039266 A2	03-04-2008
WO 9858259 A1	23-12-1998	AU 7786398 A	04-01-1999
		EP 0995119 A1	26-04-2000
		IL 121116 A	06-12-2000
		JP 2002508843 A	19-03-2002
		WO 9858259 A1	23-12-1998

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	16/522,237-Conf. #4736
Filing Date	July 25, 2019
First Named Inventor	David Wilson
Art Unit	1649
Examiner Name	Not Yet Assigned
Attorney Docket Number	Q0052.70018US03

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] 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):

Form PTO-1449
Copies of cited references

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Michael J. Pomianek/		
Printed name	Michael J. Pomianek		
Date	January 13, 2020	Reg. No.	46,190

Certificate of Electronic Filing under 37 C.F.R. § 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 C.F.R. § 1.6(a)(4).

Dated: January 13, 2020 Electronic Signature for Nathaniel W. Taylor: /Nathaniel W. Taylor/

7510713.1

DOCKET NO.: Q0052.70018US03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	David Wilson
Application No.:	16/522,237
Confirmation No.:	4736
Filed:	July 25, 2019
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:	1649

Certificate of Electronic Filing under 37 C.F.R. § 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 C.F.R. § 1.6(a)(4).

Dated: January 13, 2020 Electronic Signature for Nathaniel W. Taylor: /Nathaniel W. Taylor/

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 before the mailing date of a first Office Action on the merits in the above-identified case.

No fee or certification is required.

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.

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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 applications that may contain subject matter related to this application:

Serial No.	Filing Date	Inventor(s)	Docket No.
*15/487,889	04-14-2017	Duffy et al.	Q0052.70011US05
*16/381,614	04-11-2019	Duffy et al.	Q0052.70011US06
*15/921,429	03-14-2018	Fournier et al.	Q0052.70014US02
*15/808,969	11-10-2017	Rissin et al.	Q0052.70015US02
*14/963,421	12-09-2015	Wilson et al.	Q0052.70016US02
*14/111,331	12-18-2013	Wilson et al.	Q0052.70017US01
*15/942,379	03-30-2018	Duffy et al.	Q0052.70019US02
*14/889,982	11-09-2015	Rissin et al.	Q0052.70020US01
*15/543,401	07-13-2017	Duffy et al.	Q0052.70021US01

*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).

The undersigned would like to bring to the Examiner's attention the following other information:

Office Communication for US App. No. 14/111,331 mailed December 12, 2019 and claims pending (Q0052.70017US01)

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).

***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. 15/269,142, filed September 19, 2016, 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.

Respectfully submitted,

Electronic signature: /Michael J. Pomianek/
Michael J. Pomianek
Registration No.: 46,190
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

Docket No.: Q0052.70018US03
Date: January 13, 2020

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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
16/522,237	07/25/2019	1629	1880	Q0052.70018US03	18	1

CONFIRMATION NO. 4736

UPDATED FILING RECEIPT

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

OC000000113872144

Date Mailed: 01/14/2020

Receipt is acknowledged of this non-provisional utility 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 FIRST INVENTOR, 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 corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction 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 provided that the request is grantable.

Inventor(s)

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

Applicant(s)

Quanterix Corporation, Billerica, MA;

Assignment For Published Patent Application

Quanterix Corporation, Billerica, 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 15/269,142 09/19/2016 PAT 10393759
which is a CON of 14/111,326 06/24/2014 ABN
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.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
EX-1002
000279

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

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If Required, Foreign Filing License Granted: 08/07/2019

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/522,237**

Projected Publication Date: 04/23/2020

Non-Publication Request: No

Early Publication Request: No

Title

METHODS OF DETERMINING A TREATMENT PROTOCOL FOR AND/OR A PROGNOSIS OF A PATIENT'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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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/522,237

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	300
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	18 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	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	1720

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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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/522,237	07/25/2019	David Wilson	Q0052.70018US03

CONFIRMATION NO. 4736

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

POA ACCEPTANCE LETTER



Date Mailed: 01/14/2020

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 01/08/2020.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

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**.

/mteklemichael/

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/522,237	07/25/2019	David Wilson	Q0052.70018US03

CONFIRMATION NO. 4736

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

PUBLICATION NOTICE



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

Publication No.US-2020-0124620-A1
Publication Date:04/23/2020

NOTICE OF PUBLICATION OF APPLICATION

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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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/522,237	ATTY. DOCKET NO.: Q0052.70018US03
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 25, 2019	CONFIRMATION NO.: 4736
				FIRST NAMED INVENTOR: David Wilson	
Sheet	1	of	2	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		
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		10,725,032	B2	Duffy et al.	07-28-2020
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		2020-0393457	A1	Duffy et al.	12-17-2020

FOREIGN PATENT DOCUMENTS

Examiner's Initials *	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		EP	2 324 360	B1	Banyan Biomarkers, Inc.	01-31-2018	
		WO	91/06559	A1	E. I. Du Pont de Nemours and Company	05-16-1991	
		WO	2005/095262	A1	Nanyang Technological University	10-13-2005	
		WO	2013/103475	A2	Massachusetts Institute of Technology	07-11-2013	

OTHER ART – NON PATENT LITERATURE DOCUMENTS

Examiner's Initials *	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		KAN et al., Isolation and detection of single molecules on paramagnetic beads using sequential fluid flows in microfabricated polymer array assemblies. Lab Chip. 2012 Mar 7;12(5):977-85. Epub 2011 Dec 16.	
		KOIKE et al., Oligemic hypoperfusion differentially affects tau and amyloid-{beta}. Am J Pathol. 2010;177(1):300-310.	

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.

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000286

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 16/522,237	ATTY. DOCKET NO.: Q0052.70018US03
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 25, 2019	CONFIRMATION NO.: 4736
				FIRST NAMED INVENTOR: David Wilson	
Sheet	2	of	2	GROUP ART UNIT: 1649	EXAMINER: A. M. Weidner

		WANG et al., Cerebrovascular hypoperfusion induces spatial memory impairment, synaptic changes, and amyloid-β oligomerization in rats. J Alzheimers Dis. 2010;21(3):813-822.	

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8931142.1

Electronic Acknowledgement Receipt

EFS ID:	41868956
Application Number:	16522237
International Application Number:	
Confirmation Number:	4736
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:	Michael Pomianek/Timothy Peng
Filer Authorized By:	Michael Pomianek
Attorney Docket Number:	Q0052.70018US03
Receipt Date:	08-FEB-2021
Filing Date:	25-JUL-2019
Time Stamp:	18:29:08
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	Foreign Reference	EP2324360B1.pdf	2089897 595799850087a19618e08e7aeaafbd5a20[illegible]3824d	no	42

Warnings:

Information:					
2	Foreign Reference	WO9106559A1.pdf	930752 0be88c59673699b79a86c84fed57c66209...3dc7f	no	25
Warnings:					
Information:					
3	Foreign Reference	WO2005095262A1_QC.pdf	1929518 4ba874d8a2bd626c9af45410dbf4e71221aae...a3d8	no	34
Warnings:					
Information:					
4	Foreign Reference	WO2013103475A2.pdf	5481647 6bAe4acc1131b045a92c0fefb293e76e5b51...a655	no	97
Warnings:					
Information:					
5	Non Patent Literature	Kan_et_al_Lab_Chip_2012_v12_p977.pdf	2956509 8ba0559f67030753d4845acdcbea151008f0...b626	no	10
Warnings:					
Information:					
6	Non Patent Literature	KOIKE_et_al_Am_J_Pathol_2010_v177_p300.pdf	1614611 e2944cb0944462c7cdfcf6b65a6d37b4e72b...3f40f	no	11
Warnings:					
Information:					
7	Non Patent Literature	WANG_et_al_J_Alzheimers_Dis_2010_v21_p813.pdf	816034 62c0216d0be993ec9194b290320f90b2310...8e245	no	10
Warnings:					
Information:					
8	Miscellaneous Incoming Letter	Q005270018US03-TRN-MJP.pdf	26169 9da1e08a3bad8313f60d62b755d085c88b...e096	no	1
Warnings:					
Information:					

9	Transmittal Letter	Q005270018US03-IDSBODY-MJP.pdf	31856 6e0b6821e4d2db5fa67362667c51998e07f95e3e29	no	3
Warnings:					
Information:					
10	Information Disclosure Statement (IDS) Form (SB08)	Q005270018US03-IDS1449-MJP.pdf	35609 a73ebb986c2aece2e694a17de1c9ecf100020fa0c3e	no	2
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		Total Files Size (in bytes):	15912602		

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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.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 324 360 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
31.01.2018 Bulletin 2018/05

(21) Application number: **09807153.3**

(22) Date of filing: **11.08.2009**

(51) Int Cl.:
G01N 33/68 *(2006.01)*

(86) International application number:
PCT/US2009/053376

(87) International publication number:
WO 2010/019553 (18.02.2010 Gazette 2010/07)

(54) **BIOMARKER DETECTION PROCESS AND ASSAY OF NEUROLOGICAL CONDITION**

VERFAHREN FÜR DEN NACHWEIS VON BIOMARKERN UND TEST FÜR NEUROLOGISCHE ERKRANKUNG

PROCÉDÉ DE DÉTECTION DE BIOMARQUEURS ET TEST D'ÉTAT NEUROLOGIQUE

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK SM TR

(30) Priority: **11.08.2008 US 188554 P**
17.09.2008 US 97622 P
19.06.2009 US 218727 P

(43) Date of publication of application:
25.05.2011 Bulletin 2011/21

(73) Proprietor: **Banyan Biomarkers, Inc.**
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28036 Madrid (ES)

(56) References cited:
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US-A1- 2005 260 697

- **P. E. VOS ET AL: "Glial and neuronal proteins in serum predict outcome after severe traumatic brain injury", NEUROLOGY, vol. 62, no. 8, 27 April 2004 (2004-04-27) , pages 1303-1310, XP55030077, ISSN: 0028-3878, DOI: 10.1212/01.WNL.0000120550.00643.DC**
- **MISSLER, U., ET AL: "Measurement of Glial Fibrillary Acidic Protein in Human Blood: Analytical Method and Preliminary Clinical Results", CLINICAL CHEMISTRY, vol. 45, no. 1, 1999, pages 138-141, XP002677842,**
- **ANNALISA DEL PRETE THROMB. HAEMOST. vol. 95, 2006, pages 22 - 28, XP008144118**
- **VERCAUTEREN ET AL: "Proteomic approaches in brain research and neuropharmacology", EUROPEAN JOURNAL OF PHARMACOLOGY, ELSEVIER SCIENCE, NL, vol. 500, no. 1-3, 1 October 2004 (2004-10-01), pages 385-398, XP005480430, ISSN: 0014-2999, DOI: 10.1016/J.EJPHAR.2004.07.039**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

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EP 2 324 360 B1

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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Description

GOVERNMENTAL SUPPORT

[0001] Portions of this work were supported by grants N14-06-1-1029, W81XWH-8-1-0376 and W81XWH-07-01-0701 from the United States Department of Defense.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to US Provisional Application No. 61/188,554 filed August 11, 2008; US Provisional Application No. 61/097,622 filed September 17, 2008; U.S. Provisional Application No. 61/218,727 filed June 19, 2009; and US Provisional Application No. 61/271,135 filed July 18, 2009.

FIELD OF THE INVENTION

[0003] The present invention in general relates to determination of neurological condition of an individual, wherein said neurological condition is selected from traumatic brain injury and stroke, and in particular to measuring the quantity of a neuroprotective biomarker such as glial fibrillary acidic protein (GFAP) in concert with another biomarker of neurological condition.

BACKGROUND OF THE INVENTION

[0004] The field of clinical neurology remains frustrated by the recognition that secondary injury to a central nervous system tissue associated with physiologic response to the initial insult could be lessened if only the initial insult could be rapidly diagnosed or in the case of a progressive disorder before stress on central nervous system tissues reached a preselected threshold. Traumatic, ischemic, and neurotoxic chemical insult, along with generic disorders, all present the prospect of brain damage. While the diagnosis of severe forms of each of these causes of brain damage is straightforward through clinical response testing, computed tomography (CT), and magnetic resonance imaging (MRI), the imaging diagnostics are limited by both the high cost of spectroscopic imaging and long diagnostic time. The clinical response testing of incapacitated individuals is of limited value and often precludes a nuanced diagnosis. Additionally, owing to the limitations of existing diagnostics, situations arise wherein a subject experiences a stress to their neurological condition but are often unaware that damage has occurred or fail seek treatment as the subtle symptoms often quickly resolve. The lack of treatment of these mild to moderate challenges to neurologic condition of a subject can have a cumulative effect or otherwise result in a severe brain damage event, either of which have a poor clinical prognosis.

[0005] In order to overcome the limitations associated with spectroscopic and clinical response diagnosis of neurological condition, there is increasing attention on the use of biomarkers as internal indicators of change to molecular or cellular level health condition of a subject. As biomarker detection uses a sample obtained from a subject, typically cerebrospinal fluid, blood, or plasma, and detects the biomarkers in that sample, biomarker detection holds the prospect of inexpensive, rapid, and objective measurement of neurological condition. The attainment of rapid and objective indicators of neurological condition allows one to determine severity of a non-normal brain condition with a previously unrealized degree of objectivity, predict outcome, guide therapy of the condition, as well as monitor subject responsiveness and recovery. Additionally, such information as obtained from numerous subjects allows one to gain a degree of insight into the mechanism of brain injury.

[0006] A number of biomarkers have been identified as being associated with severe traumatic brain injury as is often seen in vehicle collision and combat wounded subjects. These biomarkers included spectrin breakdown products such as SBDP150, SBDP150i, SBDP145 (calpain mediated acute neural necrosis), SBDP120 (caspase mediated delayed neural apoptosis), UCH-L1 (neuronal cell body damage marker), and MAP2 dendritic cell injury associated marker. The nature of these biomarkers is detailed in U.S. Patents 7,291,710 and 7,396,654.

[0007] Glial Fibrillary Acidic Protein (GFAP), a member of the cytoskeletal protein family, is the principal 8-9 nanometer intermediate filament of glial cells such as mature astrocytes of the central nervous system (CNS). GFAP is a monomeric molecule with a molecular mass between 40 and 53 kDa and an isoelectric point between 5.7 and 5.8. GFAP is highly brain specific protein that is not found outside the CNS. GFAP is released into the blood and CSF soon after brain injury. In the CNS following injury, either as a result of trauma, disease, genetic disorders, or chemical insult, astrocytes become reactive in a way that is characterized by rapid synthesis of GFAP termed astrogliosis or gliosis. However, GFAP normally increases with age and there is a wide variation in the concentration and metabolic turnover of GFAP in brain tissue.

[0008] Thus, there exists a need for a process and an assay for providing improved measurement of neurological condition through the quantification of a first biomarker such as GFAP in combination with another biomarker associated with neurological condition.

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SUMMARY OF THE INVENTION

[0009] A process for determining the neurological condition of a subject, wherein said neurological condition is selected from traumatic brain injury and stroke includes measuring a sample obtained from the subject at a first time for a quantity of a first biomarker and a quantity of at least one additional neuroactive biomarker, wherein said first biomarker is GFAP and said at least one additional neuroactive biomarker is UCH-L1. Through comparison of the quantity of the first biomarker and the quantity of the at least one additional neuroactive biomarker to normal levels for each biomarker, the neurological condition of the subject is determined. When the subject have been exposed to an event that could cause mild traumatic brain injury and moderate traumatic brain injury, a process of measuring UCH-L1 and GFAP, such injuries have detection cutoffs for UCH-L1 and GFAP in serum of 0.39 nanograms per milliliter (ng/ml) and 1.4ng/ml, respectively.

[0010] An assay for determining the neurological condition of a subject is also provided. The assay includes: (a) a substrate for holding a biological sample isolated from a subject (b) a first biomarker specifically binding agent wherein a first biomarker is GFAP (c) a binding agent specific for another neuroactive biomarker wherein said another neuroactive biomarker is UCH-L1; and (d) printed instructions for reacting the first biomarker specific agent with a first portion of the sample so as to detect an amount of said first biomarker and reacting said at least one additional neuroactive biomarker specific agent with a second portion of the sample and the at least one additional neuroactive biomarker in the sample so as to detect an amount of said at least one additional neuroactive biomarker for relation to the condition of the subject or cells derived the subject.

[0011] A process for determining if a subject has suffered mild traumatic brain injury or moderate traumatic brain injury in an event comprising: measuring by ELISA a biological sample obtained from said subject for a quantity of GFAP, wherein said biological sample is whole blood, serum or plasma; and comparing the measured quantity of said GFAP to normal levels of GFAP in a control group who had not experienced any form of head injury, wherein if the measured quantity of GFAP is between 0.082 and 0.492 nanograms per milliliter, the subject has suffered mild traumatic brain injury or moderate brain injury in the event.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012]

Fig. 1 represents quantitative western blotting of UCH-L1 in rat CSF following MCAO;
Fig. 2 represents UCH-L1 levels in CSF in sham and CCI treated subjects;
Fig. 3 represents UCH-L1 levels in CSF following sham, mild MCAO challenge, and severe MCAO challenge;
Fig. 4 represents UCH-L1 levels in serum following sham or CCI at various timepoints;
Fig. 5 represents UCH-L1 levels in serum following sham, mild MCAO challenge, and severe MCAO challenge;
Fig. 6 represents SBDP145 levels in CSF and serum following sham, mild MCAO challenge, and severe MCAO challenge;
Fig. 7 represents SBDP120 levels in CSF and serum following sham, mild MCAO challenge, and severe MCAO challenge;
Fig. 8 represents MAP2 elevation in CSF and serum following sham, mild MCAO challenge, and severe MCAO challenge;
Fig. 9 are bar graphs of GFAP and other biomarkers for human control and severe TBI subjects from CSF samples;
Fig. 10 are bar graphs of GFAP and other biomarkers for human control and severe TBI subjects of Fig. 1 from serum samples;
Fig. 11 are bar graphs of GFAP and other biomarkers for human control and severe TBI subjects summarizing the data of FIGS. 9 and 10;
Fig. 12 are plots of arterial blood pressure (MABP), intracranial pressure (ICP) and cerebral profusion pressure (CPP) for a single human subject of traumatic brain injury as a function of time;
Fig. 13 are plots of inventive biomarkers from CSF and serum samples from the single human subject of traumatic brain injury of FIG. 12 as a function of time;
Fig. 14 are plots of inventive biomarkers from CSF and serum samples from another individual human subject of traumatic brain injury as a function of time;
Fig. 15 are plots of UCH-L1 amounts being present in CSF and serum post severe traumatic brain injury in a mouse subject;
Fig. 16 are bar graphs of GFAP concentration for controls, as well as individuals in the mild/moderate traumatic brain injury cohort as a function of CT scan results upon admission and 24 hours thereafter;
Fig. 17 are bar graphs of parallel assays for UCH-L1 biomarker from the samples used for FIG. 16;
Fig. 18 are bar graphs showing the concentration of UCH-L1 and GFAP as well as a biomarker not selected for diagnosis of neurological condition, S100 beta, provided as a function of injury magnitude between control, mild,

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and moderate traumatic brain injury;

Fig. 19 are bar graphs showing the concentration of the same markers as depicted in FIG. 18 with respect to initial evidence upon hospital admission as to lesions in tomography scans;

Fig. 20 represents biomarker levels in human subjects with varying types of brain injury;

Fig. 21 are plots that represent ROC analysis of UCH-L1, GFAP and SBDP145 in human CSF (severe TBI vs. Control A) First 24 hours post-injury;

Fig. 22 is a plot that represent ROC analysis of UCH-L1 and GFAP in human CSF (mild TBI vs. normal Controls) a mean of 3h35' with a range 15'-14h35 post-injury.

Fig. 23 are bar graphs of showing the elevation of brain injury biomarkers (GFAP, UCH-L1 and MAP2) in plasma from stroke patients.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0013] The present invention has utility in the diagnosis and management of abnormal neurological condition, wherein said neurological condition is selected from traumatic brain injury and stroke. Through the measurement of a biomarker such as GFAP from a subject in combination with values obtained for an additional neuroactive biomarker, a determination of subject neurological condition is provided with greater specificity than previously attainable. The present description is directed toward a first biomarker of GFAP. It is appreciated that the invention encompasses several other additional biomarkers illustratively including UCH-L1, NSE, MAP2, and SBDP, wherein at least one additional biomarker is UCH-L1. Surprisingly, by combining the detection of more than one biomarker, a synergistic result is achieved. Illustratively, combining the detection of two neuroactive biomarkers such as UCH-L1 and GFAP provides sensitive detection that is unexpectedly able to discern the level and severity of an abnormal neurological condition in a subject.

[0014] The present invention provides for the detection of a neurological condition in a subject, wherein said neurological condition is selected from traumatic brain injury an stroke. As such, it is a further object of the present invention to provide a means for detecting or diagnosing an abnormal neurological condition in a subject.

[0015] The present invention also provides an assay for detecting or diagnosing the neurological condition of a subject.

[0016] For purposes of the subject invention, brain injury is divided into two levels, mild traumatic brain injury (MTBI), and traumatic brain injury (TBI). An intermediate level of moderate TBI is also referred to herein. The spectrum between MTBI and extending through moderate TBI is also referred to synonymously mild to moderate TBI or by the abbreviation MMTBI. TBI is defined as an injury that correlates with a two-fold increase or greater of two-fold decrease or greater in molecular marker levels or activities. MTBI is defined and an injury that correlates with less than a two-fold increase or two-fold decrease in molecular marker levels or activities.

[0017] An inventive process preferably includes determining the neurological condition of a subject by assaying a sample derived from a subject at a first time for the presence of a first biomarker. A biomarker is a cell, protein, nucleic acid, steroid, fatty acid, metabolite, or other differentiator useful for measurement of biological activity or response. Biomarkers operable herein illustratively include: ubiquitin carboxyl-terminal esterase, ubiquitin carboxy-terminal hydrolase, spectrin breakdown product(s), a neuronally-localized intracellular protein, MAP-tau, C-tau, MAP2, poly (ADP-ribose) polymerase (PARP), collapsin response mediator protein, Annexin A11, Aldehyde dehydrogenase family 7, Cofilin 1, Profilin 1, α-Enolase (non-neural enolase), Enolase 1 protein, Glyceraldehyde-3-phosphate dehydrogenase, Hexokinase 1, Aconitase 2, Acetyl-CoA synthetase 2, Neuronal protein 22, Phosphoglycerate kinase 2, Phosphoglycerate kinase 1, Hsc70-ps1, Glutamate dehydrogenase 1, Aldolase A, Aldolase C, fructose-biphosphate, Dimethylarginine dimethylaminohydrolase 1, Microtubule-associated protein 2, Carbonic anhydrase, ADP-ribosylation factor 3, Transferrin, Liver regeneration-related protein, Hemoglobin α-chain, Hemoglobin β chain, Liver regeneration-related protein, Fetuin β, 3-Oxoacid-CoA transferase, Malate dehydrogenase 1, NAD (soluble), Lactate dehydrogenase B, Malate dehydrogenase, Carboxylesterase E1 precursor, Serine protease inhibitor α1, Haptoglobin, Ubiquitin carboxyl-terminal hydrolase LI, Serine protease inhibitor 2a, T-kininogen, α1 major acute phase protein, Albumin, α1 major acute phase protein prepeptide, Murinoglobulin 1 homolog, Group-specific component protein, Guanosine diphosphate dissociation inhibitor 1, Collapsin response mediator protein 2, Murinoglobulin 1 homolog, Ferroxidase, Ceruloplasmin, Spectrin α-chain, brain, C-reactive protein, Brain creatine kinase, Proteasome subunit α-type 7, 14-3-3 protein, Synaptotagmin, sublypes thereof, fragments thereof, breakdown products thereof, or combinations thereof. Other potential biomarkers illustratively include those identified by Kobeissy, FH, et al, Molecular & Cellular Proteomics, 2006; 5:1887-1898, the contents of which are incorporated herein by reference, or others known in the art.

[0018] A first biomarker is a neuroactive biomarker GFAP. Illustrative examples of neuroactive biomarkers include GFAP, ubiquitin carboxyl-terminal esterase L1 (UCH-L1), Neuron specific enolase (NSE), spectrin breakdown products (SBDP), preferably SBDP150, SBDP150i SBDP145, SBDP120, S100 calcium binding protein B (S100b), microtubule associated proteins (MAP), preferably MAP2, MAP1, MAP3, MAP4, MAP5, myelin basic protein (MBP), Tau, Neurofilament protein (NF), Cannabinoid Receptor (CB), CAM proteins, Synaptic protein, collapsin response mediator proteins (CRMP), inducible nitric oxide synthase (iNOS), Neuronal Nuclei protein (NeuN), 2',3'-cyclic nucleotide-3'-phosphohy-

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drolase (CNPase), Neuroserpin, alpha-internexin, microtubule-associated protein 1 light chain 3 (LC3), Neurofascin, the glutamate transporters (EAAT), Nestin, Cortin-1, 2', , and BIII-Tubulin.

[0019] The inventive process also includes assaying the sample for at least one additional neuroactive biomarker, wherein said at least one additional neuroactive biomarker is UCH-L1. Any of the aforementioned inventive biomarkers are operable as an additional neuroactive biomarker. Any number of biomarkers can be detected such as 2, 3, 4, 5, 6, 7, 8, 9, 10, or more. Detection can be either simultaneous or sequential and may be from the same biological sample or from multiple samples from the same or different subjects. Preferably, detection of multiple biomarkers is in the same assay chamber. The inventive process further includes comparing the quantity of the first biomarker and the quantity of the at least one additional neuroactive biomarker to normal levels of each of the first biomarker and the one additional neuroactive biomarker to determine the neurological condition of the subject.

[0020] In a preferred embodiment a biomarker is GFAP. GFAP is associated with glial cells such as astrocytes. Preferably, an additional neuroactive biomarker is associated with the health of a different type of cell associated with neural function. For example, CNPase is found in the myelin of the central nervous system, and NSE is found primarily in neurons. More preferably, the other cell type is an axon, neuron, or dendrite.

[0021] In another preferred embodiment, especially for MBTI and MMTBI, is UCH-L1 in combination with other biomarkers such as GFAP and MAP2.

[0022] It is appreciated however, that multiple biomarkers may be predictors of different modes or types of damage to the same cell type. Through the use of an inventive assay inclusive of biomarkers associated with glial cells as well as at least one other type of neural cell, the type of neural cells being stressed or killed as well as quantification of neurological condition results provides rapid and robust diagnosis of traumatic brain injury type. Measuring GFAP along with at least one additional neuroactive biomarker and comparing the quantity of GFAP and the additional biomarker to normal levels of the markers provides a determination of subject neurological condition.

[0023] Preferably, specific biomarker levels that when measured in concert with GFAP afford superior evaluation of subject neurological condition include SBDP 150, SBDP150i, a combination of SBDP145 (calpain mediated acute neural necrosis) and SBDP120 (caspase mediated delayed neural apoptosis), UCH-L1 (neuronal cell body damage marker), and MAP2. This is noted to be of particular value in measuring MMTBI and screening drug candidates or other neural cell stressor compounds with cell cultures.

[0024] A sample is preferably a biological sample. Preferred examples of biological samples are illustratively cells, tissues, cerebral spinal fluid (CSF), artificial CSF, whole blood, serum, plasma, cytosolic fluid, urine, feces, stomach fluids, digestive fluids, saliva, nasal or other airway fluid, vaginal fluids, semen, buffered saline, saline, water, or other biological fluid recognized in the art. Most preferably, a biological sample is CSF or blood serum. It is appreciated that two or more separate biological samples are optionally assayed to elucidate the neurological condition of the subject.

[0025] In addition to increased cell expression, biomarkers also appear in biological fluids in communication with injured cells. Obtaining biological fluids such as cerebrospinal fluid (CSF), blood, plasma, serum, saliva and urine, from a subject is typically much less invasive and traumatizing than obtaining a solid tissue biopsy sample. Thus, samples that are biological fluids are preferred for use in the invention. CSF, in particular, is preferred for detecting nerve damage in a subject as it is in immediate contact with the nervous system and is readily obtainable. Serum is a preferred biological sample as it is easily obtainable and presents much less risk of further injury or side-effect to a donating subject.

[0026] To provide correlations between neurological condition and measured quantities of GFAP and other neuroactive biomarkers, samples of CSF or serum are collected from subjects with the samples being subjected to measurement of GFAP as well as other neuroactive biomarkers. The subjects vary in neurological condition. Detected levels of GFAP and other neuroactive biomarkers are optionally then correlated with CT scan results as well as GCS scoring. Based on these results, an inventive assay is developed and validated (Lee et al., Pharmacological Research 23:312-328, 2006). It is appreciated that GFAP and other neuroactive biomarkers, in addition to being obtained from CSF and serum, are also readily obtained from blood, plasma, saliva, urine, as well as solid tissue biopsy. While CSF is a preferred sampling fluid owing to direct contact with the nervous system, it is appreciated that other biological fluids have advantages in being sampled for other purposes and therefore allow for inventive determination of neurological condition as part of a battery of tests performed on a single sample such as blood, plasma, serum, saliva or urine.

[0027] A biological sample is obtained from a subject by conventional techniques. For example, CSF is preferably obtained by lumbar puncture. Blood is preferably obtained by venipuncture, while plasma and serum are obtained by fractionating whole blood according to known methods. Surgical techniques for obtaining solid tissue samples are well known in the art. For example, methods for obtaining a nervous system tissue sample are described in standard neurosurgery texts such as Atlas of Neurosurgery: Basic Approaches to Cranial and Vascular Procedures, by F. Meyer, Churchill Livingstone, 1999; Stereotactic and Image Directed Surgery of Brain Tumors, 1st ed., by David G. T. Thomas, WB Saunders Co., 1993; and Cranial Microsurgery: Approaches and Techniques, by L. N. Sekhar and E. De Oliveira, 1st ed., Thieme Medical Publishing, 1999. Methods for obtaining and analyzing brain tissue are also described in Belay et al., Arch. Neurol. 58: 1673-1678 (2001); and Seijo et al., J. Clin. Microbiol. 38: 3892-3895 (2000).

[0028] After insult, nerve cells in *in vitro* culture or *in situ* in a subject express altered levels or activities of one or more

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proteins than do such cells not subjected to the insult. Thus, samples that contain nerve cells, e.g., a biopsy of a central nervous system or peripheral nervous system tissue are illustratively suitable biological samples for use in the invention. In addition to nerve cells, however, other cells express illustratively all-spectrin including, for example, cardiomyocytes, myocytes in skeletal muscles, hepatocytes, kidney cells and cells in testis. A biological sample including such cells or fluid secreted from these cells might also be used in an adaptation of the inventive methods to determine and/or characterize an injury to such non-nerve cells.

[0029] A subject illustratively includes a dog, a cat, a horse, a cow, a pig, a sheep, a goat, a chicken, non-human primate, a human, a rat, and a mouse. Subjects who most benefit from the present invention are those suspected of having or at risk for developing abnormal neurological conditions, such as victims of brain injury caused by traumatic insults (e.g., gunshot wounds, automobile accidents, sports accidents, shaken baby syndrome), ischemic events (e.g., stroke, cerebral hemorrhage, cardiac arrest), neurodegenerative disorders (such as Alzheimer's, Huntington's, and Parkinson's diseases; prion-related disease; other forms of dementia), epilepsy, substance abuse (e.g., from amphetamines, Ecstasy/MDMA, or ethanol), and peripheral nervous system pathologies such as diabetic neuropathy, chemotherapy-induced neuropathy and neuropathic pain.

[0030] Baseline levels of several biomarkers are those levels obtained in the target biological sample in the species of desired subject in the absence of a known neurological condition. These levels need not be expressed in hard concentrations, but may instead be known from parallel control experiments and expressed in terms of fluorescent units, density units, and the like. Typically, in the absence of a neurological condition SBDPs are present in biological samples at a negligible amount. However, UCH-L1 is a highly abundant protein in neurons. Determining the baseline levels of UCH-L1 in neurons of particular species is well within the skill of the art. Similarly, determining the concentration of baseline levels of MAP2, GFAP, NSE, or other biomarker is well within the skill of the art.

[0031] As used herein the term "diagnosing" means recognizing the presence or absence of a neurological or other condition such as an injury or disease. Diagnosing is optionally referred to as the result of an assay wherein a particular ratio or level of a biomarker is detected or is absent.

[0032] As used herein a "ratio" is either a positive ratio wherein the level of the target is greater than the target in a second sample or relative to a known or recognized baseline level of the same target. A negative ratio describes the level of the target as lower than the target in a second sample or relative to a known or recognized baseline level of the same target. A neutral ratio describes no observed change in target biomarker.

[0033] As used herein an injury is an alteration in cellular or molecular integrity, activity, level, robustness, state, or other alteration that is traceable to an event. Injury illustratively includes a physical, mechanical, chemical, biological, functional, infectious, or other modulator of cellular or molecular characteristics. An event is illustratively, a physical trauma such as an impact (percussive) or a biological abnormality such as a stroke resulting from either blockade or leakage of a blood vessel. An event is optionally an infection by an infectious agent. A person of skill in the art recognizes numerous equivalent events that are encompassed by the terms injury or event.

[0034] An injury is optionally a physical event such as a percussive impact. An impact is the like of a percussive injury such as resulting to a blow to the head that either leaves the cranial structure intact or results in breach thereof. Experimentally, several impact methods are used illustratively including controlled cortical impact (CCI) at a 1.6 mm depression depth, equivalent to severe TBI in human. This method is described in detail by Cox, CD, et al., J Neurotrauma, 2008; 25(11):1355-65. It is appreciated that other experimental methods producing impact trauma are similarly operable.

[0035] TBI may also result from stroke. Ischemic stroke is optionally modeled by middle cerebral artery occlusion (MCAO) in rodents. UCH-L1 protein levels, for example, are increased following mild MCAO which is further increased following severe MCAO challenge. Mild MCAO challenge may result in an increase of protein levels within two hours that is transient and returns to control levels within 24 hours. In contrast, severe MCAO challenge results in an increase in protein levels within two hours following injury and may be much more persistent demonstrating statistically significant levels out to 72 hours or more.

[0036] An exemplary process for detecting the presence or absence of GFAP and one or more other neuroactive biomarkers in a biological sample involves obtaining a biological sample from a subject, such as a human, contacting the biological sample with a compound or an agent capable of detecting of the marker being analyzed, illustratively including an antibody or aptamer, and analyzing binding of the compound or agent to the sample after washing. Those samples having specifically bound compound or agent express the marker being analyzed.

[0037] An inventive process can be used to detect GFAP and one or more other neuroactive biomarkers in a biological sample *in vitro*, as well as *in vivo*. The quantity of GFAP and one or more other neuroactive biomarkers in a sample is compared with appropriate controls such as a first sample known to express detectable levels of the marker being analyzed (positive control) and a second sample known to not express detectable levels of the marker being analyzed (a negative control). For example, *in vitro* techniques for detection of a marker illustratively include enzyme linked immunosorbent assays (ELISAs), radioimmuno assay, radioassay, western blot, Southern blot, northern blot, immunoprecipitation, immunofluorescence, mass spectrometry, RT-PCR, PCR, liquid chromatography, high performance liquid chromatography, enzyme activity assay, cellular assay, positron emission tomography, mass spectroscopy, combina-

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tions thereof, or other technique known in the art.. Furthermore, *in vivo* techniques for detection of a marker include introducing a labeled agent that specifically binds the marker into a biological sample or test subject. For example, the agent can be labeled with a radioactive marker whose presence and location in a biological sample or test subject can be detected by standard imaging techniques. Optionally, the first biomarker specifically binding agent and the agent specifically binding at least one additional neuroactive biomarker are both bound to a substrate. It is appreciated that a bound agent assay is readily formed with the agents bound with spatial overlap, with detection occurring through discernibly different detection for first biomarker and each of at least one additional neuroactive biomarkers. A color intensity based quantification of each of the spatially overlapping bound biomarkers is representative of such techniques.

[0038] Any suitable molecule that can specifically bind GFAP and any suitable molecule that specifically binds one or more other neuroactive biomarkers are operative in the invention to achieve a synergistic assay. A preferred agent for detecting GFAP or the one or more other neuroactive biomarkers is an antibody capable of binding to the biomarker being analyzed. Preferably, an antibody is conjugated with a detectable label. Such antibodies can be polyclonal or monoclonal. An intact antibody, a fragment thereof (e.g., Fab or $F(ab')_2$), or an engineered variant thereof (e.g., sFv) can also be used. Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Antibodies for numerous inventive biomarkers are available from vendors known to one of skill in the art. Illustratively, antibodies directed to inventive biomarkers are available from Santa Cruz Biotechnology (Santa Cruz, CA). Exemplary antibodies operative herein to detect a fist biomarker include anti-GFAP antibody, anti-UCH-LI antibody, anti-NSE antibody, anti-MAP2 antibody, or an anti-SBDP antibody. Other biomarkers to be targeted as part of an inventive assay different from the first biomarker include GFAP, NSE, SBDP, SBDP150, SBDP145, SBDP120, S100b, MAP2, MAP1, MAP3, MAP4, MAP5, MBP, Tau, Neurofilament protein (NF), Cannabinoid Receptor CB, CAM, Synaptic protein, CRMP, iNOS, NeuN, CSPase, Neuroserpin, alpha-internexin, LC3, Neurofascin, EAAT, Nestin, Cortin-1, or BIII-Tubulin

[0039] An antibody is optionally labeled. A person of ordinary skill in the art recognizes numerous labels operable herein. Labels and labeling kits are commercially available optionally from Invitrogen Corp, Carlsbad, CA. Labels illustratively include, fluorescent labels, biotin, peroxidase, radionucleotides, or other label known in the art. Alternatively, a detection species of another antibody or other compound known to the art is used as form detection of a biomarker bound by an antibody.

[0040] Antibody-based assays are preferred for analyzing a biological sample for the presence of GFAP and one or more other neuroactive biomarkers. Suitable western blotting methods are described below in the examples section. For more rapid analysis (as may be important in emergency medical situations), immunosorbent assays (e.g., ELISA and RIA) and immunoprecipitation assays may be used. As one example, the biological sample or a portion thereof is immobilized on a substrate, such as a membrane made of nitrocellulose or PVDF; or a rigid substrate made of polystyrene or other plastic polymer such as a microtiter plate, and the substrate is contacted with an antibody that specifically binds GFAP, or one of the other neuroactive biomarkers under conditions that allow binding of antibody to the biomarker being analyzed. After washing, the presence of the antibody on the substrate indicates that the sample contained the marker being assessed. If the antibody is directly conjugated with a detectable label, such as an enzyme, fluorophore, or radioisotope, the presence of the label is optionally detected by examining the substrate for the detectable label. Alternatively, a detectably labeled secondary antibody that binds the marker-specific antibody is added to the substrate. The presence of detectable label on the substrate after washing indicates that the sample contained the marker.

[0041] Numerous permutations of these basic immunoassays are also operative in the invention. These include the biomarker-specific antibody, as opposed to the sample being immobilized on a substrate, and the substrate is contacted with GFAP or another neuroactive biomarker conjugated with a detectable label under conditions that cause binding of antibody to the labeled marker. The substrate is then contacted with a sample under conditions that allow binding of the marker being analyzed to the antibody. A reduction in the amount of detectable label on the substrate after washing indicates that the sample contained the marker.

[0042] Although antibodies are preferred for use in the invention because of their extensive characterization, any other suitable agent (e.g., a peptide, an aptamer, or a small organic molecule) that specifically binds GFAP or another neuroactive biomarker is optionally used in place of the antibody in the above described immunoassays. For example, an aptamer that specifically binds all spectrin and/or one or more of its SBDPs might be used. Aptamers are nucleic acid-based molecules that bind specific ligands. Methods for making aptamers with a particular binding specificity are known as detailed in U.S. Patent Nos. 5,475,096; 5,670,637; 5,696,249; 5,270,163; 5,707,796; 5,595,877; 5,660,985; 5,567,588; 5,683,867; 5,637,459; and 6,011,020.

[0043] A myriad of detectable labels that are operative in a diagnostic assay for biomarker expression are known in the art. Agents used in methods for detecting GFAP or another neuroactive biomarker are conjugated to a detectable label, e.g., an enzyme such as horseradish peroxidase. Agents labeled with horseradish peroxidase can be detected by adding an appropriate substrate that produces a color change in the presence of horseradish peroxidase. Several other detectable labels that may be used are known. Common examples of these include alkaline phosphatase, horseradish peroxidase, fluorescent compounds, luminescent compounds, colloidal gold, magnetic particles, biotin, radioisotopes, and other enzymes. It is appreciated that a primary/secondary antibody system is optionally used to detect one

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or more biomarkers. A primary antibody that specifically recognizes one or more biomarkers is exposed to a biological sample that may contain the biomarker of interest. A secondary antibody with an appropriate label that recognizes the species or isotype of the primary antibody is then contacted with the sample such that specific detection of the one or more biomarkers in the sample is achieved.

[0044] The present invention employs a step of correlating the presence or amount of GFAP alone, or with one or more other neuroactive biomarker in a biological sample with the severity and/or type of nerve cell injury. GFAP measurement alone is shown herein to be highly effective in detecting MMTBI. The amount of GFAP and one or more other neuroactive biomarkers in the biological sample are associated with a neurological condition such as traumatic brain injury as detailed in the examples. The results of an inventive assay to synergistically measure GFAP and one or more other neuroactive biomarkers can help a physician or veterinarian determine the type and severity of injury with implications as to the types of cells that have been compromised. These results are in agreement with CT scan and GCS results, yet are quantitative, obtained more rapidly, and at far lower cost.

[0045] The present invention provides a step of comparing the quantity of GFAP and the amount of at least one other neuroactive biomarker to normal levels to determine the neurological condition of the subject. It is appreciated that selection of additional biomarkers allows one to identify the types of cells implicated in an abnormal neurological condition as well as the nature of cell death in the case of an axonal injury marker, namely an SBDP. The practice of an inventive process provides a test which can help a physician determine suitable therapeutics to administer for optimal benefit of the subject. While the data provided in the examples herein are provided with respect to a full spectrum of traumatic brain injury, it is appreciated that these results are applicable to ischemic events, neurodegenerative disorders, prion related disease, epilepsy, chemical etiology and peripheral nervous system pathologies. As is shown in the subsequently provided example data, a gender difference is unexpectedly noted in abnormal subject neurological condition.

[0046] An assay for analyzing cell damage in a subject or a cell culture isolated therefrom is also provided. The assay includes: (a) a substrate for holding a sample isolated from a subject suspected of having a damaged nerve cell, the sample being a fluid in communication with the nervous system of the subject prior to being isolated from the subject; (b) a GFAP specific binding agent; (c) a binding agent specific for another neuroactive biomarker, wherein said another neuroactive biomarker is UCH-L1 and (d) printed instructions for performing the assay illustratively for reacting: the specific binding agent with the biological sample or a portion of the biological sample to detect the presence or amount of biomarker, and the agent specific for another neuroactive biomarker with the biological sample or a portion of the biological sample to detect the presence or amount of the at least one biomarker in the biological sample. The inventive assay can be used to detect a neurological condition for financial renumeration.

[0047] The assay optionally includes a detectable label such as one conjugated to the agent, or one conjugated to a substance that specifically binds to the agent, such as a secondary antibody.

[0048] An inventive process illustratively includes diagnosing a neurological condition in a subject, wherein said neurological condition is selected from traumatic brain injury and stroke. The biological sample is assayed by mechanisms known in the art for detecting or identifying the presence of one or more biomarkers present in the biological sample. Based on the amount or presence of a target biomarker in a biological sample, a ratio of one or more biomarkers is optionally calculated. The ratio is optionally the level of one or more biomarkers relative to the level of another biomarker in the same or a parallel sample, or the ratio of the quantity of the biomarker to a measured or previously established baseline level of the same biomarker in a subject known to be free of a pathological neurological condition. The ratio allows for the diagnosis of a neurological condition in the subject. An inventive process also optionally administers a therapeutic to the subject that will either directly or indirectly alter the ratio of one or more biomarkers.

[0049] A process is also described for diagnosing and optionally treating a multiple-organ injury. Multiple organs illustratively include subsets of neurological tissue such as brain, spinal cord and the like, or specific regions of the brain such as cortex, hippocampus and the like. Multiple injuries illustratively include apoptotic cell death which is detectable by the presence of caspase induced SBDPs, and oncotic cell death which is detectable by the presence of calpain induced SBDPs. The inventive process illustratively includes assaying for a plurality of biomarkers in a biological sample obtained from a subject wherein the biological was optionally in fluidic contact with an organ suspected of having undergone injury or control organ when the biological sample was obtained from the subject. The inventive process determines a first subtype of organ injury based on a first ratio of a plurality of biomarkers. The inventive process also determines a second subtype of a second organ injury based on a second ratio of the plurality of biomarkers in the biological sample. The ratios are illustratively determined by processes described herein or known in the art.

[0050] The subject invention illustratively includes a composition for distinguishing the magnitude of a neurological condition in a subject. An inventive composition is either an agent entity or a mixture of multiple agents. In a preferred embodiment a composition is a mixture. The mixture optionally contains a biological sample derived from a subject. The subject is optionally suspected of having a neurological condition. The biological sample in communication with the nervous system of the subject prior to being isolated from the subject. In inventive composition also contains at least two primary agents, preferably antibodies, that specifically and independently bind to at least two biomarkers that may be present in the biological sample. In a preferred embodiment the first primary agent is in antibody that specifically

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binds GFAP. A second primary agent is preferably an antibody that specifically binds a ubiquitin carboxyl-terminal hydrolase, preferably UCH-L1, or a spectrin breakdown product.

[0051] The agents of the inventive composition are optionally immobilized or otherwise in contact with a substrate. The inventive teachings are also preferably labeled with at least one detectable label. In a preferred embodiment the detectable label on each agent is unique and independently detectable in either the same assay chamber or alternate chambers. Optionally a secondary agent specific for detecting or binding to the primary agent is labeled with at least one detectable label. In the nonlimiting example the primary agent is a rabbit derived antibody. A secondary agent is optionally an antibody specific for a rabbit derived primary antibody. Mechanisms of detecting antibody binding to an antigen are well known in the art, and a person of ordinary skill in the art readily envisions numerous methods and agents suitable for detecting antigens or biomarkers in a biological sample.

[0052] The invention employs a step of correlating the presence or amount of a biomarker in a biological sample with the severity and/or type of nerve cell (or other biomarker-expressing cell) injury. The amount of biomarker(s) in the biological sample directly relates to severity of nerve tissue injury as a more severe injury damages a greater number of nerve cells which in turn causes a larger amount of biomarker(s) to accumulate in the biological sample (e.g., CSF; serum). Whether a nerve cell injury triggers an apoptotic and/or necrotic type of cell death can also be determined by examining the SBDPs present in the biological sample. Necrotic cell death preferentially activates calpain, whereas apoptotic cell death preferentially activates caspase-3. Because calpain and caspase-3 SBDPs can be distinguished, measurement of these markers indicates the type of cell damage in the subject. For example, necrosis-induced calpain activation results in the production of SBDP150 and SBDP145; apoptosis-induced caspase-3 activation results in the production of SBDP150i and SBDP120; and activation of both pathways results in the production of all four markers. Also, the level of or kinetic extent of UCH-L1 present in a biological sample may optionally distinguish mild injury from a more severe injury. In an illustrative example, severe MCAO (2h) produces increased UCH-L1 in both CSF and serum relative to mild challenge (30 min) while both produce UCH-L1 levels in excess of uninjured subjects. Moreover, the persistence or kinetic extent of the markers in a biological sample is indicative of the severity of the injury with greater injury indicating increases persistence of GFAP, UCH-L1, or SBDP in the subject that is measured by an inventive process in biological samples taken at several time points following injury.

[0053] The results of such a test can help a physician determine whether the administration a particular therapeutic such as calpain and/or caspase inhibitors or muscarinic cholinergic receptor antagonists might be of benefit to a patient. This method may be especially important in detecting age and gender difference in cell death mechanism.

[0054] It is appreciated that other reagents such as assay grade water, buffering agents, membranes, assay plates, secondary antibodies, salts, and other ancillary reagents are available from vendors known to those of skill in the art. Illustratively, assay plates are available from Corning, Inc. (Corning, NY) and reagents are available from Sigma-Aldrich Co. (St. Louis, MO).

[0055] Methods involving conventional biological techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Immunological methods (e.g., preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, e.g., in Current Protocols in Immunology, ed. Coligan et al., John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff et al., John Wiley & Sons, New York, 1992.

[0056] Various aspects of the present invention are illustrated by the following non-limiting examples. The examples are for illustrative purposes and are not a limitation on any practice of the present invention. It will be understood that variations and modifications can be made without departing from the spirit and scope of the invention. While the examples are generally directed to mammalian tissue, specifically, analyses of mouse tissue, a person having ordinary skill in the art recognizes that similar techniques and other techniques known in the art readily translate the examples to other mammals such as humans. Reagents illustrated herein are commonly cross reactive between mammalian species or alternative reagents with similar properties are commercially available, and a person of ordinary skill in the art readily understands where such reagents may be obtained. Variations within the concepts of the invention are apparent to those skilled in the art.

Example 1: Materials for Biomarker Analyses.

[0057] Illustrative reagents used in performing the subject invention include Sodium bicarbonate (Sigma Cat #: C-3041), blocking buffer (Startingblock T20-TBS) (Pierce Cat#: 37543), Tris buffered saline with Tween 20 (TBST; Sigma Cat #: T-9039). Phosphate buffered saline (PBS; Sigma Cat #: P-3813); Tween 20 (Sigma Cat #: P5927); Ultra TMB ELISA (Pierce Cat #: 34028); and Nunc maxisorp ELISA plates (Fisher). Monoclonal and polyclonal GFAP and UCH-L1 antibodies are made in-house or are obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies directed to α-II spectrin and breakdown products as well as to MAP2 are available from Santa Cruz Biotechnology, Santa Cruz,

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CA. Labels for antibodies of numerous subtypes are available from Invitrogen, Corp., Carlsbad, CA. Protein concentrations in biological samples are determined using bicinchoninic acid microprotein assays (Pierce Inc., Rockford, IL, USA) with albumin standards. All other necessary reagents and materials are known to those of skill in the art and are readily ascertainable.

Example 2: Biomarker Assay Development

[0058] Anti-biomarker specific rabbit polyclonal antibody and monoclonal antibodies are produced in the laboratory. To determine reactivity specificity of the antibodies to detect a target biomarker a known quantity of isolated or partially isolated biomarker is analyzed or a tissue panel is probed by western blot. An indirect ELISA is used with the recombinant biomarker protein attached to the ELISA plate to determine optimal concentration of the antibodies used in the assay. Microplate wells are coated with rabbit polyclonal anti-human biomarker antibody. After determining the concentration of rabbit anti-human biomarker antibody for a maximum signal, the lower detection limit of the indirect ELISA for each antibody is determined. An appropriate diluted sample is incubated with a rabbit polyclonal antihuman biomarker antibody for 2 hours and then washed. Biotin labeled monoclonal anti-human biomarker antibody is then added and incubated with captured biomarker. After thorough wash, streptavidin horseradish peroxidase conjugate is added. After 1 hour incubation and the last washing step, the remaining conjugate is allowed to react with substrate of hydrogen peroxide tetramethyl benzadine. The reaction is stopped by addition of the acidic solution and absorbance of the resulting yellow reaction product is measured at 450 nanometers. The absorbance is proportional to the concentration of the biomarker. A standard curve is constructed by plotting absorbance values as a function of biomarker concentration using calibrator samples and concentrations of unknown samples are determined using the standard curve.

Example 3: *In vivo* model of TBI injury model:

[0059] A controlled cortical impact (CCI) device is used to model TBI on rats as previously described (Pike et al, 1998). Adult male (280-300 g) Sprague-Dawley rats (Harlan: Indianapolis, IN) are anesthetized with 4% isoflurane in a carrier gas of 1:1 O_2/N_2O (4 min.) and maintained in 2.5% isoflurane in the same carrier gas. Core body temperature is monitored continuously by a rectal thermistor probe and maintained at 37±1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals are mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. Following a midline cranial incision and reflection of the soft tissues, a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) is performed adjacent to the central suture, midway between bregma and lambda. The dura mater is kept intact over the cortex. Brain trauma is produced by impacting the right (ipsilateral) cortex with a 5 mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a 1.6 mm compression and 150 ms dwell time. Sham-injured control animals are subjected to identical surgical procedures but do not receive the impact injury. Appropriate pre-and post-injury management is preformed to insure compliance with guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and Use of Laboratory Animals. In addition, research is conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the "Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition."

Example 4: Middle cerebral artery occlusion (MCAO) injury model:

[0060] Rats are incubated under isoflurane anesthesia (5% isoflurane *via* induction chamber followed by 2% isoflurane *via* nose cone), the right common carotid artery (CCA) of the rat is exposed at the external and internal carotid artery (ECA and ICA) bifurcation level with a midline neck incision. The ICA is followed rostrally to the pterygopalatine branch and the ECA is ligated and cut at its lingual and maxillary branches. A 3-0 nylon suture is then introduced into the ICA *via* an incision on the ECA stump (the suture's path was visually monitored through the vessel wall) and advanced through the carotid canal approximately 20 mm from the carotid bifurcation until it becomes lodged in the narrowing of the anterior cerebral artery blocking the origin of the middle cerebral artery. The skin incision is then closed and the endovascular suture left in place for 30 minutes or 2 hours. Afterwards the rat is briefly reanesthetized and the suture filament is retracted to allow reperfusion. For sham MCAO surgeries, the same procedure is followed, but the filament is advanced only 10 mm beyond the internal-external carotid bifurcation and is left in place until the rat is sacrificed. During all surgical procedures, animals are maintained at 37 ± 1°C by a homeothermic heating blanket (Harvard Apparatus, Holliston, MA, U.S.A.). It is important to note that at the conclusion of each experiment, if the rat brains show pathologic evidence of subarachnoid hemorrhage upon necropsy they are excluded from the study. Appropriate pre- and post-injury management is preformed to insure compliance with all animal care and use guidelines.

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Example 5: Tissue and Sample Preparation:

[0061] At the appropriate time points (2, 6, 24 hours and 2, 3, 5 days) after injury, animals are anesthetized and immediately sacrificed by decapitation. Brains are quickly removed, rinsed with ice cold PBS and halved. The right hemisphere (cerebrocortex around the impact area and hippocampus) is rapidly dissected, rinsed in ice cold PBS, snap-frozen in liquid nitrogen, and stored at -80°C until used. For immunohistochemistry, brains are quick frozen in dry ice slurry, sectioned via cryostat (20 μm) onto SUPERFROST PLUS GOLD® (Fisher Scientific) slides, and then stored at -80°C until used. For the left hemisphere, the same tissue as the right side is collected. For Western blot analysis, the brain samples are pulverized with a small mortar and pestle set over dry ice to a fine powder. The pulverized brain tissue powder is then lysed for 90 min at 4°C in a buffer of 50 mM Tris (pH 7.4), 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT, 1x protease inhibitor cocktail (Roche Biochemicals). The brain lysates are then centrifuged at 15,000xg for 5 min at 4°C to clear and remove insoluble debris, snap-frozen, and stored at - 80°C until used.

[0062] For gel electrophoresis and electroblotting, cleared CSF samples (7 μl) are prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 2X loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H_2O. Twenty micrograms (20 μg) of protein per lane are routinely resolved by SDS-PAGE on 10-20% Tris/glycine gels (Invitrogen, Cat #EC61352) at 130 V for 2 hours. Following electrophoresis, separated proteins are laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer containing 39 mM glycine, 48 mM Tris-HCl (pH 8.3), and 5% methanol at a constant voltage of 20 V for 2 hours at ambient temperature in a semi-dry transfer unit (Bio-Rad). After electro-transfer, the membranes are blocked for 1 hour at ambient temperature in 5% non-fat milk in TBS and 0.05% Tween-2 (TBST) then are incubated with the primary polyclonal UCH-L1 antibody in TBST with 5% non-fat milk at 1:2000 dilution as recommended by the manufacturer at 4°C overnight. This is followed by three washes with TBST, a 2 hour incubation at ambient temperature with a biotinylated linked secondary antibody (Amersham, Cat # RPN1177v1), and a 30 min incubation with Streptavidin-conjugated alkaline phosphatase (BCIP/NBT reagent: KPL, Cat # 50-81-08). Molecular weights of intact biomarker proteins are assessed using rainbow colored molecular weight standards (Amersham, Cat # RPN800V). Semi-quantitative evaluation of intact GFAP, UCH-L1, or SBDP protein levels is performed via computer-assisted densitometric scanning (Epson XL3500 scanner) and image analysis with ImageJ software (NIH).

Example 6: UCH-L1 is increased in CSF following MCAO challenge.

[0063] Subjects are subjected to MCAO challenge and CSF samples analyzed by quantitative western blotting. UCH-L1 protein is readily detectable after injury at statically significant levels above the amounts of UCH-L1 in sham treated samples (FIGs. 1A, B). These UCH-L1 levels are transiently elevated (at 6 h) after mild ischemia (30 min MCAO) followed by reperfusion, while levels are sustained from 6 to 72 h after a more severe (2 h MCAO) ischemia (FIGs. 1A, B).

Example 7: ELISA readily identifies UCH-L1 levels in both CSF and serum.

[0064] ELISA is used to more rapidly and readily detect and quantitate UCH-L1 in biological samples. For a UCH-L1 sandwich ELISA (swELISA), 96-well plates are coated with 100 μl/well capture antibody (500 ng/well purified rabbit anti-UCH-LI, made in-house by conventional techniques) in 0.1 M sodium bicarbonate, pH 9.2. Plates are incubated overnight at 4°C, emptied and 300 μl/well blocking buffer (Startingblock T20-TBS) is added and incubated for 30 min at ambient temperature with gentle shaking. This is followed by either the addition of the antigen standard (recombinant UCH-L1) for standard curve (0.05 - 50 ng/well) or samples (3-10 μl CSF) in sample diluent (total volume 100 μl/well). The plate is incubated for 2 hours at room temperature, then washed with automatic plate washer (5 x 300 μl/well with wash buffer, TBST). Detection antibody mouse anti-UCH-LI-HRP conjugated (made in-house, 50 μg/ml) in blocking buffer is then added to wells at 100μL/well and incubated for 1.5 h at room temperature, followed by washing. If amplification is needed, biotinyl-tyramide solution (Perkin Elmer Elast Amplification Kit) is added for 15 min at room temperature, washed then followed by 100 μl/well streptavidin-HRP (1:500) in PBS with 0.02% Tween-20 and 1% BSA for 30 min and then followed by washing. Lastly, the wells are developed with 100μl/well TMB substrate solution (Ultra-TMB ELISA, Pierce# 34028) with incubation times of 5-30 minutes. The signal is read at 652 nm with a 96-well spectrophotometer (Molecular Device Spectramax 190).

[0065] UCH-L1 levels of the TBI group (percussive injury) are significantly higher than the sham controls ($p<0.01$, ANOVA analysis) and the naive controls as measured by a swELISA demonstrating that UCH-L1 is elevated early in CSF (2h after injury) then declines at around 24 h after injury before rising again 48 h after injury (FIG. 2).

[0066] Following MCAO challenge the magnitude of UCH-L1 in CSF is dramatically increased with severe (2h) challenge relative to a more mild challenge (30 min). (FIG. 3) The more severe 2 h MCAO group UCH-L1 protein levels are 2-5 fold higher than 30 min MCAO ($p < 0.01$, ANOVA analysis). UCH-L1 protein levels for shams are virtually indistinguishable from naive controls.

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[0067] Similar results are obtained for UCH-L1 in serum. Blood (3-4 ml) is collected at the end of each experimental period directly from the heart using syringe equipped with 21 gage needle placed in a polypropylene tube and allowed to stand for 45 min to 1 hour at room temperature to form clot. Tubes are centrifuged for 20 min at 3,000xg and the serum removed and analyzed by ELISA (FIGS. 4, 5).

[0068] UCH-L1 levels of the TBI group are significantly higher than the sham group ($p < 0.001$, ANOVA analysis) and for each time point tested 2 h through 24 h respective to the same sham time points ($p < 0.005$, Student's T-test). UCH-L1 is significantly elevated after injury as early as 2h in serum. Severe MCAO challenge produces increased serum UCH-L1 relative to mild challenge. Both mild and severe challenge are statistically higher than sham treated animals indicating that serum detection of UCH-L1 is a robust diagnostic and the levels are able to sufficiently distinguish mild from severe injury.

Example 8: Analysis of spectrin breakdown products:

[0069] Spectrin breakdown products are analyzed following rat MCAO challenge by procedures similar to those described in U.S. Patent No. 7,291,710, the contents of which are incorporated herein by reference. FIG. 6 demonstrates that levels of SBDP145 in both serum and CSF are significantly ($p<0.05$) increased at all time points studied following severe (2hr) MCAO challenge relative to mild (30 min) challenge. Similarly, SBDP120 demonstrates significant elevations following severe MCAO challenge between 24 and 72 hours after injury in CSF (FIG. 7). However, levels of SBDP120 in serum are increased following severe challenge relative to mild challenge at all time points between 2 and 120 hours. In both CSF and Serum both mild and severe MCAO challenge produces increased SPBP120 and 140 relative to sham treated subjects.

Example 9: Analysis of MAP2:

[0070] Microtubule Associated Protein 2 (MAP2) is assayed as a biomarker in both CSF and serum following mild (30 min) and severe (2 hr) MCAO challenge in subjects by ELISA or western blotting essentially as described herein. Antibodies to MAP2 (MAP-2 (E-12)) are obtained from Santa Cruz Biotechnology, Santa Cruz, CA. These antibodies are suitable for both ELISA and western blotting procedures and are crossreactive to murine and human MAP2. Levels of MAP2 are significantly ($p<0.05$) increased in subjects following mild MCAO challenge relative to naive animals in both CSF and serum (FIG. 8). Similar to UCH-L1 and SBDPs, severe challenge (2 hr) produces much higher levels of MAP2 in both samples than mild challenge (30 min).

Example 10: Severe Traumatic Brain Injury Study

[0071] A study was conducted that included 46 human subjects suffering severe traumatic brain injury. Each of these subjects is characterized by being over age 18, having a GCS of less than or equal to 8 and required ventriculostomy and neuromonitoring as part of routine care. A control group A, synonymously detailed as CSF controls, included 10 individuals also being over the age of 18 or older and no injuries. Samples are obtained during spinal anesthesia for routine surgical procedures or access to CSF associated with treatment of hydrocephalus or meningitis. A control group B, synonymously described as normal controls, totaled 64 individuals, each age 18 or older and experiencing multiple injuries without brain injury. Further details with respect to the demographics of the study are provided in Table 1.

Table 1. Subject Demographics for Severe Traumatic Brain Injury Study

		TBI	CSF Controls	Normal Controls
Number		46	10	64
	Males	34 (73.9%)	29 (65.9%)	26 (40.6%)
	Females	12 (26.1%)	15 (34.1%)	38 (59.4%
Age:	Average	50.2	58.2 1,2	30.09 2,3
	Std Dev	19.54	20.52	15.42
	Minimum	19	23	18
	Maximum	88	82	74
Race:	Caucasian			
	Black	45	38 (86.4%)	52 (81.2%)
	Asian	1	6 (13.6)	4 (6.3%)
	Other			7 (10.9%)

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	TBI	CSF Controls	Normal Controls
			1 (1.6%)
GCS in Emergency Department			
Average	5.3		
Std Dev	1.9		

[0072] The level of biomarkers found in the first available CSF and serum samples obtained in the study are provided in FIGs. 9 and 10, respectively. The average first CSF sample collected as detailed in FIG. 9 was 11.2 hours while the average time for collection of a serum sample subsequent to injury event as per FIG. 10 is 10.1 hours. The quantity of each of the biomarkers of UCH-L1, MAP2, SBDP145, SBDP120, and GFAP are provided for each sample for the cohort of traumatic brain injury sufferers as compared to a control group. The diagnostic utility of the various biomarkers within the first 12 hours subsequent to injury based on a compilation of CSF and serum data is provided in Fig.11 and indicates in particular the value of GFAP as well as that of additional markers UCH-L1 and the spectrin breakdown products. Elevated levels of UCH-L1 are indicative of the compromise of neuronal cell body damage while an increase in SPDP145 with a corresponding decrease in SBDP120 is suggestive of acute axonal necrosis.

[0073] One subject from the traumatic brain injury cohort was a 52 year old Caucasian woman who had been involved in a motorcycle accident while not wearing a helmet. Upon admission to an emergency room her GCS was 3 and during the first 24 hours subsequent to trauma her best GCS was 8. After 10 days her GCS was 11. CT scanning revealed SAH and facial fractures with a Marshall score of 11 and a Rotterdam score of 2. Ventriculostomy was removed after 5 years and an overall good outcome was obtained. Arterial blood pressure (MABP), intracranial pressure (ICP) and cerebral profusion pressure (CPP) for this sufferer of traumatic brain injury as a function of time is depicted in FIG. 12. A possible secondary insult is noted at approximately 40 hours subsequent to the injury as noted by a drop in MABP and CPP. The changes in concentration of inventive biomarkers per CSF and serum samples from this individual are noted in FIG. 13. These results include a sharp increase in GFAP in both the CSF and serum as well as the changes in the other biomarkers depicted in FIG. 13 and provide important clinical information as to the nature of the injury and the types of cells involved, as well as modes of cell death associated with the spectrin breakdown products.

[0074] Another individual of the severe traumatic brain injury cohort included a 51 year old Caucasian woman who suffered a crush injury associated with a horse falling on the individual. GCS on admission to emergency room was 3 with imaging analysis initially being unremarkable with minor cortical and subcortical contusions. MRI on day 5 revealed significant contusions in posterior fossa. The Marshall scale at that point was indicated to be 11 with a Rotterdam scale score of 3. The subject deteriorated and care was withdrawn 10 days after injury. The CSF and serum values for this individual during a period of time are provided in FIG. 14.

[0075] Based on the sandwich ELISA testing, GFAP values as a function of time are noted to be markedly elevated relative to normal controls (control group B) as a function of time.

[0076] The concentration of spectrin breakdown products, MAP2 and UCH-L1 as a function of time subsequent to traumatic brain injury has been reported elsewhere as exemplified in U.S. Patents 7,291,710 and 7,396,654 each of which is incorporated herein by reference.

[0077] An analysis was performed to evaluate the ability of biomarkers measured in serum to predict TBI outcome, specifically GCS. Stepwise regression analysis was the statistical method used to evaluate each of the biomarkers as an independent predictive factor, along with the demographic factors of age and gender, and also interactions between pairs of factors. Interactions determine important predictive potential between related factors, such as when the relationship between a biomarker and outcome may be different for men and women, such a relationship would be defined as a gender by biomarker interaction.

[0078] The resulting analysis identified biomarkers UCH-L1, MAP2, and GFAP as being statistically significant predictors of GCS (Table 2, 3). Furthermore, GFAP was shown to have improved predictability when evaluated in interaction with UCH-L1 and gender (Table 4, 5).

Table 2. Stepwise Regression Analysis 1 - Cohort includes: All Subjects >= 18 Years Old

Summary of Stepwise Selection - 48 Subjects

Variable Step Entered	Parameter Estimate	Model R-Square	F Value	p-value
Intercept	13.02579			
2 SEXCD	-2.99242	0.1580	7.29	0.0098
1 CSF_UCH_L1	-0.01164	0.2519	11.54	0.0015

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Summary of Stepwise Selection - 48 Subjects				
Variable Step Entered	Parameter Estimate	Model R-Square	F Value	p-value
3 Serum_MAP_2	0.96055	0.3226	4.59	0.0377

Table 3. Stepwise Regression Analysis 2 - Cohort includes: TBI Subjects >= 18 Years Old

Summary of Stepwise Selection - 39 Subjects				
Variable Step Entered	Parameter Estimate	Model R-Square	F Value	p-value
Intercept	5.73685			
1 Serum_UCH_L1	-0.30025	0.0821	8.82	0.0053
2 Serum_GFAP	0.12083	0.1973	5.16	0.0291

Table 4. Stepwise Regression Analysis 1 - Cohort includes: TBI and A Subjects >= 18 Years Old

Summary of Stepwise Selection - 57 Subjects				
Variable Step Entered	Parameter Estimate	Model R-Square	F Value	p-value
Intercept	8.04382			
1 Serum_UCH_L	-0.92556	0.1126	12.90	0.0007
2 Serum_MAP_2	1.07573	0.2061	5.79	0.0197
3 Serum_UCH-L1 +Serum_GFAP	0.01643	0.2663	4.35	0.0419

Table 5. Stepwise Regression Analysis 2 - Cohort includes: TBI Subjects >= 18 Years Old

Summary of Stepwise Selection - 44 Subjects				
Variable Step Entered	Parameter Estimate	Model R-Square	F Value	p-value
Intercept	5.50479			
1 Serum_UCH_L1	-0.36311	0.0737	11.95	0.0013
2 SEX_Serum_GFAP	0.05922	0.1840	5.09	0.0296
3 Serum_MAP_2	0.63072	0.2336	2.59	0.1157

Example 11:

[0079] The study of Example 10 was repeated with a moderate traumatic brain injury cohort characterized by GCS scores of between 9 and 11, as well as a mild traumatic brain injury cohort characterized by GCS scores of 12-15. Blood samples were obtained from each patient on arrival to the emergency department of a hospital within 2 hours of injury and measured by ELISA for levels of GFAP in nanograms per milliliter. The results were compared to those of a control group who had not experienced any form of injury. Secondary outcomes included the presence of intracranial lesions in head CT scans.

[0080] Over 3 months 53 patients were enrolled: 35 with GCS 13-15, 4 with GCS 9-12 and 14 controls. The mean age was 37 years (range 18-69) and 66% were male. The mean GFAP serum level was 0 in control patients, 0.107 (0.012) in patients with GCS 13-15 and 0.366 (0.126) in GCS 9-12 ($P<0.001$). The difference between GCS 13-15 and controls was significant at $P<0.001$. In patients with intracranial lesions on CT GFAP levels were 0.234 (0.055) compared to 0.085 (0.003) in patients without lesions ($P<0.001$). There is a significant increase in GFAP in serum following a MTBI compared to uninjured controls in both the mild and moderate groups. GFAP was also significantly associated with the presence of intracranial lesions on CT.

[0081] FIG. 16 shows GFAP concentration for controls as well as individuals in the mild/moderate traumatic brain injury cohort as a function of CT scan results upon admission and 24 hours thereafter. Simultaneous assays were performed in the course of this study for UCH-L1 biomarker. The UCH-L1 concentration derived from the same samples

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as those used to determine GFAP is provided FIG. 17. The concentration of UCH-L1 and GFAP as well as a biomarker not selected for diagnosis of neurological condition, S100b, is provided as a function of injury magnitude between control, mild, and moderate traumatic brain injury as shown in FIG. 18. The simultaneous analyses of UCH-L1 and GFAP from these patients illustrates the synergistic effect of the inventive process in allowing an investigator to simultaneously diagnose traumatic brain injury as well as discern the level of traumatic brain injury between mild and moderate levels of severity. FIG. 19 shows the concentration of the same markers as depicted in FIG. 18 with respect to initial evidence upon hospital admission as to lesions in tomography scans illustrating the high confidence in predictive outcome of the inventive process. FIG. 20 shows that both NSE and MAP2 are elevated in subjects with MTBI in serum both at admission and at 24 hours of follow up. These data demonstrate a synergistic diagnostic effect of measuring multiple biomarkers such as GFAP, UCH-L1, NSE, and MAP2 in a subject.

[0082] Through the simultaneous measurement of multiple biomarkers such as UCH-L1, GFAP, NSE, and MAP2, rapid and quantifiable determination as to the severity of the brain injury is obtained consistent with GSC scoring and CT scanning yet in a surprisingly more quantifiable, expeditious and economic process. Additionally, with a coupled assay for biomarkers indicative of neurological condition, the nature of the neurological abnormality is assessed and in this particular study suggestive of neuronal cell body damage. As with severe traumatic brain injury, gender variations are noted suggesting a role for hormonal anti-inflammatories as therapeutic candidates. A receiver operating characteristic (ROC) modeling of UCH-L1, GFAP and SBDP145 post TBI further supports the value of simultaneous measurement of these biomarkers, as shown in Figs. 21, 22.

[0083] In addition, Figure 22 showed that several brain biomarkers (GFAP, UCH-L1 and MAP2) in stroke patients' plasma. Samples were collected with an average post-injury time 24.2 hr (range18-30 h). Top panel shows GFAP, UCH-L1 and MAP2 levels in stroke (n=11) versus normal controls (n=30). Bottom panel further shows that UCH-L1 is elevated with both hemorrhagic and ischemic stroke populations when compared to normal control plasma.

[0084] Patent documents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains.

[0085] The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. The following claims are intended to define the scope of the invention.

Claims

1. A process for determining the neurological condition of a subject wherein said neurological condition is selected from traumatic brain injury and stroke comprising:

   measuring a biological sample obtained from the subject at a first time for a quantity of a first biomarker and a quantity of at least one additional neuroactive biomarker, wherein said first biomarker is GFAP and said at least one additional neuroactive biomarker is UCH-L1; and
   comparing the quantity of said first biomarker and the quantity of said at least one additional neuroactive biomarker to the baseline level of said first biomarker and said at least one additional neuroactive biomarker in a subject known to be free of a pathological neurological condition to determine the neurological condition of the subject;
   wherein said biological sample is cerebrospinal fluid (CSF), whole blood, serum, or plasma.

2. The process of claim 1, further comprising measuring for a quantity of a further neuroactive biomarker, wherein said further neuroactive biomarker is NSE, SBDP145, SBDP120, S100b, MAP2 or MBP.

3. The process of claim 1, further comprising measuring a second quantity of said first biomarker and a second quantity of said at least one additional neuroactive biomarker at a second time to yield a kinetic profile for said first biomarker and said at least one additional neuroactive biomarker.

4. The process of claim 1, further comprising comparing the quantity of said first biomarker and the quantity of said at least one additional neuroactive biomarker between the levels in the subject and the levels in other individuals of the same gender as the subject known to be free of a pathological neural condition.

5. The process of claim 4, wherein it is determined if the subject has been exposed to some degree of traumatic brain injury ranging from mild to severe; and wherein the process further comprises predicting mortality based on the quantity of UCH-L1 and the quantity of GFAP.

6. An assay product for determining the neurological condition, wherein said neurological condition is selected from

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traumatic brain injury and stroke of a subject comprising:

a substrate for holding a biological sample isolated from the subject;
a first biomarker specifically binding agent wherein a first biomarker is GFAP;
an agent specifically binding at least one additional neuroactive biomarker wherein said at least one additional neuroactive biomarker is UCH-L1; and
printed instructions for reacting said first biomarker specific agent with a first portion of said biological sample so as to detect an amount of said first biomarker and reacting said at least one additional neuroactive biomarker specific agent with a second portion of said biological sample and said at least one additional neuroactive biomarker in said biological sample so as to detect an amount of said at least one additional neuroactive biomarker to determine the neurological condition of the subject according to the process of claim 1.

7. The assay product of claim 6, wherein the first biomarker specific agent is an anti-GFAP antibody.

8. The assay product of claim 6, wherein said first biomarker specifically binding agent and said agent specifically binding at least one additional neuroactive biomarker are both bound to the substrate, optionally with spatial overlap.

9. The assay product of any of claims 6 or 8, further comprising a separate first biomarker detection species for said first biomarker and a separate discernable at least one additional neuroactive biomarker detection species for said at least one additional neuroactive biomarker.

10. A process for determining if a subject has suffered mild traumatic brain injury or moderate traumatic brain injury in an event comprising:

measuring by ELISA a biological sample obtained from said subject for a quantity of GFAP, wherein said biological sample is whole blood, serum or plasma; and
comparing the measured quantity of said GFAP to normal levels of GFAP in a control group who had not experienced any form of head injury, wherein if the measured quantity of GFAP is between 0.082 and 0.492 nanograms per milliliter, the subject has suffered mild traumatic brain injury or moderate traumatic brain injury in the event.

Patentansprüche

1. Prozess zum Ermitteln des neurologischen Zustands eines Subjekts, wobei der neurologische Zustand ausgewählt ist aus traumatischer Hirnverletzung und Schlaganfall umfassend:

messen einer biologischen Probe erhalten von dem Subjekt zum ersten Mal für ein Menge eines ersten Biomarkers und für eine Menge mindestens eines zusätzlichen neuroaktiven Biomarkers, wobei der erste Biomarker GFAP ist und der mindestens eine zusätzliche neuroaktive Biomarker UCH-L1 ist; und
vergleichen der Menge des ersten Biomarkers und der Menge des mindestens einen zusätzlichen neuroaktiven Biomarkers mit dem Ausgangswert des ersten Biomarkers und des mindestens einen zusätzlichen neuroaktiven Biomarker in einem Subjekt, von dem bekannt ist, dass es frei von einem pathologischen neurologischen Zustand ist, um den neurologischen Zustand des Subjekts zu ermitteln;
wobei die biologische Probe Zerebrospinalflüssigkeit (CSF), Vollblut, Serum oder Plasma ist.

2. Prozess nach Anspruch 1, weiterhin umfassend messen einer Menge eines weiteren neuroaktiven Biomarkers, wobei der weitere neuroaktive Biomarker NSE, SBDP145, SBDP120, S100b, MAP2 oder MBP ist.

3. Prozess nach Anspruch 1, weiterhin umfassend messen einer zweiten Menge des ersten Biomarkers und einer zweiten Menge des mindestens einen zusätzlichen neuroaktiven Biomarkers zum zweiten Mal, um ein kinetisches Profil für den ersten Biomarker und den mindestens einen zusätzlichen neuroaktiven Biomarker zu erbringen.

4. Prozess nach Anspruch 1, weiterhin umfassend vergleichen der Menge des ersten Biomarkers und der Menge des mindestens einen zusätzlichen neuroaktiven Biomarkers zwischen den Werten in dem Subjekt und den Werten in anderen Individuen des selben Geschlechts wie das Subjekt, von dem bekannt ist, dass es frei von einem pathologischen neuralen Zustand ist.

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5. Prozess nach Anspruch 4, wobei es ermittelt wird, falls das Subjekt einem gewissen Grad an traumatischer Hirnverletzung angefangen von leicht bis schwer ausgesetzt wurde; und wobei der Prozess weiterhin umfasst vorhersagen der Sterblichkeit basierend auf der Menge des UCH-L1 und der Menge des GFAP.

6. Assayprodukt zum Ermitteln des neurologischen Zustands, wobei der neurologische Zustand ausgewählt ist aus traumatischer Hirnverletzung und Schlaganfall eines Subjekts umfassend:

ein Substrat zum Halten einer von dem Subjekt isolierten biologischen Probe;
einen ersten Biomarker spezifisch bindenden Wirkstoff, wobei ein erster Biomarker GFAP ist;
ein Wirkstoff spezifisch mindestens einen zusätzlichen neuroaktiven Biomarker bindend, wobei der mindestens eine zusätzliche neuroaktive Biomarker UCH-L1 ist; und
ausgedruckte Anweisungen zum Reagieren des für den ersten Biomarker spezifischen Wirkstoffs mit einem ersten Teil der biologischen Probe, so dass eine Menge des ersten Biomarkers detektiert wird und zum Reagieren des für den mindestens einen zusätzlichen neuroaktiven Biomarker spezifischen Wirkstoffs mit einem zweiten Teil der biologischen Probe und dem mindestens einen zusätzlichen neuroaktiven Biomarker in der biologischen Probe, so dass eine Menge des mindestens einen zusätzlichen neuroaktiven Biomarkers detektiert wird, um den neurologischen Zustand des Subjekts nach dem Prozess nach Anspruch 1 zu ermitteln.

7. Assayprodukt nach Anspruch 6, wobei der für den ersten Biomarker spezifische Wirkstoff ein anti-GFAP Antikörper ist.

8. Assayprodukt nach Anspruch 6, wobei der erste Biomarker spezifisch bindende Wirkstoff und der Wirkstoff spezifisch mindestens einen zusätzlichen neuroaktiven Biomarker bindend beide am Substrat gebunden sind, gegebenenfalls mit räumlicher Überlagerung.

9. Assayprodukt nach einem der Ansprüche 6 oder 8, weiterhin umfassend einen getrennten ersten Biomarker Speziesnachweis für den ersten Biomarker und einen getrennten erkennbaren mindestens einen zusätzlichen neuroaktiven Biomarker Speziesnachweis für den mindestens einen zusätzlichen neuroaktiven Biomarker.

10. Prozess zum Ermitteln, falls ein Subjekt an leichter traumatischer Hirnverletzung oder an mäßiger traumatischer Hirnverletzung in einem Fall gelitten hat umfassend:

messen einer von dem Subjekt erhaltenen biologischen Probe mit Hilfe von ELISA für eine Menge von GFAP, wobei die biologische Probe Vollblut, Serum oder Plasma ist; und vergleichen der gemessenen Menge an GFAP zu normalen Werten von GFAP in einer Kontrollgruppe, die keine Form von Kopfverletzung erfahren hatte, wobei falls die gemessene Menge an GFAP zwischen 0.082 und 0.492 Nanogramm pro Milliliter ist, hat das Subjekt leichte traumatische Hirnverletzung oder mäßige traumatische Hirnverletzung in dem Fall erlitten.

Revendications

1. Procédé pour déterminer l'état neurologique d'un sujet, dans lequel ledit état neurologique est sélectionné parmi un traumatisme cérébral et un accident vasculaire cérébral, comprenant :

la mesure d'un échantillon biologique obtenu à partir du sujet à un premier moment pour une quantité d'un premier biomarqueur et une quantité d'au moins un biomarqueur neuroactif supplémentaire, ledit premier biomarqueur étant GFAP et ledit au moins un biomarqueur neuroactif supplémentaire étant UCH-L1 ; et
la comparaison de la quantité dudit premier biomarqueur et de la quantité dudit au moins un biomarqueur neuroactif supplémentaire au niveau de base dudit premier biomarqueur et dudit au moins un biomarqueur neuroactif supplémentaire chez un sujet que l'on sait dépourvu d'un état neurologique pathologique pour déterminer l'état neurologique du sujet ;
ledit échantillon biologique étant du liquide céphalorachidien (LCR), du sang total, du sérum ou du plasma.

2. Procédé de la revendication 1, comprenant en outre la mesure pour une quantité d'un autre biomarqueur neuroactif, ledit autre biomarqueur neuroactif étant NSE, SBDP145, SBDP120, S100b, MAP2 ou MBP.

3. Procédé de la revendication 1, comprenant en outre la mesure d'une seconde quantité dudit premier biomarqueur et d'une seconde quantité dudit au moins un biomarqueur neuroactif supplémentaire à un second moment pour

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fournir un profil cinétique pour ledit premier biomarqueur et ledit au moins un biomarqueur neuroactif supplémentaire.

4. Procédé de la revendication 1, comprenant en outre la comparaison de la quantité dudit premier biomarqueur et de la quantité dudit au moins un biomarqueur neuroactif supplémentaire entre les niveaux chez le sujet et les niveaux chez d'autres individus du même sexe que le sujet que l'on sait être dépourvus d'un état neurologique pathologique.

5. Procédé de la revendication 4, dans lequel on détermine si le sujet a été exposé à un certain degré de traumatisme cérébral allant de léger à grave ; et dans lequel le procédé comprend en outre la prédiction de la mortalité sur la base de la quantité d'UCH-L1 et de la quantité de GFAP.

6. Produit de test pour déterminer l'état neurologique, dans lequel ledit état neurologique est sélectionné parmi un traumatisme cérébral et un accident vasculaire cérébral d'un sujet, comprenant :

un substrat pour maintenir un échantillon biologique isolé à partir du sujet ;
un agent se liant spécifiquement au premier biomarqueur, un premier biomarqueur étant GFAP ;
un agent se liant spécifiquement à au moins un biomarqueur neuroactif supplémentaire, ledit au moins un biomarqueur neuroactif supplémentaire étant UCH-L1 ; et
des instructions imprimées pour faire réagir ledit agent spécifique au premier biomarqueur avec une première partie dudit échantillon biologique de façon à détecter une quantité dudit premier biomarqueur et faire réagir ledit agent spécifique au au moins un biomarqueur neuroactif supplémentaire avec une seconde partie dudit échantillon biologique et dudit au moins un biomarqueur neuroactif supplémentaire dans ledit échantillon biologique de façon à détecter une quantité dudit au moins un biomarqueur neuroactif supplémentaire pour déterminer l'état neurologique du sujet selon le procédé de la revendication 1.

7. Produit de test de la revendication 6, dans lequel l'agent spécifique au premier biomarqueur est un anticorps anti-GFAP.

8. Produit de test de la revendication 6, dans lequel ledit agent se liant spécifiquement au premier biomarqueur et ledit agent se liant spécifiquement à au moins un biomarqueur neuroactif supplémentaire sont tous les deux liés au substrat, facultativement avec un chevauchement spatial.

9. Produit de test de l'une quelconque des revendications 6 ou 8, comprenant en outre une espèce distincte de détection du premier biomarqueur pour ledit premier biomarqueur et une espèce distincte, discernable, de détection du au moins un biomarqueur neuroactif supplémentaire pour ledit au moins un biomarqueur neuroactif supplémentaire.

10. Procédé pour déterminer si un sujet a subi un traumatisme cérébral léger ou un traumatisme cérébral modéré lors d'un événement, comprenant :

la mesure par ELISA d'un échantillon biologique obtenu à partir dudit sujet pour une quantité de GFAP, ledit échantillon biologique étant du sang total, du sérum ou du plasma ; et
la comparaison de la quantité mesurée de ladite GFAP à des niveaux normaux de GFAP dans un groupe témoin qui n'avait subi aucune forme de traumatisme crânien, où, si la quantité mesurée de GFAP est entre 0,082 et 0,492 nanogramme par millilitre, le sujet a subi un traumatisme cérébral léger ou un traumatisme cérébral modéré lors de l'événement.

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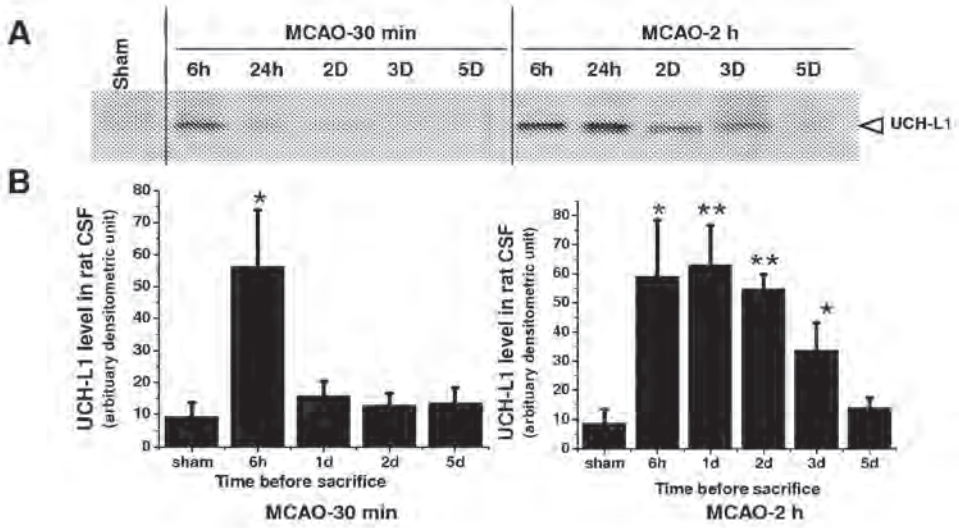


Figure 1

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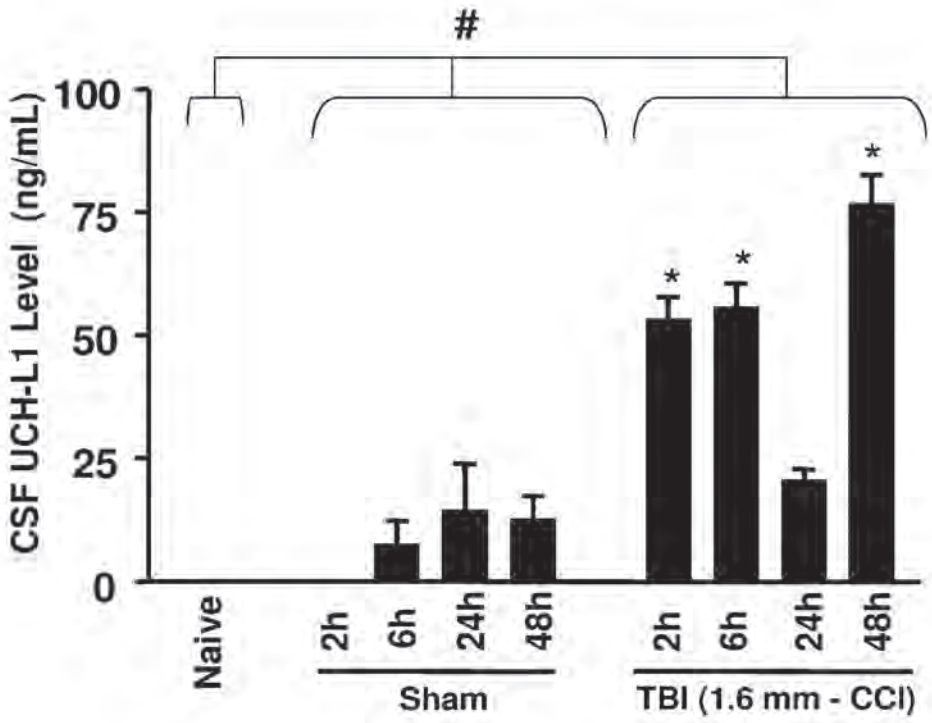


Figure 2

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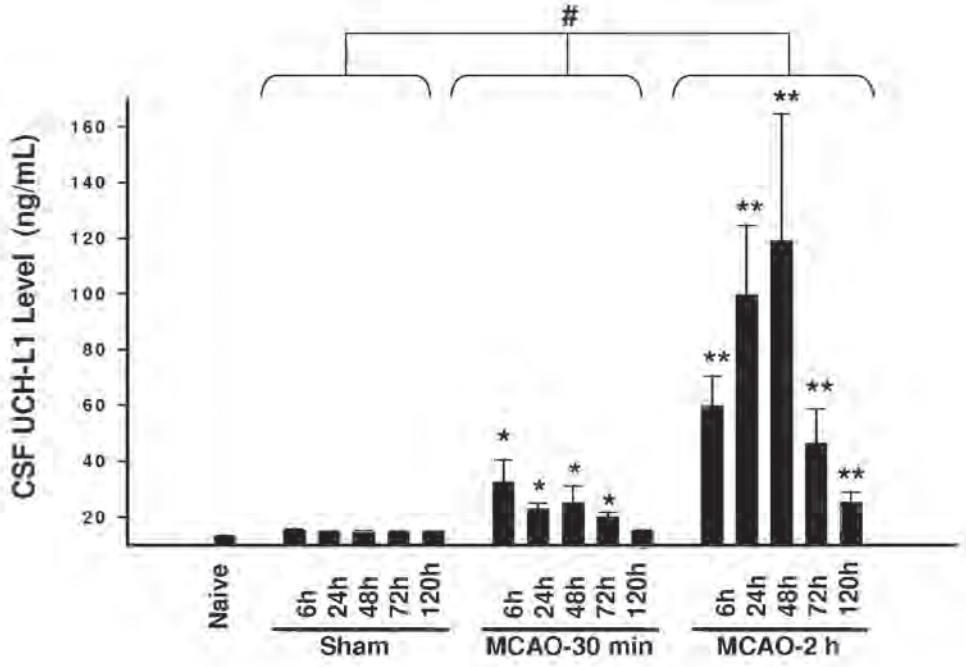


Figure 3

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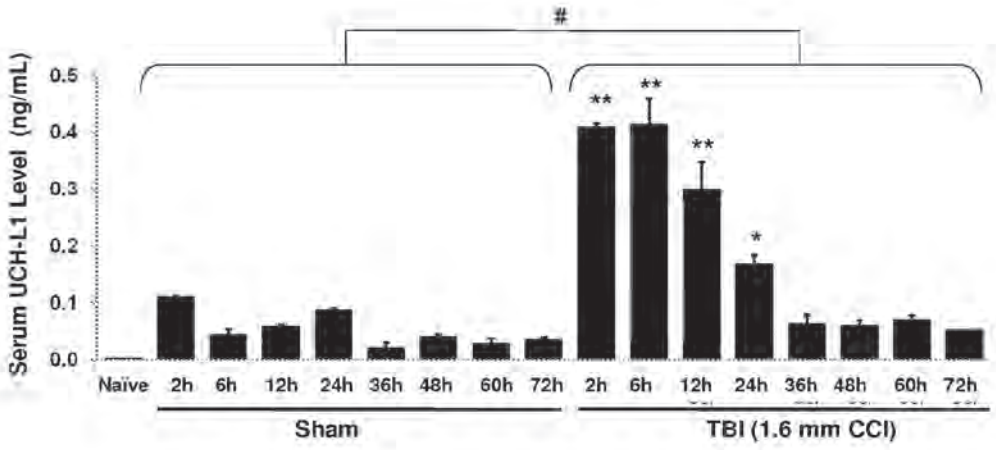


Figure 4

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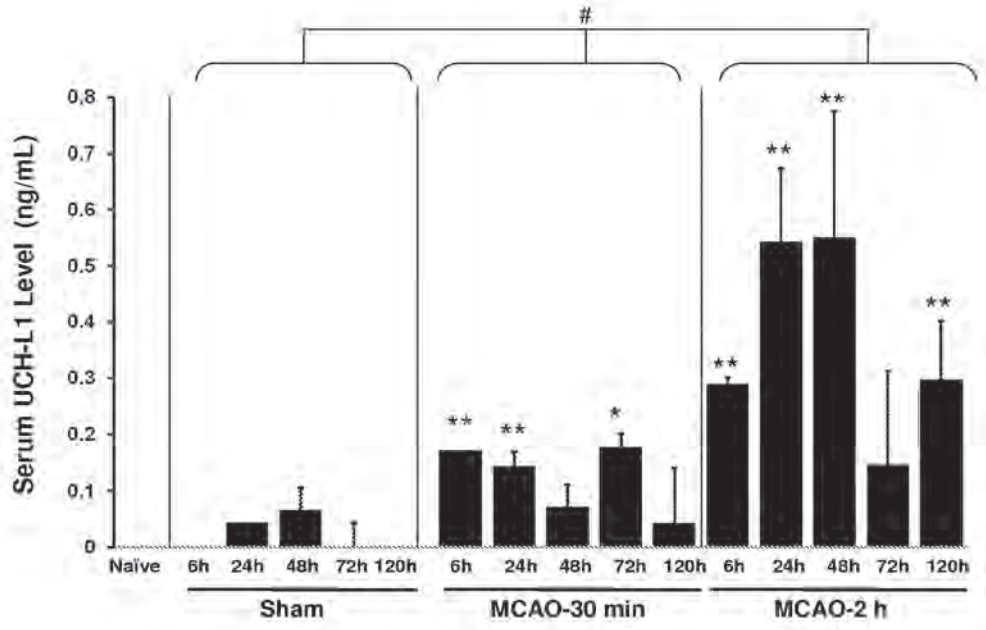
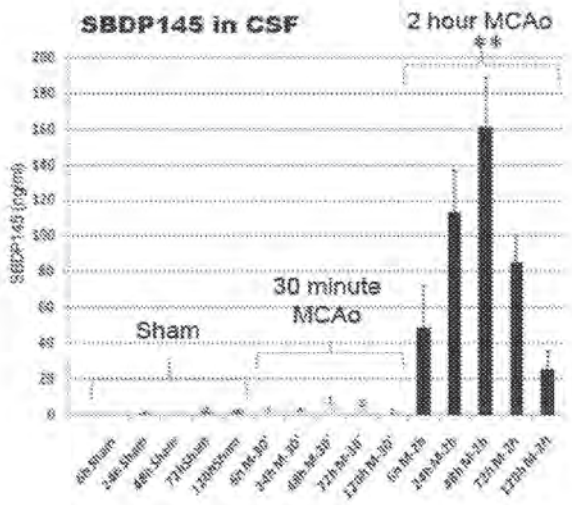


Figure 5

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A



B

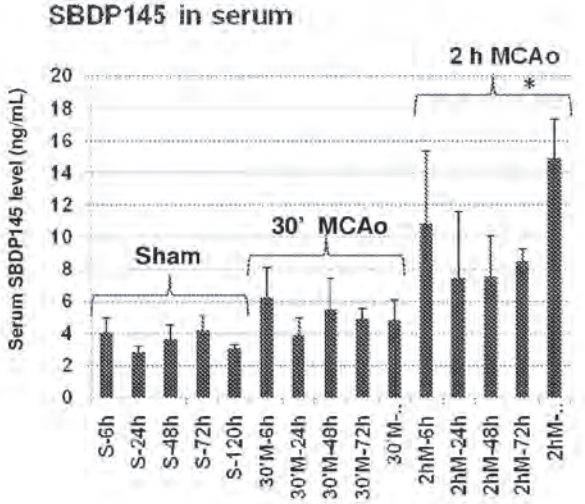


Figure 6

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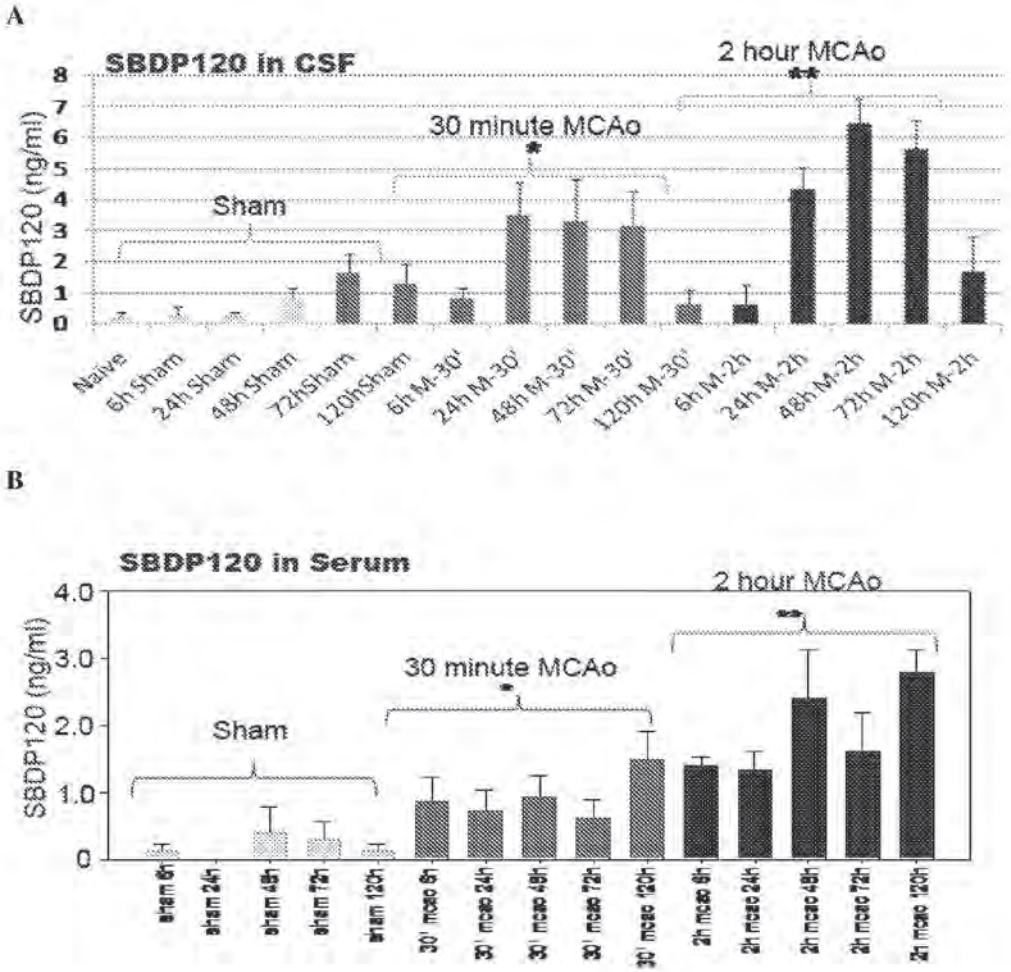


Figure 7

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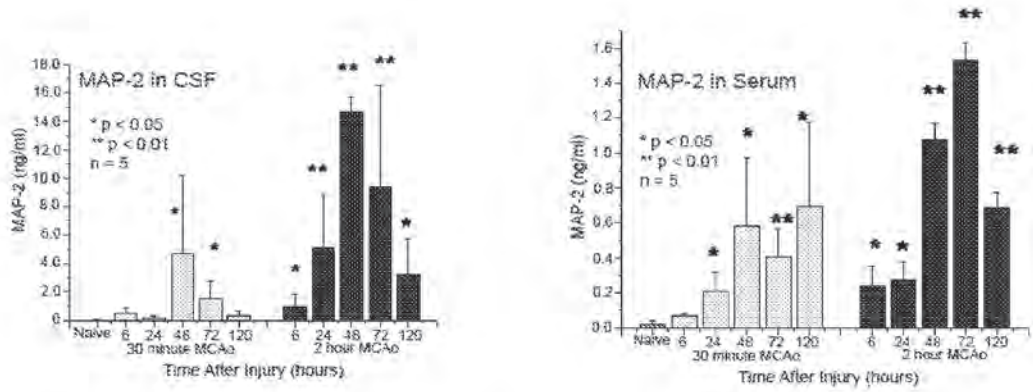


Figure 8

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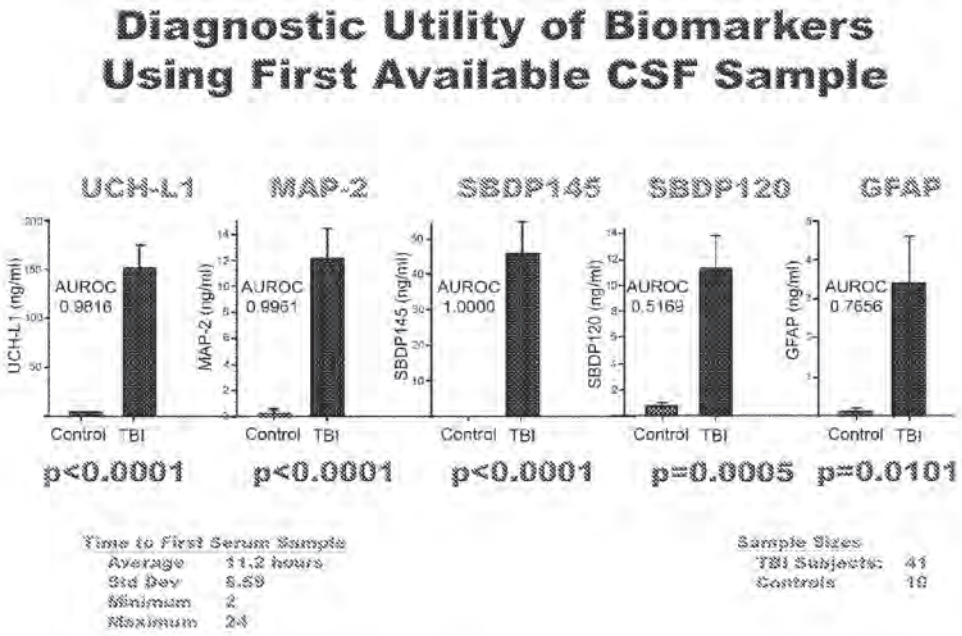
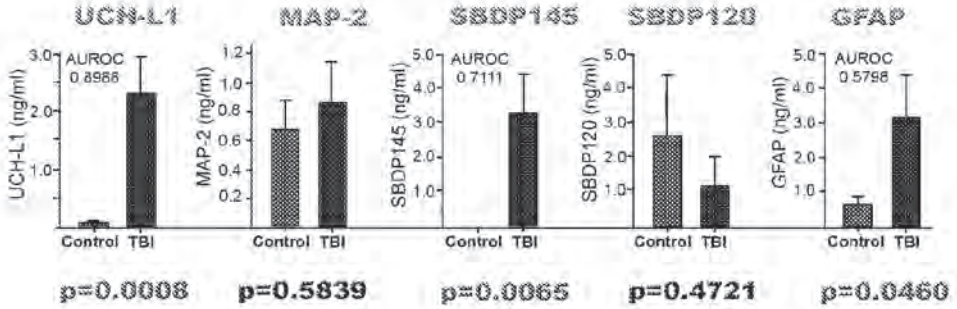


Figure 9

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Diagnostic Utility of Biomarkers Using First Available Serum Sample



Time to First Serum Sample	
Average	10.1 hours
Std Dev	6.96
Minimum	2
Maximum	24

Sample Sizes	
TBI Subjects:	46
Controls (MAP-2, UCH-L1, GFAP)	54
Controls (SBDP145, SBDP120)	10

Figure 10

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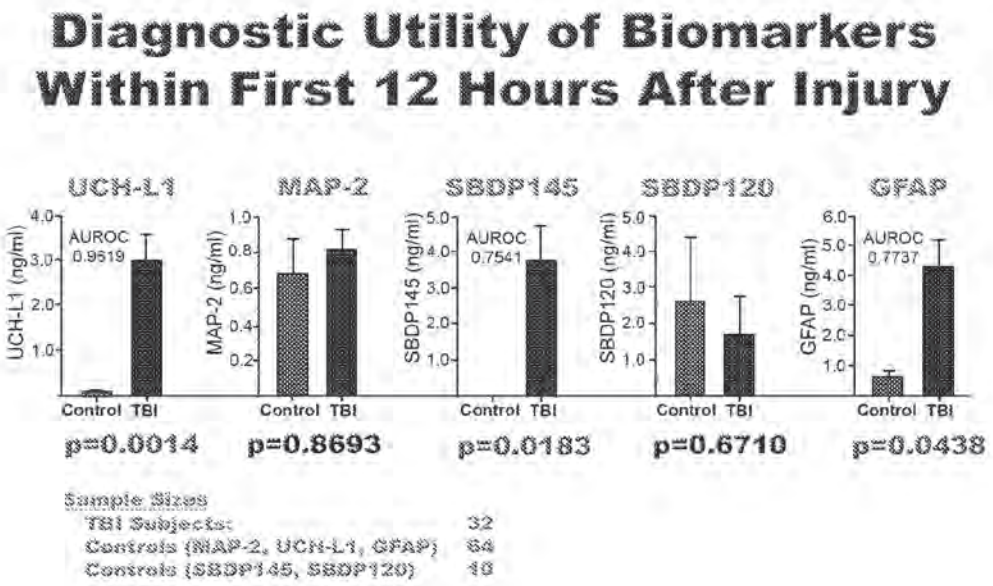


Figure 11

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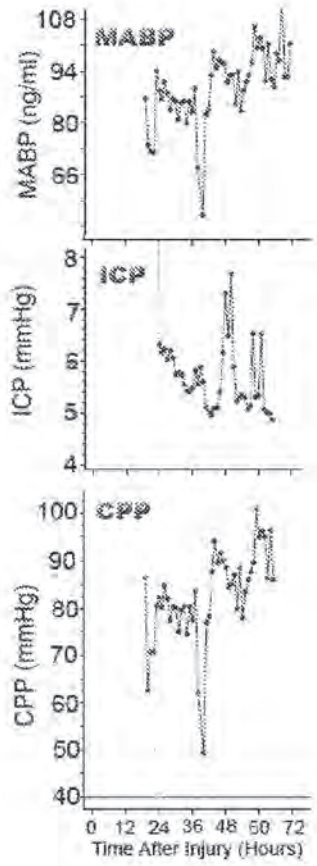


Figure 12

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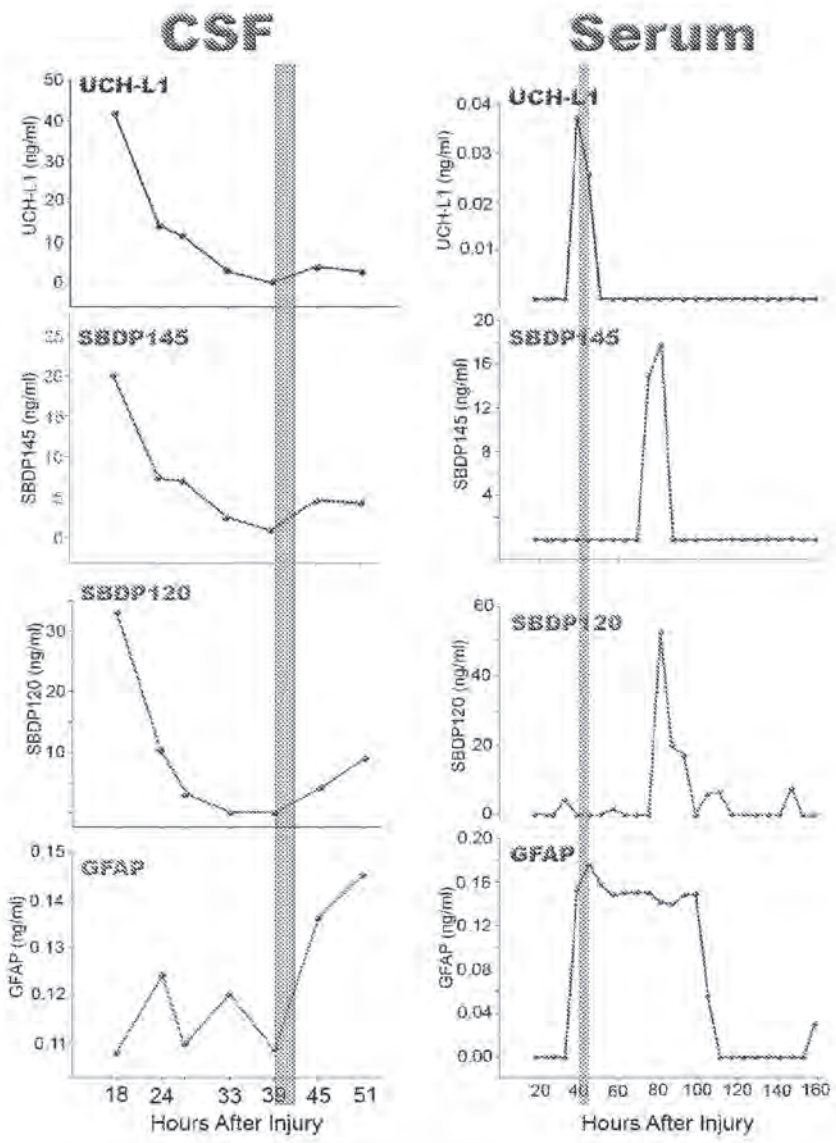


Figure 13

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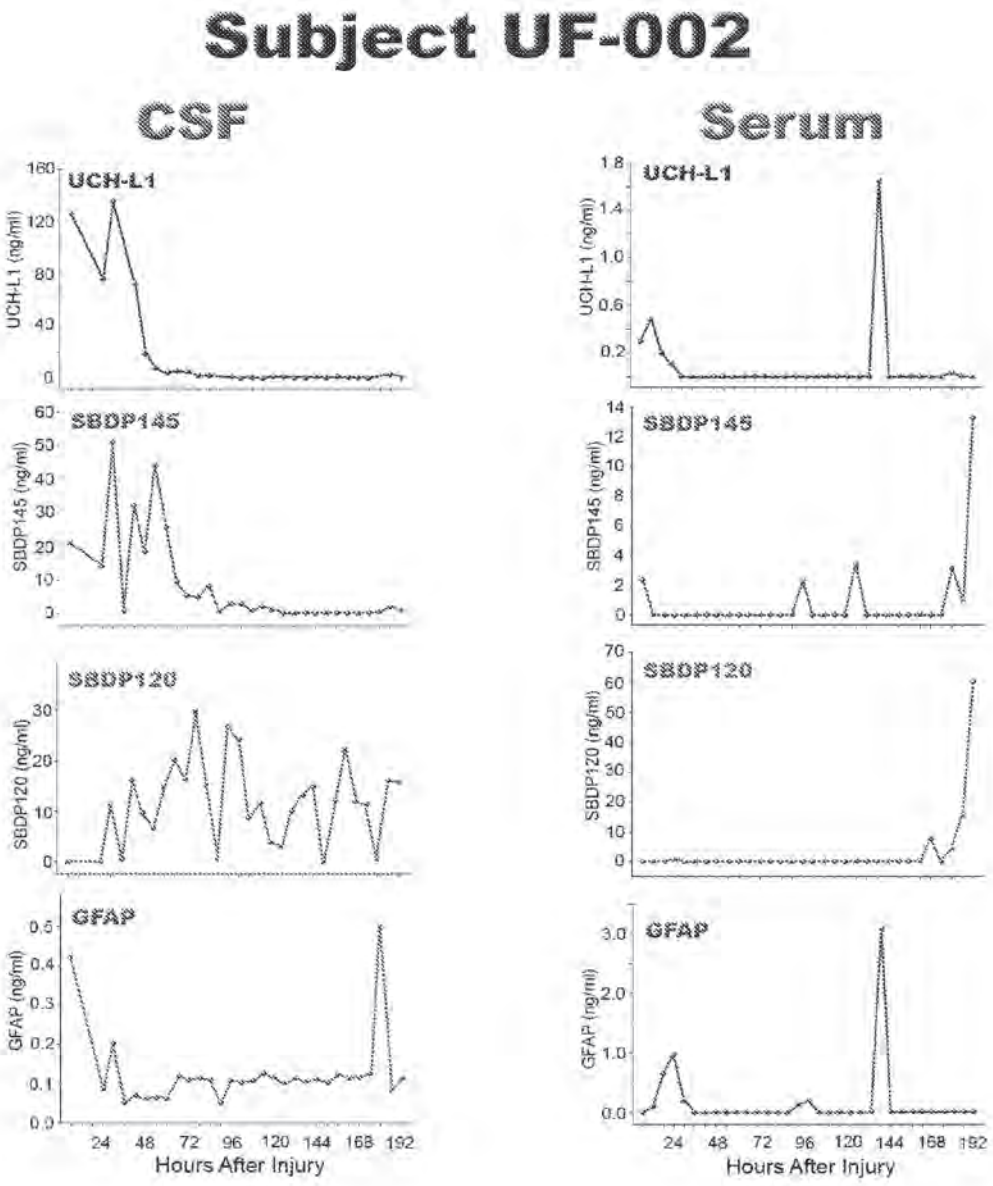


Figure 14

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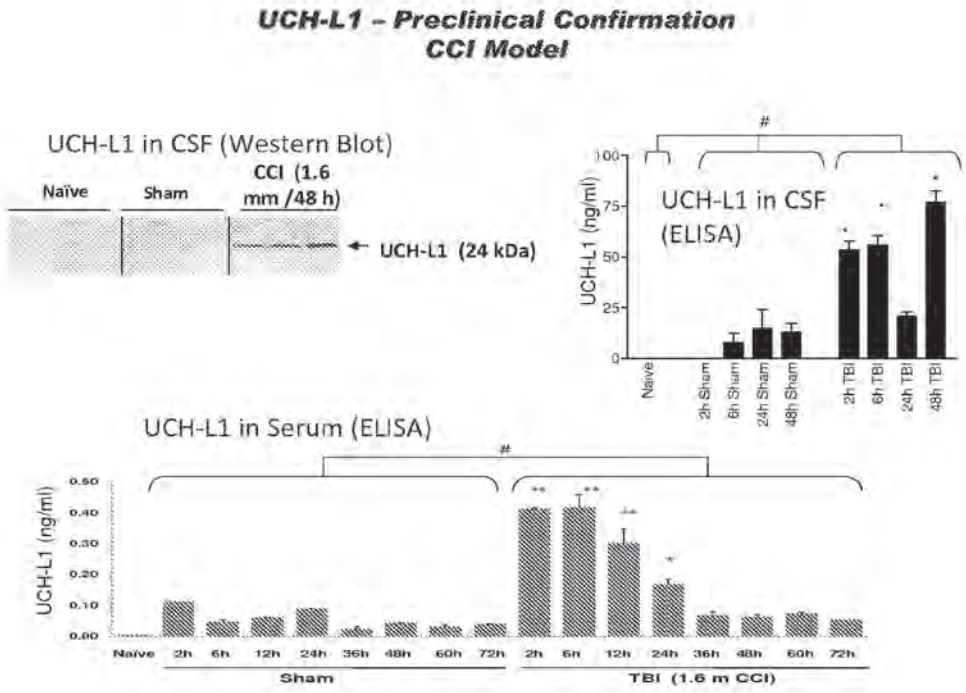


Figure 15

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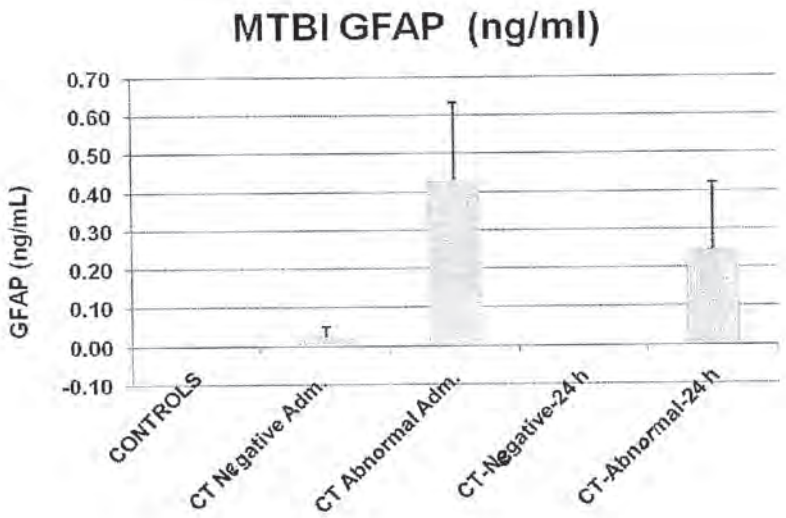


Figure 16

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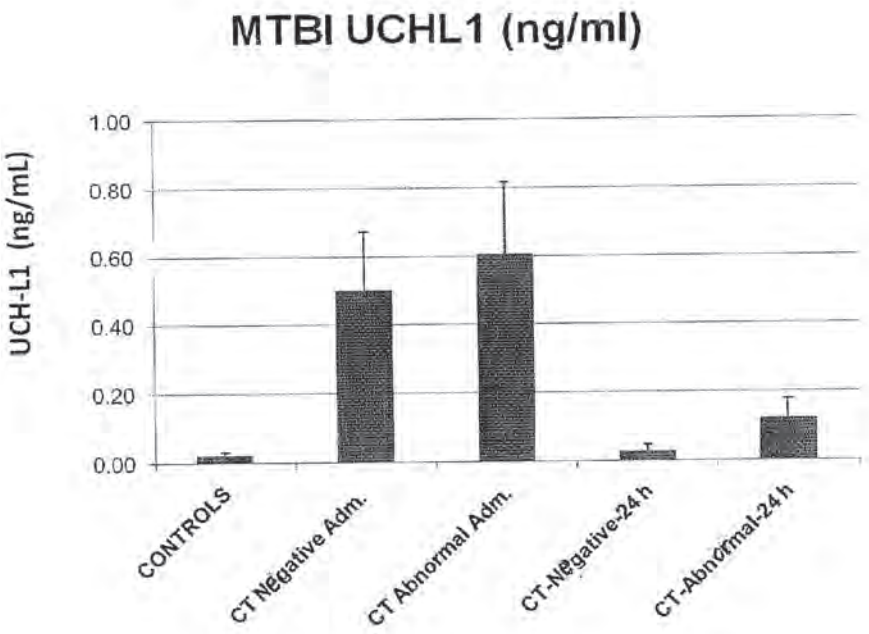


Figure 17

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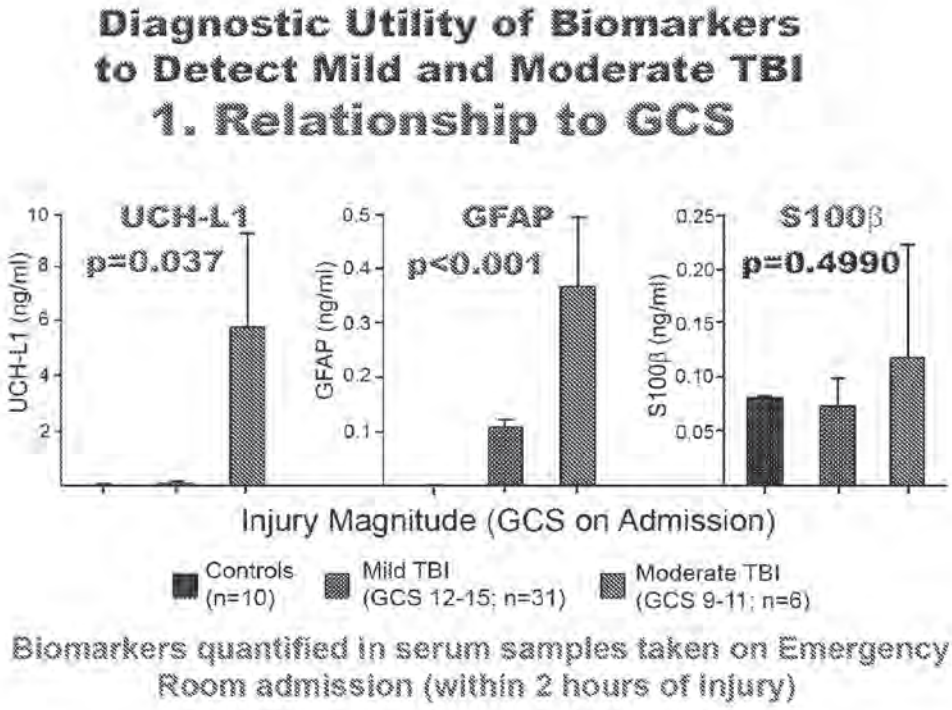


Figure 18

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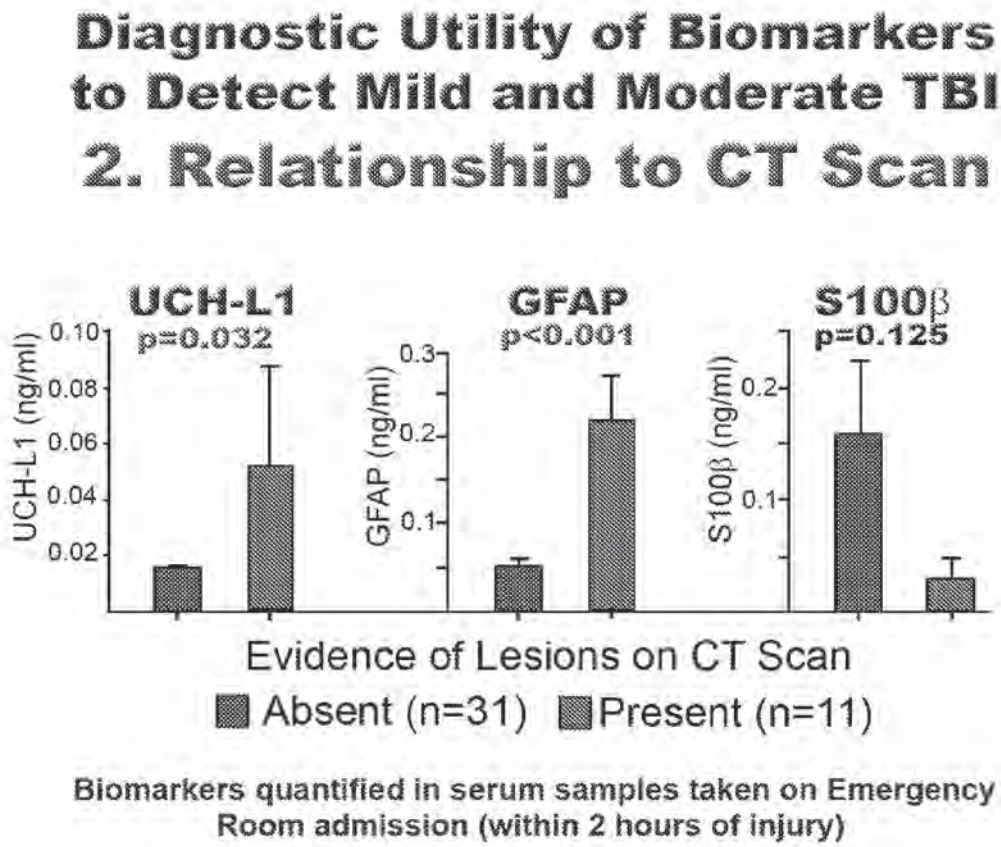
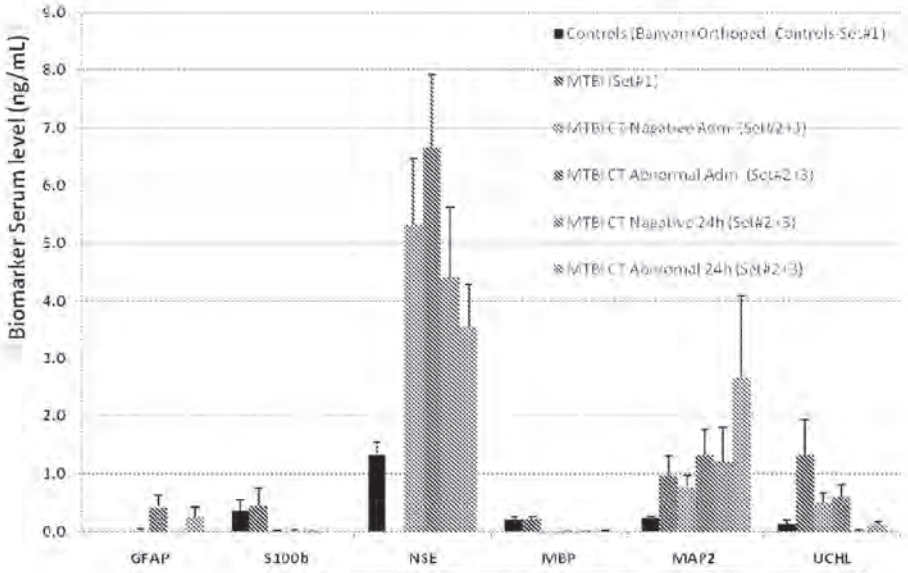


Figure 19

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		GFAP ng/mL	S100b ng/mL	NSE ng/mL	MBP ng/mL	MAP2 ng/mL	UCHL1 ng/mL
Normal Controls	n=	8	16	8	18	15	33
MTBI CT negative adm. (Set#2+3)	n=	15	15	15	8	15	8
MTBI CT abnormal adm.(Set#2+3)	n=	12	12	12	8	12	10
MTBI CT-negative 24 h (Set#2+3)	n=	11	11	11	6	11	6
MTBI CT-abnormal 24 h (Set#2+3)	n=	9	8	10	5	9	7
MTBI (Set #1, adm.)	n=	0	17	0	17	17	17

Figure 20

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ROC analysis of **UCH-L1, GFAP and SBDP145** in human CSF (severe TBI vs. Control
A) First 24 hours post-injury

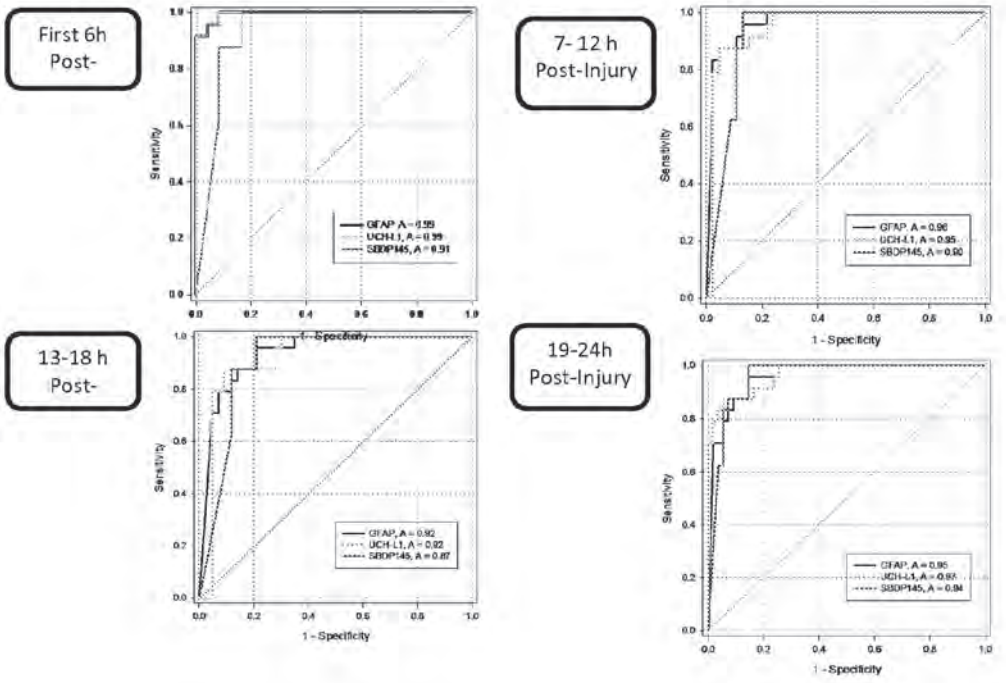


Fig. 21

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ROC Curves: Biomarkers in Mild TBI

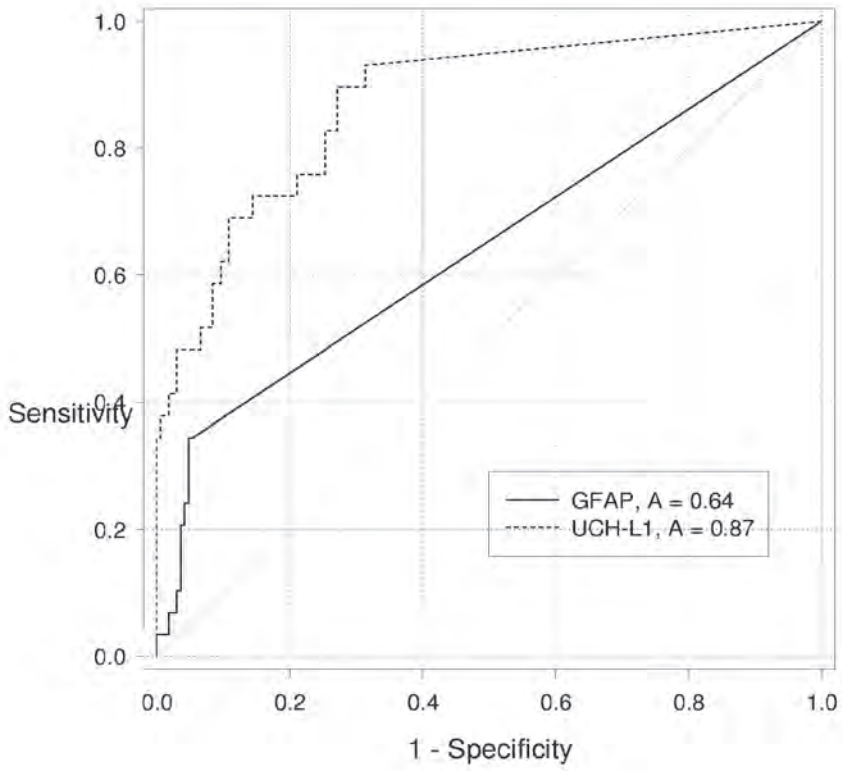
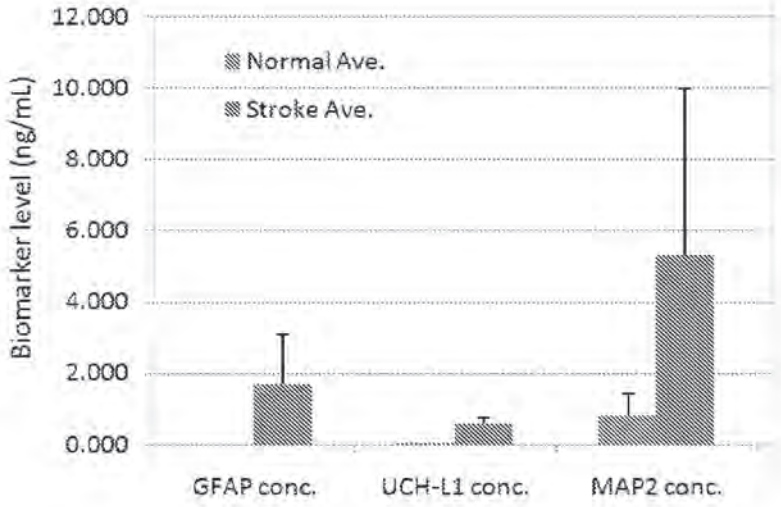


Fig. 22

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Elevation of brain injury biomarkers (GFAP, UCH-L1 and MAP2) in plasma in stroke patients.



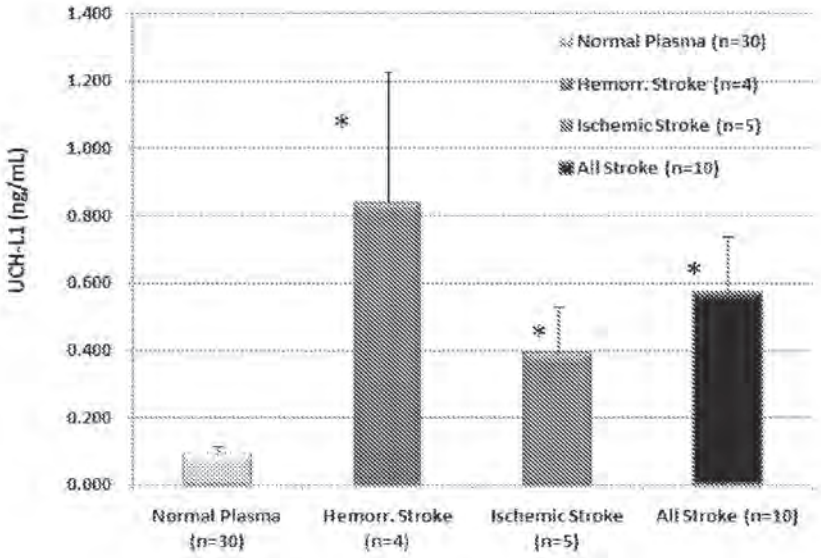


Fig. 23

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REFERENCES CITED IN THE DESCRIPTION

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(54) Title: USE OF POLYMERIZED IMMUNOGLOBULIN TO REDUCE THE INCIDENCE OF FALSE-POSITIVE RESPONSES IN IMMUNOASSAYS

(57) Abstract

The present invention relates to a method to reduce false positive results in immunoassays. The method uses polymerized immunoglobulin to overcome binding which is commonly referred to as non-specific binding. The falsely elevated signal generated in the presence of human serum in assays for infectious disease markers, cancer related antigens and peptide hormones is reduced in assays performed in the presence of the polymerized immunoglobulin. The polymerized immunoglobulin can be prepared using different methods of polymerization.

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TITLE

USE OF POLYMERIZED IMMUNOGLOBULIN TO REDUCE THE INCIDENCE OF FALSE-POSITIVE RESPONSES IN IMMUNOASSAYS

TECHNICAL FIELD

This invention relates to a method for reducing false positive responses in enzyme immunoassays, more specifically to the method of using polymerized immunoglobulin for removing false positive responses in immunoassays.

BACKGROUND ART

Immunometric assays are widely in use for detection of antigens having multiple epitopes. Typically, such an assay involves the use of at least two antibodies, a capture antibody being immobilized onto a solid support and a detector antibody conjugated to a label. One of the most commonly used labels is an enzyme while colorometric, radioactive, fluorescent or chemiluminescent and other labels are available.

An enzyme label offers high sensitivity, ready availability and stability compared to a radioactive label. However, the enzyme being a large protein contributes to non-specific protein-protein interactions which induce false positive responses, also refered to as non-specific responses. By a false positive response is meant a response that is not specifically associated with the binding of the capture or the detector antibody to the antigen of interest. Although the problem of false positives is less frequent in immunoradiometric or competitive radioimmunoassays, it manifests itself in high sensitivity assays requiring large sample size and long incubation step [Hunter et al, Lancet, Vol. ii, 1136, 1980]. Thus, it remains a universal problem to be solved.

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The false positive response can be observed in assays for many different types of clinical parameters, both quantitive and qualitative in nature, including, for example, hormones of peptide nature, such as hCG, LH, somatotropin, FSH, ACTH, LPH, prolactin, MSH, beta-endorphinns, enkephalines, vasopressin, oxitocin, and the like. In addition, false positive results can be observed in determination of markers which are indicators of malignancy, such as, carcinoembryonic antigen (CEA), alpha-feto protein, prostatic specific antigen, cancer antigens ca 19.9, ca 125, ca 15-3, beta-2-microglobulin, ferritin, prostatic acid phosphatase, and the like. Assays for specific microbial and viral disease markers are also subject to false positive results. Such markers are hepatitis B surface antigen, hepatitis B core antigen, antibody to hepatitis B core antigen, antibody to HIV, p17 antigen, p24 antigen, HTLV 1 and HTLV 2 antigen and specific peptide markers of infection with HTLV 1 and HTLV 2, markers for hepatitis A, C, D and E.

One form of non-specific interaction involves IgM rheumatoid factor (RF). Methods for removal of RF interference using non-specific aggregated IgG are well known in the art. It is also well known that aggregated IgG has enhanced reactivity with RF compared to unmodified monomeric IgG [Oreskes et al, Immunology, Vol. 51, 115-121, 1984, and Dissanayake et al, Immunology, Vol. 32, 309-318, 1977]. In fact, Hallgren, US 4,184,847 and US, 4,153,417 discloses a method for detection of RF using labeled aggregated IgG.

Most of the methods for removing RF interference involve a sample pretreatment process. Sakai et al, US 4,680,274, issued July 14, 1987 disclose the use of ultrafine particles of less than 0.2 micrometers in size having immobilized immunoglobulins thereon to absorb

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non-specific factors from the sample. Vejtorp, J. Virol. Methods, Vol. 1, 1-10, 1980 describes the use of latex particles coated with aggregated human IgG as means for removing RF from the sample in an ELISA based rubella IgM antibody assay. Forghani et al, J. Clin. Microbiol. Vol. 18, 652-657, 1983, also describe a sample pretreatment method using polymerized human IgG. Here, the sample is pretreated with glutaraldehyde-aggregated human IgG followed by centrifugation to remove the interferant. These references do not discuss nor suggest the use of polymerized immunoglobulin, which is of the same animal species of the capture and detector antibodies, in sample or conjugate diluent.

Briantais et al, J. Virol. Methods, Vol. 9, 15-26, 1984, describe the use of a radiolabeled (Fab')2 antibody to overcome RF interference in rubella and hepatitis B core IgM assays. In their tests for human IgM, they describe specific IgG binding non-specifically with the immobilized IgG antibody. This type of interference is said to be reduced only by the addition to the dilution medium of non-specific human IgG or fetal calf serum (FCS). They use unmodified IgG to reduce the IgG-IgG interaction in an IgM capture assay. The advantageous use of polymerized Immunoglobulin as in assay milieu is not recognized.

Gerlich et al, J. Med. Virol. Vol. 4, 227-238, 1979, describe the use of excess heat-aggregated human IgG in a sample diluent to remove false positive responses in a hepatitis B core IgM antibody assay. Vejtorp, Acta Path. Microbiol. Scan., B, Vol. 89, 123-128, 1981, also describes the additon of heat-aggregated human IgG to the serum diluent eliminating RF interference in a rubella IgM assay. These are methods for reducing RF interference applicable only in IgM assays. The present invention is useful in immunoassays

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in which the analyte of interest can be viral markers, hormones or tumor markers, when both RF-positive and RF-negative samples are tested.

A mechanism for false positive responses in two site immunoassays has recently been proposed by Levinson et al, Clin. Immunol. Letter, Vol. 9, 101-116, 1988. It involves endogeneous immunoglobulins (heterophile antibodies) in a sample having broad specificities toward animal antibodies. These heterophile antibodies are capable of bridging capture antibodies with detector antibodies in the absence of an antigen, producing a non-specific reponse. Removal of this type of interference requires either a sample pretreatment with immunoglobulins from several species or addition of these immunoglobulins to the assay milieu.

The addition of non-specific immunoglobulins derived from the same species as the antigen-specific antibody is commonly used to block non-specific binding caused by heterophile antibodies. For example, the addition of a non-specific murine monoclonal antibody that is of the same subclass as those of the specific monoclonal antibodies is shown to reduce non-specific interactions in a sandwich assay using murine monoclonal antibodies.[Fukuyama et al, JP Application, 63-12,960, published January 20, 1988]

R. C. McCarthy et. al., Arch. Pathol. Lab. Med., Vol. 112, pp. 901-907, 1988, describe the nature of "heterophile antibody" as primarily human IgM having reactivity to mouse IgG. They were able to inhibit this type of interference by adding an excess of unmodified mouse IgG in the sample (final concentration of IgG: 1.3 mg/mL). They also report that the false positive responses can be reduced, but not completely eliminated, by using Fab' or (Fab')2 of IgG as the detector antibody. The present invention employs polymerized IgG

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with a defined range of molecular weight in the assay media, which is much more effective in eliminating the false positive responses.

These cited methods do not solve all of the false positive problems. For example, it is often observed in correlating enzyme-immunoassay (EIA) to radio-immunoassay (RIA) that EIA values in certain samples are always higher than the RIA values. Furthermore, due to the complex nature of protein-protein interactions involved, the incidence of false positives is highly dependent on the assay configuration. For example, a forward sandwich assay, wherein a sample is incubated with the capture antibody on a solid support, washed to remove all of the unwanted components, then incubated with the detector antibody, is less prone to non-specific interactions.

The present invention provides a method useful for reducing non-specific interactions in immunoassays wherein the capture antibody and detector antibody are derived from the same or different animal species. It is useful regardless whether the false positive sample is RF-positive or RF-negative. The method can be used either with a whole antibody or (Fab')2 fragment.

SUMMARY OF INVENTION

The present invention provides a method for reducing false positive responses in an immunoassay for detection of an antigen comprising:

(a) forming a heat or chemically polymerized immunoglobulin that has no specific reactivity with said antigen,

(b) treating a sample suspected of containing said antigen with said polymerized immunoglobulin, and

(c) subjecting the treated sample to an immunoassay employing antibodies of the same animal species as the polymeric immunoglobulin.

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EXAMPLE I: SYNTHESIS OF POLYMERIC IMMUNOGLOBULIN (POLY IG)

The polymeric Ig can be generated by heating as described in the references mentioned in Background Art. Alternatively, it can be synthesized under defined chemical conditions as follow. In all cases the procedures are optimized with the goal of maintaining the solubility of the Poly Ig in buffered aqueous solution.

I.1 Reaction with S-Acetylmercaptosuccinic Anhydride (SAMSA) and N,N'-1,4-Phenylenedimaleimide (PDM):

Sulfhydryl groups are introduced into a monoclonal IgG by treatment with SAMSA at 50 molar excess in phosphate buffer, pH 7.0. The sulfur protecting group is cleaved with hydroxylamine and the protein fraction is obtained by chromatography on Sephadex G-25, eluting with phosphate buffer pH 6.5.

The thiolated IgG is treated with a 20 molar excess of PDM and the reaction mixture is allowed to rock at room temperature. The progress of the polymerization reaction is monitored by HPLC analysis on a Zorbax(R) Bio Series GF-250 column (9.4 X 250 mm). When the yield of IgG polymer of optimal size reaches a maximum the reaction is quenched with 2-mercaptoethylamine or directly fractionated by preparative HPLC on a Zorbax(R) Bio Series GF-250XL column (21.1 X 250 mm).

I.2 Reaction with Disuccinimidyl Suberate (DSS):

A purified monoclonal IgG (IgG1 subclass) at approximately 5 mg/ml concentration, is reacted with DSS at 12 to 25 fold molar excess to the antibody. The mixture is stirred magnetically for 2 hours.

A sodium azide solution is added to the reaction mixture in order to achieve a final sodium azide concentration of 0.1%. After stirring for 0.5 hour, the mixture is transferred to a dialysis bag and dialyzed

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against a buffer solution containing 0.1 M Tris, 0.15 M Sodium chloride and 0.1% azide for 24 hours.

I.3 Reaction with Glutaraldehyde:

Monoclonal IgG solution at a concentration of 4 to 10 mg/ml is reacted with a 0.005% to 0.03% solution of glutaraldehyde at 20 to 24°C for 16 to 24 hours. Preferred conditions are IgG at 5 mg/ml, reacting with 0.02% glutaraldehyde at 22°C for 20 hours.

The solution is dialyzed against phosphate buffered saline overnight.

I.4 Molecular Weight Distribution:

The polymerized IgG preparations are analyzed by molecular sieving chromatography. The solution of polymer is fractionated in a glass column (approximately 2.5 cm in diameter and 85 cm in length) packed with a gel such as Sephacryl S300 (Pharmacia Co.) and equilibrated with 0.1 M phosphate buffered saline solution, pH 7.4.

In addition to monomeric IgG, four major polymeric ractions are obtained. By comparing to standard molecular weight markers, the median molecular weights of the four fractions are estimated to be 1,500,000 to 2,000,000; 1,000,000; 700,000 and 400,000 Daltons. These values translate to polymers of 10-15, 6-8, 4-6 and 3-4 IgG molecules respectively. All fractions are soluble in aqueous buffer, such as phosphate buffered saline.

Poly Ig prepared by different procedures described above consists of somewhat different proportions of the four fractions. However, the effectiveness in reducing false positivity can not be differentiated. The preferred poly Ig preparation therefore consists of 3 to 15 IgG molecules.

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EXAMPLE II: USE OF POLYMERIZED IMMUNOGLOBULIN IN AN ENZYME IMMUNOASSAY FOR HUMAN ANTIGEN (HBSAG ASSAY)

An enzyme immunoassay for human hepatitis B surface antigen is used to demonstrate the efficacy of Poly Ig in reducing the incidence of false-positive responses. The assay steps are described as follows:

II.1 TWO-STEP FORWARD FORMAT:

1. Antigen Capture:

In a plastic test tube, serum sample (200 ul) is first incubated with chromium dioxide particles (100-200 ug), that are coated with anti-HBsAg monoclonal antibodies (IgG1 subclass), and a sample diluent (100 ul) containing 0.1 M sodium citrate, and 0.15 M sodium chloride. The incubation is allowed to proceed for 30 minutes in a 37°C heating block.

2. Wash:

A wash solution containing 0.1 M Tris and 0.15 M sodium chloride, pH 7.8 was added to the reaction mixture. After mixing the slurry by vortexing, the test tube is placed in a rack equipped with small cylindrical magnets situated at both sides of the tube. The chromium dioxide particles are attracted to the side walls of the tube. The wash solution is then removed by suction. The wash, particle separation and aspiration steps are repeated two more times.

3. Enzyme Labelling:

A solution (50-250 ul) containing covalently conjugate of anti-HBsAg monoclonal antibody (or its (Fab')2 fragment, IgG1 subclass) and calf intestinal alkaline phosphatase, 0.1 M Tris and 5% bovine serum albumin, pH 7.8 is added to the washed particles. The mixture is allowed to incubate at 37°C for 30 minutes.

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4. Wash:

The same wash steps as described in Step (2) are repeated.

5. Substrate Reaction and Measurement:

A substrate solution (240 ul) containing 4-methylumbelliferyl phosphate in diethyleneamine, pH 8.9, is added to the washed chromium dioxide particles. The substrate reacts with alkaline phosphatase to generate fluorescent 4-methylumbelliferone. The reaction is allowed to proceed for precisely 5 minutes at which time 0.5 M EDTA solution is added to stop the reaction.

The particles are separated from the solution in the magnetic rack and the clear supernatant is transferred to a IMMUNLON-2(R) microtiter plate. The fluorescent signals are measured on a Dynatech MicroFLUOR(R) reader equipped with a 365 nm excitation filter and a 450 nm emission filter.

Poly IgG, when used, is added either in the sample diluent (Step 1) or in the conjugate reagent (Step 3) at a concentration specified by each experiment.

II.2 SIMULTANEOUS FORMAT:

Alternately, the assay can be performed by the simultaneous incubation of sample, sample diluent, antibody-coated chromium dioxide particles and conjugate reagent. This format necessarily omits the first wash step (Step 2 of II.1). After the incubation, the assay proceeds to Steps 4 and 5 as described above.

Again, Poly Ig, when used, is added either in the sample diluent or in the conjugate reagent at a concentration specified by each experiment.

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EXAMPLE III: USE OF SAMSA/PDM POLYMERIZED MONOCLONAL IGG (Poly IgG) IN HBSAG ASSAY

III.1 Efficacy on normal serum samples:

Table 1 exhibits correlative decrease of the fluorescent signals produced by sample No. 630 with the increasing concentration of Poly IgG added. This sample is rheumatoid factor (RF) negative. However, it produces a false-positive response with pepsin-fragmented anti-HBsAg/alkaline phosphatase conjugate [(Fab')2-AP]. When other normal HBsAg-negative serum samples are tested with the inclusion of Poly IgG no significant effect is observed.

The response of a HBsAg-containing positive control is not affected by poly IgG (see control values in Tables 1). This is also apparent by the results shown in Table 3.

Table 1 also indicates that a minimum of 7 ug/test Poly IgG is needed to reduce the assay response to negative, but additional amounts do not induce much further change. The preferred quantity of Poly IgG used in this type of heterogeneous enzyme immunoassay is 7 to 15 ug/test (200 ul serum or plasma sample per test).

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Table 1: Effect of the Quantity of Poly IgG on HBSAG Assay Results##

Poly IgG added**	0 ug/test	7 ug/test	14 ug/test
Pos. Control(RFU)#	1882	1907	1885
Neg. Control(RFU)	45	42	49
Cut Off*	165	162	169
No. 630 (+/-)	264(+)	91(-)	61(-)

Footnotes:

A two-step forward format and (Fab')2-AP conjugate are used in this experiment.

** Poly IgG is synthesized by SAMSA/DPM procedure.

RFU: Relative Fluorescent Unit

* Cut off = Neg. Control + 120, Values greater than Cut off are assigned as positive.

Table 2 summarizes a study comparing no addition, addition of monomeric IgG and addition of poly IgG in the HBSAG assay. While a sample (#34) with normal response is not affected, false positive samples are reduced to negative much more effectively by Poly IgG than monomeric IgG.

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Table 2: Comparison of Monomeric IgG to Poly IgG in Reducing False Positive Responses in HBSAG Assay*

Samples	No Additive	Monomeric IgG Added**	Poly IgG Added**
Pos. Control(RFU)	N/T	N/T	1504
Neg. Control(RFU)	N/T	N/T	49
Cut Off#	-	-	169
No. 34	98(-)	91(-)	89(-)
No. 55	423(+)	189(+)	63(-)
No. 57	1260(++)	214(+)	114(-)

Footnotes:

* A two-step forward format and intact IgG-alkaline phosphatase conjugate are used in this study.

** Twenty (20) ug/test of either monomeric or polymeric IgG is used. Poly IgG is synthesized by the SAMSA/DPM Procedures.

Cut off = Neg. Control+120, Values greater than Cut off are assigned as positive.

III.2 Efficacy of Poly IgG on rheumatoid factor positive samples:

Rheumatoid factors are autoimmune antibodies which are known to interfer with enzyme-linked immunoassays and cause sporadic false-positive responses. Such false-positive responses are not directly correlated to the RF titers. The use of pepsin-fragmented IgG [(Fab')2] to prepare the antibody-enzyme conjugate has been reported to help reduce the incidence of false-positive responses associated with RF-positivity.

Data presented in Table 3 indicate that certain RF-positive samples still produce false-positive

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results, even with the use of (Fab')2-enzyme conjugate. These interferences are eliminated by the use of Poly IgG.

Table 3: Effect of Poly IgG on Rheumatoid Factor Positive Samples when (Fab')2-AP is Used as Enzyme Conjugate

CONTROL VALUES:					
Pos Control*		Neg Control		Cut-Off(Neg+120)	
1189		92		212	
		No PolyIgG		PolyIgG#	
Samples	RF Titer	RFU	+/-	RFU	+/-
129	N/T	76	-	101	-
137	10,240	143	-	90	-
142	10,240	111	-	73	-
147	10,240	94	-	99	-
173	10,240	101	-	78	-
176	10,240	71	-	113	-
177	10,240	767	+	76	-
187	10,240	108	-	139	-
385	10,240	333	+	115	-
190	10,240	97	-	90	-
279	10,240	87	-	78	-
327	>10,240	76	-	80	-
False positive		2/12		0/12	

Footnotes:

* A two-step forward format and a (Fab')2-AP conjugate are used.

Poly IgG prepared by SAMSA/DPM Procedure, 15 ug/test used.

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EXAMPLE IV: EFFECT OF DSS-POLYMERIZED IGG IN HBSAG ASSAY

Table 4 summarizes a study involving 80 negative samples that are identified as likely to cause false positive responses and 7 HBsAg-positive samples.

When the assay is conducted without Poly IgG, 24 samples exhibit falsely positive responses. With the addition of DSS-polymerized Poly IgG, all samples are statistically indistinguishable from the negative control. The positive samples are not affected by the Poly IgG.

Table 4: Effect of DSS-polymerized IgG on HBSAG Assay*

	No Poly IgG	Poly IgG
Total Negative Samples Tested**	80	80
Total False Positive	24	0
Percent False Positive	30%	0%
False Positive Mean (St. Dev.)	1266(1305)	71(28)
Negative Control (St. Dev.)	65(6)	65(6)
Delta (False Positive mean-Neg Control)	1,201	6
Total Positives Tested	7	7
Total True Positive(T/P)	7	7
T/P Mean(St. Dev.)	3162(976)	3115(1009)

Footnotes:

* A two-step forward format and an intact IgG-Alkaline Phosphatase conjugate are used in this study. Poly IgG is used at 15 ug/test.

** These are samples identified as likely to cause false positive responses.

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EXAMPLE V: EFFECT OF GLUTARALDEHYDE-POLYMERIZED IGG ON HBSAG ASSAY

Table 5 exhibits the results of using two Poly IgG preparations in the HBSAG assay with five identified false positive samples. These two lots were synthesized from IgG generated from two mouse monoclonal cell lines. Both preparations reduce the false positive responses to negative range.

This study indicates that glutaraldehyde-polymerized IgG performs as well as the SAMSA/DPM- and DSS- polymerized material in reducing the false positive responses. It also shows that Poly IgG prepared from two lots of IgG perform equally well.

Table 5: Effect of Poly IgG Synthesized by Glutaraldehyde Procedures on HBSAG Assay**

Control Values:			
Positive Control			1086
Negative Control			58
Cut-Off(=Neg Ctl+120)			178

SAMPLE ID*	No PolyG	Poly IgG #0619	Poly IgG #0620
9388	174	73	93
8523VS	1066	67	44
L155951	1180	82	76
E47458	4000	70	47
182999	74	46	63

* These are samples identified as likely to cause false positive responses in the HBSAG assay.

These two lots of Poly IgG are synthesized from two mouse monoclonal cell lines using the Glutaraldehyde Procedure. Poly IgG is used at 15 ug/test.

** A simultaneous format and intact IgG-Alkaline Phosphatase conjugate are used in this study.

EXAMPLE VI: EFFECT OF POLY IGG IN A HETEROGENEOUS IMMUNOASSAY FOR HUMAN CARCINOEMBRYONIC ANTIGEN (CEA)

The heterogeneous immunoassay for CEA is based on the same principle as outlined in Example II. In the assay, 50 ul of serum sample, 50 ul of anti-CEA-alkaline phosphatase conjugate solution, and 25 ul of an anti-CEA antibody coated chromium dioxide particle suspension are incubated at 37°C for 30 minutes. The particles are then washed and treated with the fluorescent substrate solution in the same manner as described in Example II.

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Fluorescent signals are then measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Many serum samples possess interfering material which resulted in falsely elevated CEA results. In the case of a quantitative determination such as measurement of CEA, this false positive result is determined by comparison of the result in a commercially available assay with that obtained in the new assay. Since normal humans will have from 5 to 8 ng/ml of CEA in their sera, values which are greater than this are indicative of disease. Discrepancies between two assays where the value is above the normal range will be considered to be the result of an interferring substance. The interference is usually seen as a value higher in analyte level that the reference, commercially available assay. Inclusion of Poly IgG in the conjugate solution at a concentration of 19 ug/test effectively eliminated such false-positive responses. Table 6 exhibits a summary of CEA tests which demonstrate such effect.

Table 6: Effect of Poly IgG on False-Positive Samples in CEA Assay

Sample ID	[CEA] No Poly IgG	[CEA] With Poly IgG	Tandem(r) [CEA]*
102	8.8 ng/mL	0.9 ng/mL	4.9 ng/mL
109	8.0	0.3	1.0
7721	10.1	2.7	4.2
E47458	48.9	10.7	8.6
L215797	65.8	2.5	3.0

* Manufactured and sold by Hybritech Inc. San Diego, CA.

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EXAMPLE VII: EFFECT OF POLY IGG IN A HETEROGENEOUS IMMUNOASSAY FOR HUMAN THYROID STIMULATING HORMONE (TSH)

The immunoassay for human TSH is based on the same principle described in Example II. It is performed by a simultaneous incubation of 50 ul of serum sample, 50 ul of anti-TSH-alkaline phosphatase conjugate solution and 25 ul of anti-TSH coated chromium dioxide particles. The washing, substrate reaction and fluorescent measurement are performed in the same manner as described in Example II.

Table 7 shows the effect of including Poly IgG in the TSH test, when false positive samples are involved. While true TSH concentrations are correctly measured in samples 8523, L(10) and L(50), those exhibiting falsely high results are corrected by the use of Poly IgG.

Table 7: Effect of Poly IgG on False-Positive Samples in TSH assay

Sample ID	[TSH] No PolyIgG	[TSH] With PolyIgG#
L215797	2.92 mIU/L	0.85 mIU/L
8523	1.70	1.69
393ms	6.13	2.66
L155951	2.77	1.64
L(10)*	9.99	10.02
L(50)*	49.50	50.02

* Calibrators which have been spiked with purified TSH to concentrations of 10 mIU/L and 50 mIU/L, respectively.

Poly IgG used at 16 ug/test.

CLAIMS:

1. A method for reducing false positive responses in an immunoassay for detection of an antigen in a sample, said method comprising not in any order, the steps of:

forming a polymerized immunoglobulin from an immunoglobulin that has no specific reactivity with said antigen;

contacting said sample suspected of containing said antigen with said polymerized immunoglobulin; and

subjecting the sample to an immunoassay employing antibodies derived from the same animal species as that of the polymeric immunoglobulin.

2. The method of Claim 1 wherein the immunoglobuln is IgG.

3. The method of Claim 2 wherein the polymerized IgG comprises about 3 to 15 IgG molecules.

4. The method of Claim 3 wherein the polymerized IgG is present in an amount sufficient to provide about 7 to 20 µg per immunoassay.

5. The method of Claim 1 wherein the immunoglobulin in IgM.

6. The method of Claim 2 wherein said sample is pretreated with the polymerized IgG.

7. The method of Claim 1 wherein the polymerized immunoglobulin is a component of the reagents used in the assay.

8. The method of Claim 1 wherein the antigen to be detected is selected from the group consisting of hCG, LH, TSH, FSH, alpha-feto protein, prostatic specific antigen, carcinoembrionic antigen (CEA), cancer antigens ca 19.9, ca 125 and ca 15-3, human hepatitis B surface antigen, hepatitis B core antigen and hepatitis C, D and E.

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9. The method of Claim 1 wherein the antigen to be detected is a virus or viral marker.

10. The method of Claim 1 wherein the antigen to be detected is a macromolecular hormone.

11. The method of Claim 1 wherein the antigen to be detected is a molecular component of human malignant tumor cells.

12. The method of Claim 8 wherein the antigen to be detected is human hepatitis B surface antigen.

13. The method of Claim 8 wherein the antigen to be detected is human thyroid stimulating hormone (TSH).

14. The method of Claim 8 wherein the antigen to be detected is carcinoembryonic antigen (CEA).

15. A method of performing an enzyme immunoassay employing two antibodies of the same animal species for capture and detection of an antigen in a sample to be assayed, said method comprising the steps of:

a) forming a polymerized immunoglobulin of the same species as those in the assay, said polymerized immunoglobulin having no reactivity to the antigen to be detected;

b) contacting said sample with a solution containing the polymerized immunoglobulin of step a) while performing said assay;

c) performing the assay to determine the presence or absence of antigen in said sample.

16. The method of Claim 15 wherein the immunoglobulin is IgG.

17. The method of Claim 16 wherein said sample is pretreated with the polymerized IgG.

18. The method of Claim 15 wherein the polymerized immunoglobulin is a component of the reagents used in the assay.

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19. The method of Claim 15 wherein the detector antibody to enzyme conjugate is comprised of a pepsin-hydrolysis produced antibody fragment.

20. The method of Claim 15 wherein the detector antibody to enzyme conjugate is comprised of a papain produced antibody fragment.

21. The method of Claim 19 wherein the fragment is (Fab')2, F(ab)2 or Fab.

22. The method of Claim 15 wherein the antigen to be detected is a virus or viral marker.

23. The method of Claim 15 wherein the antigen to be detected is a macromolecular hormone.

24. The method of Claim 1 wherein the antigen to be detected is selected from the group consisting of hCG, LH, TSH, FSH, alpha-feto protein, prostatic specific antigen, carcinoembryonic antigen (CEA), cancer antigens ca 19.9, ca 125 and ca 15-3, human hepatitis B surface antigen, hepatitis B core antigen and hepatitis C, D and E.

25. The method of Claim 24 wherein the antigen to be detected is human hepatitis B surface antigen.

26. The method of Claim 24 wherein the antigen to be detected is human thyroid stimulation hormone (TSH).

27. The method of Claim 24 wherein the antigen to be detected is carcinoembryonic antigen (CEA).

28. The method of Claim 15 wherein the antigen to be detected is a molecular component of human malignant tumor cells.

29. A method for forming polymeric immunoglobulin comprising treating immunoglobulin with a heterobifunctional or a homobifunctional chemical linking reagent whereby the resulting polymeric molecules exist in soluble form; and wherein the polymeric molecules are comprised of 3 to 15 monomeric IgG molecules.

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30. The method of Claim 18 wherein the heterobifunctional reagents is S-acetylmercaptosuccinic anhydride (SAMSA).

31. The method of Claim 18 wherein the homobifunctional agent is disuccinimidyl suberate (DSS) or glutaraldehyde or their derivatives.

32. The method of Claim 7 wherein the amount of polymeric immunoglobulin in the assay is in the range of about 2 µg to 25 µg per test.

33. A diagnostic test kit for conducting an immunoassay for the detection of an antigen and for reducing false positive responses in said immunoassay, said test kit comprising:

a selected quantity of polymerized IgG comprising about 3 to 15 IgG molecules in an amount sufficient to provide a minimum of about 7 µg per test, said polymerized IgG having no specific reactivity with the antigen to be detected; and

a labelled antibody to the antigen to be detected.

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International Application No PCT/US90/05943

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.CL.: 435/4,5,7.1,7.9,7.94; 436/512,536,547,825,826; 530/387,389,402
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II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.CL.	435/4,5,7.1,7.9,7.94; 436/512,536,547,825,826; 530/387,389,402

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

APS, CAS, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,X P,Y	US, A, 4,914,040 (LENZ et al.) 03 April 1990, see column 3, line 1-column 2, line 43, column 5, lines 1-32, column 6, lines 17-30 and 55-62 and claims 1-9, 19-20, 23-27, 34-35, and 37.	1-2,4-5, 7-16,18-29, and 31-33 3,6,17 and 30
Y	US, A, 4,220,565 (KATZ) 02 September 1980, see column 2, lines 57-66, column 7, lines 7-42 and claim 4.	30 and 31

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

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International Searching Authority [1]	**Signature of Authorized Officer** [20]
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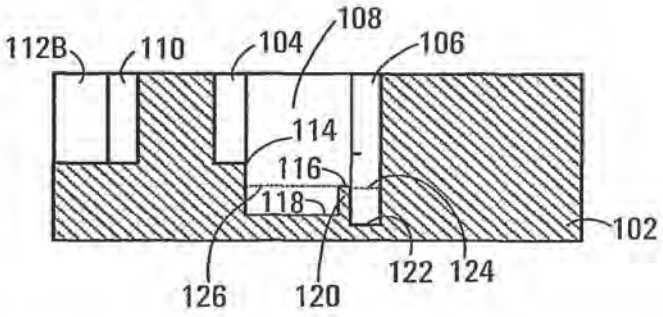
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MICROCHIP AND METHOD FOR DETECTING MOLECULES AND MOLECULAR INTERACTIONS



(57) Abstract: A microchip with flow-through inlet (104) and outlet (106) channels and test channels (108). The test channels (108) are in fluid communication with the inlet (104) and outlet (106) channels, through inlets (114) and outlets (116) respectively. Each test channel (108) has one test site therein for detection of specific molecules or molecular interactions. The inlet (114) in a test channel (108) is elevated from the outlet (116) of the test channel (108) and the outlet (116) is elevated from a fluid level (124) in the outlet channel (106). Back diffusion from outlet channel (106) to the test channels (108) and from the test channels (108) to the inlet channel (104) can thus be inhibited to reduce or eliminate cross-interference between different test sites. The microchip can be useful as a flow-through high density enzyme immunoassay array device with high-throughput.

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MICROCHIP AND METHOD FOR DETECTING MOLECULES AND MOLECULAR INTERACTIONS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit and priority from U.S. provisional patent application no. 60/558,116 filed April 1, 2004 and U.S. provisional patent application no. 60/558,118 filed April 1, 2004, the contents of each of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to detection of molecules and molecular interactions, and more particularly to microchips and methods for detecting molecules and their interactions.

BACKGROUND

[0003] Detection of biomolecules or their interactions has applications in many biological and biochemical industries. For example, it has been widely used for medical diagnostic applications.

[0004] Known detection devices of biomolecules and their interactions include microchips or biochips with arrays of addressable test sites thereon. Such devices are powerful analytical tools because hundreds or thousands of unique test sites can be analyzed simultaneously, with high throughput. In a typical conventional microchip, target molecules can be captured and immobilized at different spots on the microchip for detection. Different test sites may be used to detect different target molecules or the same molecules in different samples. Various techniques may be used to detect the target molecules. For example, the target molecules may be detected optically or electrically. Further, biomolecules or biomolecular interactions may be detected by the enzyme immunoassay (EIA) or ‹ed immunosorbent assay (ELISA) techniques, with extremely high ınd specificity.

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[0005] However, conventional microchips suffer certain drawbacks. For example, in known devices the test sites are often immersed in liquid during detection and cross-interference between different test sites can occur due to diffusion of the molecules to be detected from one test site to another. Such cross-interference can lead to false or inaccurate detection results. The molecules to be detected can be either the target molecules or reaction products which are to be detected to indicate the presence of the target molecules.

[0006] A known approach to avoid cross-interference due to diffusion is to increase the distance between different test sites, to reduce the effect of diffusion. For example, Ying Ding et al. disclosed a device based on the EIA electrochemical technique (EIA-EC) in "Feasibility studies of simultaneous multianalyte amperometric immunoassay based on spatial resolution", Journal of Pharmaceutical and Biomedical Analysis, (1999), vol. 19, p. 153, the contents of which are incorporated herein by reference. In this detection device, each test site has a working electrode and the distance between two adjacent electrodes is 2.5 mm. This spatial separation is found to be effective for avoiding cross-interference caused by diffusion when the detection measurement is completed within a certain time period after introducing the enzyme substrate. However, such an approach also has some drawbacks. One problem is that only a small number of test sites can be formed on a microchip device when the distance between adjacent electrodes is so large.

[0007] An alternative known approach of optical ELISA or EIA is to provide an array of isolated test wells in a chip to avoid cross-interference caused by diffusion. However, these well-based microchips have their own limitations. One problem is that well-density is limited, due to either manufacturing or operation requirements, so that it is difficult to form high-density test sites. Another problem is that a well-based microchip does not allow flow-through operation, thus making automated operation difficult. For example, automated sampling, washing, or dilution on such a chip may require expensive equipments such as robotic devices.

[0008] Thus, there remains a need of improved devices and methods for detection of molecules and molecular interactions.

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SUMMARY OF THE INVENTION

[0009] In summary, a microchip with flow-through inlet and outlet channels and test channels is disclosed. The test channels are in fluid communication with the inlet and outlet channels, through inlets and outlets respectively. Each test channel has one test site therein for detection of specific molecules or molecular interactions. The inlet in a test channel is elevated from the outlet of the test channel and the outlet is elevated from a fluid level in the outlet channel.

[0010] Advantageously, back flow or diffusion from the outlet channel to the test channels and from the test channels to the inlet channel can be inhibited to reduce or eliminate cross-interference between different test sites.

[0011] Accordingly, an aspect of the present invention relates to a microchip. The microchip comprises a substrate, an inlet channel in the substrate, an outlet channel in the substrate for guiding fluid below a fluid level, and a plurality of test channels in the substrate for guiding fluid flow from the inlet channel to the outlet channel. Each one of the test channels has an inlet for fluid communication with the inlet channel and an outlet for fluid communication with the outlet channel. The inlet is elevated from the outlet for inhibiting back flow through the inlet. The outlet is elevated from the fluid level for inhibiting back flow through the outlet. One test site is in each one of the test channels for detection of a specific molecule or molecular interaction at the test site.

[0012] Another aspect of the present invention relates to a method of detecting molecules or molecular interaction. In this method, a microchip is provided. The microchip has a substrate, an inlet channel in the substrate, an outlet channel in the substrate, a plurality of test channels in the substrate each having an inlet for fluid communication with the inlet channel and an outlet for fluid communication with the outlet channel. The inlet is elevated from the outlet. One test site is in each one of the plurality of test channels. A fluid is introduced to the test channels through the inlet channel. Back flow through the inlets and outlets is inhibited by allowing overflow of the fluid from the test channels to the outlet channel through the outlets and maintaining the fluid level in the outlet channel

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below the outlets, thus inhibiting diffusion of molecules in the fluid from one of the test sites to another one of the test sites. After the fluid has been introduced to the test channels, a molecule or molecular interaction is detected at a test site within the plurality of test sites.

[0013] In optical detection, the probe molecules may be directly immobilized on the bottom surface of the detection channel, i.e. detection well; in electronic detection, two (working and counter electrodes) or three electrodes (working, counter and reference electrodes are fabricated in the detection channels, where in all working and counter electrodes can be interconnected respectively for addressable electronic detection.

[0014] Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying Figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] In the figures, which illustrate exemplary embodiments of the invention,

[0016] **FIG. 1A** is a plan view of a microchip, exemplary of an embodiment of the present invention;

[0017] **FIG. 1B** is a cross-sectional view of the microchip of **FIG. 1A** taken along the line **B-B**;

[0018] **FIG. 2A** is a plan view of another microchip exemplary of a further embodiment of the present invention;

[0019] **FIG. 2B** is a partial cross-sectional view of the microchip of **FIG. 2A** taken along the line **B-B**;

[0020] **FIG. 3A** is a partial cross-sectional view of a variation of the microchip of **FIG. 2A** and **2B**;

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[0021] **FIG. 3B** is a partial plan view of the microchip of **FIG. 3A**;

[0022] **FIG. 4** is a partial plan view of a variation of the microchip of **FIG. 3A**;

[0023] **FIGS. 5A** and **5B** are schematic diagrams illustrating exemplary enzyme immunoassay techniques;

[0024] **FIG. 6A** is a partial image of a mold for forming a microchip; and

[0025] **FIG. 6B** is a partial image of a microchip formed from the mold of **FIG. 6A**.

DETAILED DESCRIPTION

[0026] **FIGS. 1A** and **1B** illustrate a microchip or microfluidic biochip **100**, exemplary of an embodiment of the present invention.

[0027] Biochip **100** includes a substrate **102**. An inlet channel **104**, an outlet channel **106**, and a plurality of test channels **108** are formed in substrate **102**. The test channels **108** may be substantially parallel to each other. Inlet channel **104** is in fluid communication with, and can receive fluid from, a dilution channel **110**. Dilution channel **110** has multiple inlets **112A** to **112D** (also collectively and individually referred to as **112**), each for introducing or feeding a fluid into dilution channel **110**. Each test channel **108** has an inlet **114** and an outlet **116**. Each test channel **108** is in fluid communication with inlet channel **104** through inlet **114** and with outlet channel **106** through outlet **116**. While four test channels **108** are depicted in **FIG. 1A**, the number of test channels **108** may vary depending on the particular application and manufacturing considerations. Each test channel **108** has one test site therein for detection of a specific molecule or molecular interaction at the test site, as will be further described below.

[0028] As depicted in **FIG. 1B**, a test channel **108** has a bottom **118** and an overflow wall **120** which forms the lower end of outlet **116**. However, as will become apparent, wall **120** is optional and can be omitted. With wall **120**, a test well is formed in each channel **108**. The well structure may be advantageous in some

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applications, as will become clear below.

[0029] As can be better seen in **FIG. 1B**, outlet channel **106** has a bottom **122** below outlet **116** for guiding fluid below a fluid level, such as indicated by the dashed line **124**. For each test channel **108**, inlet **114** is elevated from outlet **116**, which is in turn elevated from the fluid level **124** in outlet channel **106**. As can be understood, inlet channel **104** can thus guide fluid at a fluid level above the fluid level in each test channel **108**. A test channel **108** can hold fluid at a fluid level up to outlet **116**, as indicated by the dashed line **126** in **FIG. 1B**, which is at a level above the fluid level **124** in outlet channel **106**. As can be understood, back flow or diffusion through inlet **114** or outlet **116** can therefore be inhibited, thus limiting or eliminating cross-interference due to diffusion of molecules in the fluid from one test site to another test site.

[0030] A sample fluid may be diluted in dilution channel **110**. Inlets **112** can be conveniently used to dilute the sample fluid multiple times. The number of inlets **112** may vary, for example, depending on the sample concentration and the desired amount of dilution. One of the inlets **112,** such as inlet **112A** may be adapted for connection to a sample source (not shown) and the other inlets such as **112B** to **112D** may be adapted for connection to a dilution solution source (not shown). Inlets **112** may also be connected to one or both of a substrate solution source and a washing solution source (not shown). Outlet channel **106** may be connected to a waste dispenser (not shown).

[0031] Substrate **102** can be made of a suitable material such as plastic, ceramic, graphite, glass, rubber, fabric, printed circuit board, silicon, suitable polymer, or the like. A combination of two or more of these materials may also be used. Substrate **102** can be prepared in a suitable manner known to persons skilled in the art.

[0032] Channels **104**, **106**, **108**, and **110** can each has a width or depth on the order of 0.02 μm to 2000 μm, depending on the application. They can have different widths and depths. For example, inlet channel **104** can have a width of about 200 μm and a depth of about 50 μm; outlet channel **106** can have a width of

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about 300 μm and a depth of about 200 μm; and each test channel **108** can have a width of about 100 μm and a well depth of about 150 μm, wherein all depths are relative to the top surface of substrate **102**. As can be appreciated, in this case, the liquid flow rate is faster in outlet channel **106** than in inlet channel **104**, which can be advantageous because it reduces the chance for fluid to overflow from test channels **108** back into inlet channel **104**. Channels **110** is shaped and sized for supplying a fluid to inlet channel **104** at a desired rate and for effectively diluting the fluid by a desired ratio. The size of channel **110** can be readily determined by a person skilled in the art for a given application.

[0033] Channels **104**, **106**, **108** and **110** may be formed using conventional microchip fabrication techniques including etching, embossing, molding, or the like. For example, a silicon mold can be fabricated using a soft-lithography method to make a master device and then a rubbery polymer (such as polydimethylsiloxane (PDMS)) substrate can be formed from the mold.

[0034] Channel surfaces may be coated with a polymeric, metallic, or ceramic material, or other materials if desired. In particular, the test site of each test channel **108** may have a surface, such as bottom surface **118**, suitable for immobilizing molecules thereon. For example, for immobilizing antigens directly onto a bottom surface **118**, the surface can be made of a plastic such as polyvinyl, polystyrene, or cellulose. Probe molecules (not shown) may be immobilized at the test site in each test channel **108**. Probe molecules may be immobilized using a conventional immobilization technique, including physical adsorption, cross-linking, entrapment, covalent bonding, grafting, or a combination of the foregoing techniques. Probe molecules can be immobilized with a robotic nano- or pico-dispenser, or by introducing a fluid containing the probe molecules to test channels **108**. The test channel surface may be coated with a surface that is suitable for selectively immobilizing probe molecules. For example, the surface may be coated with aminopropyltriethoxysilane (APTES). The coated surface may then be treated with a suitable cross-linker for capturing the probe molecules. For example, homo-bifunctional cross-linkers such as glutaraldehyde, or heterobifunctional cross-linkers such as 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) may be applied to the APTES surface for selectively binding probe proteins. Immobilization of probe

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molecules can be optionally performed during use before detection. After probe immobilization, a thorough wash of the channels including the test channels can be conducted. The channel surfaces outside the test sites can be coated with blocking agents such as gelatin to eliminate non-specific binding of molecules to these surfaces. The washing and blocking can be conducted by automatic operation by connecting biochip **100** to a pumping system.

[0035] A cover (not shown), such as a transparent cover plate, may be provided on the top of substrate **102** to seal the channels and the test channels for flow-through operation. The cover can be made of a glass, plastic, or acrylic sheet. A film with one adhesive side may be used as the cover. The cover can prevent contamination during use. A transparent cover may allow detection of optical signals originated from the test channels **108**.

[0036] Other components may be incorporated in or connected to biochip **100**. For example, fluid sources (not shown) such as sample sources, substrate sources, washing fluid sources may be provided or linked to dilution channel **110**, such as through one or more of inlets **112**. In an alternative embodiment, some fluid sources may be connected directly to inlet channel **104**, bypassing dilution channel **110**. Fluid pumps (not shown) and valves (not shown) for controlling fluid flow in the channels and for supplying fluid to, or withdrawing fluid from, the channels may be provided. Electrical interconnects (not shown) and circuitry (not shown) may be provided for controlling the operation of biochip **100** and/or for measuring or detecting a signal such as an optical or electrical signal from the test sites in test channels **108**.

[0037] In operation, a fluid, such as a sample fluid containing target molecules or a washing buffer solution, is introduced into test channels **108** through inlet channel **106** and inlets **116**.

[0038] The sample fluid may be fed into dilution channel **110** through one of inlets **112**, such as inlet **112A**. Dilution fluids may be fed into dilution channel **110** through other inlets **112**, such as inlets **112B** to **112D**, to dilute the sample fluid. The sample fluid can be diluted multiple times by introducing a dilution fluid at each one of a number of inlets **112**. The dilution ratio at each inlet **112** can be

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individually controlled by adjusting the flow rates at inlets **112**. The diluted sample fluid is received in inlet channel **104** and then in test channels **108**. However, when dilution is not required or desired, a sample solution may be introduced into test channels **108** through inlet channel **104** without dilution.

[0039] Overflow of the fluid from the test channels **108** is received in outlet channel **106**. The fluid levels in the inlet, outlet and test channels are maintained such that the fluid in test channels **108** is below inlet **114** and the fluid level in outlet channel **106** is below outlets **116**. Therefore, back flow through inlet **114** and outlet **116** are inhibited. As can be appreciated, such fluid levels can be maintained conveniently when the fluid flows at a greater rate in outlet channel **106** than in inlet channel **104**.

[0040] Different fluids may be introduced into each test channel **108** depending on the detection technique used. The fluids introduced may include probe solutions, sample solutions, washing solutions, substrate solutions, solutions containing labelling or signal generating molecules, and the like, as will be understood by persons skilled in the art. A continuous fluid flow may be maintained. Alternatively, fluid flow may be stopped for a certain period of time to allow certain interactions to occur, such as for incubation.

[0041] A molecule or molecular interaction is then detected at one, some or each of the test sites. The molecule detected can be a target molecule, or a signal generating molecule for indirect detection of the target molecule. The molecule or molecular interaction can be detected using various techniques, including conventional detection techniques for detecting molecules and molecular interactions. For example, the molecule or molecular interaction may be detected by detecting an optical, electrical, magnetic, radiation or electromagnetic signal indicative of the presence of the target molecule or occurrence of the molecular interaction. Example detection techniques include enzyme immunoassay (EIA) techniques. An EIA technique can be performed with an electrochemical detection technique (electrochemical EIA or EIA-EC) or with an optical detection technique. Possible EIA techniques include enzyme-linked immunosorbent assay (ELISA), direct EIA, competitive EIA, competitive inhibition EIA, sandwich EIA, and the like.

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These EIA techniques can be carried out in manners known to a person skilled in the art. In such techniques, the signal generating molecules can be product molecules produced from enzyme-catalysed reactions, which may be mobile in a fluid. The enzyme-catalyzed interaction or its product can be detected. For example, the interaction may generate heat, light, radiation, or sound. The reaction product may be electrochemically active or fluorescent. Detection of the enzyme-catalyzed interaction or its product can thus indicate the presence of the target molecules. For example, when the products are fluorescent, detection of fluorescent light from a test channel can indicate the presence of the target molecules in the sample solution. It can then be determined that the target molecules are present in the sample solution. The intensity of the fluorescent light can also indicate the concentration of the target molecules in the sample solution. Since back flow or diffusion is inhibited, cross-interference due to diffusion of these product molecules can be avoided or reduced.

[0042] The target molecule may be captured by probe molecules immobilized at the test site. Probe molecules may be pre-immobilized or immobilized during use. Different probe molecules may be immobilized at different test sites for detecting different target molecules.

[0043] For instance, probe proteins can be pre-immobilized on the bottom **118** of a test channel **108**, thus defining the test site.

[0044] If the target protein allows for enzyme labelling, a direct EIA or ELISA detection may be conducted. In a direct EIA technique, the probe proteins can selectively capture target molecules, which are labelled with enzymes, in a sample fluid introduced into the test channels. A substrate fluid can be then introduced into the test channel. The substrates in the substrate fluid can produce detectable products in reactions catalysed by the enzymes attached to the target molecules at a test site. The higher the concentration of target molecules in the sample fluid, the more target molecules will be captured and thus producing a stronger signal.

[0045] In a competitive ELISA technique, the probe molecules can selectively capture a specific protein in a complex, which can contain either a target

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protein or an enzyme-conjugated competing protein. A sample fluid containing unknown concentrations of target molecules can be first introduced into a test channel **108**. The target molecules are captured at some of the binding sites. A fluid containing known concentrations of competing proteins is then introduced into test channel **108**. The competing proteins are captured at the remaining binding sites. By detecting how many competing proteins are captured, the presence or concentration of the target proteins can be indirectly detected.

[0046] In a competitive inhibition EIA technique, the probe molecules immobilized on the bottom surface of a test channel **108** can be the same type of molecules as the target molecules, such as the same antigens. During detection, both a sample fluid and a fluid containing enzyme-labelled molecules such as antibodies of a known concentration can be introduced into the test channel. An enzyme-labelled molecule can bind to either a target molecule or a probe molecule. There is thus competition between target molecules and probe molecules for binding with the enzyme-labelled molecules. Enzyme-labelled molecules attached to the target molecules cannot interact with the probe molecules. The presence of target molecules at the test site can inhibit a signal that would have resulted from interactions between enzyme-labelled molecules and probe molecules. Thus, the intensity of a detected signal can be inversely proportional to the concentration of the target molecules in the test channel. This technique can be particularly useful for detection of small proteins without a second epitope for enzyme labels.

[0047] In a sandwich-ELISA technique, the target protein may be captured by the probe protein. The test channels may be washed after introducing the sample fluid. Next, a fluid containing some other proteins labelled with enzymes may be introduced into the test channel to allow the other proteins to bind to the target proteins. After incubation, the test channels may be washed before conducting the ELISA detection. As can be appreciated, a capture target protein is sandwiched between two proteins, the probe protein and the enzyme-labelled protein.

[0048] The captured molecules or proteins including target molecules, such as antibodies or antigens, can be detected, for example, after introducing a fluid

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containing substrate molecules that can be converted by enzymatic reactions to coloured products for optical detection or electrochemically active products for the electrochemical detection.

[0049] As mentioned above, after a fluid is introduced into test channels **108**, or after certain reactions are allowed to occur, the channels may be washed with a washing fluid, which may be fed into inlet channel **104** through dilution channel **110**.

[0050] As can be appreciated by persons skilled in the art, samples containing same or different types of target molecules may be transported into different test channels **108** for simultaneous detection of multi-analytes when the different test channels **108** have different probe molecules immobilized therein.

[0051] In other techniques, the target molecules may be detected without an enzyme attached thereto. For example, the target molecules may be labeled with radioactive tracers and detected by sensing a radiation from each test channel **108**. In yet other techniques, the target molecules may be detected without a label. For example, certain interaction of a specific molecule with another molecule may produce detectable heat or light.

[0052] In any event, advantageously, back diffusion can be inhibited or limited, and multiple sample tests can be simultaneously performed on biochip **100**.

[0053] Further, biochip **100** can be conveniently used for analysis of both low and high concentration samples. For high concentration sample, the sample fluid can be conveniently diluted on biochip **100**. Saturation in the test channels can be avoided even when the sample initially has very high concentrations of target molecules. Dilution can be carried out in multiple steps thus reducing the concentration at each step. As can be understood, when the dilution ratio is very high in a single dilution step, large errors can result. Thus, diluting at a low dilution ratio at each step can reduce the errors than may result from dilution. Dilution on biochip **100** can be carried out automatically.

[0054] **FIGS. 2A** and **2B** illustrate a microfluidic biochip **200**, exemplary of

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another embodiment of the present invention, which has an X-Y addressable array of test sites. Microchip or biochip **200** has a substrate **202** and inlet, outlet and test channels **204**, **206**, and **208** formed therein. Test channels **208** are arranged in rows and columns. Multiple columns of test channels **208** are formed in substrate **202**. The number of test channels **208** may vary and can be selected depending on the particular application and manufacturing considerations. Each column of test channels **208** are separated into multiple rows by sidewalls **210** and share a common inlet channel **204** and a common outlet channel **206**. Rows of test channels **208** in different columns are aligned for convenient addressing. One test site is formed in each test channel **208**, which comprises a pair of electrodes **212**, one of which is a working electrode and the other a counter or counter/reference electrode. As can be appreciated, counter electrodes may be replaced by reference electrodes. Alternatively, a reference electrode may be added in each test channel **208**. As depicted, electrodes **212** are formed on the opposite sidewalls **210** within each test channel **208**.

[0055] As can be better seen in **FIG. 2B**, each channel **204**, **206** and **208** has a substantially flat bottom **214**, **216** or **218**. Bottom **214** is elevated from bottom **218**, which is in turn elevated from bottom **216**. Therefore, as with microchip **100**, back diffusion from one test site to anther test site can be inhibited in microchip **200**. The respective amount of elevation can be readily determined by persons skilled in the art depending on the application. For example, each elevation may vary between 25 and 100 μm. However, in an alternative embodiment, the channels may also be structured similar to that of microchip **100**. In particular, a well may be formed in each test channels.

[0056] The detection of molecules or molecular interactions at each test site can be conducted using electrodes **212**. As indicated in **FIG. 2A** by dot-dashed lines, the working electrodes **212** in each row and column can be respectively interconnected. The counter electrodes **212** in each row and column can be respectively interconnected. Each row of electrodes can have an X address, such as X1 to X4, and each column of electrodes can have a Y address, such as Y1 to Y4. Thus, the electrodes in each test channel can be addressed with an X-Y addressing technique. Conventional X-Y addressing techniques such as

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multiplexing techniques can be used. Such a technique can be readily implemented by a person skilled in the art. In microchip **200**, electrical interconnections or I/O lines can be limited with simple multiplexing, as can be understood by a person of skill in the art.

[0057] The substrate and channels of microchip **200** may be formed as described above for microchip **100**.

[0058] Electrodes **212** may be formed using any suitable technique including conventional techniques for forming electrodes. Electrodes **212** may have any suitable shape or size. Different electrodes may have different shapes and sizes or made of different materials. Working electrodes in different test channels may be spaced apart at distances on the order of 0.01 μm to 1000 μm, depending on the application and manufacturing limitations. Electrodes **212** may be made of a solid or porous material such as gold, silver, platinum, copper, titanium, chromium, aluminum, metal oxide, metal carbide, carbon, graphite, fullerene, conductive plastic, conductive polymer, metal impregnated polymers, or the like. A combination of two or more of these materials can be used.

[0059] Probe molecules (not shown) may be immobilized on working electrodes **212** or on bottoms **218** of test channels **208** proximate electrodes **212**.

[0060] A cover (not shown), such as a transparent cover plate, may be optionally provided on the top of the substrate to cover the channels for sealing the flow-through channels.

[0061] Other components may be incorporated in or connected to biochip **200**. For example, fluid sources (not shown) such as sample sources, substrate sources, washing solution sources may be provided or linked to input channel **204**. Fluid pumps (not shown) and valves (not shown) for controlling fluid flow in the channels and for supplying fluids to the channels may be provided. Electrical interconnects (not shown) and circuitry (not shown) may be provided for biasing electrodes **212** and for measuring electrical signals such as voltages and currents at electrodes **212**.

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[0062] Biochip **200** may be operated in a similar manner as for biochip **100**. Conveniently, an electrical signal can be sensed using electrodes **212** for detecting molecules or molecular interactions at each test site.

[0063] For example, the reaction products of the molecular interactions may comprise electrochemically active molecules. Each pair of electrodes **212** in a test channel **208** is biased to monitor the electrochemical reactivity at the test site in the test channel. As can be appreciated, when electrochemically active products are present in the liquid in the test channel, an electrical current can flow between the pair of electrodes **212**.

[0064] As can be understood, in an EIA technique, a highly concentrated substrate solution can be used to produce a high concentration of electrochemically active products even when the target molecules have a very low concentration in the sample solution. Thus, such a technique can be highly sensitive. Alternatively, the product concentration can be significantly increased for high sensitivity detection by increasing the incubation time for the enzymatic reaction.

[0065] The amount of electrochemically active products produced at a test site can be detected by monitoring an electrical signal at electrodes **212**, such as a current through an electrode **212**. The concentration of the target molecules can be determined, for example, by comparing the detected current with a calibration curve of current vs. concentration, which can be obtained by measuring samples with known concentration of target molecules. In an amperimetric approach, the current due to generation of the electrochemically active products produced from the enzymatic reaction can be proportional to the concentration of the product, and can be approximately proportional to the target concentration in the sample solution.

[0066] As can be understood, as the test sites are separated by the sidewalls **210** and back flow from outlet channels **206** into test channels **208** and from test channels **208** into inlet channels **204** can be inhibited, diffusion of molecules from one test site to another can be limited or inhibited. In addition, when a liquid flow is maintained in the channels, upstream diffusion of molecules within test channels **208** is further limited. If the liquid can flow faster in outlet channel **206** than in inlet channel **208**, backflow from outlet channel **206** into a test channel **208** can be

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further limited. As such, the chances of products produced at one test site moving to another test site can be reduced.

[0067] To guide fluid towards electrodes **212**, a section of each test channel **208** proximate the test site, i.e. electrodes **212**, may be narrowed, as illustrated in **FIGS. 3A** and **3B**.

[0068] **FIGS. 3A** and **3B** illustrate a biochip **300** which is a variation of biochip **200**. As in biochip **200**, biochip **300** includes a substrate **302**, and an inlet channel **304**, an outlet channel **306**, and a plurality of test channels **308** formed therein. Inlet channel **304** has a bottom surface **314**. Outlet channel **306** has a bottom surface **316**. Test channels **308** are defined by sidewalls **310** and bottom surface **318**.

[0069] Biochip **300** has a panel **320** extending between sidewalls **310** and elevated from bottom surface **318**, thus defining an opening **322** to allow the liquid to flow through. The narrowed section at opening **322** guides fluid towards the test site for effective contact with electrodes **212** or a surface having probe molecules immobilized thereon. Panel **320** can also further prevent backflow. Panel **320** may be shifted vertically to vary opening **322** for controlling the flow rate.

[0070] Electrodes **212** may be disposed in a test channel differently than as shown in **FIGS. 3A** and **3B**. For example, as illustrated in **FIG. 4**, electrodes, such as electrodes **412**, can be formed on bottoms **318** of test channels **308**. A pair of working and counter/reference electrodes **412** may be disposed at bottom **318** adjacent opposite sidewalls, as depicted.

[0071] As discussed above, probe molecules can be immobilized on a surface in a test channel. Probe molecules may include antibodies, antigens, peptides, aptomers, DNAs, and the like, which can specifically capture and bind to the target molecules. Probe molecules can capture target molecules contained in a sample solution introduced into the test channels. The sample solution may be flown through each test channel to allow the target molecules and/or enzyme-labelled molecules (reporter molecules) to be captured by the probe molecules. The reporter molecules in a competition detection scheme are labelled with enzyme molecules. A reporter molecule can be an antibody, antigen or any molecule with

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affinity to the probe molecule. The target molecules may be directly labelled before they react with the probe molecules. In each of the above steps, a liquid may be flown through the channels to attach a next molecule to the already immobilized molecule. To ensure accuracy and efficiency, the channels may be washed with a washing solution before feeding the next liquid. In particular, the channels may be washed before providing substrate solutions to the test channels to ensure that mobile enzymes are removed from the test channels.

[0072] A specific example of a sandwich immunoassay technique is illustrated in **FIG. 5A**. A probe antibody is immobilized on the surface of a working electrode or surface close to the working electrode. The target molecule is an antigen of interest and is bond to the probe antibody. An enzyme (such as ALP)-labelled second antibody is transported to bind to the target antigen after washing. Then the substrate, such as a PAPP including dephosphorylase-PAPP, is delivered into the detection spots for an enzymatic reaction, for example by flowing a substrate solution through the test channels. The substrate molecule reacts under enzyme catalysis at the enzyme active site to produce an electrochemically active product, PAP. Advantageously, PAP can be detected electrochemically when the electrodes are biased to a low potential such as 300 mv with reference to an Ag/Ag reference electrode.

[0073] Similarly, in the example shown in **FIG. 5B**, a probe DNA is first immobilized on the electrode surface. The target DNA is then bond to the probe DNA. The target DNA is labelled with an ALP enzyme. The enzyme can catalyse conversion of PAPP substrates to PAP products as in **FIG. 5A** for electrochemical detection.

[0074] **FIG. 6A** shows a partial image of a silicon mold for forming a biochip exemplary of embodiments of the present invention. The mold was prepared with a soft-lithography method. The test channels (seen as parallel narrow channels) have widths of about 100 μm.

[0075] **FIG. 6B** shows a partial image of a biochip formed from the mold shown in **FIG. 6A**. The biochip was formed by pouring a rubbery polymer (PDMS) over the mold, curing the polymer, and then separating the cured polymer from the

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mold. The biochip is suitable for ELISA analysis or detection. Probe antigens were immobilized in the test channels. A transparent polyacrylic sheet was attached to the biochip as the cover for sealing the device.

[0076] To test biochips such as the one shown in **FIG. 6B**, target proteins were immobilized as follows on the biochips. A thin aminopropyltriethoxysilane (APTES) film was coated or grafted on the PDMS surface of the test channels. The APTES surface was treated with homo-bifunctional cross-linkers (glutaraldehyde) for capturing immunoglobulin proteins on the APTES film. Alternatively, succinic acid anhydride generated carboxyl groups were applied onto the APTES surface, and heterobifunctional cross-linkers (1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC)) were then applied to the surface to covalently bind proteins onto the PDMS-APTES surface through the carboxyl groups.

[0077] A PDMS-APTES surface can be prepared by first cleaning the PDMS surface and then immersing the surface in an ethanol solution containing about 10% v/v APTES for about 10 minutes at room temperature. The APTES treated PDMS is rinsed with 96% ethanol, followed by air-drying, and then heated at 80 °C in a vacuum oven for about 2 hours.

[0078] Covalent immobilization can be realized via EDC as follows. Succinic acid anhydride (SAA) can be first used to modify the APTES surface by immersing the PDMS-APTES surface in a 50g/ml SAA solution for about 2 hours at room temperature. The pH value of the solution is adjusted by using a 3M NaOH solution to keep the solution stable at pH 6.0. Then surface is rinsed by phosphate-citrate buffer and dried under nitrogen flow. EDC and protein mixture are dropped onto the modified solid surface for cross-linking. The concentration of the EDC is 20µg/ml. The reaction buffer has 0.01M of phosphate-citrate, with a pH of 4.6. The cross-linking process lasts for about 1 hour at room temperature. The cross-linking process is terminated by immersing the surface in a Tris-HCl buffer. The protein coated surface is then immersed in a 1% bovine serum albumin (BSA) for blocking unoccupied surface for about 2 hours at about 37°C or for about 8 hours at room temperature.

[0079] Covalent immobilization can also be realized via glutaraldehyde

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(GA) as follows. The APTES treated PDMS surface is modified by GA to covalently immobilize protein on the PDMS surface. The amino groups on the surface are activated by 2.5% GA for 1 hour at room temperature. The surface is rinsed with a pH 8.0 Tris-HCl buffer, and dried with nitrogen gas. Protein probes in Tris-HCl are added onto the glutaraldehyde-activated surface. The cross-linking reaction can take place at room temperature for about 2 hour. After washing, the protein-coated surface is surface-blocked with the block reagent described above.

[0080] Example biochips fabricated as above have shown good performance. For example, rabbit IgG were immobilized in the test channels as described in the preceding paragraph. Sample solutions containing varying concentrations of enzymes labeled with anti-rabbit IgG were flown through the channels and test channels to immobilize the enzymes in the test channels by antibody-antigen binding. After feeding each sample solution, the channels and test channels was washed with a washing solution. A substrate solution was then flown through the test channels. Fluorescent light emitted from the test channels was detected. The intensities of the fluorescent light were substantially linearly dependent on the concentration of the sample solutions. The linear dependence indicates that cross-interference between test channels was very low. It has been found that as low as about 5.6 pg/ml of enzyme concentration can be detected.

[0081] Exemplary embodiments disclosed herein are useful for detecting low level of antigens, antibodies, peptides, DNA, proteins and other biomolecules in fluid or tissue samples. Low levels of DNA, RNA and other biomolecules such as glucose, which can be enzymatically converted to electrochemically active species, can be detected using embodiments of the present invention. Embodiments of the present invention can be incorporated into existing optical detection systems based on enzyme-catalyzed bio-reactions, in which PCR amplification can be eliminated. The exemplary biochips disclosed herein may provide a basic platform for a variety of applications, for example in diagnostics, drug discovery, target validation and pathogen detection.

[0082] Embodiments of the invention can be used in competition and displacement immunoassays where target antigens are labeled with enzymes for

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competition binding or displacement for detection. Radioactive tracers can be used to label the target molecules or binding molecules as reporters. The sandwich immunoassay is suitable for use with high molecular weight antigens, which possess at least two antigenic determinants or epitopes, but the competition and displacement immunoassay can be used to detect different antigens.

[0083] As can be appreciated, the operation of the biochips described herein can be automated. The biochips can be connected to utility modules. The operation of a biochip can be controlled by a computer or a microprocessor. The liquid flow rate and flow time can be conveniently adjusted.

[0084] As the substrate solution can continuously flow through the test sites generating more and more product molecules, even a low concentration of target molecules in the analyte solution can be detected. The substrate solution can also have a high concentration of substrate molecules. The liquid flow rate and flow time can be conveniently adjusted to control the enzyme reaction, for example, for achieving different detection sensitivities.

[0085] As can be appreciated, target molecules can be immobilized or labelled in other suitable manners. For example, the surface of an electrode may have a coating that can selectively capture the target molecules. In a further example, when the target molecule is itself an enzyme, it may not be necessary to attach another enzyme to it.

[0086] As can be understood, the enzyme reactions or target molecules present at different test sites can be identified using electrode addressing techniques including conventional addressing techniques. For example, the test sites may form an array of rows and columns and X-Y addressing techniques may be used.

[0087] Advantageously, high-density array biochips disclosed herein can be made compact without creating substantial cross-interference for a flow-through system. It can also be fabricated inexpensively and easily, for example, using inexpensive and matured printed circuit board technology to fabricate a plastic based microarray chip with microchannels, or embossing fabrication process.

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[0088] As can be appreciated, working and counter or reference electrodes **212** may be interchangeable. In alternative embodiments, separate counter and reference electrodes, thus more than two electrodes, can be provided in each test channel **208**. The reference electrodes can be interconnected with each other. The implementation and operation of electrodes for detecting electrochemical reactions including the use of reference electrodes can be readily understood by persons skilled in the art.

[0089] Embodiments of the invention may also be useful in protein expression profiling. Measurement of the variation in the expression of known proteins within tissues or cells over time, or in response to challenge by drugs, toxins, injury or disease, may require high throughput operation and can be conveniently performed using embodiments of the present invention.

[0090] Embodiments of the invention can be used in high density array-sensors with high sensitivity and specificity. Accuracy and reliability of the embodiments disclosed herein can be high because cross-interference due to diffusion can be inhibited or limited. It is also convenient to perform multi-fold dilution of sample, automatic post-immobilization probe treatment, and washing.

[0091] Advantageously, array dispensers are not necessary for operating biochips disclosed herein. Total assay time can be reduced compared to conventional biochips. The requirement of the samples amount is significantly decreased.

[0092] Other features, benefits and advantages of the present invention not expressly mentioned above can be understood from this description and the drawings by those skilled in the art.

[0093] The above described embodiments are intended to be illustrative only and in no way limiting. The described embodiments are susceptible to many modifications of form, arrangement of parts, details and order of operation. The invention, rather, is intended to encompass all such modification within its scope, as defined by the claims.

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WHAT IS CLAIMED IS:

1. A microchip comprising:

   a substrate;

   an inlet channel in said substrate;

   an outlet channel in said substrate for guiding fluid below a fluid level;

   a plurality of test channels in said substrate for guiding fluid flow from said inlet channel to said outlet channel, each one of said test channels having an inlet for fluid communication with said inlet channel and an outlet for fluid communication with said outlet channel, said inlet elevated from said outlet for inhibiting back flow through said inlet, said outlet elevated from said fluid level for inhibiting back flow through said outlet; and

   one test site in each one of said test channels, for detection of at least one of a specific molecule and a molecular interaction at said test site.

2. The microchip of claim 1, wherein each one of said channels has a substantially flat bottom, said bottom of said inlet channel elevated from bottoms of said test channels, said bottoms of said test channels elevated from said bottom of said outlet channel.

3. The microchip of claim 1 or claim 2, wherein at least one of said test channels comprises a well for holding fluid therein.

4. The microchip of any one of claims 1 to 3, wherein said inlet and outlet channels are adapted to allow a liquid to flow at a greater rate in said outlet channel than in said inlet channel.

5. The microchip of any one of claims 1 to 4, wherein each one of said test sites comprises a surface suitable for immobilizing specific molecules thereon.

6. The microchip of any one of claims 1 to 5, further comprising probe molecules immobilized at each one of said test sites, said probe molecules having specific affinity to selected target molecules.

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7. The microchip of claim 6, wherein different probe molecules are immobilized at different ones of said test sites for detecting different target molecules.

8. The microchip of any one of claims 1 to 7, further comprising a plurality of electrodes, one of said electrodes at each one of said test sites.

9. The microchip of claim 8, wherein at least one of said test channels comprises a narrowed section proximate said test site in said at least one test channel for guiding fluid towards said test site.

10. The microchip of claim 8 or claim 9, wherein said plurality of electrodes are first electrodes, said microchip further comprising a plurality of second electrodes, one of said second electrodes in each one of said test channels.

11. The microchip of claim 10, wherein said first electrodes are interconnected and said second electrodes are interconnected, such that each pair of said electrodes in each one of said test channels is uniquely addressable.

12. The microchip of claim 10 or claim 11, further comprising probe molecules immobilized proximate said first electrodes.

13. The microchip of any one of claims 1 to 12, wherein said test channels extend substantially in parallel.

14. The microchip of any one of claims 1 to 13, further comprising a dilution channel formed in said substrate, said dilution channel in fluid communication with said inlet channel and having a first inlet for reception of a first fluid and at least one second inlet for reception of a second fluid to dilute said first fluid.

15. The microchip of claim 14, wherein said at least one second inlet comprises a plurality of second inlets.

16. The microchip of any one of claims 1 to 15, wherein said substrate comprises polydimethylsiloxane (PDMS).

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17. The microchip of claim 16, wherein a PDMS surface in each one of at least one of said plurality of test sites is coated with aminopropyltriethoxysilane (APTES), thus forming a PDMS-APTES surface.

18. The microchip of claim 17, wherein probe molecules are covalently immobilized on said PDMS-APTES surface through cross-linkers.

19. The microchip of claim 18, wherein said cross-linkers comprise at least one of glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide.

20. The microchip of any one of claims 1 to 19, wherein at least one of said channels is adapted for connection to at least one of a pump and a valve for regulating fluid flow.

21. A method of detecting molecules or molecular interaction, comprising:

providing a microchip having a substrate, an inlet channel in said substrate, an outlet channel in said substrate, a plurality of test channels in said substrate each having an inlet for fluid communication with said inlet channel and an outlet for fluid communication with said outlet channel, said inlet elevated from said outlet, and one test site in each one of said plurality of test channels;

introducing a fluid to said test channels through said inlet channel;

inhibiting back flow of said fluid through said inlets and outlets by allowing overflow of said fluid from said test channels to said outlet channel through said outlets and maintaining the fluid level in said outlet channel below said outlets, thus inhibiting diffusion of molecules in said fluid from one of said test sites to another one of said test sites; and

after said fluid has been introduced to said test channels, detecting at least one of a molecule and a molecular interaction at a test site within said plurality of test sites.

22. The method of claim 21, wherein said detecting comprises detecting a signal indicative of at least one of the presence of said molecule and the occurrence of said molecular interaction, said signal comprising at least one of optical, electrical, magnetic, radiation, and electromagnetic signals.

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23. The method of claim 21 or claim 22, wherein probe molecules are immobilized at said test site for selectively capturing at least one of target molecules and reporter molecules.

24. The method of any one of claims 21 to 23, wherein said at least one of said molecule and molecular interaction is detected at said test site by enzyme immunoassaying (EIA).

25. The method of claim 24, wherein said EIA comprises enzyme-linked immunosorbent assaying (ELISA).

26. The method of claim 24 or claim 25, wherein said EIA comprises electrochemical EIA (EIA-EC).

27. The method of any one of claims 24 to claim 26, wherein said EIA comprises one of direct EIA, competitive EIA, competitive inhibition EIA, and sandwich EIA.

28. The method of any one of claims 21 to 27, wherein said detecting comprises detecting an electrical signal and said microchip comprises a plurality of electrodes, at least one of said electrodes at each one of said test sites for detecting said electric signal.

29. The method of claim 28, wherein said electric signal comprises at least one of a current, a voltage, and impedance.

30. The method of any one of claims 21 to 29, wherein said microchip further comprises a dilution channel in fluid communication with said inlet channel and having a first inlet and at least one second inlet, said introducing a fluid comprising feeding said fluid into said dilution channel through said first inlet and feeding a dilution fluid into said dilution channel through said at least one second inlet so that a diluted fluid is received in said inlet channel and thus said test channels.

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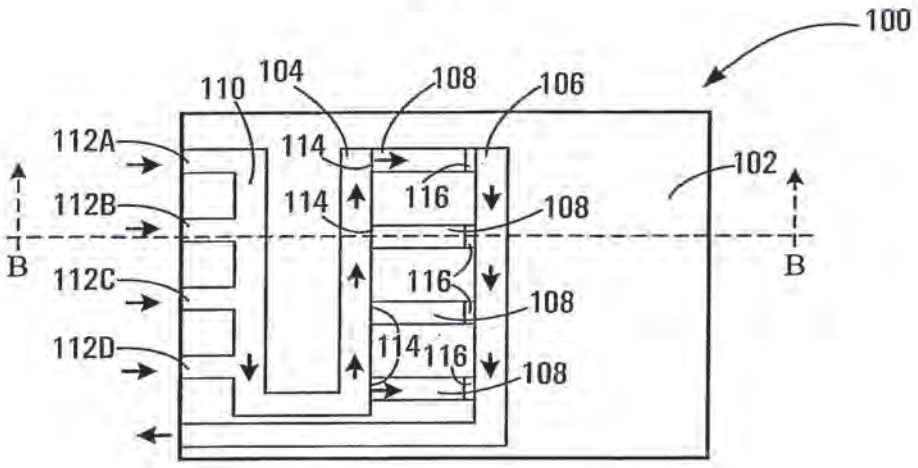

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FIG. 1A

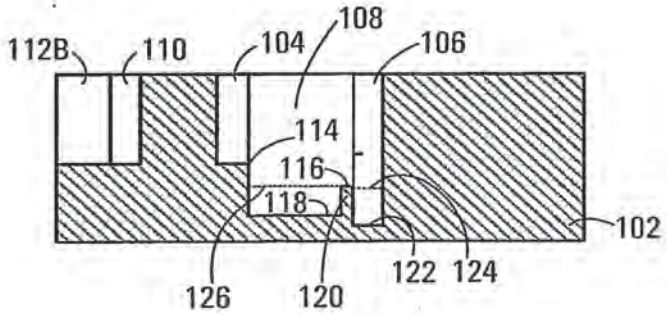

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FIG. 1B

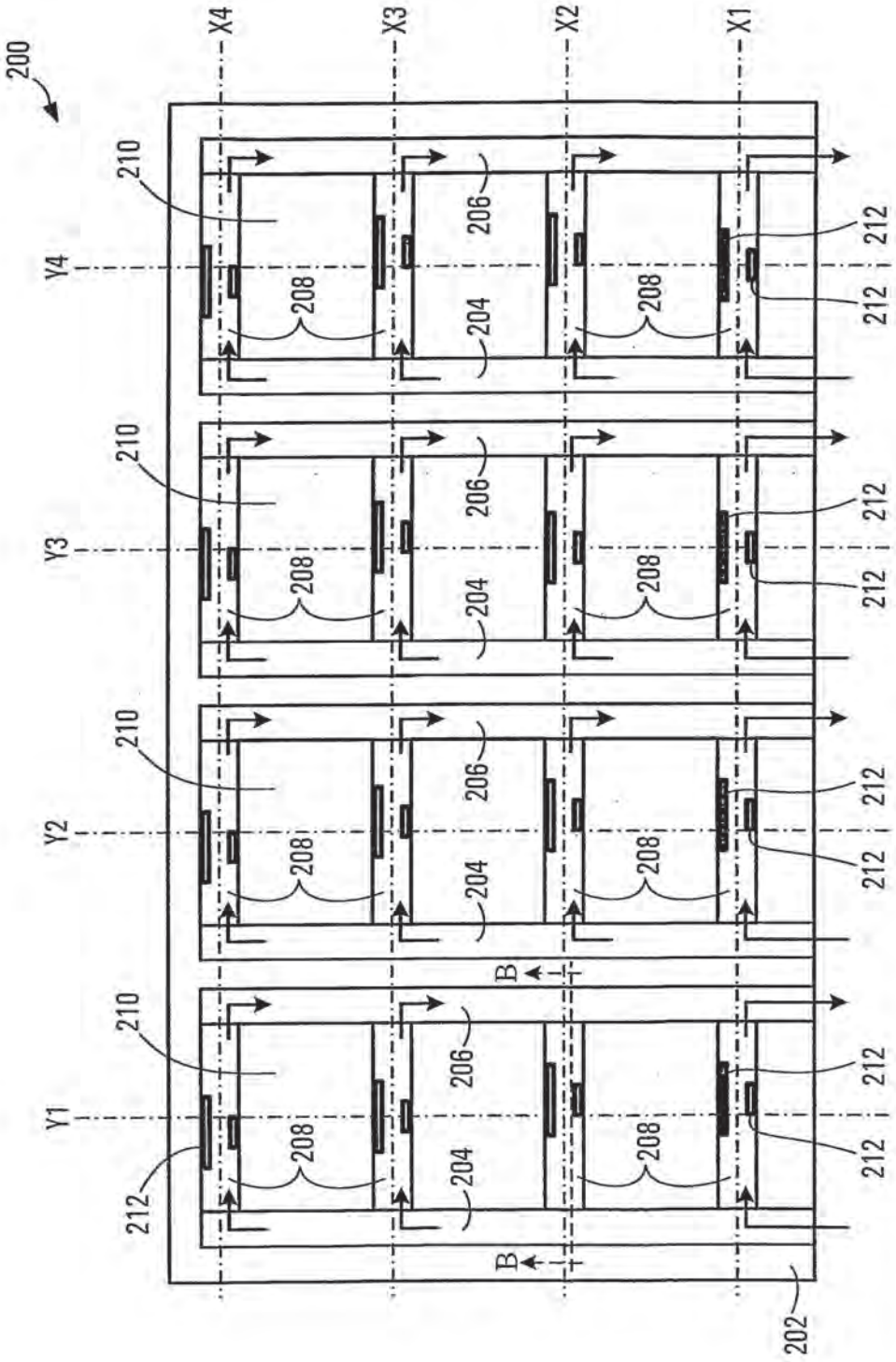


FIG. 2A

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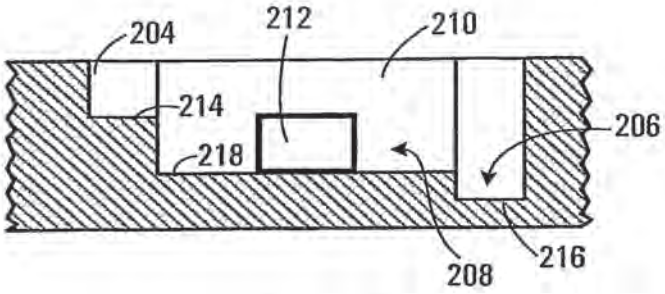


FIG. 2B

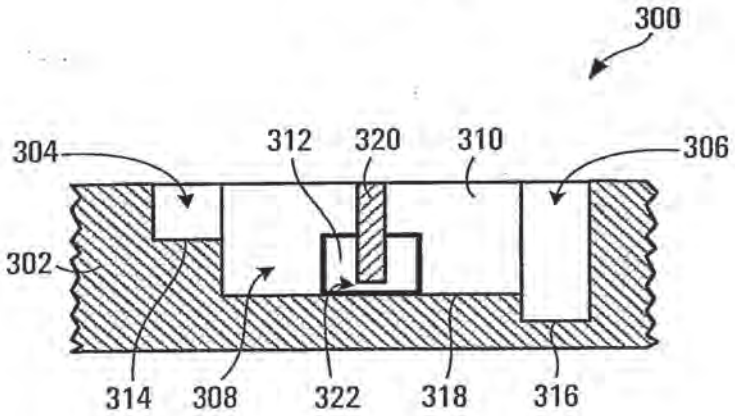


FIG. 3A

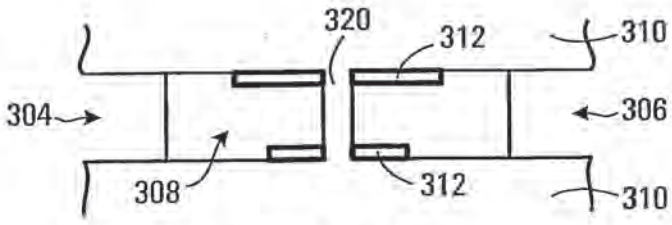


FIG. 3B

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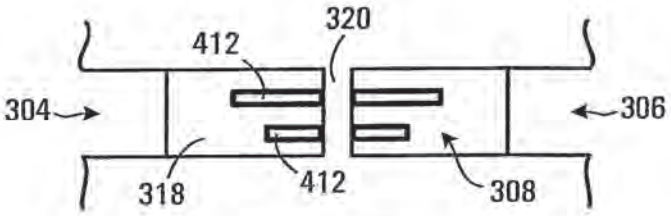


FIG. 4

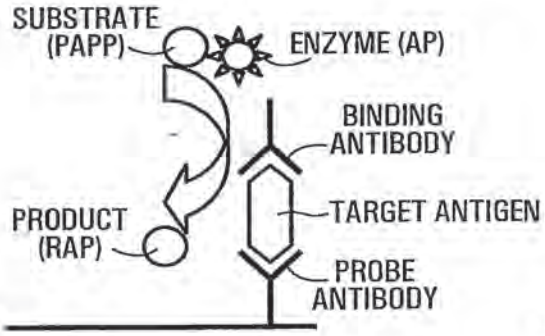


FIG. 5A

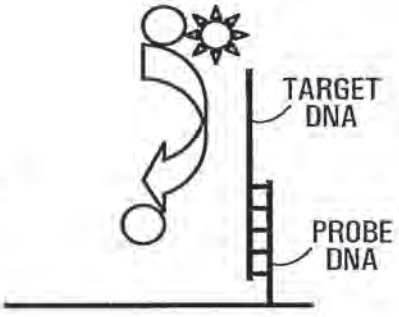


FIG. 5B

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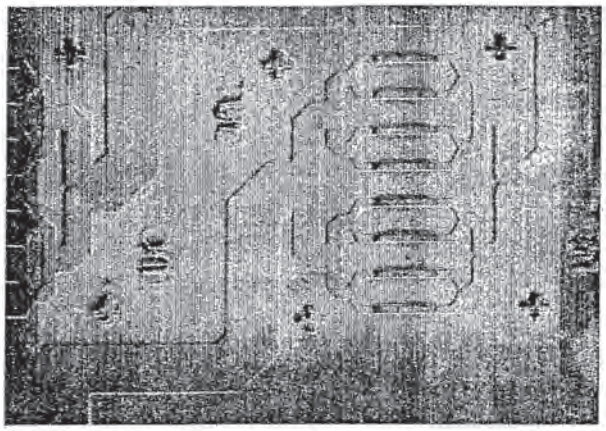


FIG. 6A

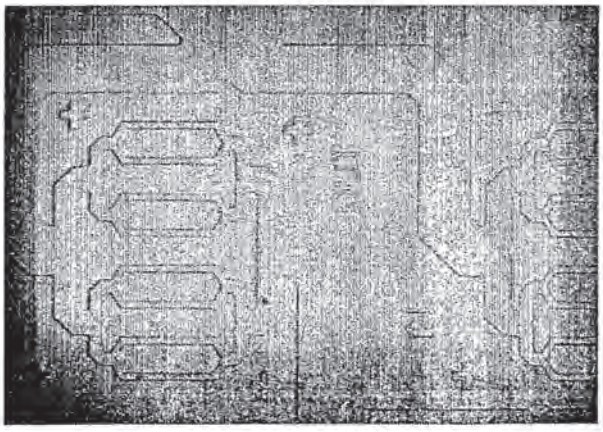


FIG. 6B

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2005/000112

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: B81B 1/00, G01N 1/28, B01L 3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DWPI & keywords: microfluidic, microchip, lab on chip, microchannel; test, assay, site; backflow, back diffusion; elevate, higher, raised, height; inlet, outlet, waste, drain; and other similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2003/035229 A2 (NTU VENTURES PTE LTD) 1 May 2003 See paragraphs [0047] – [0067]	1, 3–7, 13, 14, 16, 20–25, 30
Y	EP 1 357 178 A1 (IBIDI GMBH) 29 October 2003 See paragraph [0012] and figure 1	1, 3–7, 13, 14, 16, 20-25, 30
A	WO 2003/052428 A1 (BIOMICRO SYSTEMS, INC) 26 June 2003 See entire document	
A	US 2002/0185183 A1 (O'CONNOR ET AL.) 12 December 2002 See entire document	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 June 2005	16 JUN 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **LYNN BLOOMFIELD** Telephone No : (02) 6283 2851

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2005/000112

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2002/068823 A1 (NANOSTREAM INC.) 6 September 2002 See entire document	

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/SG2005/000112

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	03035229	EP	1440168	EP	1461454	US	2003138819
		US	2003138941	WO	03035909		
EP	1357178	AU	2003222824	DE	10224725	EP	1397483
		WO	03089565				
WO	03052428	AU	64268/99	AU	2002327550	BR	9914554
		CA	2347182	CN	1326549	EP	1125129
		US	6296020	US	6591852	US	6601613
		US	6637463	US	2002036018	WO	0022436
		WO	03098218	ZA	200102964		
US	2002185183	AU	13075/02	AU	81076/01	AU	2002349871
		AU	2002351291	AU	2003202958	AU	2003210909
		AU	2003213071	AU	2003217199	CA	2445806
		CA	2445816	CA	2472945	CN	1511256
		EP	1309404	EP	1333915	EP	1392435
		EP	1392436	EP	1393060	EP	1395365
		EP	1453606	EP	1453758	EP	1463579
		EP	1474236	EP	1474238	US	6536477
		US	6676835	US	6729352	US	6811695
		US	6814859	US	6827095	US	6848462
		US	6877892	US	6880576	US	6890093
		US	2002097633	US	2002113009	US	2002124896
		US	2002185184	US	2002185431	US	2002186263
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		US	2003150792	US	2003150806	US	2003198130
		US	2003223913	US	2004238052	US	2005006293
		WO	0211888	WO	0230560	WO	02100543
		WO	02100544	WO	02101383	WO	03008101
		WO	03045559	WO	03050035	WO	03059498
		WO	03059499	WO	03068401	WO	03068402
WO	02068823	EP	1334279	US	6644944	US	2002081222

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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International Bureau

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WIPO | PCT

(10) International Publication Number
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(51) International Patent Classification:
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(30) Priority Data:
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13/338,920 28 December 2011 (28.12.2011) US

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(72) Inventors; and
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(72) Inventor: **FELD, Michael** *(deceased)*.

(74) Agents: **HOOVER, Thomas, O.** et al.; McCarter & English, LLP, 265 Franklin Street, Boston, MA 02110 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with information concerning one or more priority claims considered void (Rule 26bis.2(d))*

(54) **Title**: PORTABLE OPTICAL FIBER PROBE-BASED SPECTROSCOPIC SCANNER FOR RAPID CANCER DIAGNOSIS

(57) **Abstract**: A multimodal probe system for spectroscopic scanning of tissue for disease diagnosis. The system can use diffuse reflectance spectroscopy, fluorescence spectroscopy and Raman spectroscopy for the detection of cancerous tissue, such as tissue margin assessment.

WO 2013/103475 A2

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TITLE OF THE INVENTION
PORTABLE OPTICAL FIBER PROBE-BASED SPECTROSCOPIC SCANNER FOR RAPID CANCER DIAGNOSIS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant No. RR002594 awarded by the National Institutes of Health. The government has certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATION

This application claims the priority to U.S. Provisional Application No. 61/569,095, filed on December 9, 2011, and is a continuation-in-part application of U.S. Patent Application No. 13/338,920, filed on December 28, 2011, which is a continuation-in-part application of U.S. Patent Application No. 11/492,301, filed July 25, 2006, entitled MULTI MODAL SPECTROSCOPY, which claims priority to U.S. Provisional Application No. 60/702,248, filed July 25, 2005. The entire contents of the above applications are being incorporated herein by reference.

BACKGROUND OF THE INVENTION

Techniques capable of evaluating human disease in a safe, minimally invasive and reproducible way are of importance for clinical disease diagnosis, risk assessment, therapeutic decision-making, and evaluating the effects of therapy, and for investigations of disease pathogenesis and pathophysiology. Among the clinical methods available to diagnose tissue lesions, pathologic examination of cytology preparations, biopsies and surgical specimens is the present day standard.

Pathologists have traditionally based their diagnoses primarily on tissue morphology. However, as the field of diagnostic pathology has evolved, assessment of tissue morphology has become more sophisticated, including such techniques as morphometry (or quantitative image analysis) and ploidy analysis. Pathologic diagnosis has also begun to move from complete dependence on morphology to inclusion of a host of adjunct techniques that provide biochemical and molecular information as well. This is particularly true for the diagnosis of cancer, where routine diagnosis begins with morphology but usually also includes such molecular diagnostic techniques such as immunohistochemistry and *in situ* hybridization that identify specific molecular signatures.

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This molecular information is not only of use for diagnosis, but is also of use for risk assessment and therapeutic decision-making, for example, in qualifying patients for molecular therapies, such as gene therapy or therapy with monoclonal antibodies directed against specific molecular targets. This molecular information has also greatly advanced the understanding of the pathogenesis and pathophysiology of many diseases, particularly cancer. But this evolution toward a focus on molecular events is not unique to the diagnosis of cancer. Recent molecular studies are also beginning to shed light on the pathogenesis and pathophysiology of cardiovascular disease, not only atherosclerosis but other disease (such as the cardiomyopathies) as well.

Intraoperative tissue diagnosis is an important component of successful cancer surgery in a variety of organ systems and tissues. Yet there continues to exist a significant clinical need for rapid and reliable intraoperative margin assessment of excised surgical specimens. Currently, intraoperative margin assessment is done by visual inspection and palpation, followed by selective assessment of any suspicious areas by rapid histology or cytology evaluation, which can be time consuming and inaccurate due to limited sampling. It is not unusual for the result of this pathologic margin assessment to come after the surgical wound is closed and the patient moved to the recovery room. Further, there are frequent discrepancies between the selective intraoperative and more comprehensive postoperative pathology margin assessment, necessitating a further operation to achieve negative margins. In breast-conserving surgery, for example, a second operation for positive margins discovered after surgery is required in up to 50% of cases. Furthermore, breast cancer recurs locally in the surgical site in ~10% of patients with negative margins on postoperative pathology margin assessment which, while more comprehensive than intraoperative pathology assessment, is still subject to sampling limitations. Intraoperative assessment of surgical margins is an important aspect in surgical management of cancer.

SUMMARY OF THE INVENTION

Diseases are more reliably identified by biochemical signatures than purely morphological markers. The present invention relates to the use of Raman spectroscopy in combination with other spectroscopic methods to provide biochemical and morphologic information and to further provide molecular information reflective of the metabolic state of tissue. This combination of biochemical, morphologic and metabolic information is used as the basis of more robust diagnostic methods. These types of molecular signature can be used for disease diagnosis, the disease progression and response to therapy.

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Thus, in a preferred embodiment Raman and fluorescence can be used in combination to measure tissue in vivo using a probe or can be used to measure excised tissue samples. In a further embodiment Raman and reflected light can be used in combination for measurements on a human or animal body with a probe or on biological samples. Additionally, Raman, fluorescence and reflectance measurements can be made using a probe for in vivo or ex vivo measurements. A common light delivery and light collection probe can be used in preferred embodiments of the invention.

The combination of modalities in the modal spectroscopy (TMS) has several advantages over the single modalities alone. First, fluorescence spectroscopy provides information about tissue metabolites, such and NADH, that are not provided by Raman spectroscopy. Second, TMS uses diffuse reflectance spectroscopy (DRS) to overcome distortion of fluorescence signatures by the effects of tissue absorption and scattering, and extract the intrinsic fluorescence signature (IFS). Third, in addition to its value in extracting IFS, DRS provides critical information about the tissue absorbers and scatterers themselves. Finally, while DRS provides information about tissue components responsible for diffusive scattering, light scattering spectroscopy (LSS) provides information about tissue components responsible for single backscattering. The combination of techniques into TMS, therefore, provides a wealth of information about tissue fluorophores, absorbers and scatterers, which creates a much more complete biochemical, morphologic and metabolic tissue profile.

TMS and Raman methods have been applied to specific diseases based on the strengths of each spectroscopic modality for detecting the primary biochemical or morphologic hallmarks of that disease. For example, cancer is a characterized by rapid cellular proliferation that is reflected in increased cellular metabolism. TMS, which provides IFS and DRS information about key cellular metabolites such as NADH and oxy- and deoxy-hemoglobin is, thus, a natural choice of modality for the diagnosis of cancer. TMS also provides information about key morphologic cellular changes, such as the nuclear enlargement and pleomorphism (variation in size and shape), that are characteristic of cancer. On the other hand, vulnerable atherosclerotic plaque is the end result of an inflammatory process that leads to thinning and rupture of the fibrous cap, leading to the release of thrombogenic necrotic lipid core material into the blood stream. Atherosclerotic plaques are also subject to calcific mineralization of the fibrous cap and necrotic core. Most lipids and calcium salts are strong Raman scatterers and, thus, Raman spectroscopy is a natural choice of modality for the diagnosis of vulnerable atherosclerotic plaque.

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The combination of spectroscopic modalities in multimodal spectroscopy (MMS) can provide information not provided by each modality. The whole (MMS) is also greater than the sum of the various individual modalities, because the biochemical and morphologic information provided is complementary – that is – the information provided by one technique often answers a question raised by the results of another. For example, for vulnerable atherosclerotic plaque, Raman spectroscopy provides information about the aggregate spectral contribution of foam cells and necrotic core, but raises questions about their individual contributions. Both DRS and light scattering answer those questions by providing specific information about the contribution of foam cells. So by combining the modalities in MMS one can decipher the separate contributions of both foam cells and necrotic core.

Measurements show that for vulnerable plaque, in some cases, two or more modalities are needed to fully characterize the contribution of a single tissue component. For example, as discussed above, oxy- and deoxy-hemoglobin are metabolites that may be key to the spectroscopic diagnosis of cancer. Hemoglobin is a strong tissue absorber and, therefore, it is a potential cause of distortion of tissue fluorescence signatures. This problem has been addressed in part by the use of TMS to derive undistorted IFS signatures. However, measurements in surgical breast biopsies have shown that in extremely bloody operative fields it is not be possible to account for all the absorbance effects of hemoglobin and achieve accurate diagnosis using TMS. On the other hand, hemoglobin is a weak Raman scatterer at NIR excitation wavelengths, and excellent model fits can be achieved for spectra acquired in bloody fields/tissues.

The combination of TMS and Raman spectroscopy in MMS provides a more complete and complementary biochemical, morphologic and metabolic tissue profiles than either TMS or Raman spectroscopy alone resulting in better diagnostic accuracy. Another key advantage in combining both techniques is the potential for depth sensing. TMS and Raman spectroscopy can use different excitation wavelengths, and therefore sample different tissue volumes because of wavelength-dependent differences in absorption and scattering. A Raman source preferably emits in a range of 750 nm to 1000 nm while the fluorescence source can employ one or more laser sources or a filtered white light source. Reflectance measurements preferably use a broadband source such as xenon flash lamp.

This difference in sampling volume can be exploited to provide information about the depth (or thickness) of certain tissue structures or layers. For example, the thickness of the fibrous cap is used for the diagnosis of vulnerable atherosclerotic plaque. The fibrous cap is composed largely of collagen. IFS and Raman spectroscopy both provide information about

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the contribution of collagen to tissue spectra. Comparison of the results from these two techniques, which use different excitation wavelengths and sample different tissue volumes, may provide information about the thickness of the fibrous cap. DRS and Raman spectroscopy both provide information about the contribution of deoxy-hemoglobin to the tissue spectra. Comparison of the results of these two techniques, which again use different excitation wavelengths and sample different tissue volumes, can provide depth-sensitive information useful in mapping cancers and pre-cancers of breast tissue.

Multimodal spectroscopy (MMS) is a system for spectral diagnosis and efficacy of combining spectroscopic results from TMS and Raman spectroscopy to provide better diagnostic detail and a more comprehensive picture of the biochemical, morphological and metabolic changes that occur in diseased tissues. The probe used in such measurements can be an endoscope or a small diameter probe for insertion through an endoscope channel or a small diameter catheter for insertion in the arterial system, for example.

The Raman methods for the diagnosis of breast cancer are based on a linear combination model similar to that used for peripheral arteries, that yields fit coefficients for epithelial cell nuclei and cell cytoplasm, fat cells, stromal collagen fibers, β-carotene, calcium oxalate and hydroxyapatite and cholesterol-like deposits (corresponding to tissue necrosis). The diagnostic procedure makes use of fit coefficients collagen and fat, two components of the tumor stroma.

Breast cancer, like most cancers, is characterized by abnormal cell proliferation and differentiation as well as increased cell metabolism. Autofluorescence, reflectance and LSS provide information about cell metabolism and tissue scatterers such as cell nuclei that is not provided by Raman spectroscopy. Therefore, by combining Raman spectroscopy with autofluorescence, reflectance and/or LSS, a method for the diagnosis of breast cancer incorporates contributions from both the malignant epithelial cells and the stroma.

Preferred embodiments of the invention relate to a portable, quantitative, optical fiber probe-based, spectroscopic tissue scanner designed for intraoperative diagnostic imaging of surgical margins. The tissue scanner combines diffuse reflectance spectroscopy (DRS) and intrinsic fluorescence spectroscopy (IFS), and has hyperspectral imaging capability, acquiring full DRS and IFS spectra for each scanned image pixel. A preferred embodiment can incorporate Raman detection into the probe used for scanning the region of interest. Modeling of the DRS and IFS spectra yields quantitative parameters that reflect the metabolic, biochemical and morphological state of tissue, which are translated into disease diagnosis. The tissue scanner

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has high spatial resolution (0.25mm) over a wide field of view (10 X 10 cm), for example, and both high spectral resolution (2nm) and high spectral contrast, readily distinguishing tissues with widely varying optical properties (bone, skeletal muscle, fat and connective tissue). Tissue-simulating phantom measurements confirm that the tissue scanner can quantitatively measure spectral parameters, such as hemoglobin concentration, in a physiologically relevant range with a high degree of accuracy (<5% error). Measurements using human breast tissues showed that the tissue scanner can detect small foci of breast cancer in a background of normal breast tissue. This tissue scanner is simpler in design, images a larger field of view at higher resolution and provides a more physically meaningful tissue diagnosis than existing spectroscopic imaging systems. This spectroscopic tissue scanner can provide real-time, comprehensive diagnostic imaging of surgical margins in excised tissues, overcoming the sampling limitation in current histopathology margin assessment. Preferred embodiments can use a fiber optic probe for manual scanning of surgical sites.

The challenge in addressing this need is to develop an imaging system with a wide enough field of view to image large surgical specimens with high enough resolution to detect small foci of cancer at the surgical margin in a clinically useful time frame. It is desirable, for example, to scan a tissue sample exceeding 10 cm^2 in area in less than 20 minutes to achieve this objective.

Optical fiber probe-based diffuse reflectance spectroscopy (DRS), intrinsic fluorescence spectroscopy (IFS) and Raman spectroscopy are used as tools for the real time diagnosis of cancer, and have advantages over other approaches to intraoperative and comprehensive assessment of surgical sites and margins. DRS, IFS and Raman measurements depend on the inherent optical properties of tissue and, as such, do not require exogenous imaging probes or contrast agents. The combination of DRS, IFS and Raman measurements provide information on the metabolic, biochemical and morphological state of tissue, which can be translated into disease diagnosis. DRS and IFS have relatively shallow (≤ 1mm) tissue penetration, and thus interrogate only the margin of the excised tissue specimen. Unlike traditional pathology diagnosis, spectroscopic diagnosis can be performed in real time.

Spectroscopic techniques are also quantitative and therefore more objective than the traditional methods. Conventional optical fiber probe-based spectroscopy techniques only examine a small area of tissue (~1mm) at a time, and so can suffer from under sampling and easily miss the lesion of interest. Spectroscopic imaging techniques can examine the entire

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margin of the excised tissue specimen, and so are not prone to the sampling limitations inherent in traditional pathology examination.

A preferred embodiment of the present invention includes a portable, quantitative, optical fiber probe-based, spectroscopic tissue scanner that can provide real-time comprehensive assessment of surgical margins in excised tissue specimens. The scanner significantly extends existing optical fiber probe-based spectroscopy instruments, which can be employed in the diagnosis of oral, esophageal, cervical, skin and breast cancer but previously unavailable in a wide field, high resolution imaging system. This tissue scanner is simpler in design, images a larger field of view at higher resolution and provides a more physically meaningful tissue diagnosis. The tissue scanner can provide fast, accurate, diagnostic images of the entire margin of excised surgical specimens, overcoming the sampling limitation in current pathology margin assessment.

A preferred embodiment can employ an inverted geometry with the sample placed on an optically transparent support and scanned by providing relative movement between a fiber optic light delivery and collection system and the tissue sample. Either the tissue sample or the fiber optic probe can be scanned to achieve the desired scan area, resolution and scan time. These scan parameters can be selected based on the size and geometry of the tissue sample. Note that pressure can be applied uniformly across the tissue surface to provide contact with the scanned surface. This provides precise spot size and distance to the probe to achieve this required pixel by pixel registration of images.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is a schematic illustration of an MMS system in accordance with a preferred embodiment of the invention;

Fig. 1B is a scatter plot of Raman data;

Fig. 2A and 2B are basis spectra;

Figs. 3A-3C are scatter plots of an MMS system in accordance with the invention;

Figs. 4A-4C are plots generated by an MMS system;

Figs. 5A and 5B graphically shows Raman basis spectra in accordance with the invention;

Fig. 6A-6C show spectra and fits for MMS modes;

Fig. 7 is a bar graph for hemoglobin concentration;

Fig. 8 shows scattering parameter A for DRS;

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Fig. 9 is a plot of the coefficients ratio for IFS;

Fig. 10 is a plot of the Raman parameter for artery samples;

Fig. 11A is a graph of coefficients for artery tissue;

Figs. 11B-D are Raman, reflectance, and fluorescence data of an artery;

Fig. 12 shows sampling depths used in accordance with embodiments of the invention;

Figs. 13A and 13B include side and end views, respectively, of a Raman Probe;

Fig. 13C is a side cross-sectional view of a side looking probe;

Fig. 13D is an end view of an MMS probe in accordance with the invention;

Fig. 13E is a forward looking MMS probe with a ball lens;

Fig. 13F is a forward looking MMS probe with a half ball lens;

Fig. 13G graphically illustrates a distal filter system for an MMS probe;

Fig. 14A is a schematic of an MMS system;

Fig. 14B is another embodiment of an MMS system;

Fig. 15A is a schematic diagram of the tissue scanner in accordance with preferred embodiment of the invention;

Fig. 15B is a cross-sectional view of a fiber optic probe used in conjunction with preferred embodiments of the invention;

Fig. 15C is a schematic view of a housing for a portable tissue scanner in accordance with a preferred embodiments of the invention;

Fig. 16A is an image of the resolution target and a randomly selected 2D 2.5cm x 2.5cm DRS spectral intensity map of zoomed target at 510nm;

Fig. 16B is a photograph of the porcine lower leg tissue cross section that was scanned;

Fig. 16C is an image of the tissue cross section on the glass plate during scanning;

Figs. 16D-16H are 9cm x 9cm DRS images of the tissue cross section at different wavelengths; note that the color bar is for all DRS images and is in arbitrary units;

Figs. 17A-17C show fifteen liquid phantoms composed of 2% intralipid and varying Hb concentrations in glass vials; O-rings filled with liquid phantoms and spectralon standards (10% and 20%) on the glass plate, with the scanning field of view marked with yellow tape; 2D DRS scan of the phantoms and spectralon standards at 490nm (field of view = 10x10 cm), respectively;

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