allowing said molten substrate to cool in the presence of said particle, thereby making a solid-phase support comprising said particle embedded in said substrate.

33. A method of separating a liquid and solid-phase, comprising:

providing a solid-phase support, said solid phase support comprising a particle embedded in a surface of a substrate; and

removing said liquid from said solid-phase support by centrifugation around an axis of rotation, wherein said surface of said substrate is oriented away from said axis of rotation and wherein said particles remain embedded in said surface.

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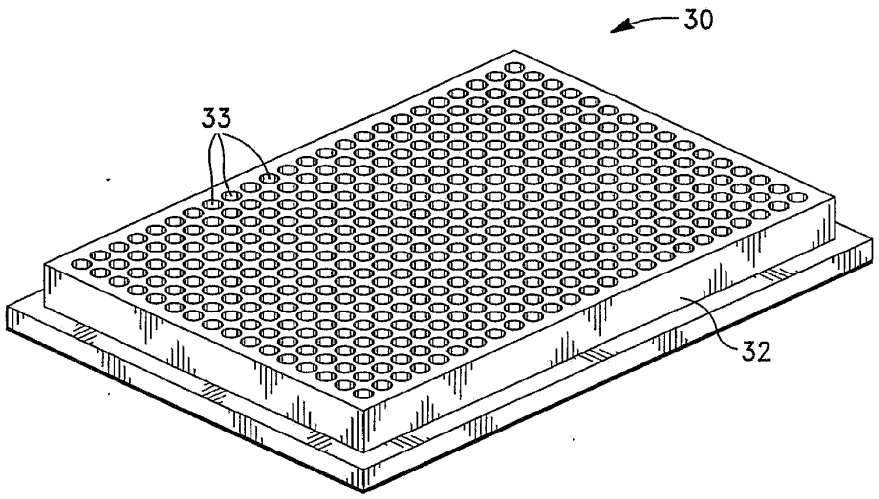


FIG.—1

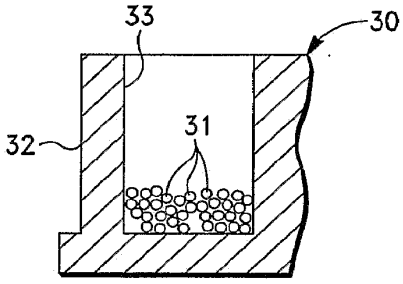


FIG.—2

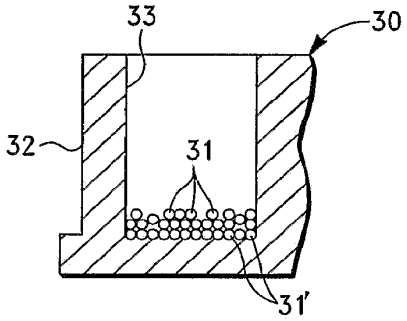


FIG.—3

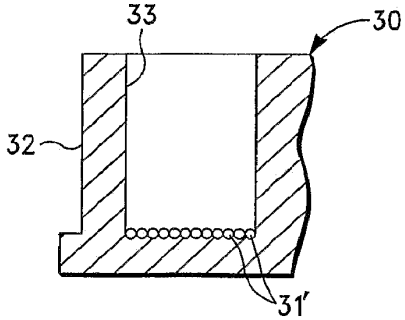


FIG.—4

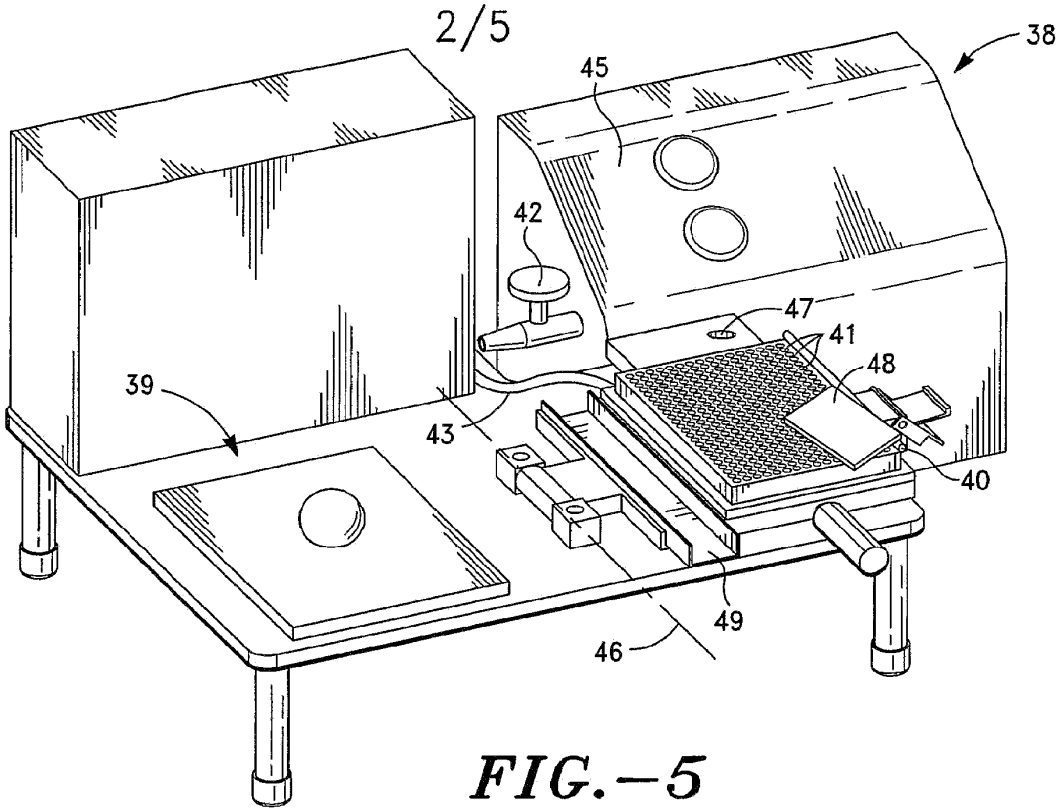


FIG.—5

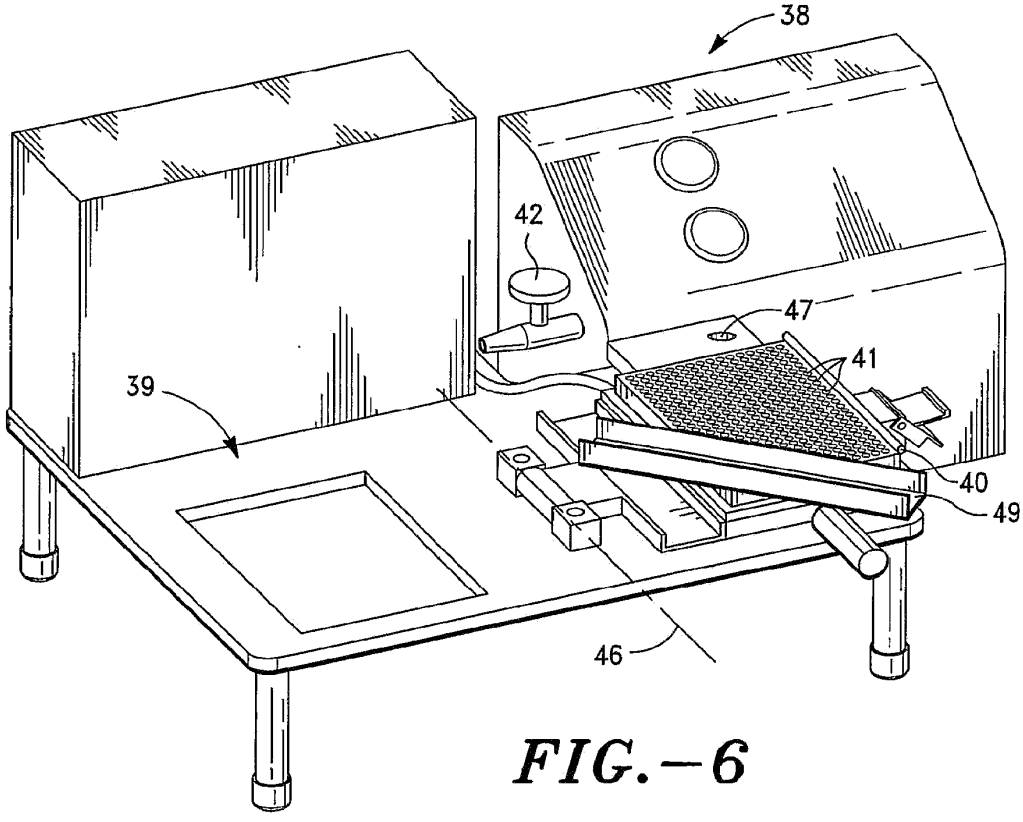


FIG.—6

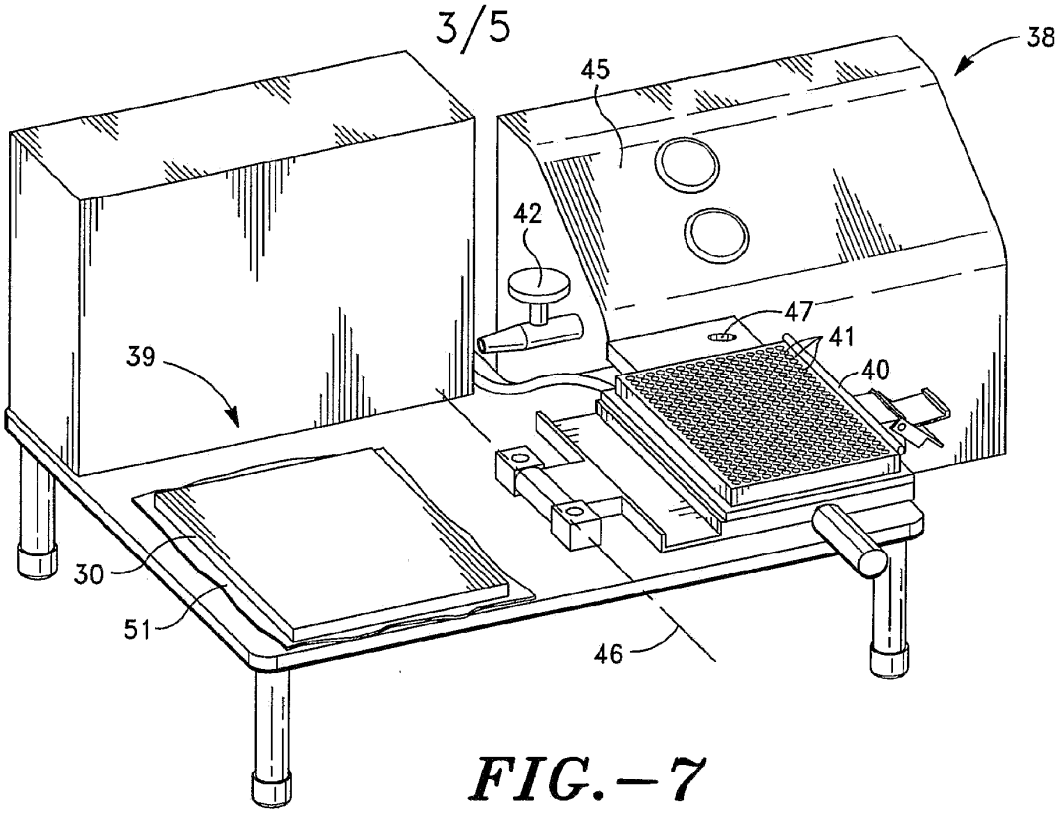


FIG.–7

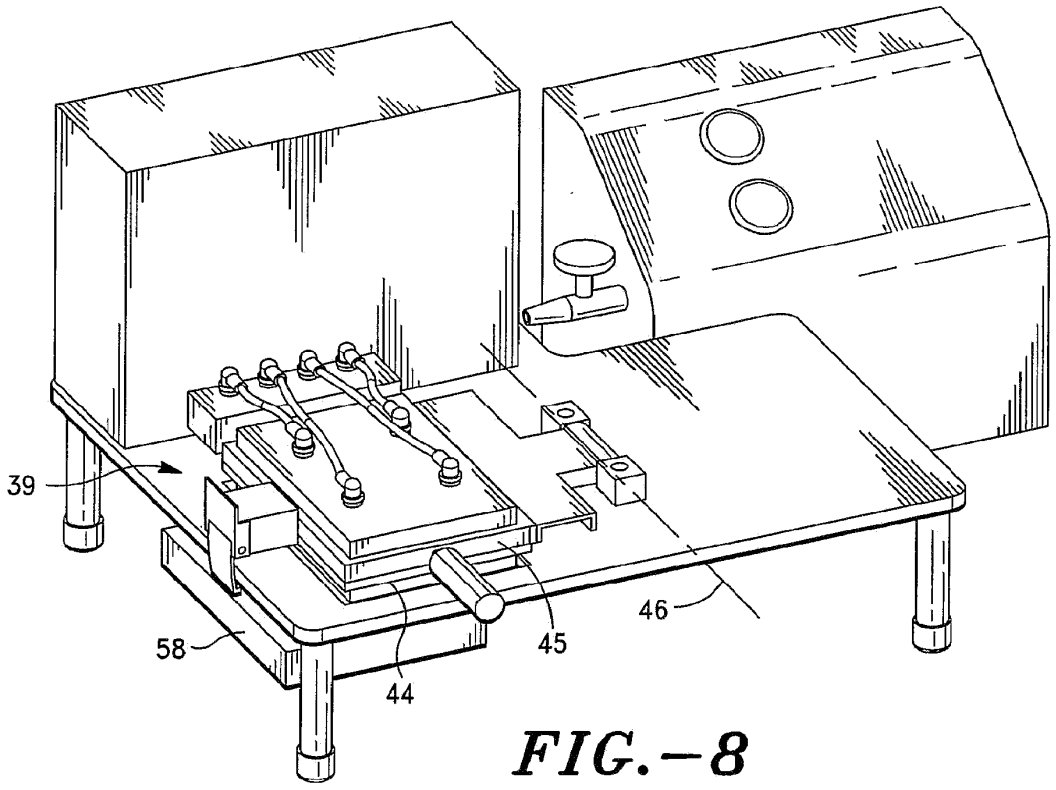


FIG.–8

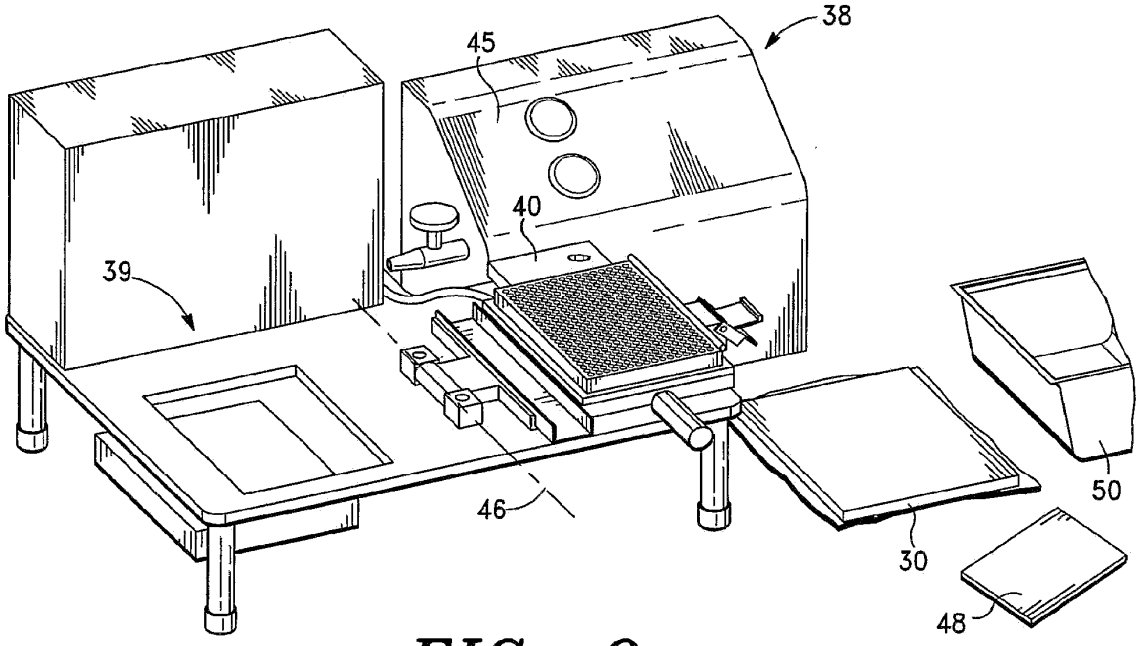


FIG.—9

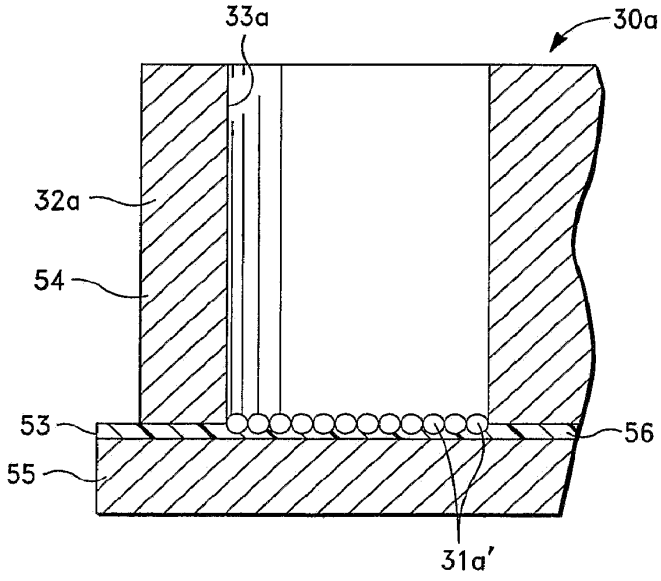


FIG.—10

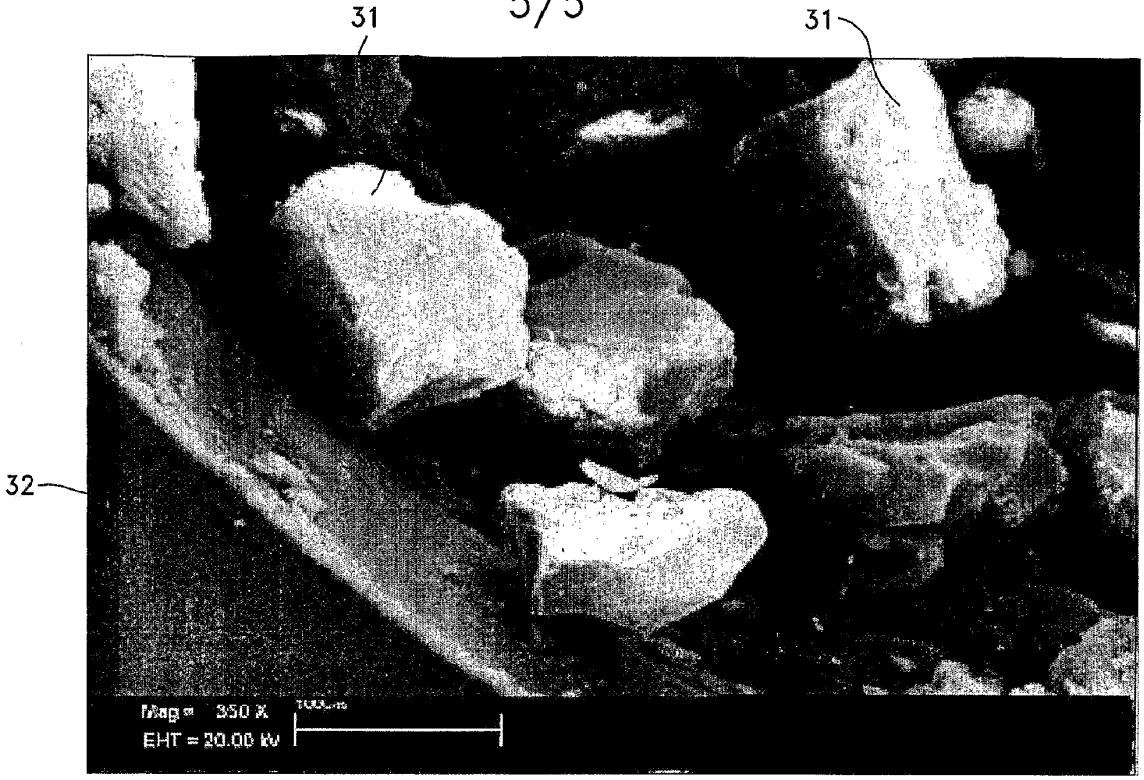


FIG.—11

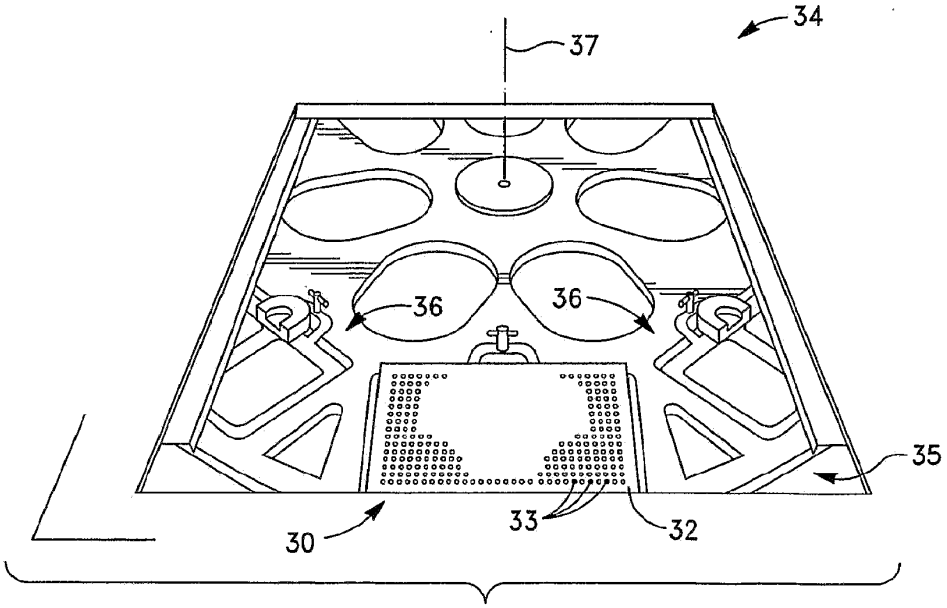


FIG.—12

INTERNATIONAL SEARCH REPORT

Ir[illegible]pplication No
I[illegible]04/027599

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01J19/00

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)
IPC 7 B01J

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 practical, search terms used)

EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/016868 A (CARLSON EDWARD ; DICKINSON TODD (US); ILLUMINA INC (US); COBLENTZ KENN) 27 February 2003 (2003-02-27)	1-5,32
Y	abstract page 2, paragraph 5 - page 3, paragraph 1 page 3, paragraph 2 - paragraph 3 page 7, paragraph 3 - page 16, paragraph 1 page 24, paragraph 3 - page 25, paragraph 1 page 25, paragraph 7 - page 26, paragraph 1 page 26, paragraph 4 - page 28, paragraph 4; figures 1,2	6-16, 23-31,33

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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"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

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"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.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2004	14/01/2005

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nazario, L

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INTERNATIONAL SEARCH REPORT

Inte pplication No
PC[illegible]04/027599

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	abstract column 4, line 25 - column 5, line 59 column 7, line 56 - column 8, line 2 column 8, line 24 - line 37	23-31,33
X	WO 03/031054 A (SURMODICS INC) 17 April 2003 (2003-04-17)	1,2,4,5
Y	page 2, line 14 - page 3, line 4 page 15, line 18 - page 20, line 32 claims; figures	6-16, 23-31,33
Y	WO 01/60520 A (GLAXO GROUP LTD ; NORTHROP JEFFREY PAUL (US); CHAN SAM (US); GAVIN ROB) 23 August 2001 (2001-08-23) abstract page 5, line 24 - page 13, line 30; figures	6-16
Y	WO 99/25470 A (TREGA BIOSCIENCES INC) 27 May 1999 (1999-05-27) abstract page 3, line 10 - line 24 page 6, line 19 - page 8, line 35 figures 3-9	23-31,33

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INTERNATIONAL SEARCH REPORT

Application No
2004/027599

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(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
9. August 2001 (09.08.2001)

PCT

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(51) Internationale Patentklassifikation[7]: G01N 33/00

(21) Internationales Aktenzeichen: PCT/EP01/01333

(22) Internationales Anmeldedatum:
7. Februar 2001 (07.02.2001)

(25) Einreichungssprache: Deutsch

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(30) Angaben zur Priorität:
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(71) Anmelder und
(72) Erfinder: **TRUTNAU, Hans-Heinrich** [DE/DE]; Bertelestr. 77, 81479 München (DE).

(74) Anwalt: **HANSMANN & VOGESER**; Albert-Rosshaupter-Str. 65, 81369 München (DE).

(81) Bestimmungsstaaten *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

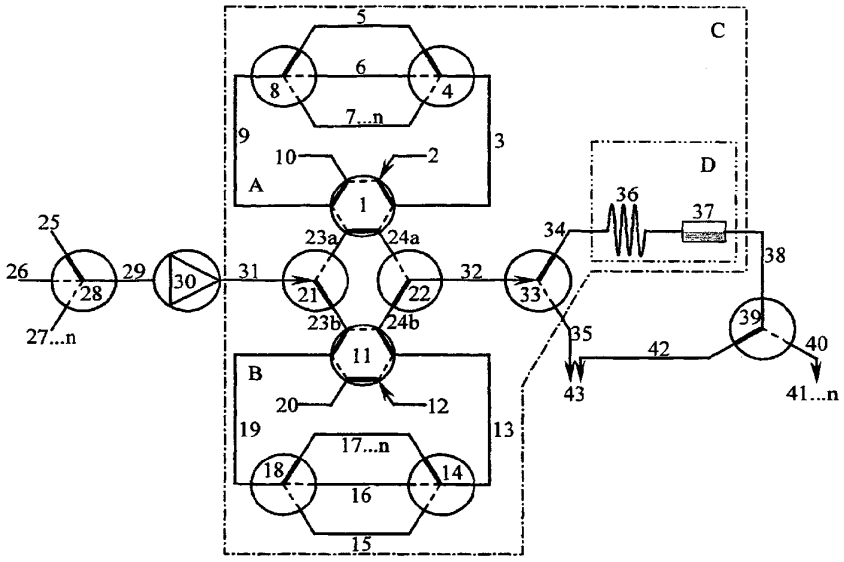
(84) Bestimmungsstaaten *(regional)*: ARIPO-Patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI-Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:
— *ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts*

[Fortsetzung auf der nächsten Seite]

(54) Title: ANALYTICAL MEASURING AND EVALUATION METHOD FOR MOLECULAR INTERACTIONS

(54) Bezeichnung: ANALYTISCHES MESS- UND AUSWERTEVERFAHREN FÜR MOLEKULARE INTERAKTIONEN



(57) Abstract: The invention relates to an analytical measuring and evaluation method for determining the interaction parameters between an analyte and a ligand, preferably in a biosensor. According to the inventive method, the concentration of the analyte is gradually changed at defined intervals t_i and the initial association or dissociation rates or association and dissociation constants are determined. The invention further relates to a device for carrying out the inventive method.

[Fortsetzung auf der nächsten Seite]

WO 01/57520 A2

Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(57) Zusammenfassung: Die Erfindung betrifft ein Mess- und Auswerteverfahren zur Bestimmung von Interaktionsparametern zwischen einem Analyt und einem Ligand, vorzugsweise in einem Biosensor. Dazu wird zu bestimmten Zeitpunkten t_i die Konzentration des Analyten schrittweise verändert und die initialen Assoziations- oder Dissoziationsraten bzw. Assoziations- oder Dissoziationsgeschwindigkeitskonstanten ermittelt. Ausserdem wird eine Vorrichtung zur Durchführung des Verfahrens beschrieben.

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Analytisches Meß- und Auswerteverfahren für molekulare Interaktionen

1. Anwendungsgebiet

Die Erfindung bezieht sich auf ein Meß- und Auswerteverfahren zur Bestimmung von Interaktionsparametern zwischen einem Analyt und einem Ligand wie unter anderem Geschwindigkeitskonstanten oder Bindungspartner-Aktivitäten. Die Aufnahme der Meßwerte erfolgt z. B. mit Biosensoren, in denen ein i. allg. immobilisierter erster Bindungspartner (Ligand L) mit einem zweiten Bindungspartner (Analyt A) versetzt wird und die Bildung des Ligand-Analyt-Komplexes LA im zeitlichen Verlauf erfaßt wird.

2. Technischer Hintergrund

Sogenannte Biosensoren zur Erfassung der zeitlichen Verläufe der Bildung von Analyt-Ligand-Komplexen sind in den unterschiedlichsten Ausführungen bekannt, wobei auch andere Vorrichtungen wie u. a. Array-Systeme zur Erfassung derartiger Reaktionen verwendbar sind. Dabei werden sowohl die zeitlichen Verläufe der Assoziation des Analyten am Liganden erfaßt, wenn die Analytkonzentration durch die Zugabe einer Analytlösung bestimmter Konzentration erhöht wird, sowie die Dissoziation bei Zugabe einer geringer konzentrierten Lösung oder mit einer Konzentration von Null. I. allg. wird der zeitliche Verlauf der Komplexbildung mit der Funktion R(t) oder R_t beschrieben, wobei die Konzentration des Komplexes bzw. seine zeitliche Änderung mit $c_t(LA)$ bzw. $dc_t(LA)/dt$ bezeichnet wird. In erster Näherung wird für den zeitlichen Verlauf der Funktion R ein exponentieller Verlauf erster Ordnung mit dem Exponenten k_{on} bei der Assoziation und k_{off} bei der Dissoziation des Komplexes angenommen. Zur Erfassung dieser Werte ist es bisher

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üblich, die Reaktion, z. B. die Komplexbildung, teilweise oder bis zum Erreichen eines Gleichgewichtswertes R_{eq}, bei dem die Assoziation des Komplexes aus Analyt und Ligand im Gleichgewicht steht mit dessen Dissoziation, auszumessen. Es folgt eine Dissoziation des Komplexes und eine Regenerations- und Waschphase. Dies ist ein sehr zeitaufwendiges Verfahren, insbesondere wenn hintereinander mehrere Messungen mit unterschiedlichen Konzentrationsänderungen vorgenommen werden sollen. Eine weitere Auswertung der erhaltenen Exponenten führt zu den eigentlich interessierenden kinetischen Geschwindigkeitskonstanten, das Verfahren bedarf jedoch einer konstanten Analytkonzentration.

Bei den bisherigen Auswertungsverfahren der Meßwerte wird meistens davon ausgegangen, daß die Konzentration des zu dem Liganden zugegebenen Analyten trotz der Komplexbildung konstant ist. Dies wird z. B. dadurch angenähert, daß die Analytkonzentration vielfach höher ist als die Ligandkonzentration oder durch ständigen Austausch im Durchflußsystem. Tatsächlich jedoch tritt z. B. bei der Assoziation im Küvettensystem immer eine Verarmung des Analyten oder eine Konzentrationsänderung auf, die dazu führt, daß der Gleichgewichtswert R'_{eq}, der tatsächlich erreicht wird, sich von dem hypothetischen Wert R_{eq} bei konstanter Analytkonzentration unterscheidet. Eine Analyse der Meßwerte mit einer Näherungsfunktion zweiter Ordnung ergibt zwar einen genaueren Wert des Koeffizienten der Exponentialfunktion, ist aber aufwendig und bedarf der zusätzlichen Ermittlung bzw. Berücksichtigung einer Reihe von Randbedingungen des Tests.

Zur Konzentrationsbestimmung werden initiale Raten herangezogen. Die initialen Raten werden dadurch erhalten, daß in den Anfang der Assoziationskurve eine Ausgleichsgerade gelegt wird, deren Steigung die initiale Rate aber unterbewertet, da die Gerade die Krümmung der Kurve nicht berücksichtigt. Desweiteren ist es bekannt, daß beim Auftragen der initialen Raten in einem Diagramm über die Konzentration des Analyten c(A), bei mehrmaliger Messung mit jeweils unterschiedlichen Konzentrationen, theoretisch eine Gerade durch den Ursprung mit der Steigung $R_{max} * k_{ass}$ erhalten wird, wobei R_{max} die maximal mögliche Reaktion z. B. des Biosensors auf die Zugabe eines Analyten im Überschuß darstellt. Die Steigung dieser Geraden ist aber durch die o.g. Unterbewertung ebenfalls verfälscht, so daß diese Art von Diagramm nicht zur Bestimmung von k_{ass} herangezogen wird.

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Zur Erfassung der Meßwerte werden u. a. Biosensoren nach dem Durchflußprinzip oder dem Küvettenprinzip verwendet, wobei die unterschiedlichsten Meßverfahren im Stand der Technik bekannt sind. Bei Durchflußsystemen wird eine Sensoroberfläche, auf der der Ligand immobilisiert ist, pro Analyse mit einem konstanten Strom einer Analytlösung beaufschlagt. Hierbei ist die Näherung einer vorgewählten konstanten Analytkonzentration im Prinzip gegeben. Bei Küvettensystemen wird eine Meßzelle mit einer Analytlösung gefüllt und die Reaktion mit dem Liganden an einer Sensoroberfläche erfaßt. Besonders hier tritt eine Verfälschung der Meßergebnisse auf, da bei tatsächlichen Reaktionen die Analytkonzentration verändert wird. Werden mehrere Meßvorgänge mit unterschiedlichen Konzentrationen hintereinander durchgeführt, so wird normalerweise die Küvette mit einer Pufferlösung gespült oder auf andere Weise regeneriert, und anschließend eine Lösung mit geänderter Konzentration eingebracht.

Bei mehrfachen Titrationen, d. h. Änderung der Konzentration, werden die Meßkurven bisher so lange aufgenommen, bis die Komplexbildung einen Gleichgewichtszustand erreicht hat. Es werden lediglich die Gleichgewichtszustände zur Bestimmung weiterer Größen, nicht jedoch der Kinetik oder initialer Raten, verwendet. Vielmehr wird bei solchen Mehrschritt-Titrationen ein Zugang zur Kinetik ausdrücklich ausgeschlossen.

Für Durchfluß-Systeme sind sequentiell bzw. hintereinander angeordnete sogenannte Probenschleifen bekannt, die nacheinander befüllt und in die Meßkammer ausgespült werden können. Jedoch weisen die Probenschleifen jeweils ein produktionstechnisch aufwendiges exakt definiertes Volumen und zwischen ihnen ein undefiniertes Extravolumen, das auch als Totvolumen bezeichnet wird, auf. Im Totvolumen kann es zu unerwünschten Durchmischungen verschiedener Lösungen kommen. Die Schleifen sind weder von ihrer Auslaß-Seite her noch teilweise und auch nicht unabhängig voneinander befüllbar; und sie sind nicht unabhängig voneinander in die Meßkammer spülbar.

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3. Darstellung der Erfindung

a) Technische Aufgabe

Ausgehend vom Stand der Technik hat sich die Erfindung die Aufgabe gestellt, ein Verfahren anzugeben, mit dem die Meßzeiten bei der Erfassung der Meßwerte bei der Komplexbildung verkürzbar sind, die tatsächlichen Verhältnisse bei der Komplexbildung, d. h. Änderung der Analytkonzentration, berücksichtigt werden, und die Durchführung mehrerer Messungen hintereinander ohne aufwendige Spülvorgänge z. B. in einer Küvette möglich ist, sowie es möglich ist, die kinetischen Konstanten aus den initialen Raten, und unter idealen Verhältnissen auch aus den Kurvenexponenten zu ermitteln.

b) Lösung der Aufgabe

Erfindungsgemäß wird diese Aufgabe durch die Verfahrensmerkmale des Anspruchs 1 und die Vorrichtungsmerkmale des Anspruchs 13 gelöst.

Vorteilhafte Ausgestaltungen der Erfindung sind jeweils Gegenstand von Unteransprüchen.

Der Kerngedanke der Erfindung besteht darin, daß die Änderung der Analytkonzentration sowohl bei der Assoziation als auch der Dissoziation des Komplexes berücksichtigt wird. Dadurch nimmt die Funktion R einen Gleichgewichtswert R'_{eq} an, der sich vom Wert R_{eq} unterscheidet. Bei der Assoziation, d.h. Zugabe eines Analyten erhöhter Konzentration zu dem Liganden, tritt während der Assoziation eine Verarmung des Analyten auf, die dazu führt, daß R'_{eq} kleiner ist als der theoretische Wert R_{eq}. Im ersten Moment der Reaktion jedoch, d. h. wenn die Analytlösung z. B. in die Küvette eines Biosensors eingebracht wird, ist die initiale Rate der Funktion R unabhängig davon, ob im späteren Verlauf eine Analytverarmung durch Bindung des Analyten an den Liganden auftritt oder konstante Analytkonzentration angenommen wird. Zum Zeitpunkt t = 0 hat noch keine Reaktion zwischen Analyt und Ligand stattgefunden. Durch die

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Berücksichtigung des tatsächlichen Reaktionsverlaufs ändert sich auch der Exponentialkoeffizient von k_{on} in k'_{on}. Tatsächlich jedoch bleibt die Beziehung zwischen den einzelnen Größen der Exponentialfunktion erhalten, d. h.:

$$k'_{on} * R'_{eq} = \frac{dR_{ass}(t=0)}{dt} \quad (1)$$

und

$$k'_{off} * \Delta R' = \frac{dR_{diss}(t=0)}{dt} \quad (2)$$

wobei $\Delta R'$ die Differenz zwischen dem Gleichgewichtswert der Dissoziation und dem Startwert R_{st} der Funktion R ist.

Das bedeutet, daß aus den tatsächlichen Meßwerten z. B. die initiale Rate ermittelbar ist. Dies gilt ebenso, wenn die Exponentialfunktion zwar nach 1. Ordung, aber mit zwei Koeffizienten $k'_{on(1)}$ und $k'_{on(2)}$ verläuft, wobei sich die beiden korrespondierenden initialen Raten zu einer Gesamtrate addieren.

Dabei ist es im Rahmen der Erfindung möglich, daß die Meßkurve der Funktion R bis zum Erreichen des Gleichgewichtswertes R'_{eq} aufgenommen wird, um z. B. mit Hilfe eines mathematischen Programms den Exponenten k'_{on} oder k'_{off} zu ermitteln und daraus die initialen Raten der Assoziation oder Dissoziation. Vorzugsweise wird lediglich ein Teil der Kurve erfaßt, z. B. kann die Erfassung der Funktion R zu einem früheren Zeitpunkt abgebrochen werden, um aus einer Extrapolation des Kurvenabschnitts die Gleichgewichtslage zu ermitteln. Der Exponent k wird dabei mit einer non-linearen Näherung erhalten, kann aber auch über die Lineare Regression der Ableitung der Funktion R bestimmt werden.

Aus den initialen Raten können dann die jeweiligen Assoziations- und Dissoziationsgeschwindigkeitskonstanten k_{ass} und k_{diss} ermittelt werden gemäß:

$$\frac{dR_{ass}(t=0)}{dt} = k_{ass} * R_{max} * c_0(A) \quad (3)$$

im Falle der Komplexassoziation mit der Startkonzentration des Analyten $c_0(A)$ bzw.:

$$\frac{dR_{diss}(t=0)}{dt} = k_{diss} * (-R_{st}) \quad (4)$$

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im Falle der Komplexdissoziation, wobei die Größe R_{st} den Startwert der Funktion R bei der Dissoziation angibt. Die Startkonzentration $c_0(A)$ ist i. allg. durch den Versuch von außen vorgegeben.

Der Vorteil der Erfindung besteht darin, daß mehrmals hintereinander, z. B. in einer Küvette, eine Messung mit jeweils schrittweise veränderter Analytkonzentration durchführbar ist, wobei es nicht notwendig ist, daß jede Messung bis zum Erreichen des Gleichgewichtswertes zu Ende geführt wird, sondern bereits vorher abgebrochen werden kann bzw. die Konzentration des Analyten erhöht oder erniedrigt werden kann. Auch die Durchführung von Meßserien mit mehrmaliger auch abwechselnd aufeinanderfolgender Analyterhöhung und -verminderung zur Erfassung von Assoziations- und Dissoziationskurven ist möglich.

Obschon sich die Start-Raten von multiplen, i-fachen Assoziationsschritten aus der Summe der vorangegangenen und der neuen Assoziation bilden, lassen sich die Netto-Start-Raten der jeweiligen Assoziationsschritte basierend auf den eingangs formulierten Gleichungen (1) und (3) für die Assoziation ermitteln gemäß:

$$k'_{on,i} * (R'_{eq,ass,i} - R'_{st,ass,i}) = (dR_{ass,i}/dt)_{t=0} \quad (5)$$

und:

$$(dR_{ass,i}/dt)_{t=0} - (dR_{ass,i-1}/dt)_{t=st,ass,i} = (dR_{ass,i,net}/dt)_{t=0} \quad (6)$$

und auswerten gemäß:

$$(dR_{ass,i,net}/dt)_{t=0} / c_{0,i,net}(A) = -k_{ass} * R_{st,ass,i} + k_{ass} * R_{max} \quad (7)$$

was einer Geradengleichung entspricht und sich entsprechend auftragen lässt.

Der Quotient aus initialer Netto-Assoziationsrate und Analyt-Netto-Startkonzentration, aufgetragen gegen das Assoziations-Startsignal $R_{st,ass,i}$, liefert linear gefittet eine Gerade mit der Steigung = $-k_{ass}$ und einen x-Achsenabschnitt R_{max}. Die einzelnen Phasen multipler Dissoziationsschritte aus Küvettensystemen können direkt nach den Gleichungen (2) und (4) für die Dissoziation ausgewertet werden.

Die Berechnung von sogenannten Fit-Kurven, mit denen die erhaltenen Meßergebnisse angenähert oder im Falle des vorzeitigen Abbruchs der Messung extrapoliert werden, erfolgt mit mathematischen Annäherungsprogrammen, die eine Exponentialfunktion, auch eine Doppelt-Exponentialfunktion, erster oder höherer Ordnung ermitteln, die die Meßergebnisse mit größtmöglicher Genauigkeit annähert.

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Die Berechnung einer derartigen Kurve kann durch die Auswertungseinheit z. B. eines Biosensors selbsttätig während der Messung erfolgen.

Die schrittweise Änderung der Konzentration in der Meßkammer kann zu beliebigen Zeitpunkten während der Aufnahme der Meßergebnisse erfolgen. Insbesondere wird jedoch die Halbwertszeit der Funktion R vorgeschlagen, zu der die Funktion, beispielsweise bei einer Assoziation die Hälfte ihres Endwertes R_{eq} erreicht hat.

Wird die Ableitung der Funktion R(t) in einem Diagramm gegen die Funktion R(t) aufgetragen, so wird, wenn die aufgetragenen Werte z. B. mit einer linearen Regression genähert werden, eine Gerade erhalten, deren y-Achsenabschnitt gerade der initialen Rate entspricht, die sich auch, und in Übereinstimmung mit der Gleichung (1), aus dem absoluten Wert der Geradensteigung (-k'on) und dem x-Achsenabschnitt R'_{eq} ergibt.

Zur Vereinfachung der Auswertung wird angenommen, daß die Reaktionen unter idealen Bedingungen ablaufen, d. h., daß insbeondere keine Analytverarmung auftritt. Dies kann z. B. bei Biosensoren mit Durchflußsystemen angenähert werden, indem ein stetiger Durchsatz der Lösung in der Meßkammer erfolgt. damit vereinfachen sich die Näherungsfunktionen, da z. B. eine Exponentialfunktion erster Ordnung für den Verlauf der Funktion R angenommen werden kann.

Gemäß obenstehender Formel (3) zur Bestimmung der Assoziationsgeschwindigkeitskonstante kann, wenn die anderen Größen bekannt sind, auch die Anfangskonzentration $c_0(A)$ einer neu in eine Meßkammer eingebrachten Lösung bestimmt werden. Dazu kann z. B. in einer vorhergehenden Versuchsreihe mit bekannten $c_0(A)$-Werten aus den ermittelten initialen Raten eine Kalibrationsgerade erstellt werden gemäß initiale Rate = $f(c_0(A))$; der unbekannte $c_0(A)$-Wert einer Probe kann dann mit der aus den Meßwerten ermittelten initialen Rate aus der Kalibrationsgeraden berechnet werden.

Ebenso ist es, besonders unter Verwendung von Küvettensystemen, möglich, daß aus den aus einem Assoziationsvorgang erhaltenen Werten die Dissoziationsgeschwindigkeitskonstante erhalten wird und umgekehrt. Dies beruht

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darauf, daß z. B. bei einer Assoziation im ersten Moment, zu dem die Reaktion beginnt, noch keine Dissoziation erfolgt. Da die Funktion R aber einen Gleichgewichtswert annimmt, bei dem Assoziation und Dissoziation im Gleichgewicht stehen, enthält dieser Wert also auch Informationen über k_{diss}. Die Umkehrung dieses Ansatzes führt zu einem analogen Ergebnis für die Dissoziation. Die entsprechenden Formeln lauten:

$$k_{ass} = \frac{\frac{(dR_{diss}/dt)_{t=0}}{\Delta R_{eq-st\_diss}}}{\left(\frac{c_{eq'\_diss}(A)}{R_{eq'\_diss}}\right)_{c_{eq'\_diss}=0}} \cdot R_{max} \qquad (8)$$

wobei ΔReq-st_diss der Differenz zwischen dem Endwert, der idealerweise 0 ist, und den Anfangswerten von R bei der Dissoziation entspricht, $c_{eq'\_diss}$ (A) die Konzentration von A nach der Dissoziation im Gleichgewicht ist und $R_{eq'\_diss}$ der Gleichgewichtswert von R nach der Dissoziation ist, und in den Nenner der Grenzwert für $c_{eq'\_diss} = 0$ eingesetzt wird, und:

$$k_{diss} = \frac{\frac{(dR_{ass}/dt)_{t=0}}{c_0(A)}}{\left(\frac{R_{eq'\_ass}}{c_{eq'\_ass}(A)}\right)_{R_{eq'\_ass}=0}} \qquad (9)$$

mit den entsprechenden Werten für die Assoziation.

Ebenso kann das Verfahren zur Auswertung bei anderen Affinitätsbiosensoren oder Array-Systemen in Lösung angewendet werden, sowie bei zeitaufgelösten ELISA- oder RIA-Techniken oder anderen festphasengestützten Systemen, die dem Stand der Technik entnehmbar sind.

Zur Realisierung einer ständig die Meßkammer durchströmenden Lösung wird in einem Küvettensystem die Konzentration der Lösung durch ständige Zugabe und Entnahme eines Teils der Lösung konstant gehalten. Dazu sind z. B. zwei Kanülen mit Lösungen vorhanden, mit denen entweder ständig frische Lösung in der Meßkammer umgewälzt wird, oder mit denen ein Teil der Lösung ständig entnommen und wieder zugeführt wird, um ein Durchflußsystem mit konstanter Konzentration zu simulieren.

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In diesem Fall, wie auch in idealen Durchfluß-Systemen mit konstanter Konzentration der Lösung in der Meßzelle, wird die Funktion R durch eine Exponentialfunktion erster Ordnung angenähert und es gilt folgende Beziehung:

$$k'_{on,i} = k_{ass} * c_{0,i}(A) + k_{diss} \quad (10)$$

wobei der Index i die i-te schrittweise Änderung der Konzentration in einem Mehrschritt-Experiment angibt. Nach diesem Verfahren lassen sich beide Geschwindigkeitskonstanten aus derselben Beziehung ermitteln.

Für ideale Durchfluß-Systeme mit konstant gehaltener Analyt-Startkonzentration während einer Assoziationsphase ist als bekannt gezeigt worden, daß die Startkonzentration genau dann gleich der Dissoziations-Gleichgewichtskonstante $K_D = k_{diss}/k_{ass}$ ist, wenn mit dieser Konzentration die Hälfte der immobilisierten Ligandenmoleküle mit Analytmolekülen gebunden werden, d.h. ½ R_{max} erreicht wird. Für diesen Fall läßt sich darüber hinaus aber zeigen, daß die Assoziationsphase bis zur Erreichung des Gleichgewichtswertes gerade halb so lange dauert wie die sich anschließende Dissoziationsphase, um wieder auf den Ausgangswert Null zurückzukehren. Bei höheren Analyt-Startkonzentrationen verläuft die Assoziation schneller. Unter dieser Voraussetzung schneidet die Dissoziationskurve, wenn sie mit der vorausgelaufenen Assoziationskurve überlagert wird, diese Assoziationskurve exakt im Goldenen Schnitt des Assoziations-Gleichgewichtswertes, was dem Wert 0,618...* R_{eq_ass} entspricht. D.h. durch die Applikation verschiedener Analyt-Startkonzentrationen und eine darauf folgende Regression auf den oben beschriebenen Schnittmeßwert läßt sich der K_D-Wert extrapolieren. Umgekehrt muß eine Analyt-Startkonzentrationen, numerisch im K_D-Wert appliziert, den oben beschriebenen Schnittmeßwert ergeben; andernfalls verläuft die aufgezeichnete Interaktion nicht ideal. Prinzipiell erlaubt die Multi-Schritt-Kinetik einen analogen Ansatz.

Generell, insbesonders für Küvettensysteme mit variabler Analytkonzentration in der Meßzelle während eines Meßschrittes, gelten desweiteren die folgenden Beziehungen:

$$k'_{on} = k_{ass} * c'_{eqass}(A) + k'_{offmax} \quad (11)$$

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mit der Geradensteigung k_{ass}, wobei $c'_{eqass}(A)$ die Analytgleichgewichtskonzentration nach der Assoziation und k'_{offmax} der Maximalwert des Koeffizienten der Exponentialfunktion bei der Dissoziation ist; und

$$k'_{off} = k_{diss} * (-R_{stdiss})/(R'_{eq} - R_{stdiss}) \quad (12)$$

mit der Geradensteigung k_{diss}, wobei der Index stdiss den Startwert bei der Dissoziation bedeutet.

c) Ausführungsbeispiele des Biosensors

Zur Durchführung des Verfahrens wird ein an sich bekannter Biosensor verwendet, der mit einer Sensoroberfläche ausgestattet ist, an der die Liganden immobilisiert d. h. gebunden sind. Diese Bindung kann z. B. wie im Stand der Technik bekannt über eine chemische Bindung des Liganden oder mit einem auf den Liganden abgestimmten Rezeptor erfolgen. Über der Sensoroberfläche ist eine Meßkammer oder Küvette ausgebildet, die mit einer Lösung des Analyten beaufschlagbar ist. Zur Erfassung der Komplexbildung, d. h. der Bindung des Analyten am Liganden, sowie deren zeitlicher Verlauf können verschiedene Meßverfahren verwendet werden, z. B. die Erfassung des Reflexionsvermögens der Rückseite des Sensors, das sich mit dem Grad der Komplexbildung c(LA) ändert. Zum Einbringen der Lösung in die Meßkammer dient eine Kanüle, die mit der Meßkammer verbunden ist und entweder die Lösung zugibt und/oder absaugt. Die Meßkammer kann auch mit zwei getrennten Kanülen zur Zugabe/Herausnahme ausgestattet sein. Auch die Beaufschlagung mit einer Spülflüssigkeit über eine der Kanülen ist möglich. Zur Durchmischung der Lösung in der Meßkammer, um Konzentrationsgradienten in der Lösung während der Reaktion zu vermeiden, ist eine Vorrichtung zur Durchmischung der Lösung vorhanden, dies kann z. B. ein Vibrationsrührer sein. Die Ausstattung des Biosensors mit einer vorzugsweise elektronischen Steuerung sowie geeigneten Meßgeräten nebst Datenerfassungssoftware ist dem Stand der Technik entnehmbar.

Erfindungsgemäß ist der Biosensor derart ausgebildet, daß die Lösung in der Meßkammer zumindest teilweise instantan mehrmals mit Lösungen unterschiedlicher Analytkonzentration austauschbar ist, wobei das Flüssigkeitsvolumen im wesentlichen konstant gehalten wird. Das bedeutet, daß zumindest ein Teil der

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Lösung in der Meßkammer, oder die gesamte Lösung, entnommen und im wesentlichen gleichzeitig eine neue Lösung mit einer anderen Konzentration zugegeben wird. Durch den Austausch zumindest eines Teils der Lösung durch eine andere Lösung mit gewünschter Konzentration wird die Gesamtkonzentration in der Meßkammer in definierter Weise verändert. Die Art des Austauschens der Flüssigkeit kann im Rahmen der Erfindung beliebig ausgeführt oder wie im folgenden dargelegt vorgenommen werden. In einem Durchflußsystem wird die komplette Lösung in der Meßkammer ausgetauscht, bei einem Küvettensystem zumindest ein Teil davon. Mit dem mehrmaligen Austauschen der Lösung wird eine schrittweise Änderung der Konzentration in der Meßkammer ermöglicht, um das erfindungsgemäße Verfahren durchführen zu können.

Der Vorteil des erfindungsgemäßen Biosensors besteht darin, daß z.B. bei Küvettensystemen das Volumen der Lösung im wesentlichen konstant gehalten ist und damit die Anzahl der Parameter bei der mathematischen Auswertung der Meßergebnisse verringert wird. Der instantane Austausch der Lösungen führt zudem besonders bei Durchflußsystemen zu scharfen Übergängen zwischen einzelnen Assoziations- bzw. Dissoziationsphasen, was für die Bestimmung der initialen Raten notwendig ist.

Vorzugsweise erfolgt der Austausch zumindest eines Teils der Lösung in der Meßkammer über eine oder über mehrere Kanülen gleichzeitig. Insbesondere wird die Lösung mit einer Kanüle entnommen und mit einer anderen Kanüle eine neue Lösung mit einer beliebig wählbaren Konzentration zugegeben.

Werden die Kanülen getrennt voneinander an der Meßkammer angeordnet, z. B. einander gegenüberliegend, kann die Entnahme und Zuführung auch gleichzeitig erfolgen, wobei zum vollständigen Austausch der Lösung in einem Küvettensystem die Entnahme bzw. Zuführung derart erfolgt, daß die Meßkammer durchgespült wird, um das Verbleiben unerwünschter Rückstände zu vermeiden. Die Kanülen können auch koaxial angeordnet werden, d. h. eine verläuft in der anderen, wobei die innere entweder die Lösung entnimmt oder zuführt.

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Zur Förderung der Lösungen durch die Kanülen in die Meßkammer hinein oder heraus sind Pumpen vorgesehen, mit denen die Menge der jeweils geförderten Lösung dosierbar ist. Dazu werden sogenannte Mikropumpen verwendet, die eine Dosierung mit der gewünschten Genauigkeit erlauben, um wohl definierte Mengen an Lösung der Meßkammer zu entnehmen oder ihr zuzuführen. Diese Pumpen sind unter anderem als Schlauchpumpen, Spritzenpumpen oder piezoelektrische Pumpen auslegbar, wobei die Betätigung mit Stellmotoren, elektrischen Antrieben etc. erfolgt und sie ebenfalls elektronisch angesteuert sind.

Die Durchmischung der Lösung in der Meßkammer kann auch dadurch erfolgen, daß mit einer oder mehreren Kanülen ein Teil der Lösung angesaugt und sofort wieder zurückgepumpt wird, so daß die Lösung ständig umgewälzt und durchmischt wird. Vorzugsweise erfolgt eine Umwälzung mit mindestens 1 µl/min, um eine homogene Konzentration der Lösung in der Meßkammer während der Komplexbildung zu gewährleisten.

Eine weitere vorteilhafte Ausgestaltung der Erfindung besteht darin, daß an der Meßkammer in bestimmter Weise zwei oder mehr sogenannte Probenschleifen beliebigen Volumens vorhanden sind, die manuell und/oder mittels eines Probengebers automatisch befüllt werden können. Vorzugsweise sind die Probenschleifen parallel angeordnet zwischen zwei Mehrfach-Wahlventilen, wobei diese gesamte Anordnung über ein Schaltventil in den Durchfluß einschaltbar ist. Die Probenschleifen können so außerhalb des Durchflusses einerseits über die Wahlventile unabhängig voneinander und andererseits über das Schaltventil von ihrem Ausflußende her ganz oder nur teilweise befüllt werden, um z. B. Analytlösung zu sparen. Nach der Befüllung steht an der Schnittstelle zum Durchfluß am Schaltventil ohne andere Zwischenvolumina, d.h. ohne sogenanntes Totvolumen, die Lösung der zuletzt befüllten Probenschleife an. Das bedeutet, daß die Meßkammer mit mehreren unterschiedlichen Lösungen aus den einzelnen Probenschleifen über die Wahlventile nacheinander, unabhängig voneinander und totvolumenfrei im Durchfluß beaufschlagbar ist, und ohne daß eine beliebige Probenschleife vollständig ausgespült bzw. der Fluß durch diese in eine andere Schleife geleitet werden muß. Damit ist eine variable, instantane, praktisch durchmischungsfreie und probensparende schrittweise Konzentrationsänderung gewährleistet. Zudem kann

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jeweils die Konzentration des Analyten in der Lösung in der Meßkammer in erster Näherung konstant gehalten werden, um eine Verarmung zu verhindern. Vorzugsweise ist in jeder Probenschleife eine Lösung unterschiedlicher Konzentration vorhanden, um Messungen mit unterschiedlichen Konzentrationen auch unmittelbar hintereinander ausführen zu können oder durch die gezielte Ansteuerung von Probenschleifen eine Zwischenkonzentration zwischen den Werten in den einzelnen Probenschleifen zu erhalten. Die Zahl der Probenschleifen ist praktisch zwar nicht begrenzt; zwecks höherer Flexibilität kann jedoch die o.g. gesamte Anordnung mitsamt des Schaltventils z.B. zwischen zwei im Durchfluß positionierten Zweifach-Wahlventilen ohne weiteres dupliziert werden. So kann der eine Probenschleifensatz abgearbeitet werden, während der andere Satz befüllt wird und praktisch totvolumenfrei in den Fluß geschaltet werden kann, noch bevor die letzte Schleife des ersten Satzes vollständig ausgespült worden ist.

Eine Ausgestaltungsmöglichkeit der Probenaufgabe z.B. in einem Durchfluß-Meßsystem ist in Figur 1 wiedergegeben, die im folgenden beschrieben ist.

Mit Ausnahme der Pumpe 30, die z.B. eine Spritzenpumpe oder eine peristaltische Pumpe sein kann, symbolisieren alle übrigen Kreise Ventile, die wie hier beschrieben ausgelegt sein können. Die Ventile 1 und 11 sind Schaltventile, mit denen je nach Schaltung der beiden Schaltpositionen die entsprechenden Ein- bzw. Auslässe der Ventile verbunden werden; wobei in Figur 1 die aktuellen Verbindungen fett gezeichnet dargestellt sind und die alternativen Verbindungen gestrichelt. Die Ventile 4, 8, 14, 18, 28 und wenn gewünscht auch 21, 22, 33 und 39 sind Wahlventile, mit denen ein zentraler Anschluß, der mit normaler Linienbreite dargestellt ist, mit einem von beliebig vielen anderen Anschlüssen verbunden werden kann. Die Ventile 21, 22, 33 und 39 sind hier beispielhaft als Zweifach-Wahlventile gezeigt. Alle Ventilpositionen können manuell oder automatisch und auch vorprogrammiert mit z.B. elektrischem oder Druck-Antrieb gestellt werden. Alle Ventile sind vorzugsweise im Typ der in der Chromatographie verwendeten Drehventile ausgelegt, z.B. in volumenarmer Mikroausführung, können aber auch z.B. als Magnetventile ausgelegt werden. 36 ist eine beliebig dimensionierbare Temperierungs-Kapillare, 37 ist die Interaktions-Detektionseinheit, kurz Detektor, z.B. vom Typ eines Affinitäts-Biosensors. Alle mit normaler durchgezogener Linie angezeigten Verbindungen

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bestehen aus Kapillaren, gefertigt z.B. aus Tefzel, PE, PEEK oder Edelstahl, mit beliebigen Dimensionen bezüglich Innendurchmesser / Außendurchmesser / Länge, z.B. 1 mm / $^{1}/_{16}$ " / 0,5 m für die Verbindung 31. Eine beliebige Lösung, aus Vorräten 25 bis 27 oder folgenden n ausgewählt, wird mittels der Pumpe 30 über Ventil 33 entweder für Waschzwecke in den Abfall 43 oder durch den Detektor 37 gepumpt und über Ventil 39 in den Abfall 43 geleitet. Aus dem Detektor 37 ausgespülte Lösungen von weiterem Interesse können über das Ventil 39 im Sammler 41 gesammelt, in mehreren Gefäßen n fraktioniert aufgefangen oder mit anderen Techniken, z.B. Massenspektrometrie, direkt gekoppelt werden. Die in C enthaltenen Komponenten, mit Strich-Punkt umrahmt, können gemeinsam temperiert werden, z.B. mittels eines hier nicht gezeigten Peltier-Elementes.

Das erfindungsgemäß Besondere dieses Teils ist die Art der Probenaufgabe, die für eine repetitive Probenaufgabe und besonders für die Multischritt-Kinetik von Vorteil ist. Die Komponenten 1 bis 10 sind als Probenaufgabe A zusammengefaßt und über Ventil 1 entweder direkt in Verbindung 31 oder beliebig oft dupliziert über die Ventile 21 und 22 in die Verbindungen 31/32 einschaltbar; dargestellt ist hier eine Duplizierung von A, mit analoger Probenaufgabe B zusammengefaßt. In A befindet sich Ventil 1 in der Position Ladung für die Probenschleifen 5 bis 7, die über Probeneinlaß 2 entweder manuell oder mittels eines Probengebers automatisch mit beliebigen Lösungen, d.h. auch mit Analytlösungen jeweils fallender Konzentration, befüllt werden können, wobei die verdrängten Lösungen in Abfall 10 gespült werden. Minimal-Volumina, z.B. für Mikro- oder Test-Analysen, lassen sich realisieren, indem lediglich Verbindung 3 teilweise befüllt wird. Theoretisch unbegrenzte Maximal-Volumina einer Lösung, z.B. für sogenannte Präparationen mit besonders großen Sensoroberflächen, lassen sich realisieren, indem alle Probenschleifen mit derselben Lösung befüllt werden.

Nach jeweils synchronem Schalten der Ventile 4 und 8 können die Probenschleifen 5 bis 7 nacheinander und unabhängig voneinander sowie jeweils vollständig oder zwecks Proben-Ersparnis nur teilweise befüllt werden, und zwar in jedem Fall "rückwärts", d.h. von ihrer späteren Auslaßseite her. Damit ist gewährleistet, daß, nach dem Schalten von Ventil 1 in die Position Fluß, an die Verbindung 24a totvolumenfrei die Lösung der letzten Befüllung ansteht und sich daran nach zeitlich

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beliebigem, schrittweisen und jeweils synchronen Schalten der Ventile 4 und 8 übergangslos und unabhängig voneinander die Lösungen der anderen Probenschleifen auf den Detektor 37 beaufschlagen lassen. Diese Beaufschlagung ist in B gezeigt, wo sich die Komponenten11 bis 20 spiegelbildlich zu A in Position Fluß befinden. Durch bevorzugt synchrones Schalten der Ventile 1, 11, 21 und 22 können A und B wechselseitig in Position Fluß bzw. Ladung umgeschaltet werden, so daß sich A im Fluß befindet, während B befüllt wird bzw. umgekehrt. Alle Verbindungskapillaren und Schleifen... 3, 9, 13, 19, 23a,b, 24a,b, 29, 31, 32, 34, 35, 38, 40, 42 und 5, 6, 7, 15, 16, 17 können je nach Anwendung in ihren Dimensionen besonders optimiert ausgelegt werden. In 24a und/oder 24b befindet sich normalerweise Spül- oder sogenannter Lauf-Puffer vom letzten Spül- oder Analysengang. Bei Bedarf können diese Verbindungskapillaren auch höhervolumiger ausgestaltet werden, um nach der Beaufschlagung der Lösungen aus B bzw. A und der gleichzeitigen Umschaltung der Lösungen aus A bzw. B in die Position Fluß zunächst eine Wasch- oder Dissoziationsphase in dem Detektor 37 durchzuführen, deren Dauer durch das Volumen von 24a bzw. 24b und der Flußrate bestimmt wird, was je nach Anwendung von Interesse sein kann. Die Anordnung ist darüber hinaus offen für die Positionierung weiterer Ventile und/oder Pumpen zwecks Waschung / Spülung beliebiger Abschnitte oder zwecks Einbringung weiterer Lösungen. So können z.B. B und/oder weitere Duplikate von B über Ventil 11 wahlweise z.B. in die Verbindungen 3, 5, 6, 7 oder 32 positioniert werden.

Desweiteren wird durch ein häufiges Schalten bestimmter, je nach Anwendung unterschiedlicher Ventile, eine Mischung von Lösungen ermöglicht, bei Bedarf auch durch Installation eines Flußteilers z.B. in Verbindung 31 anstelle von oder zusätzlich zu Ventil 21 und/oder einer sogenannten Mikro-Mischkammer z.B. in Verbindung 32 anstelle von oder zusätzlich zu Ventil 22. Diese Anordnung ergibt insgesamt eine größtmögliche Flexibilität der Lösungs-Beaufschlagung auf die Meßzelle. Ein Mischen der Vorräte 25 bis 27 oder mehr kann auch durch häufiges Schalten von Ventil 28 erreicht werden. Für Lösungen, die temperatursensitiv sind, können alle oder nur Teile der in C enthaltenen Komponenten, exklusive z.B. der in D enthaltenen mit Strich-Punkt-Punkt umrahmten Komponenten, gesondert temperiert werden, während die z.B. in D enthaltenen Komponenten ihrerseits gesondert auf eine Arbeitstemperatur temperiert werden.

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Index-Liste

ass	Assoziation
diss	Dissoziation
i	i-ter Schritt
st	jeweiliger Startwert einer Assoziation oder Dissoziation
eq	jeweiliger Gleichgewichtswert einer Assoziation oder Dissoziation
net	Netto
k_{on}	Exponentialkoeffizient bei Assoziation
k_{off}	Exponentialkoeffizient bei Dissoziation

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Patentansprüche

1. Analytisches Mess- und Auswerteverfahren für molekulare Interaktionen von chemischen Gleichgewichts-Bindungspaaren oder –Bindungssystemen, wobei Assoziations- und/oder Dissoziationskurven aufgenommen werden, und wobei ein erster Bindungspartner („Ligand") mit einem in Lösung befindlichen zweiten Bindungsparter („Analyt") versetzt wird, der mit dem Liganden reversibel unter Komplexbildung reagiert, die zeitliche Entwicklung der Komplexbildung sowie deren Änderung (c_t (LA); $\frac{dc_t(LA)}{dt}$) erfaßt und der Reaktionsverlauf in 1. Näherung mit einer exponentiell verlaufenden Funktion R(t) mit einem Exponenten k'_{on} oder k'_{off} ,die auf einen Gleichgewichtswert R'_{eq} zuläuft, beschrieben wird, wobei $\frac{dR(t)}{dt}$ die Assoziations- oder Dissoziationsrate des Analyten am Liganden angibt,
dadurch gekennzeichnet, daß
zu bestimmten Zeitpunkten t_i die Konzentration c_{ti} (A) einmal oder mehrmals schrittweise erhöht oder erniedrigt wird, und aus den jeweiligen Differenzen zwischen den Gleichgewichtswerten $R'_{eq,ass,i}$ und $R'_{st,ass,i}$ oder den Differenzen zwischen den Werten $R'_{eq,diss,i}$ und $R'_{st,diss,i}$ und den jeweiligen Exponenten $k'_{on,i}$ oder $k'_{off,i}$ die jeweiligen initialen Assoziations- oder Dissoziationsraten und aus diesen Raten die jeweilige, Assoziations- oder Dissoziationsgeschwindigkeitskonstante k_{ass} oder k_{diss} ermittelt werden.

2. Verfahren nach Anspruch 1,
dadurch gekennzeichnet, daß
die Änderung der Konzentration zur Halbwertszeit der Funktion $R_i(t)$ erfolgt.

3. Verfahren nach Anspruch 1 oder 2,
dadurch gekennzeichnet, daß
die initiale Assoziations- oder Dissoziationsrate aus einer linearen Beziehung zwischen $\frac{dR(t)}{dt}$ und R(t) oder (R(t)- $R_{st}(t)$) ermittelt wird.

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4. Verfahren nach einem der vorhergehenden Ansprüche,
dadurch gekennzeichnet, daß
die Ermittlung der initialen Raten unter der Annahme idealer Bedingungen erfolgt, insbesondere der Annahme, daß bei der Assoziation keine Analytverarmung und bei der Dissoziation keine Analytanreicherung erfolgt.

5. Verfahren nach einem der vorhergehenden Ansprüche,
dadurch gekennzeichnet, daß
die unbekannte Analytkonzentration $c_0(A)$ zu Beginn der Reaktion ermittelt wird.

6. Verfahren nach einem der vorhergehenden Ansprüche,
dadurch gekennzeichnet, daß
die Dissoziationsgeschwindigkeitskonstante k_{diss} aus den initialen Assoziationsraten und den jeweiligen Assoziationsgleichgewichtswerten der Funktion R(t) und die Assoziationsgeschwindigkeitskonstante k_{ass} aus den initialen Dissoziationsraten und den jeweiligen Dissoziationsgleichgewichtswerten der Funktion R(t) erhalten wird.

7. Verfahren nach einem der vorhergehenden Ansprüche
dadurch gekennzeichnet, daß
das Verfahren mit an sich bekannten Affinitätsbiosensoren und/oder Array-Systemen in Lösung und/oder zeitaufgelösten ELISA – oder RIA-Techniken oder anderen festphasengestützten Systemen durchgeführt wird.

8. Verfahren nach einem der vorhergehenden Ansprüche
dadurch gekennzeichnet, daß
in einem Küvettensystem die Konzentration der Lösung in der Meßkammer durch kontinuierliche Entnahme eines Teils der Lösung und Zugabe einer Lösung mit einer entsprechenden Konzentration über eine oder mehrere Kanülen während des Assoziations- oder Dissoziationsvorgangs im wesentlichen konstant gehalten wird.

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9. Verfahren nach nach einem der vorhergehenden Ansprüche
dadurch gekennzeichnet, daß
im Falle von jeweils im wesentlichen konstanter Analytkonzentration bei Mehr-Schritt-Analysen die Werte für k_{ass} und k_{diss} aus der Beziehung zwischen k'_{on} und der Analytstartkonzentration ermittelt werden.

10. Verfahren nach einem der vorhergehenden Ansprüche
dadurch gekennzeichnet, daß
bei konstanter Konzentration der Lösung in der Meßkammer derjenige $c_0(A)$-Wert bestimmt wird, bei dem sich die Assoziationskurve oder ein Abschnitt davon mit der Dissoziationskurve oder einem Abschnitt davon im goldenen Schnitt des gesamten oder des jeweiligen Teilwerts des Assoziationswertes schneidet.

11. Verfahren nach Anspruch 10,
dadurch gekennzeichnet, daß
die Konzentration in der Meßkammer mehrmals schrittweise geändert wird, und die jeweiligen Startkonzentrationen des Analyten ermittelt werden.

12. Verfahren nach einem der vorhergehenden Ansprüche
dadurch gekennzeichnet, daß
die Assoziationsgeschwindigkeitskonstante k_{ass} aus der Beziehung zwischen k'_{on} und der Analytgleichgewichtskonzentration und die Dissoziationsgeschwindigkeits-konstante k_{diss} aus der Beziehung zwischen k'_{off} und dem Gleichgewichtswert der Funktion R(t) bei der Dissoziation ermittelt wird.

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13. Vorrichtung zur Durchführung des Verfahrens nach einem der vorhergehenden Ansprüche, mit einer Sensoroberfläche zur Bindung des in Lösung befindlichen Analyten am Liganden, mit einer Meßkammer auf der Sensoroberfläche, die mit dem Analyt in Lösung befüllt ist, mit jeweils einer Kanüle zur Zugabe und/oder Herausnahme der Lösung und/oder einer Spülflüssigkeit, mit einer Vorrichtung zur Durchmischung der Lösung in der Meßkammer

dadurch gekennzeichnet, daß

die Lösungen in der Meßkammer zumindest teilweise in vorbestimmter Menge instantan mehrmals gegen Lösungen anderer Konzentration austauschbar sind, wobei das Volumen der Lösung im wesentlichen konstant gehalten ist.

14. Vorrichtung nach Anspruch 13,

dadurch gekennzeichnet, daß

der Austausch der Lösungen in der Meßkammer über eine oder mehrere Kanülen erfolgt, insbesondere eine Kanüle die Lösung entnimmt und eine andere Kanüle eine neue Lösung zugibt.

15. Vorrichtung nach Anspruch 13 oder 14,

dadurch gekennzeichnet, daß

die Kanülen getrennt an der Meßkammer angeordnet sind oder die eine koaxial innerhalb der anderen.

16. Vorrichtung nach einem der Ansprüche 13 bis 15,

dadurch gekennzeichnet, daß

eine oder mehrere Pumpen zur Dosierung der Menge der auszutauschenden Lösungen an den Kanülen vorgesehen sind, insbesondere Schlauchpumpen, Spritzenpumpen oder piezoelektrische Pumpen.

17. Vorrichtung nach einem der Ansprüche 13 bis 16,

dadurch gekennzeichnet, daß

über mehrere Kanülen die Lösung in der Meßkammer durch zumindest teilweise Absaugung und wieder Zugabe in die Meßkammer durchmischt wird, insbesondere mindestens 1µl/min umgewälzt wird.

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18. Vorrichtung nach einem der Ansprüche 13 bis 17,
dadurch gekennzeichnet, daß
mehrere Probenschleifen vorhanden sind und ihr Inhalt entgegen seiner Befüllrichtung und unabhängig in die Meßkammer einbringbar ist, insbesondere daß die Probenschleifen mit jeweils unterschiedlichen Lösungen befüllt sind.

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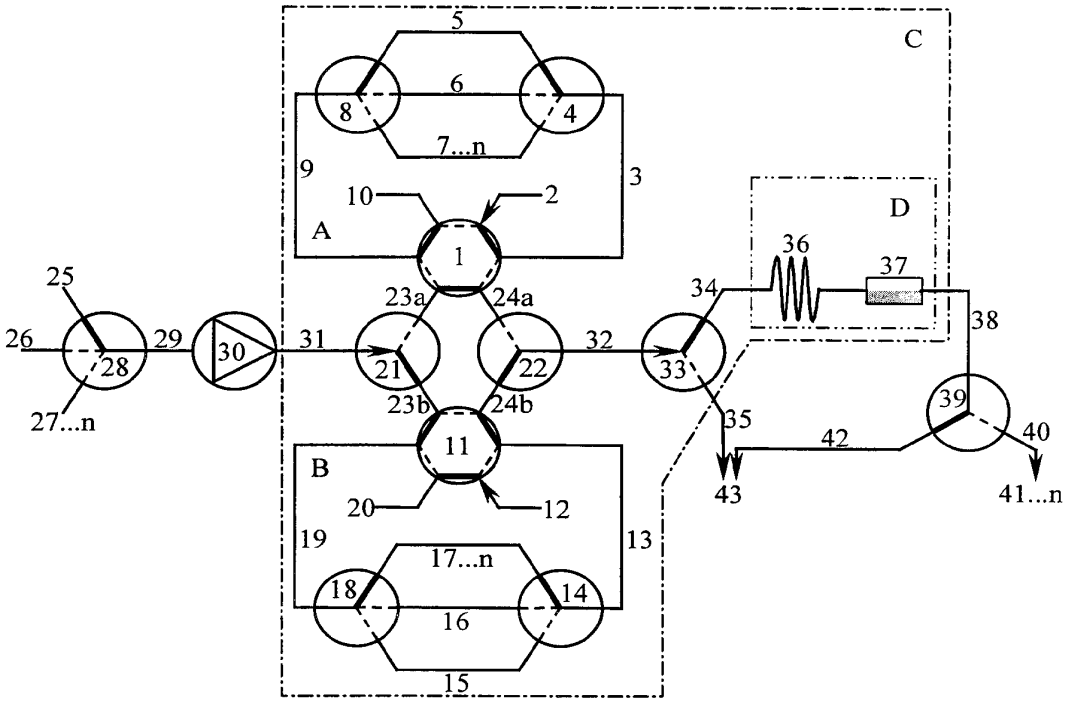

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Fig. 1

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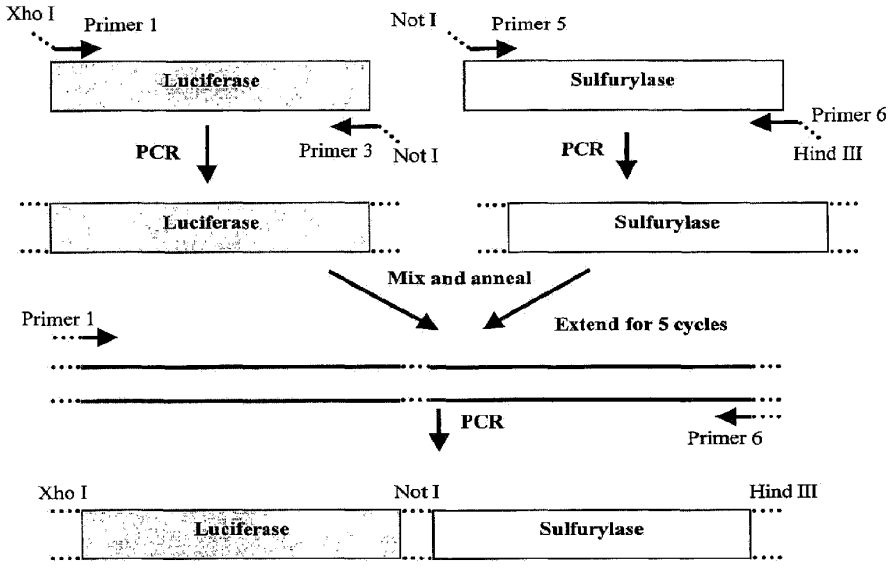
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[Continued on next page]

(54) Title: NOVEL SULFURYLASE-LUCIFERASE FUSION PROTEINS AND THERMOSTABLE SULFURYLASE



(57) Abstract: The present invention relates to the field of DNA recombinant technology. More specifically, this invention relates to fusion proteins comprising an ATP generating polypeptide joined to a polypeptide that converts ATP into a detectable entity. Accordingly, this invention focuses on sulfurylase-luciferase fusion proteins. This invention also relates to pharmaceutical compositions containing the fusion proteins and methods for using them.

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NOVEL SULFURYLASE-LUCIFERASE FUSION PROTEINS AND THERMOSTABLE SULFURYLASE

FIELD OF THE INVENTION

The invention relates generally to fusion proteins that are useful as reporter proteins, in particular to fusion proteins of ATP sulfurylase and luciferase which are utilized to achieve an efficient conversion of pyrophosphate (PPi) to light. This invention also relates to a novel thermostable sulfurylase which can be used in the detection of inorganic pyrophosphate, particularly in the sequencing of nucleic acid.

BACKGROUND OF THE INVENTION

ATP sulfurylase has been identified as being involved in sulfur metabolism. It catalyzes the initial reaction in the metabolism of inorganic sulfate (SO_4^{-2}); see *e.g.*, Robbins and Lipmann, 1958. *J. Biol. Chem.* **233**: 686-690; Hawes and Nicholas, 1973. *Biochem. J.* **133**: 541-550). In this reaction SO_4^{-2} is activated to adenosine 5'-phosphosulfate (APS). ATP sulfurylase is also commonly used in pyrophosphate sequencing methods. In order to convert pyrophosphate (PPi) generated from the addition of dNMP to a growing DNA chain to light, PPi must first be converted to ATP by ATP sulfurylase.

ATP produced by an ATP sulfurylase can also be hydrolyzed using enzymatic reactions to generate light. Light-emitting chemical reactions (*i.e.*, chemiluminescence) and biological reactions (*i.e.*, bioluminescence) are widely used in analytical biochemistry for sensitive measurements of various metabolites. In bioluminescent reactions, the chemical reaction that leads to the emission of light is enzyme-catalyzed. For example, the luciferin-luciferase system allows for specific assay of ATP. Thus, both ATP generating enzymes, such as ATP sulfurylase, and light emitting enzymes, such as luciferase, could be useful in a number of different assays for the detection and/or concentration of specific substances in fluids and gases. Since high physical and chemical stability is sometimes required for enzymes involved in sequencing reactions, a thermostable enzyme is desirable.

Because the product of the sulfurylase reaction is consumed by luciferase, proximity between these two enzymes by covalently linking the two enzymes in the form of a fusion protein would provide for a more efficient use of the substrate. Substrate channeling is a

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phenomenon in which substrates are efficiently delivered from enzyme to enzyme without equilibration with other pools of the same substrates. In effect, this creates local pools of metabolites at high concentrations relative to those found in other areas of the cell. Therefore, a fusion of an ATP generating polypeptide and an ATP converting peptide could benefit from the phenomenon of substrate channeling and would reduce production costs and increase the number of enzymatic reactions that occur during a given time period.

All patents and publications cited throughout the specification are hereby incorporated by reference into this specification in their entirety in order to more fully describe the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

The invention provides a fusion protein comprising an ATP generating polypeptide bound to a polypeptide which converts ATP into an entity which is detectable. In one aspect, the invention provides a fusion protein comprising a sulfurylase polypeptide bound to a luciferase polypeptide. This invention provides a nucleic acid that comprises an open reading frame that encodes a novel thermostable sulfurylase polypeptide. In a further aspect, the invention provides for a fusion protein comprising a thermostable sulfurylase joined to at least one affinity tag.

In another aspect, the invention provides a recombinant polynucleotide that comprises a coding sequence for a fusion protein having a sulfurylase poylpeptide sequence joined to a luciferase polypeptide sequence. In a further aspect, the invention provides an expression vector for expressing a fusion protein. The expression vector comprises a coding sequence for a fusion protein having: (i) a regulatory sequence, (ii) a first polypeptide sequence of an ATP generating polypeptide and (iii) a second polypeptide sequence that converts ATP to an entity which is detectable. In an additional embodiment, the fusion protein comprises a sulfurylase polypeptide and a luciferase polypeptide. In another aspect, the invention provides a transformed host cell which comprises the expression vector. In an additional aspect, the invention provides a fusion protein bound to a mobile support. The invention also includes a kit comprising a sulfurylase-luciferase fusion protein expression vector.

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The invention also includes a method for determining the nucleic acid sequence in a template nucleic acid polymer, comprising: (a) introducing the template nucleic acid polymer into a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added; (b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released; (c) separately recovering each of the feedstocks from the polymerization environment; and (d) measuring the amount of PPi with an ATP generating polypeptide-ATP converting polypeptide fusion protein in each of the recovered feedstocks to determine the identity of each nucleotide in the complementary polymer and thus the sequence of the template polymer. In one embodiment, the amount of inorganic pyrophosphate is measured by the steps of: (a) adding adenosine-5'-phosphosulfate to the feedstock; (b) combining the recovered feedstock containing adenosine-5'-phosphosulfate with an ATP generating polypeptide-ATP converting polypeptide fusion protein such that any inorganic pyrophosphate in the recovered feedstock and the adenosine-5'-phosphosulfate will react to the form ATP and sulfate; (c) combining the ATP and sulfate-containing feedstock with luciferin in the presence of oxygen such that the ATP is consumed to produced AMP, inorganic pyrophosphate, carbon dioxide and light; and (d) measuring the amount of light produced.

In another aspect, the invention includes a method wherein each feedstock comprises adenosine-5'-phosphosulfate and luciferin in addition to the selected nucleotide base, and the amount of inorganic pyrophosphate is determined by reacting the inorganic pyrophosphate feedstock with an ATP generating polypeptide-ATP converting polypeptide fusion protein thereby producing light in an amount proportional to the amount of inorganic pyrophosphate, and measuring the amount of light produced.

In another aspect, the invention provides a method for sequencing a nucleic acid, the method comprising: (a) providing one or more nucleic acid anchor primers; (b) providing a plurality of single-stranded circular nucleic acid templates disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the

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reaction chambers have a center to center spacing of between 5 to 200 μm; (c) annealing an effective amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex; (d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template; (e) annealing an effective amount of a sequencing primer to one or more copies of said covalently linked complementary nucleic acid; (f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a sequencing reaction byproduct; and (g) identifying the sequencing reaction byproduct with the use of a ATP generating polypeptide-ATP converting polypeptide fusion protein, thereby determining the sequence of the nucleic acid.

In one aspect, the invention provides a method for sequencing a nucleic acid, the method comprising: (a) providing at least one nucleic acid anchor primer; (b) providing a plurality of single-stranded circular nucleic acid templates in an array having at least 400,000 discrete reaction sites; (c) annealing a first amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex; (d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template; (e) annealing a second amount of a sequencing primer to one or more copies of the covalently linked complementary nucleic acid; (f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, when the predetermined nucleotide triphosphate is incorporated onto the 3' end of the sequencing primer, to yield a sequencing reaction byproduct; and (g) identifying the sequencing reaction byproduct with the use of a ATP generating polypeptide-ATP converting polypeptide fusion protein, thereby determining the sequence of the nucleic acid at each reaction site that contains a nucleic acid template.

In another aspect, the invention includes a method of determining the base sequence of a plurality of nucleotides on an array, the method comprising the steps of: (a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, (b) adding an activated nucleotide 5'-triphosphate

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precursor of one known nitrogenous base to a reaction mixture in each reaction chamber, each reaction mixture comprising a template-directed nucleotide polymerase and a single-stranded polynucleotide template hybridized to a complementary oligonucleotide primer strand at least one nucleotide residue shorter than the templates to form at least one unpaired nucleotide residue in each template at the 3'-end of the primer strand, under reaction conditions which allow incorporation of the activated nucleoside 5'-triphosphate precursor onto the 3'-end of the primer strands, provided the nitrogenous base of the activated nucleoside 5'-triphosphate precursor is complementary to the nitrogenous base of the unpaired nucleotide residue of the templates; (c) determining whether or not the nucleoside 5'-triphosphate precursor was incorporated into the primer strands through detection of a sequencing byproduct with a ATP generating polypeptide-ATP converting polypeptide fusion protein, thus indicating that the unpaired nucleotide residue of the template has a nitrogenous base composition that is complementary to that of the incorporated nucleoside 5'-triphosphate precursor; and (d) sequentially repeating steps (b) and (c), wherein each sequential repetition adds and, detects the incorporation of one type of activated nucleoside 5'-triphosphate precursor of known nitrogenous base composition; and

(e) determining the base sequence of the unpaired nucleotide residues of the template in each reaction chamber from the sequence of incorporation of said nucleoside precursors.

In one aspect, the invention includes a method for determining the nucleic acid sequence in a template nucleic acid polymer, comprising: (a) introducing a plurality of template nucleic acid polymers into a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, each reaction chamber having a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added; (b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released; (c) detecting the formation of inorganic pyrophosphate with an ATP generating polypeptide-ATP

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converting polypeptide fusion protein to determine the identify of each nucleotide in the complementary polymer and thus the sequence of the template polymer.

In one aspect, the invention provides a method of identifying the base in a target position in a DNA sequence of sample DNA including the steps comprising: (a) disposing sample DNA within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, said DNA being rendered single stranded either before or after being disposed in the reaction chambers, (b) providing an extension primer which hybridizes to said immobilized single-stranded DNA at a position immediately adjacent to said target position; (c) subjecting said immobilized single-stranded DNA to a polymerase reaction in the presence of a predetermined nucleotide triphosphate, wherein if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer then a sequencing reaction byproduct is formed; and (d) identifying the sequencing reaction byproduct with a ATP generating polypeptide-ATP converting polypeptide fusion protein, thereby determining the nucleotide complementary to the base at said target position.

The invention also includes a method of identifying a base at a target position in a sample DNA sequence comprising: (a) providing sample DNA disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, said DNA being rendered single stranded either before or after being disposed in the reaction chambers; (b) providing an extension primer which hybridizes to the sample DNA immediately adjacent to the target position; (c) subjecting the sample DNA sequence and the extension primer to a polymerase reaction in the presence of a nucleotide triphosphate whereby the nucleotide triphosphate will only become incorporated and release pyrophosphate (PPi) if it is complementary to the base in the target position, said nucleotide triphosphate being added either to separate aliquots of sample-primer mixture or successively to the same sample-primer mixture; (d) detecting the release of PPi with an ATP generating polypeptide-ATP converting polypeptide fusion protein to indicate which nucleotide is incorporated.

In one aspect, the invention provides a method of identifying a base at a target position in a single-stranded sample DNA sequence, the method comprising: (a) providing an extension primer which hybridizes to sample DNA immediately adjacent to the target position, said sample DNA disposed within a plurality of cavities on a planar surface, each

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cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 um, said DNA being rendered single stranded either before or after being disposed in the reaction chambers; (b) subjecting the sample DNA and extension primer to a polymerase reaction in the presence of a predetermined deoxynucleotide or dideoxynucleotide whereby the deoxynucleotide or dideoxynucleotide will only become incorporated and release pyrophosphate (PPi) if it is complementary to the base in the target position, said predetermined deoxynucleotides or dideoxynucleotides being added either to separate aliquots of sample-primer mixture or successively to the same sample-primer mixture, (c) detecting any release of PPi with an ATP generating polypeptide-ATP converting polypeptide fusion protein to indicate which deoxynucleotide or dideoxynucleotide is incorporated;characterized in that, the PPi-detection enzyme(s) are included in the polymerase reaction step and in that in place of deoxy- or dideoxy adenosine triphosphate (ATP) a dATP or ddATP analogue is used which is capable of acting as a substrate for a polymerase but incapable of acting as a substrate for a said PPi—detection enzyme.

In another aspect, the invention includes a method of determining the base sequence of a plurality of nucleotides on an array, the method comprising: (a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, (b) converting PPi into light with an ATP generating polypeptide-ATP converting polypeptide fusion protein; (c) detecting the light level emitted from a plurality of reaction sites on respective portions of an optically sensitive device; (d) converting the light impinging upon each of said portions of said optically sensitive device into an electrical signal which is distinguishable from the signals from all of said other regions; (e) determining a light intensity for each of said discrete regions from the corresponding electrical signal; (f) recording the variations of said electrical signals with time.

In one aspect, the invention provides a method for sequencing a nucleic acid, the method comprising:(a) providing one or more nucleic acid anchor primers; (b) providing a plurality of single-stranded circular nucleic acid templates disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm;(c) converting PPi into a detectable entity with the use of an ATP generating polypeptide-ATP converting

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polypeptide fusion protein; (d) detecting the light level emitted from a plurality of reaction sites on respective portions of an optically sensitive device; (e) converting the light impinging upon each of said portions of said optically sensitive device into an electrical signal which is distinguishable from the signals from all of said other regions; (f) determining a light intensity for each of said discrete regions from the corresponding electrical signal; (g) recording the variations of said electrical signals with time.

In another aspect, the invention includes a method for sequencing a nucleic acid, the method comprising: (a) providing at least one nucleic acid anchor primer; (b) providing a plurality of single-stranded circular nucleic acid templates in an array having at least 400,000 discrete reaction sites; (c) converting PPi into a detectable entity with an ATP generating polypeptide-ATP converting polypeptide fusion protein; (d) detecting the light level emitted from a plurality of reaction sites on respective portions of an optically sensitive device; (e) converting the light impinging upon each of said portions of said optically sensitive device into an electrical signal which is distinguishable from the signals from all of said other regions; (f) determining a light intensity for each of said discrete regions from the corresponding electrical signal; (g) recording the variations of said electrical signals with time.

In another aspect, the invention includes an isolated polypeptide comprising an amino acid sequence selected from the group consisting of: (a) a mature form of an amino acid sequence of SEQ ID NO: 2; (b) a variant of a mature form of an amino acid sequence of SEQ ID NO: 2; an amino acid sequence of SEQ ID NO: 2; (c) a variant of an amino acid sequence of SEQ ID NO: 2, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 5% of amino acid residues from said amino acid sequence; (d) and at least one conservative amino acid substitution to the amino acid sequences in (a), (b), (c) or (d). The invention also includes an antibody that binds immunospecifically to the polypeptide of (a), (b), (c) or (d).

In another aspect, the invention includes an isolated nucleic acid molecule comprising a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence selected from the group consisting of: (a) a mature form of an amino acid sequence of SEQ ID NO: 2; (b) a variant of a mature form of an amino acid sequence of SEQ ID NO: 2, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 5% of the amino acid residues from

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the amino acid sequence of said mature form; (c) an amino acid sequence of SEQ ID NO: 2; (d) a variant of an amino acid sequence of SEQ ID NO: 2, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 15% of amino acid residues from said amino acid sequence; a nucleic acid fragment encoding at least a portion of a polypeptide comprising an amino acid sequence of SEQ ID NO: 2, or a variant of said polypeptide, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 5% of amino acid residues from said amino acid sequence; (e) and a nucleic acid molecule comprising the complement of (a), (b), (c), (d) or (e). In a further aspect, the invention provides a nucleic acid molecule wherein the nucleic acid molecule comprises nucleotide sequence selected from the group consisting of: (a) a first nucleotide sequence comprising a coding sequence differing by one or more nucleotide sequences from a coding sequence encoding said amino acid sequence, provided that no more than **20%** of the nucleotides in the coding sequence in said first nucleotide sequence differ from said coding sequence; an isolated second polynucleotide that is a complement of the first polynucleotide; (b) and a nucleic acid fragment of (a) or (b). The invention also includes a vector comprising the nucleic acid molecule of (a) or (b). In another aspect, the invention includes a cell comprising the vector.

In a further aspect, the invention includes a method for determining the nucleic acid sequence in a template nucleic acid polymer, comprising: (a) introducing the template nucleic acid polymer into a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added; (b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released; (c) separately recovering each of the feedstocks from the polymerization environment; and (d) measuring the amount of PPi with an ATP sulfurylase and a luciferase in each of the recovered feedstocks to determine the identity of each nucleotide in the complementary polymer and thus the sequence of the template polymer.

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In another aspect, the invention provides a method for sequencing a nucleic acid, the method comprising: (a) providing one or more nucleic acid anchor primers; (b) providing a plurality of single-stranded circular nucleic acid templates disposed within a plurality of cavities in an array on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm and at least 400,000 discrete sites; (c) annealing an effective amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex; (d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template; (e) annealing an effective amount of a sequencing primer to one or more copies of said covalently linked complementary nucleic acid; (f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a sequencing reaction byproduct; and (g) identifying the sequencing reaction byproduct with the use of an ATP sulfurylase and a luciferase, thereby determining the sequence of the nucleic acid.

In another aspect, the invention provides a method for sequencing a nucleic acid, the method comprising: (a) providing at least one nucleic acid anchor primer; (b) providing a plurality of single-stranded circular nucleic acid templates in an array having at least 400,000 discrete reaction sites; (c) annealing a first amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex; (d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template; (e) annealing a second amount of a sequencing primer to one or more copies of the covalently linked complementary nucleic acid; (f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, when the predetermined nucleotide triphosphate is incorporated onto the 3' end of the sequencing primer, to yield a sequencing reaction byproduct; and (g) identifying the sequencing reaction byproduct with the use of a thermostable sulfurylase and a luciferase, thereby determining the sequence of the nucleic acid at each reaction site that contains a nucleic acid template.

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In a further aspect, the invention includes a method of determining the base sequence of a plurality of nucleotides on an array, the method comprising: (a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, (b) adding an activated nucleotide 5'-triphosphate precursor of one known nitrogenous base to a reaction mixture in each reaction chamber, each reaction mixture comprising a template-directed nucleotide polymerase and a single-stranded polynucleotide template hybridized to a complementary oligonucleotide primer strand at least one nucleotide residue shorter than the templates to form at least one unpaired nucleotide residue in each template at the 3'-end of the primer strand, under reaction conditions which allow incorporation of the activated nucleoside 5'-triphosphate precursor onto the 3'-end of the primer strands, provided the nitrogenous base of the activated nucleoside 5'-triphosphate precursor is complementary to the nitrogenous base of the unpaired nucleotide residue of the templates; (c) detecting whether or not the nucleoside 5'-triphosphate precursor was incorporated into the primer strands through detection of a sequencing byproduct with a thermostable sulfurylase and luciferase, thus indicating that the unpaired nucleotide residue of the template has a nitrogenous base composition that is complementary to that of the incorporated nucleoside 5'-triphosphate precursor; and (d) sequentially repeating steps (b) and (c), wherein each sequential repetition adds and, detects the incorporation of one type of activated nucleoside 5'-triphosphate precursor of known nitrogenous base composition; and (e) determining the base sequence of the unpaired nucleotide residues of the template in each reaction chamber from the sequence of incorporation of said nucleoside precursors.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is one embodiment for a cloning strategy for obtaining the luciferase-sulfurylase sequence.

FIG. 2A and 2B show the preparative agarose gel of luciferase and sulfurylase as well as sulfurylase-luciferase fusion genes.

FIG. 3 shows the results of experiments to determine the activity of the luciferase-sulfurylase fusion protein on NTA-agarose and MPG-SA solid supports.

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DETAILED DESCRIPTION OF THE INVENTION

This invention provides a fusion protein containing an ATP generating polypeptide bound to a polypeptide which converts ATP into an entity which is detectable. As used herein, the term "fusion protein" refers to a chimeric protein containing an exogenous protein fragment joined to another exogenous protein fragment. The fusion protein could include an affinity tag to allow attachment of the protein to a solid support or to allow for purification of the recombinant fusion protein from the host cell or culture supernatant, or both.

In a preferred embodiment, the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote. The eukaryote could be an animal, plant, fungus or yeast. In some embodiments, the animal is a mammal, rodent, insect, worm, mollusk, reptile, bird and amphibian. Plant sources of the polypeptides include but are not limited to *Arabidopsis thaliana*, *Brassica napus*, *Allium sativum*, *Amaranthus caudatus*, *Hevea brasiliensis*, *Hordeum vulgare*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Oryza sativum*, *Pisum sativum*, *Populus trichocarpa*, *Solanum tuberosum*, *Secale cereale*, *Sambucus nigra*, *Ulmus americana* or *Triticum aestivum*. Examples of fungi include but are not limited to *Penicillum chrysogenum*, *Stachybotrys chartarum*, *Aspergillus fumigatus*, *Podospora anserina* and *Trichoderma reesei*. Examples of sources of yeast include but are not limited to *Saccharomyces cerevisiae*, *Candida tropicalis*, *Candida lypolitica*, *Candida utilis*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida spp.*, *Pichia spp.* and *Hansenula spp.*.

The prokaryote source could be bacteria or archaea. In some embodiments, the bacteria is *E. coli*, *B. subtilis*, *Streptococcus gordonii*, flavobacteria or green sulfur bacteria. In other embodiments, the archaea is *Sulfolobus*, *Thermococcus*, *Methanobacterium*, *Halococcus*, *Halobacterium* or *Methanococcus jannaschii*.

The ATP generating polypeptide can be a ATP sulfurylase, hydrolase or an ATP synthase. In a preferred embodiment, the ATP generating polypeptide is ATP sulfurylase. In one embodiment, the ATP sulfurylase is a thermostable sulfurylase cloned from *Bacillus stearothermophilus* (Bst) and comprising the nucleotide sequence of SEQ ID NO:1. This putative gene was cloned using genomic DNA acquired from ATCC (Cat. No. 12980D). The gene is shown to code for a functional ATP sulfurylase that can be expressed as a fusion

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protein with an affinity tag. The disclosed Bst sulfurylase nucleic acid (SEQ ID NO:1) includes the 1247 nucleotide sequence. An open reading frame (ORF) for the mature protein was identified beginning with an ATG codon at nucleotides 1-3 and ending with a TAA codon at nucleotides 1159-1161. The start and stop codons of the open reading frame are highlighted in bold type. The putative untranslated regions are underlined and found upstream of the initiation codon and downstream from the termination codon.

Bst Thermostable Sulfurylase Nucleotide Sequence (SEQ ID NO:1)

GTTATGAAC**ATG**AGTTTGAGCATTCCGCATGGCGGCACATTGATCAACCGTTGGAATCCG 60
GATTACCCAATCGATGAAGCAACGAAAACGATCGAGCTGTCCAAAGCCGAACTAAGCGAC 120
CTTGAGCTGATCGGCACAGGCGCCTACAGCCCGCTCACCGGGTTTTTAACGAAAGCCGAT 180
TACGATGCGGTCGTAGAAACGATGCGCCTCGCTGATGGCACTGTCTGGAGCATTCCGATC 240
ACGCTGGCGGTGACGGAAGAAAAAGCGAGTGAACTCACTGTCGGCGACAAAGCGAAACTC 300
GTTTATGGCGGCGACGTCTACGGCGTCATTGAAATCGCCGATATTTACCGCCCGGATAAA 360
ACGAAAGAAGCCAAGCTCGTCTATAAAACCGATGAACTCGCTCACCCGGGCGTGCGCAAG 420
CTGTTTGAAAAACCAGATGTGTACGTCGGCGGAGCGGTTACGCTCGTCAAACGGACCGAC 480
AAAGGCCAGTTTGCTCCGTTTTATTTCGATCCGGCCGAAACGCGGAAACGATTTGCCGAA 540
CTCGGCTGGAATACCGTCGTCGGCTTCCAAACACGCAACCCGGTTCACCGCGCCCATGAA 600
TACATTCAAAAATGCGCGCTTGAAATCGTGGACGGCTTGTTTTTAAACCCGCTCGTCGGC 660
GAAACGAAAGCGGACGATATTCCGGCCGACATCCGGATGGAAAGCTATCAAGTGCTGCTG 720
GAAAACTATTATCCGAAAGACCGCGTTTTCTTGGGCGTCTTCCAAGCTGCGATGCGCTAT 780
GCCGGTCCGCGCGAAGCGATTTTCCATGCCATGGTGCGGAAAAACTTCGGCTGCACGCAC 840
TTCATCGTCGGCCGCGACCATGCGGGCGTCGGCAACTATTACGGCACGTATGATGCGCAA 900
AAAATCTTCTCGAACTTTACAGCCGAAGAGCTTGGCATTACACCGCTCTTTTTCGAACAC 960
AGCTTTTATTGCACGAAATGCGAAGGCATGGCATCGACGAAAACATGCCCGCACGACGCA 1020
CAATATCACGTTGTCCTTTCTGGCACGAAAGTCCGTGAAATGTTGCGTAACGGCCAAGTG 1080
CCGCCGAGCACATTCAGCCGTCCGGAAGTGGCCGCCGTTTTGATCAAAGGGCTGCAAGAA 1140
CGCGAAACGGTCACCCCGTCGACACGC**TAA**AGGAGGAGCGAGATGAGCACGAATATCGTT 1200
TGGCATCATACATCGGTGACAAAAGAAGATCGCCGCCAACGCAACGG 1247

The Bst sulfurylase polypeptide (SEQ ID NO:2) is 386 amino acid residues in length and is presented using the three letter amino acid code.

Bst Sulfurylase Amino Acid Sequence (SEQ ID NO:2)

Met Ser Leu Ser Ile Pro His Gly Gly Thr Leu Ile Asn Arg Trp Asn
1 5 10 15

Pro Asp Tyr Pro Ile Asp Glu Ala Thr Lys Thr Ile Glu Leu Ser Lys
20 25 30

Ala Glu Leu Ser Asp Leu Glu Leu Ile Gly Thr Gly Ala Tyr Ser Pro
35 40 45

Leu Thr Gly Phe Leu Thr Lys Ala Asp Tyr Asp Ala Val Val Glu Thr

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50 55 60

Met Arg Leu Ala Asp Gly Thr Val Trp Ser Ile Pro Ile Thr Leu Ala
65 70 75

Val Thr Glu Glu Lys Ala Ser Glu Leu Thr Val Gly Asp Lys Ala Lys
80 85 90 95

Leu Val Tyr Gly Gly Asp Val Tyr Gly Val Ile Glu Ile Ala Asp Ile
100 105 110

Tyr Arg Pro Asp Lys Thr Lys Glu Ala Lys Leu Val Tyr Lys Thr Asp
115 120 125

Glu Leu Ala His Pro Gly Val Arg Lys Leu Phe Glu Lys Pro Asp Val
130 135 140

Tyr Val Gly Gly Ala Val Thr Leu Val Lys Arg Thr Asp Lys Gly Gln
145 150 155

Phe Ala Pro Phe Tyr Phe Asp Pro Ala Glu Thr Arg Lys Arg Phe Ala
160 165 170 175

Glu Leu Gly Trp Asn Thr Val Val Gly Phe Gln Thr Arg Asn Pro Val
180 185 190

His Arg Ala His Glu Tyr Ile Gln Lys Cys Ala Leu Glu Ile Val Asp
195 200 205

Gly Leu Phe Leu Asn Pro Leu Val Gly Glu Thr Lys Ala Asp Asp Ile
210 215 220

Pro Ala Asp Ile Arg Met Glu Ser Tyr Gln Val Leu Leu Glu Asn Tyr
225 230 235

Tyr Pro Lys Asp Arg Val Phe Leu Gly Val Phe Gln Ala Ala Met Arg
240 245 250 255

Tyr Ala Gly Pro Arg Glu Ala Ile Phe His Ala Met Val Arg Lys Asn
260 265 270

Phe Gly Cys Thr His Phe Ile Val Gly Arg Asp His Ala Gly Val Gly
275 280 285

Asn Tyr Tyr Gly Thr Tyr Asp Ala Gln Lys Ile Phe Ser Asn Phe Thr
290 295 300

Ala Glu Glu Leu Gly Ile Thr Pro Leu Phe Phe Glu His Ser Phe Tyr
305 310 315

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Cys Thr Lys Cys Glu Gly Met Ala Ser Thr Lys Thr Cys Pro His Asp
320 325 330 335

Ala Gln Tyr His Val Val Leu Ser Gly Thr Lys Val Arg Glu Met Leu
340 345 350

Arg Asn Gly Gln Val Pro Pro Ser Thr Phe Ser Arg Pro Glu Val Ala
355 360 365

Ala Val Leu Ile Lys Gly Leu Gln Glu Arg Glu Thr Val Thr Pro Ser
370 375 380

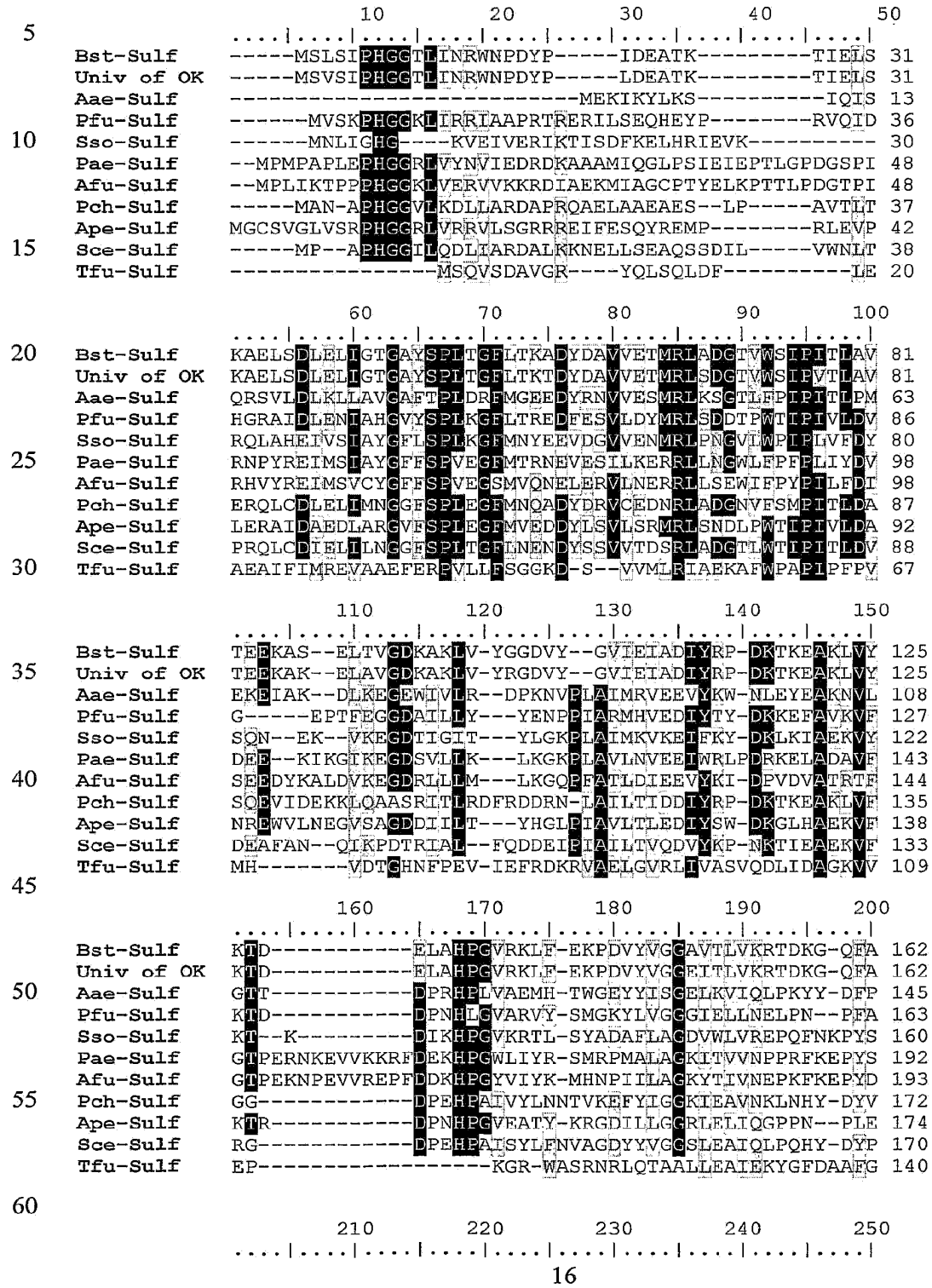
Thr Arg
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In one embodiment, the thermostable sulfurylase is active at temperatures above ambient to at least 50°C. This property is beneficial so that the sulfurylase will not be denatured at higher temperatures commonly utilized in polymerase chain reaction (PCR) reactions or sequencing reactions. In one embodiment, the ATP sulfurylase is from a thermophile. The thermostable sulfurylase can come from thermophilic bacteria, including but not limited to, *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

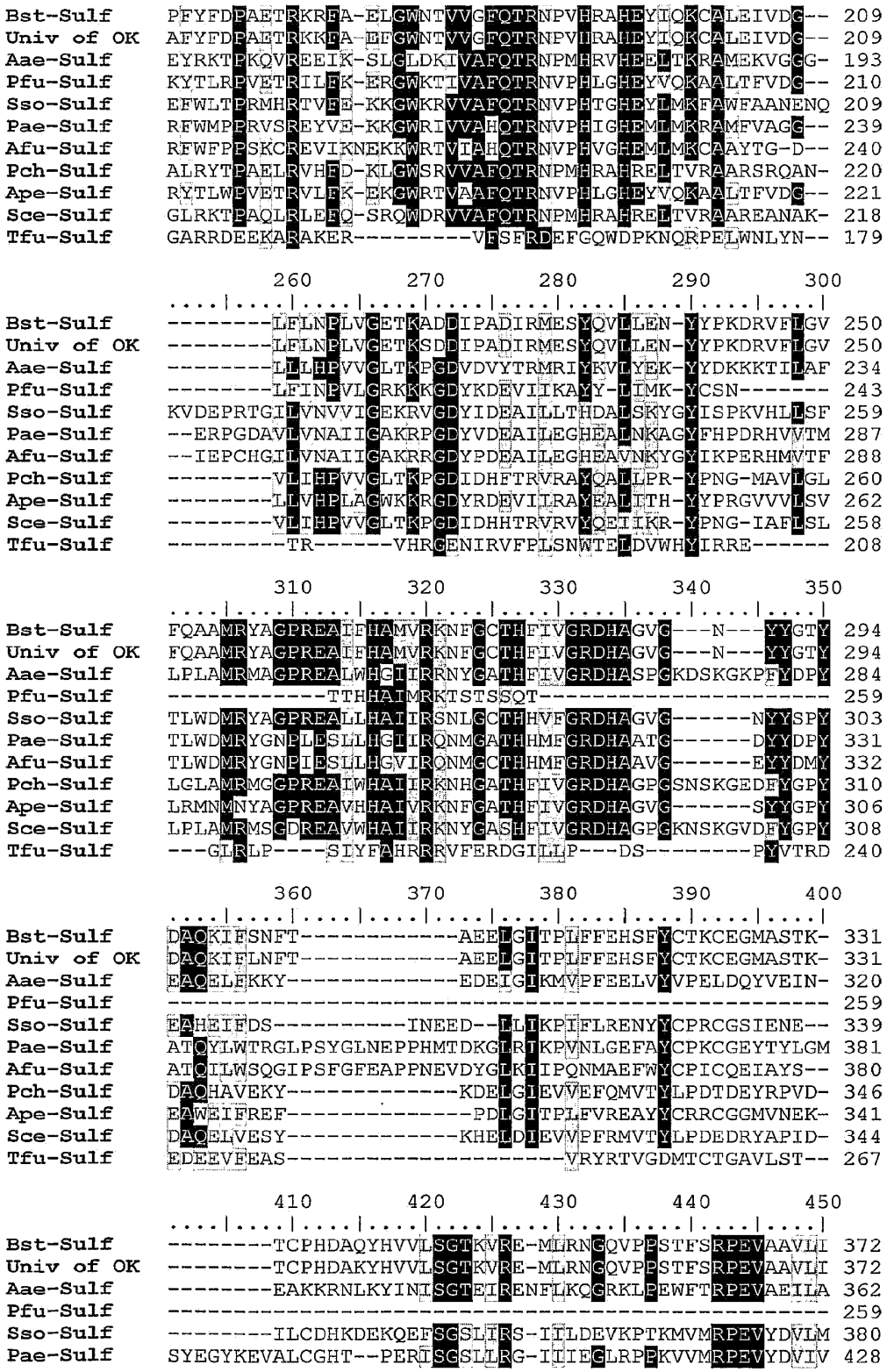
The homology of twelve ATP sulfurylases can be shown graphically in the ClustalW analysis in Table1. The alignment is of ATP sulfurylases from the following species: *Bacillus stearothermophilus* (Bst), University of Oklahoma – Strain 10 (Univ of OK), *Aquifex aeolicus* (Aae), *Pyrococcus furiosus* (Pfu), *Sulfolobus solfataricus* (Sso), *Pyrobaculum aerophilum* (Pae), *Archaeoglobus fulgidus* (Afu), *Penicillium chrysogenum* (Pch), *Aeropyrum pernix* (Ape), *Saccharomyces cerevisiae* (Sce), and *Thermomonospora fusca* (Tfu).

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Table 1: ClustalW Analysis of ATP Sulfurylase Amino Acid Sequence



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Afu-Sulf    --------ENCGHTDAKQKFSGSFLRG-MVAEGVFPPRVVMRPEVYKQIV 421
Pch-Sulf    --------QVPAG-VKTLNISGTELRR-RLRSGAHIPEWFSYPEVVKILR 386
Ape-Sulf    --------VCPHGDEYRVRISGTRLRE-MLGRGERPPEYMMRPEVADAII 382
Sce-Sulf    --------QIDTTKTRTLNISGTELRR-RLRVGGEIPEWFSYPEVVKILR 385
Tfu-Sulf    --------AT-TLDEVIAEIAATRITE---RGQTRADDRGSEAAMEERKR 305

                    460       470       480       490       500
                    ....|....|....|....|....|....|....|....|....|
Bst-Sulf    KGLQER-----------------ETVT----------------PSTR--- 386
Univ of OK  KGLQER-----------------ETVA----------------PSAR--- 386
Aae-Sulf    ETYVPKHKQGFCVWLTGLPCAGKSTIA-EILATMLQARGRKVTLLDGDVV 411
Pfu-Sulf    -------------------------------------------------- 259
Sso-Sulf    KAAEQYGFGS--------PFVTEEYLE--------------KRQSILG-- 406
Pae-Sulf    KWWRVYGY----------PYVTDKYLR--------------IKEQELEVE 454
Afu-Sulf    KWWKVYNY----------PFVNRKYLE--------------LKNKELEID 447
Pch-Sulf    ESNPPRATQGFTIFLTGYMNSGKDAIARALQVTLNQQGGRSVSLLLGDTV 436
Ape-Sulf    SHPDPFI----------------------------------------- 389
Sce-Sulf    ESNPPRPKQGFSIVLGNSLTVSREQLSIALLSTFLQFGGGRYYKIFEHNN 435
Tfu-Sulf    EGYF-------------------------------------------- 309

                    510       520       530       540       550
                    ....|....|....|....|....|....|....|....|....|
Bst-Sulf    -------------------------------------------------- 386
Univ of OK  -------------------------------------------------- 386
Aae-Sulf    RTHLSRGLGFSKEDRITNILRVGFVASEIVKHNGVVICALVSPYRSARN- 460
Pfu-Sulf    -------------------------------------------------- 259
Sso-Sulf    -------------------------------------------------- 406
Pae-Sulf    L------------------------------------------------- 455
Afu-Sulf    LPAMEVPKA---------------------------------------- 456
Pch-Sulf    RHELSSELGFTREDRHTNIQRIAFVATELTRAGAAVIAAPIAPYEESRKF 486
Ape-Sulf    -------------------------------------------------- 389
Sce-Sulf    KTELLSLI----------------QDFIGSGSGLIIP--NQWEDD--- 462
Tfu-Sulf    -------------------------------------------------- 309

                    560       570       580       590       600
                    ....|....|....|....|....|....|....|....|....|
Bst-Sulf    -------------------------------------------------- 386
Univ of OK  -------------------------------------------------- 386
Aae-Sulf    QVRNMMEEGKFIEVFVDAPVEVCEERDVKGLYKKAKEGLIKGFTGVDDPY 510
Pfu-Sulf    -------------------------------------------------- 259
Sso-Sulf    -------------------------------------------------- 406
Pae-Sulf    -------------------------------------------------- 455
Afu-Sulf    -------------------------------------------------- 456
Pch-Sulf    ARDAVSQAGSFFLVHVATPLEHCEQSDKRGIYAAARRGEIKGFTGVDDPY 536
Ape-Sulf    -------------------------------------------------- 389
Sce-Sulf    -KDSVVGKQNVYLLDTSS------------------------------- 479
Tfu-Sulf    -------------------------------------------------- 309

                    610       620       630
                    ....|....|....|....|....|....|..
Bst-Sulf    ---------------------------------- 386 (SEQ ID NO:2)
Univ of OK  ---------------------------------- 386 (SEQ ID NO:22)
Aae-Sulf    EPPVAPEVRVDTTKLTPEESALKILEFLKKEGFIKD- 546 (SEQ ID NO:23)
Pfu-Sulf    ---------------------------------- 259 (SEQ ID NO:24)
Sso-Sulf    ---------------------------------- 406 (SEQ ID NO:25)
Pae-Sulf    ---------------------------------- 455 (SEQ ID NO:26)
Afu-Sulf    ---------------------------------- 456 (SEQ ID NO:27)
Pch-Sulf    ETPEKADLVVDFSKQSVRSIVHEIILVLESQGFLERQ 573 (SEQ ID NO:28)
Ape-Sulf    ---------------------------------- 389 (SEQ ID NO:29)
Sce-Sulf    ----SADIQLESADEPISHIVQKVVLFLEDNGFFVF- 511 (SEQ ID NO:30)
Tfu-Sulf    ---------------------------------- 309 (SEQ ID NO:31)
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A thermostable sulfurylase polypeptide is encoded by the open reading frame ("ORF") of a thermostable sulfurylase nucleic acid. An ORF corresponds to a nucleotide sequence that could potentially be translated into a polypeptide. A stretch of nucleic acids comprising an ORF is uninterrupted by a stop codon. An ORF that represents the coding sequence for a full protein begins with an ATG "start" codon and terminates with one of the three "stop" codons, namely, TAA, TAG, or TGA. For the purposes of this invention, an ORF may be any part of a coding sequence, with or without a start codon, a stop codon, or both. For an ORF to be considered as a good candidate for coding for a *bona fide* cellular protein, a minimum size requirement is often set, *e.g.*, a stretch of DNA that would encode a protein of 50 amino acids or more.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequences shown in SEQ ID NO:1 due to degeneracy of the genetic code and thus encode the same thermostable sulfurylase proteins as that encoded by the nucleotide sequences shown in SEQ ID NO:1. In another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO:2. In addition to the thermostable sulfurylase nucleotide sequence shown in SEQ ID NO:1 it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the thermostable sulfurylase polypeptides may exist within a population (*e.g.*, the bacterial population). Such genetic polymorphism in the thermostable sulfurylase genes may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a thermostable sulfurylase protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the thermostable sulfurylase genes. Any and all such nucleotide variations and resulting amino acid polymorphisms in the thermostable sulfurylase polypeptides, which are the result of natural allelic variation and that do not alter the functional activity of the thermostable sulfurylase polypeptides, are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding thermostable sulfurylase proteins from other species, and thus that have a nucleotide sequence that differs from the sequence SEQ ID

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NO:1 are intended to be within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and homologues of the thermostable sulfurylase cDNAs of the invention can be isolated based on their homology to the thermostable sulfurylase nucleic acids disclosed herein using the human cDNAs, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. The invention further includes the nucleic acid sequence of SEQ ID NO:1 and mature and variant forms thereof, wherein a first nucleotide sequence comprising a coding sequence differing by one or more nucleotide sequences from a coding sequence encoding said amino acid sequence, provided that no more than 11% of the nucleotides in the coding sequence differ from the coding sequence.

Another aspect of the invention pertains to nucleic acid molecules encoding a thermostable sulfurylase protein that contains changes in amino acid residues that are not essential for activity. Such thermostable sulfurylase proteins differ in amino acid sequence from SEQ ID NO:2 yet retain biological activity. In separate embodiments, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 96%, 97%, 98% or 99% homologous to the amino acid sequence of SEQ ID NO:2. An isolated nucleic acid molecule encoding a thermostable sulfurylase protein homologous to the protein of SEQ ID NO: 2 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein.

Mutations can be introduced into SEQ ID NO:2 by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted, non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined within the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan,

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histidine). Thus, a predicted non-essential amino acid residue in the thermostable sulfurylase protein is replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a thermostable sulfurylase coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for thermostable sulfurylase biological activity to identify mutants that retain activity. Following mutagenesis of SEQ ID NO:1, the encoded protein can be expressed by any recombinant technology known in the art and the activity of the protein can be determined.

The relatedness of amino acid families may also be determined based on side chain interactions. Substituted amino acids may be fully conserved "strong" residues or fully conserved "weak" residues. The "strong" group of conserved amino acid residues may be any one of the following groups: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW, wherein the single letter amino acid codes are grouped by those amino acids that may be substituted for each other. Likewise, the "weak" group of conserved residues may be any one of the following: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, VLIM, HFY, wherein the letters within each group represent the single letter amino acid code.

The thermostable sulfurylase nucleic acid of the invention includes the nucleic acid whose sequence is provided herein, or fragments thereof. The invention also includes mutant or variant nucleic acids any of whose bases may be changed from the corresponding base shown herein while still encoding a protein that maintains its sulfurylase-like activities and physiological functions, or a fragment of such a nucleic acid. The invention further includes nucleic acids whose sequences are complementary to those just described, including nucleic acid fragments that are complementary to any of the nucleic acids just described. The invention additionally includes nucleic acids or nucleic acid fragments, or complements thereto, whose structures include chemical modifications. Such modifications include, by way of nonlimiting example, modified bases, and nucleic acids whose sugar phosphate backbones are modified or derivatized. These modifications are carried out at least in part to enhance the chemical stability of the modified nucleic acid, such that they may be used, for example, as antisense binding nucleic acids in therapeutic applications in a subject.

A thermostable sulfurylase nucleic acid can encode a mature thermostable sulfurylase polypeptide. As used herein, a "mature" form of a polypeptide or protein disclosed in the

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present invention is the product of a naturally occurring polypeptide or precursor form or proprotein. The naturally occurring polypeptide, precursor or proprotein includes, by way of nonlimiting example, the full-length gene product, encoded by the corresponding gene. Alternatively, it may be defined as the polypeptide, precursor or proprotein encoded by an ORF described herein. The product "mature" form arises, again by way of nonlimiting example, as a result of one or more naturally occurring processing steps as they may take place within the cell, or host cell, in which the gene product arises. Examples of such processing steps leading to a "mature" form of a polypeptide or protein include the cleavage of the N-terminal methionine residue encoded by the initiation codon of an ORF, or the proteolytic cleavage of a signal peptide or leader sequence. Thus a mature form arising from a precursor polypeptide or protein that has residues 1 to N, where residue 1 is the N-terminal methionine, would have residues 2 through N remaining after removal of the N-terminal methionine. Alternatively, a mature form arising from a precursor polypeptide or protein having residues 1 to N, in which an N-terminal signal sequence from residue 1 to residue M is cleaved, would have the residues from residue M+1 to residue N remaining. Further as used herein, a "mature" form of a polypeptide or protein may arise from a step of post-translational modification other than a proteolytic cleavage event. Such additional processes include, by way of non-limiting example, glycosylation, myristoylation or phosphorylation. In general, a mature polypeptide or protein may result from the operation of only one of these processes, or a combination of any of them.

The term "isolated" nucleic acid molecule, as utilized herein, is one, which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5'- and 3'-termini of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated thermostable sulfurylase nucleic acid molecules can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell/tissue from which the nucleic acid is derived (*e.g.*, brain, heart, liver, spleen, etc.). Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or of chemical precursors or other chemicals when chemically synthesized.

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A nucleic acid molecule of the invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 or a complement of this aforementioned nucleotide sequence, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or a portion of the nucleic acid sequence of SEQ ID NO:1 as a hybridization probe, thermostable sulfurylase molecules can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook, *et al.*, (eds.), MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Ausubel, *et al.*, (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, NY, 1993.)

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to thermostable sulfurylase nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

As used herein, the term "complementary" refers to Watson-Crick or Hoogsteen base pairing between nucleotides units of a nucleic acid molecule, and the term "binding" means the physical or chemical interaction between two polypeptides or compounds or associated polypeptides or compounds or combinations thereof. Binding includes ionic, non-ionic, van der Waals, hydrophobic interactions, and the like. A physical interaction can be either direct or indirect. Indirect interactions may be through or due to the effects of another polypeptide or compound. Direct binding refers to interactions that do not take place through, or due to, the effect of another polypeptide or compound, but instead are without other substantial chemical intermediates.

Fragments provided herein are defined as sequences of at least 6 (contiguous) nucleic acids or at least 4 (contiguous) amino acids, a length sufficient to allow for specific hybridization in the case of nucleic acids or for specific recognition of an epitope in the case of amino acids, respectively, and are at most some portion less than a full length sequence. Fragments may be derived from any contiguous portion of a nucleic acid or amino acid sequence of choice. Derivatives are nucleic acid sequences or amino acid sequences formed from the native compounds either directly or by modification or partial substitution. Analogs are nucleic acid sequences or amino acid sequences that have a structure similar to, but not

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identical to, the native compound but differs from it in respect to certain components or side chains. Analogs may be synthetic or from a different evolutionary origin and may have a similar or opposite metabolic activity compared to wild type. Homologs are nucleic acid sequences or amino acid sequences of a particular gene that are derived from different species.

Derivatives and analogs may be full length or other than full length, if the derivative or analog contains a modified nucleic acid or amino acid, as described below. Derivatives or analogs of the nucleic acids or proteins of the invention include, but are not limited to, molecules comprising regions that are substantially homologous to the nucleic acids or proteins of the invention, in various embodiments, by at least about 89% identity over a nucleic acid or amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, or whose encoding nucleic acid is capable of hybridizing to the complement of a sequence encoding the aforementioned proteins under stringent, moderately stringent, or low stringent conditions. See *e.g.* Ausubel, *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, NY, 1993, and below.

A "homologous nucleic acid sequence" or "homologous amino acid sequence," or variations thereof, refer to sequences characterized by a homology at the nucleotide level or amino acid level as discussed above. Homologous nucleotide sequences encode those sequences coding for isoforms of thermostable sulfurylase polypeptides. Isoforms can be expressed in different tissues of the same organism as a result of, for example, alternative splicing of RNA. Alternatively, isoforms can be encoded by different genes. In the invention, homologous nucleotide sequences include nucleotide sequences encoding for a thermostable sulfurylase polypeptide of species other than humans, including, but not limited to: vertebrates, and thus can include, *e.g.*, frog, mouse, rat, rabbit, dog, cat cow, horse, and other organisms. Homologous nucleotide sequences also include, but are not limited to, naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. Homologous nucleic acid sequences include those nucleic acid sequences that encode conservative amino acid substitutions in SEQ ID NO:1, as well as a polypeptide possessing thermostable sulfurylase biological activity. Various biological activities of the thermostable sulfurylase proteins are described below.

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The thermostable sulfurylase proteins of the invention include the sulfurylase protein whose sequence is provided herein. The invention also includes mutant or variant proteins any of whose residues may be changed from the corresponding residue shown herein while still encoding a protein that maintains its sulfurylase-like activities and physiological functions, or a functional fragment thereof. The invention further encompasses antibodies and antibody fragments, such as F_{ab} or $(F_{ab})_2$, that bind immunospecifically to any of the proteins of the invention. This invention also includes a variant or a mature form of the amino acid sequence of SEQ ID NO:2, wherein one or more amino acid residues in the variant differs in no more than 4% of the amino acic residues from the amino acid sequence of the mature form.

Several assays have been developed for detection of the forward ATP sulfurylase reaction. The colorimetric molybdolysis assay is based on phosphate detection (see *e.g.*, Wilson and Bandurski, 1958. *J. Biol. Chem.* **233**: 975-981), whereas the continuous spectrophotometric molybdolysis assay is based upon the detection of NADH oxidation (see *e.g.*, Seubert, *et al.*, 1983. *Arch. Biochem. Biophys.* **225**: 679-691; Seubert, *et al.*, 1985. *Arch. Biochem. Biophys.* **240**: 509-523). The later assay requires the presence of several detection enzymes.

Suitable enzymes for converting ATP into light include luciferases, *e.g.*, insect luciferases. Luciferases produce light as an end-product of catalysis. The best known light-emitting enzyme is that of the firefly, *Photinus pyralis* (*Coleoptera*). The corresponding gene has been cloned and expressed in bacteria (see *e.g.*, de Wet, *et al.*, 1985. *Proc. Natl. Acad. Sci. USA* **80**: 7870-7873) and plants (see *e.g.*, Ow, *et al.*, 1986. *Science* **234**: 856-859), as well as in insect (see *e.g.*, Jha, *et al.*, 1990. *FEBS Lett.* **274**: 24-26) and mammalian cells (see *e.g.*, de Wet, *et al.*, 1987. *Mol. Cell. Biol.* 7: 725-7373; Keller, *et al.*, 1987. *Proc. Natl. Acad. Sci. USA* **82**: 3264-3268). In addition, a number of luciferase genes from the Jamaican click beetle, *Pyroplorus plagiophihalamus* (*Coleoptera*), have recently been cloned and partially characterized (see *e.g.*, Wood, *et al.*, 1989. *J. Biolumin. Chemilumin.* **4**: 289-301; Wood, *et al.*, 1989. *Science* **244**: 700-702). Distinct luciferases can sometimes produce light of different wavelengths, which may enable simultaneous monitoring of light emissions at different wavelengths. Accordingly, these aforementioned characteristics are unique, and add new dimensions with respect to the utilization of current reporter systems.

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Firefly luciferase catalyzes bioluminescence in the presence of luciferin, adenosine 5'-triphosphate (ATP), magnesium ions, and oxygen, resulting in a quantum yield of 0.88 (see *e.g.,* McElroy and Selinger, 1960. *Arch. Biochem. Biophys.* **88**: 136-145). The firefly luciferase bioluminescent reaction can be utilized as an assay for the detection of ATP with a detection limit of approximately $1x10^{-13}$ M (see *e.g.,* Leach, 1981. *J. Appl. Biochem.* **3**: 473-517). In addition, the overall degree of sensitivity and convenience of the luciferase-mediated detection systems have created considerable interest in the development of firefly luciferase-based biosensors (see *e.g.,* Green and Kricka, 1984. *Talanta* **31**: 173-176; Blum, *et al.,* 1989. *J. Biolumin. Chemilumin.* **4**: 543-550).

The development of new reagents have made it possible to obtain stable light emission proportional to the concentrations of ATP (see *e.g.,* Lundin, 1982. Applications of firefly luciferase In; *Luminescent Assays* (Raven Press, New York). With such stable light emission reagents, it is possible to make endpoint assays and to calibrate each individual assay by addition of a known amount of ATP. In addition, a stable light-emitting system also allows continuous monitoring of ATP-converting systems.

In a preferred embodiment, the ATP generating-ATP converting fusion protein is attached to an affinity tag. The term "affinity tag" is used herein to denote a peptide segment that can be attached to a polypeptide to provide for purification or detection of the polypeptide or provide sites for attachment of the polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract or a biotin carboxyl carrier protein (BCCP) domain, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), substance P, Flag.™. peptide (Hopp et al., Biotechnology 6:1204-1210, 1988; available from Eastman Kodak Co., New Haven, Conn.), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, N.J.).

As used herein, the term "poly-histidine tag," when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine

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residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate or IDA column.

In some embodiments, the fusion protein has an orientation such that the sulfurylase polypeptide is N-terminal to the luciferase polypeptide. In other embodiments, the luciferase polypeptide is N-terminal to the sulfurylase polypeptide. As used herein, the term sulfurylase-luciferase fusion protein refers to either of these orientations. The terms "amino-terminal" (N-terminal) and "carboxyl-terminal" (C-terminal) are used herein to denote positions within polypeptides and proteins. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide or protein to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a protein is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete protein.

The fusion protein of this invention can be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, *e.g.*, by employing blunt-ended or "sticky"-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, Ausubel et al. (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, 1992). The two polypeptides of the fusion protein can also be joined by a linker, such as a unique restriction site, which is engineered with specific primers during the cloning procedure. In one embodiment, the sulfurylase and luciferase polypeptides are joined by a linker, for example an ala-ala-ala linker which is encoded by a Not1 restriction site.

In one embodiment, the invention includes a recombinant polynucleotide that comprises a coding sequence for a fusion protein having an ATP generating polypeptide sequence and an ATP converting polypeptide sequence. In a preferred embodiment, the recombinant polynucleotide encodes a sulfurylase-luciferase fusion protein. The term "recombinant DNA molecule" or "recombinant polynucleotide" as used herein refers to a

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DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques. The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

In one aspect, this invention discloses a sulfurylase-luciferase fusion protein with an N-terminal hexahistidine tag and a BCCP tag. The nucleic acid sequence of the disclosed N-terminal hexahistidine-BCCP luciferase-sulfurylase gene (His6-BCCP L-S) gene is shown below:

His6-BCCP L-S Nucleotide Sequence (SEQ ID NO:3):

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ATGCGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGGAAGCGCCAGCAGCA    60
GCGGAAATCAGTGGTCACATCGTACGTTCCCCGATGGTTGGTACTTTCTACCGCACCCCA   120
AGCCCGGACGCAAAAGCGTTCATCGAAGTGGGTCAGAAAGTCAACGTGGGCGATACCCTG   180
TGCATCGTTGAAGCCATGAAAATGATGAACCAGATCGAAGCGGACAAATCCGGTACCGTG   240
AAAGCAATTCTGGTCGAAAGTGGACAACCGGTAGAATTTGACGAGCCGCTGGTCGTCATC   300
GAGGGATCCGAGCTCGAGATCCAAATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCG   360
CCATTCTATCCTCTAGAGGATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGA   420
TACGCCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGAACATCACG   480
TACGCGGAATACTTCGAAATGTCCGTTCGGTTGGCAGAAGCTATGAAACGATATGGGCTG   540
AATACAAATCACAGAATCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTTATGCCGGTG   600
TTGGGCGCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAACGACATTTATAATGAACGT   660
GAATTGCTCAACAGTATGAACATTTCGCAGCCTACCGTAGTGTTTGTTTCCAAAAAGGGG   720
TTGCAAAAAATTTTGAACGTGCAAAAAAAATTACCAATAATCCAGAAAATTATTATCATG   780
GATTCTAAAACGGATTACCAGGGATTTCAGTCGATGTACACGTTCGTCACATCTCATCTA   840
CCTCCCGGTTTTAATGAATACGATTTTGTACCAGAGTCCTTTGATCGTGACAAAACAATT   900
GCACTGATAATGAATTCCTCTGGATCTACTGGGTTACCTAAGGGTGTGGCCCTTCCGCAT   960
AGAACTGCCTGCGTCAGATTCTCGCATGCCAGAGATCCTATTTTTGGCAATCAAATCATT  1020
CCGGATACTGCGATTTTAAGTGTTGTTCCATTCCATCACGGTTTTGGAATGTTTACTACA  1080
CTCGGATATTTGATATGTGGATTTCGAGTCGTCTTAATGTATAGATTTGAAGAAGAGCTG  1140
TTTTTACGATCCCTTCAGGATTACAAAATTCAAAGTGCGTTGCTAGTACCAACCCTATTT  1200
TCATTCTTCGCCAAAAGCACTCTGATTGACAAATACGATTTATCTAATTTACACGAAATT  1260
GCTTCTGGGGGCGCACCTCTTTCGAAAGAAGTCGGGGAAGCGGTTGCAAAACGCTTCCAT  1320
CTTCCAGGGATACGACAAGGATATGGGCTCACTGAGACTACATCAGCTATTCTGATTACA  1380
CCCGAGGGGGATGATAAACCGGGCGCGGTCGGTAAAGTTGTTCCATTTTTTGAAGCGAAG  1440
GTTGTGGATCTGGATACCGGGAAAACGCTGGGCGTTAATCAGAGAGGCGAATTATGTGTC  1500
AGAGGACCTATGATTATGTCCGGTTATGTAAACAATCCGGAAGCGACCAACGCCTTGATT  1560
GACAAGGATGGATGGCTACATTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTC  1620
TTCATAGTTGACCGCTTGAAGTCTTTAATTAAATACAAAGGATATCAGGTGGCCCCCGCT  1680
GAATTGGAATCGATATTGTTACAACACCCCAACATCTTCGACGCGGGCGTGGCAGGTCTT  1740
CCCGACGATGACGCCGGTGAACTTCCCGCCGCCGTTGTTGTTTTGGAGCACGGAAAGACG  1800
ATGACGGAAAAAGAGATCGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAAAAGTTG  1860
CGCGGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGGAAAACTCGACGCA  1920
AGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGTCCAAATTGGCGGCC  1980
GCTATGCCTGCTCCTCACGGTGGTATTCTACAAGACTTGATTGCTAGAGATGCGTTAAAG  2040
AAGAATGAATTGTTATCTGAAGCGCAATCTTCGGACATTTTAGTATGGAACTTGACTCCT  2100
AGACAACTATGTGATATTGAATTGATTCTAAATGGTGGGTTTTCTCCTCTGACTGGGTTT  2160
TTGAACGAAAACGATTACTCCTCTGTTGTTACAGATTCGAGATTAGCAGACGGCACATTG  2220
TGGACCATCCCTATTACATTAGATGTTGATGAAGCATTTGCTAACCAAATTAAACCAGAC  2280
ACAAGAATTGCCCTTTTCCAAGATGATGAAATTCCTATTGCTATACTTACTGTCCAGGAT  2340
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GTTTACAAGCCAAACAAAACTATCGAAGCCGAAAAAGTCTTCAGAGGTGACCCAGAACAT 2400
CCAGCCATTAGCTATTTATTTAACGTTGCCGGTGATTATTACGTCGGCGGTTCTTTAGAA 2460
GCGATTCAATTACCTCAACATTATGACTATCCAGGTTTGCGTAAGACACCTGCCCAACTA 2520
AGACTTGAATTCCAATCAAGACAATGGGACCGTGTCGTAGCTTTCCAAACTCGTAATCCA 2580
ATGCATAGAGCCCACAGGGAGTTGACTGTGAGAGCCGCCAGAGAAGCTAATGCTAAGGTG 2640
CTGATCCATCCAGTTGTTGGACTAACCAAACCAGGTGATATAGACCATCACACTCGTGTT 2700
CGTGTCTACCAGGAAATTATTAAGCGTTATCCTAATGGTATTGCTTTCTTATCCCTGTTG 2760
CCATTAGCAATGAGAATGAGTGGTGATAGAGAAGCCGTATGGCATGCTATTATTAGAAAG 2820
AATTATGGTGCCTCCCACTTCATTGTTGGTAGAGACCATGCGGGCCCAGGTAAGAACTCC 2880
AAGGGTGTTGATTTCTACGGTCCATACGATGCTCAAGAATTGGTCGAATCCTACAAGCAT 2940
GAACTGGACATTGAAGTTGTTCCATTCAGAATGGTCACTTATTTGCCAGACGAAGACCGT 3000
TATGCTCCAATTGATCAAATTGACACCACAAAGACGAGAACCTTGAACATTTCAGGTACA 3060
GAGTTGAGACGCCGTTTAAGAGTTGGTGGTGAGATTCCTGAATGGTTCTCATATCCTGAA 3120
GTGGTTAAAATCCTAAGAGAATCCAACCCACCAAGACCAAAACAAGGTTTTTCAATTGTT 3180
TTAGGTAATTCATTAACCGTTTCTCGTGAGCAATTATCCATTGCTTTGTTGTCAACATTC 3240
TTGCAATTCGGTGGTGGCAGGTATTACAAGATCTTTGAACACAATAATAAGACAGAGTTA 3300
CTATCTTTGATTCAAGATTTCATTGGTTCTGGTAGTGGACTAATTATTCCAAATCAATGG 3360
GAAGATGACAAGGACTCTGTTGTTGGCAAGCAAAACGTTTACTTATTAGATACCTCAAGC 3420
TCAGCCGATATTCAGCTAGAGTCAGCGGATGAACCTATTTCACATATTGTACAAAAAGTT 3480
GTCCTATTCTTGGAAGACAATGGCTTTTTTGTATTTTAA 3519

The amino acid sequence of the disclosed His6-BCCP L-S polypeptide is presented using the three letter amino acid code (SEQ ID NO:4).

His6-BCCP L-S Amino Acid Sequence (SEQ ID NO:4)

Met Arg Gly Ser His His His His His His Gly Met Ala Ser Met Glu
1 5 10 15

Ala Pro Ala Ala Ala Glu Ile Ser Gly His Ile Val Arg Ser Pro Met
20 25 30

Val Gly Thr Phe Tyr Arg Thr Pro Ser Pro Asp Ala Lys Ala Phe Ile
35 40 45

Glu Val Gly Gln Lys Val Asn Val Gly Asp Thr Leu Cys Ile Val Glu
50 55 60

Ala Met Lys Met Met Asn Gln Ile Glu Ala Asp Lys Ser Gly Thr Val
65 70 75 80

Lys Ala Ile Leu Val Glu Ser Gly Gln Pro Val Glu Phe Asp Glu Pro
85 90 95

Leu Val Val Ile Glu Gly Ser Glu Leu Glu Ile Gln Met Glu Asp Ala
100 105 110

Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro Leu Glu Asp Gly

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115 120 125

Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg Tyr Ala Leu Val
130 135 140

Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu Val Asn Ile Thr
145 150 155 160

Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala Glu Ala Met Lys
165 170 175

Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val Cys Ser Glu Asn
180 185 190

Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu Phe Ile Gly Val
195 200 205

Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg Glu Leu Leu Asn
210 215 220

Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val Ser Lys Lys Gly
225 230 235 240

Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro Ile Ile Gln Lys
245 250 255

Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly Phe Gln Ser Met
260 265 270

Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe Asn Glu Tyr Asp
275 280 285

Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile Ala Leu Ile Met
290 295 300

Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val Ala Leu Pro His
305 310 315 320

Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp Pro Ile Phe Gly
325 330 335

Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val Val Pro Phe His
340 345 350

His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu Ile Cys Gly Phe
355 360 365

Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu Phe Leu Arg Ser

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370 375 380

Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val Pro Thr Leu Phe
385 390 395 400

Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr Asp Leu Ser Asn
405 410 415

Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys Glu Val Gly
420 425 430

Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile Arg Gln Gly Tyr
435 440 445

Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr Pro Glu Gly Asp
450 455 460

Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe Phe Glu Ala Lys
465 470 475 480

Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val Asn Gln Arg Gly
485 490 495

Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly Tyr Val Asn Asn
500 505 510

Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly Trp Leu His Ser
515 520 525

Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe Phe Ile Val Asp
530 535 540

Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val Ala Pro Ala
545 550 555 560

Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile Phe Asp Ala Gly
565 570 575

Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu Pro Ala Ala Val
580 585 590

Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys Glu Ile Val Asp
595 600 605

Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu Arg Gly Gly Val
610 615 620

Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly Lys Leu Asp Ala

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625 630 635 640

Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys Gly Gly Lys Ser
645 650 655

Lys Leu Ala Ala Ala Met Pro Ala Pro His Gly Gly Ile Leu Gln Asp
660 665 670

Leu Ile Ala Arg Asp Ala Leu Lys Lys Asn Glu Leu Leu Ser Glu Ala
675 680 685

Gln Ser Ser Asp Ile Leu Val Trp Asn Leu Thr Pro Arg Gln Leu Cys
690 695 700

Asp Ile Glu Leu Ile Leu Asn Gly Gly Phe Ser Pro Leu Thr Gly Phe
705 710 715

Leu Asn Glu Asn Asp Tyr Ser Ser Val Val Thr Asp Ser Arg Leu Ala
720 725 730 735

Asp Gly Thr Leu Trp Thr Ile Pro Ile Thr Leu Asp Val Asp Glu Ala
740 745 750

Phe Ala Asn Gln Ile Lys Pro Asp Thr Arg Ile Ala Leu Phe Gln Asp
755 760 765

Asp Glu Ile Pro Ile Ala Ile Leu Thr Val Gln Asp Val Tyr Lys Pro
770 775 780

Asn Lys Thr Ile Glu Ala Glu Lys Val Phe Arg Gly Asp Pro Glu His
785 790 795

Pro Ala Ile Ser Tyr Leu Phe Asn Val Ala Gly Asp Tyr Tyr Val Gly
800 805 810 815

Gly Ser Leu Glu Ala Ile Gln Leu Pro Gln His Tyr Asp Tyr Pro Gly
820 825 830

Leu Arg Lys Thr Pro Ala Gln Leu Arg Leu Glu Phe Gln Ser Arg Gln
835 840 845

Trp Asp Arg Val Val Ala Phe Gln Thr Arg Asn Pro Met His Arg Ala
850 855 860

His Arg Glu Leu Thr Val Arg Ala Ala Arg Glu Ala Asn Ala Lys Val
865 870 875

Leu Ile His Pro Val Val Gly Leu Thr Lys Pro Gly Asp Ile Asp His

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880 885 890 895

His Thr Arg Val Arg Val Tyr Gln Glu Ile Ile Lys Arg Tyr Pro Asn
900 905 910

Gly Ile Ala Phe Leu Ser Leu Leu Pro Leu Ala Met Arg Met Ser Gly
915 920 925

Asp Arg Glu Ala Val Trp His Ala Ile Ile Arg Lys Asn Tyr Gly Ala
930 935 940

Ser His Phe Ile Val Gly Arg Asp His Ala Gly Pro Gly Lys Asn Ser
945 950 955

Lys Gly Val Asp Phe Tyr Gly Pro Tyr Asp Ala Gln Glu Leu Val Glu
960 965 970 975

Ser Tyr Lys His Glu Leu Asp Ile Glu Val Val Pro Phe Arg Met Val
980 985 990

Thr Tyr Leu Pro Asp Glu Asp Arg Tyr Ala Pro Ile Asp Gln Ile Asp
995 1000 1005

Thr Thr Lys Thr Arg Thr Leu Asn Ile Ser Gly Thr Glu Leu Arg Arg
1010 1015 1020

Arg Leu Arg Val Gly Gly Glu Ile Pro Glu Trp Phe Ser Tyr Pro Glu
1025 1030 1035

Val Val Lys Ile Leu Arg Glu Ser Asn Pro Pro Arg Pro Lys Gln Gly
1040 1045 1050 1055

Phe Ser Ile Val Leu Gly Asn Ser Leu Thr Val Ser Arg Glu Gln Leu
1060 1065 1070

Ser Ile Ala Leu Leu Ser Thr Phe Leu Gln Phe Gly Gly Gly Arg Tyr
1075 1080 1085

Tyr Lys Ile Phe Glu His Asn Asn Lys Thr Glu Leu Leu Ser Leu Ile
1090 1095 1100

Gln Asp Phe Ile Gly Ser Gly Ser Gly Leu Ile Ile Pro Asn Gln Trp
1105 1110 1115

Glu Asp Asp Lys Asp Ser Val Val Gly Lys Gln Asn Val Tyr Leu Leu
1120 1125 1130 1135

Asp Thr Ser Ser Ser Ala Asp Ile Gln Leu Glu Ser Ala Asp Glu Pro

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1140 1145 1150

Ile Ser His Ile Val Gln Lys Val Val Leu Phe Leu Glu Asp Asn Gly
1155 1160 1165

Phe Phe Val Phe
1170

Accordingly, in one aspect, the invention provides for a fusion protein comprising a thermostable sulfurylase joined to at least one affinity tag. The nucleic acid sequence of the disclosed N-terminal hexahistidine-BCCP Bst ATP Sulfurylase (His6-BCCP Bst Sulfurylase) gene is shown below:

His6-BCCP Bst Sulfurylase Nucleotide Sequence (SEQ ID NO:5)

```
ATGCGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGGAAGCGCCAGCAGCA    60
GCGGAAATCAGTGGTCACATCGTACGTTCCCCGATGGTTGGTACTTTCTACCGCACCCCA   120
AGCCCGGACGCAAAAGCGTTCATCGAAGTGGGTCAGAAAGTCAACGTGGGCGATACCCTG   180
TGCATCGTTGAAGCCATGAAAATGATGAACCAGATCGAAGCGGACAAATCCGGTACCGTG   240
AAAGCAATTCTGGTCGAAAGTGGACAACCGGTAGAATTTGACGAGCCGCTGGTCGTCATC   300
GAGGGATCCGAGCTCGAGATCTGCAGCATGAGCGTAAGCATCCCGCATGGCGGCACATTG   360
ATCAACCGTTGGAATCCGGATTACCCAATCGATGAAGCAACGAAAACGATCGAGCTGTCC   420
AAAGCCGAACTAAGCGACCTTGAGCTGATCGGCACAGGCGCCTACAGCCCGCTCACCGGG   480
TTTTTAACGAAAGCCGATTACGATGCGGTCGTAGAAACGATGCGCCTCGCTGATGGCACT   540
GTCTGGAGCATTCCGATCACGCTGGCGGTGACGGAAGAAAAAGCGAGTGAACTCACTGTC   600
GGCGACAAAGCGAAACTCGTTTATGGCGGCGACGTCTACGGCGTCATTGAAATCGCCGAT   660
ATTTACCGCCCGGATAAAACGAAAGAAGCCAAGCTCGTCTATAAAACCGATGAACTCGCT   720
CACCCGGGCGTGCGCAAGCTGTTTGAAAAACCAGATGTGTACGTCGGCGGAGCGGTTACG   780
CTCGTCAAACGGACCGACAAAGGCCAGTTTGCTCCGTTTTATTTCGATCCGGCCGAAACG   840
CGGAAACGATTTGCCGAACTCGGCTGGAATACCGTCGTCGGCTTCCAAACACGCAACCCG   900
GTTCACCGCGCCCATGAATACATTCAAAAATGCGCGCTTGAAATCGTGGACGGCTTGTTT   960
TTAAACCCGCTCGTCGGCGAAACGAAAGCGGACGATATTCCGGCCGACATCCGGATGGAA  1020
AGCTATCAAGTGCTGCTGGAAAACTATTATCCGAAAGACCGCGTTTTCTTGGGCGTCTTC  1080
CAAGCTGCGATGCGCTATGCCGGTCCGCGCGAAGCGATTTTCCATGCCATGGTGCGGAAA  1140
AACTTCGGCTGCACGCACTTCATCGTCGGCCGCGACCATGCGGGCGTCGGCAACTATTAC  1200
GGCACGTATGATGCGCAAAAAATCTTCTCGAACTTTACAGCCGAAGAGCTTGGCATTACA  1260
CCGCTCTTTTTCGAACACAGCTTTTATTGCACGAAATGCGAAGGCATGGCATCGACGAAA  1320
ACATGCCCGCACGACGCACAATATCACGTTGTCCTTTCTGGCACGAAAGTCCGTGAAATG  1380
TTGCGTAACGGCCAAGTGCCGCCGAGCACATTCAGCCGTCCGGAAGTGGCCGCCGTTTTG  1440
ATCAAAGGGCTGCAAGAACGCGAAACGGTCGCCCCGTCAGCGCGCTAA              1488
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The amino acid sequence of the His6-BCCP Bst Sulfurylase polypeptide is presented using the three letter amino acid code in Table 6 (SEQ ID NO:6).

His6-BCCP Bst Sulfurylase Amino Acid Sequence (SEQ ID NO:6)

Met Arg Gly Ser His His His His His His Gly Met Ala Ser Met Glu
1 5 10 15

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Ala Pro Ala Ala Ala Glu Ile Ser Gly His Ile Val Arg Ser Pro Met
20 25 30

Val Gly Thr Phe Tyr Arg Thr Pro Ser Pro Asp Ala Lys Ala Phe Ile
35 40 45

Glu Val Gly Gln Lys Val Asn Val Gly Asp Thr Leu Cys Ile Val Glu
50 55 60

Ala Met Lys Met Met Asn Gln Ile Glu Ala Asp Lys Ser Gly Thr Val
65 70 75 80

Lys Ala Ile Leu Val Glu Ser Gly Gln Pro Val Glu Phe Asp Glu Pro
85 90 95

Leu Val Val Ile Glu Gly Ser Glu Leu Glu Ile Cys Ser Met Ser Val
100 105 110

Ser Ile Pro His Gly Gly Thr Leu Ile Asn Arg Trp Asn Pro Asp Tyr
115 120 125

Pro Ile Asp Glu Ala Thr Lys Thr Ile Glu Leu Ser Lys Ala Glu Leu
130 135 140

Ser Asp Leu Glu Leu Ile Gly Thr Gly Ala Tyr Ser Pro Leu Thr Gly
145 150 155 160

Phe Leu Thr Lys Ala Asp Tyr Asp Ala Val Val Glu Thr Met Arg Leu
165 170 175

Ala Asp Gly Thr Val Trp Ser Ile Pro Ile Thr Leu Ala Val Thr Glu
180 185 190

Glu Lys Ala Ser Glu Leu Thr Val Gly Asp Lys Ala Lys Leu Val Tyr
195 200 205

Gly Gly Asp Val Tyr Gly Val Ile Glu Ile Ala Asp Ile Tyr Arg Pro
210 215 220

Asp Lys Thr Lys Glu Ala Lys Leu Val Tyr Lys Thr Asp Glu Leu Ala
225 230 235 240

His Pro Gly Val Arg Lys Leu Phe Glu Lys Pro Asp Val Tyr Val Gly
245 250 255

Gly Ala Val Thr Leu Val Lys Arg Thr Asp Lys Gly Gln Phe Ala Pro
260 265 270

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Phe Tyr Phe Asp Pro Ala Glu Thr Arg Lys Arg Phe Ala Glu Leu Gly
275 280 285

Trp Asn Thr Val Val Gly Phe Gln Thr Arg Asn Pro Val His Arg Ala
290 295 300

His Glu Tyr Ile Gln Lys Cys Ala Leu Glu Ile Val Asp Gly Leu Phe
305 310 315 320

Leu Asn Pro Leu Val Gly Glu Thr Lys Ala Asp Asp Ile Pro Ala Asp
325 330 335

Ile Arg Met Glu Ser Tyr Gln Val Leu Leu Glu Asn Tyr Tyr Pro Lys
340 345 350

Asp Arg Val Phe Leu Gly Val Phe Gln Ala Ala Met Arg Tyr Ala Gly
355 360 365

Pro Arg Glu Ala Ile Phe His Ala Met Val Arg Lys Asn Phe Gly Cys
370 375 380

Thr His Phe Ile Val Gly Arg Asp His Ala Gly Val Gly Asn Tyr Tyr
385 390 395 400

Gly Thr Tyr Asp Ala Gln Lys Ile Phe Ser Asn Phe Thr Ala Glu Glu
405 410 415

Leu Gly Ile Thr Pro Leu Phe Phe Glu His Ser Phe Tyr Cys Thr Lys
420 425 430

Cys Glu Gly Met Ala Ser Thr Lys Thr Cys Pro His Asp Ala Gln Tyr
435 440 445

His Val Val Leu Ser Gly Thr Lys Val Arg Glu Met Leu Arg Asn Gly
450 455 460

Gln Val Pro Pro Ser Thr Phe Ser Arg Pro Glu Val Ala Ala Val Leu
465 470 475 480

Ile Lys Gly Leu Gln Glu Arg Glu Thr Val Ala Pro Ser Ala Arg
485 490 495

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding an ATP generating polypeptide and an ATP converting

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polypeptide, or derivatives, fragments, analogs or homologs thereof. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, that is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner that allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the

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design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce a fusion protein.

The recombinant expression vectors of the invention can be designed for expression of the fusion protein in prokaryotic or eukaryotic cells. For example, a sulfurylase-luciferase fusion protein can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: (1) to increase expression of recombinant protein; (2) to increase the solubility of the recombinant protein; and (3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein.

In another embodiment, the ATP generating-ATP converting fusion protein expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari, *et al.*, (1987) *EMBO J* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (InVitrogen Corp, San Diego, Calif.).

Alternatively, the fusion protein can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, SF9 cells) include the pAc series (Smith *et al.* (1983) *Mol Cell Biol* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors

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include pCDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J* 6: 187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells. See, *e.g.*, Chapters 16 and 17 of Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv Immunol* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, *e.g.*, the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev* 3:537-546).

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. The invention also includes a kit comprising a sulfurylase-luciferase fusion protein expression vector.

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A host cell can be any prokaryotic or eukaryotic cell. For example, the sulfurylase-luciferase fusion protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (MOLECULAR CLONING: A LABORATORY MANUAL. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Various selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding ORFX or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) the fusion protein. Accordingly, the invention further provides methods for producing the fusion protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding the fusion protein has been introduced) in a suitable medium such that the fusion protein is produced. In another embodiment, the method further comprises isolating the fusion protein from the medium or the host cell.

The invention also includes a fusion protein bound to a mobile support. In a preferred embodiment, the fusion gene is a sulfurylase-luciferase fusion gene. In another embodiment,

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the mobile support is bound to strepavidin. The mobile support could be a bead or optical fiber. In a preferred embodiment, the bead is a nickel-agarose bead or a MPG-Streptavidin bead. In one embodiment, the sulfurylase-luciferase fusion protein is bound to the beads in a 1:3 ratio of protein to bead. It can be attached to the solid support via a covalent or non-covalent interaction. In general, any linkage recognized in the art can be used. Examples of such linkages common in the art include any suitable metal (*e.g.*, Co^{2+}, Ni^{2+})-hexahistidine complex, a biotin binding protein, *e.g.*, NEUTRAVIDIN™ modified avidin (Pierce Chemicals, Rockford, IL), streptavidin/biotin, avidin/biotin, glutathione S-transferase (GST)/glutathione, monoclonal antibody/antigen, and maltose binding protein/maltose, and pluronic coupling technologies. Samples containing the appropriate tag are incubated with the sensitized substrate so that zero, one, or multiple molecules attach at each sensitized site.

Acetyl-CoA carboxylase (ACCase) catalyzes the first committed step in de novo fatty acid biosynthesis. It belongs to a group of carboxylases that use biotin as cofactor and bicarbonate as a source of the carboxyl group. There are two types of ACCase: prokaryotic ACCase (e.g., *E. coli*, *P. aeruginosa*, *Anabaena, Synechococcus* and probably pea chloroplast) in which the three functional domains: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and carboxyltransferase (CT) are located on separable subunits and eukaryotic ACCase (e.g., rat, chicken, yeast, diatom and wheat) in which all the domains are located on one large polypeptide. It is known that a BCCP as a subunit of acetyl CoA carboxylase from *E. coli* is biotinated at the Lys residue at the 122-position by the action of biotin holoenzyme synthetase in *E. coli* (Journal of Biological Chemistry, 263, 6461 (1988)). In a preferred embodiment of this invention, the fusion protein is bound to a BCCP domain which is then utilized for binding avidins; therefore, it can bind to a streptavidin mobile support. One biotin-(strept-)avidin-based anchoring method uses a thin layer of a photoactivatable biotin analog dried onto a solid surface. (Hengsakul and Cass, 1996. *Bioconjugate Chem.* 7: 249-254). The biotin analog is then exposed to white light through a mask, so as to create defined areas of activated biotin. Avidin (or streptavidin) is then added and allowed to bind to the activated biotin. The avidin possesses free biotin binding sites which can be utilized to "anchor" the biotinylated proteins through a biotin-(strept-)avidin linkage.

Alternatively, the fusion protein can be attached to the solid support with a biotin derivative possessing a photo-removable protecting group. This moiety is covalently bound

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to bovine serum albumin (BSA), which is attached to the solid support, *e.g.*, a glass surface. See Pirrung and Huang, 1996. *Bioconjugate Chem.* 7: 317-321. A mask is then used to create activated biotin within the defined irradiated areas. Avidin may then be localized to the irradiated area, with a biotinylated sulfurylase-luciferase fusion protein subsequently attached through a BSA-biotin-avidin-biotin link.

Another method of attachment is with the use of a pluronics based attachment. Pluronics attach to hydrophobic surfaces by virtue of the reaction between the hydrophobic surface and the polypropylene oxide. The remaining polyethylene oxide groups extend off the surface, thereby creating a hydrophilic environment. Nitrilotriacetic acid (NTA) can be conjugated to the terminal ends of the polyethylene oxide chains to allow for hexahistidine tagged proteins to be attached.

This invention provides methods of sequencing which utilize and ATP generating polypeptide-ATP converting polypeptide fusion protein for detection. In a preferred embodiment, the nucleotide sequence of the sequencing product is determined by measuring inorganic pyrophosphate (PPi) liberated from a nucleotide triphosphate (dNTP) as the dNMP is incorporated into an extended sequence primer. This method of sequencing is termed Pyrosequencing™ technology (PyroSequencing AB, Stockholm, Sweden). It can be performed in solution (liquid phase) or as a solid phase technique. Various sequencing methods, including PPi sequencing methods, are described in, *e.g.,* WO9813523A1, Ronaghi, *et al.,* 1996. *Anal. Biochem.* 242: 84-89, and Ronaghi, *et al.*, 1998. *Science* 281: 363-365 (1998), US patent 6,274,320 and the patent application USSN 10/104,280 which was filed on March 21, 2001 (21465-501CIP3). These disclosures of sequencing are incorporated herein in their entirety, by reference.

Pyrophosphate released under these conditions can be detected enzymatically (*e.g.,* by the generation of light in the luciferase-luciferin reaction). Such methods enable a nucleotide to be identified in a given target position, and the DNA to be sequenced simply and rapidly while avoiding the need for electrophoresis and the use of potentially dangerous radiolabels.

The invention also provides a method for sequencing nucleic acids which generally comprises (a) providing one or more nucleic acid anchor primers and a plurality of single-stranded circular nucleic acid templates disposed within a plurality of reaction chambers or cavities; (b) annealing an effective amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template

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complex; (c) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template; (d) annealing an effective amount of a sequencing primer to one or more copies of said covalently linked complementary nucleic acid; (e) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a sequencing reaction byproduct; and (f) identifying the PPi sequencing reaction byproduct with the use of an ATP generating polypeptide-ATP converting polypeptide fusion protein, thereby determining the sequence of the nucleic acid. In one embodiment, a dATP or ddATP analogue is used in place of deoxy- or dideoxy adenosine triphosphate. This analogue is capable of acting as a substrate for a polymerase but incapable of acting as a substrate for a PPi-detection enzyme. This method can be carried out in separate parallel common reactions in an aqueous environment.

In another aspect, the invention includes a method of determining the base sequence of a plurality of nucleotides on an array, which generally comprises (a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface; (b) adding an activated nucleotide 5'-triphosphate precursor of one known nitrogenous base to a reaction mixture in each reaction chamber, each reaction mixture comprising a template-directed nucleotide polymerase and a single-stranded polynucleotide template hybridized to a complementary oligonucleotide primer strand at least one nucleotide residue shorter than the templates to form at least one unpaired nucleotide residue in each template at the 3'-end of the primer strand, under reaction conditions which allow incorporation of the activated nucleoside 5'-triphosphate precursor onto the 3'-end of the primer strands, provided the nitrogenous base of the activated nucleoside 5'-triphosphate precursor is complementary to the nitrogenous base of the unpaired nucleotide residue of the templates; (c) utilizing an ATP generating polypeptide-ATP converting polypeptide fusion protein to detect whether or not the nucleoside 5'-triphosphate precursor was incorporated into the primer strands in which incorporation of the nucleoside 5'-triphosphate precursor indicates that the unpaired nucleotide residue of the template has a nitrogenous base composition that is complementary to that of the incorporated nucleoside 5'-triphosphate precursor; and (d) sequentially repeating steps (b) and (c), wherein each sequential repetition adds and, detects the

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incorporation of one type of activated nucleoside 5'-triphosphate precursor of known nitrogenous base composition; and (e) determining the base sequence of the unpaired nucleotide residues of the template in each reaction chamber from the sequence of incorporation of said nucleoside precursors.

The anchor primers of the invention generally comprise a stalk region and at least one adaptor region. In a preferred embodiment the anchor primer contains at least two contiguous adapter regions. The stalk region is present at the 5' end of the anchor primer and includes a region of nucleotides for attaching the anchor primer to the solid substrate.

The adaptor region(s) comprise nucleotide sequences that hybridize to a complementary sequence present in one or more members of a population of nucleic acid sequences. In some embodiments, the anchor primer includes two adjoining adaptor regions, which hybridize to complementary regions ligated to separate ends of a target nucleic acid sequence. In additional embodiments, the adapter regions in the anchor primers are complementary to non-contiguous regions of sequence present in a second nucleic acid sequence. Each adapter region, for example, can be homologous to each terminus of a fragment produced by digestion with one or more restriction endonucleases. The fragment can include, *e.g.*, a sequence known or suspected to contain a sequence polymorphism. Additionally, the anchor primer may contain two adapter regions that are homologous to a gapped region of a target nucleic acid sequence, *i.e.*, one that is non-contiguous because of a deletion of one or more nucleotides. When adapter regions having these sequences are used, an aligning oligonucleotide corresponding to the gapped sequence may be annealed to the anchor primer along with a population of template nucleic acid molecules.

The anchor primer may optionally contain additional elements such as one or more restriction enzyme recognition sites, RNA polymerase binding sites, *e.g.*, a T7 promoter site, or sequences present in identified DNA sequences, *e.g.*, sequences present in known genes. The adapter region(s) may also include sequences known to flank sequence polymorphisms. Sequence polymorphisms include nucleotide substitutions, insertions, deletions, or other rearrangements which result in a sequence difference between two otherwise identical nucleic acid sequences. An example of a sequence polymorphism is a single nucleotide polymorphism (SNP).

In general, any nucleic acid capable of base-pairing can be used as an anchor primer. In some embodiments, the anchor primer is an oligonucleotide. As utilized herein the term

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oligonucleotide includes linear oligomers of natural or modified monomers or linkages, *e.g.*, deoxyribonucleosides, ribonucleosides, anomeric forms thereof, peptide nucleic acids (PNAs), and the like, that are capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions. These types of interactions can include, *e.g.*, Watson-Crick type of base-pairing, base stacking, Hoogsteen or reverse-Hoogsteen types of base-pairing, or the like. Generally, the monomers are linked by phosphodiester bonds, or analogs thereof, to form oligonucleotides ranging in size from, *e.g.*, 3-200, 8-150, 10-100, 20-80, or 25-50 monomeric units. Whenever an oligonucleotide is represented by a sequence of letters, it is understood that the nucleotides are oriented in the 5' $\longrightarrow$ 3' direction, from left-to-right, and that the letter "A" donates deoxyadenosine, the letter "T" denotes thymidine, the letter "C" denotes deoxycytosine, and the letter "G" denotes deoxyguanosine, unless otherwise noted herein. The oligonucleotides of the present invention can include non-natural nucleotide analogs. However, where, for example, processing by enzymes is required, or the like, oligonucleotides comprising naturally occurring nucleotides are generally required for maintenance of biological function.

Anchor primers are linked to the solid substrate at the sensitized sites. They can be linked by the same method of linkage as described for the fusion protein to the solid support. A region of a solid substrate containing a linked primer is referred to herein as an anchor pad. Thus, by specifying the sensitized states on the solid support, it is possible to form an array or matrix of anchor pads. The anchor pads can be, *e.g.*, small diameter spots etched at evenly spaced intervals on the solid support. The anchor pads can be located at the bottoms of the cavitations or wells if the substrate has been cavitated, etched, or otherwise micromachined as discussed above.

In one embodiment, the anchor primer is linked to a particle. The anchor primer can be linked to the particle prior to formation of the extended anchor primer or after formation of the extended anchor primer.

Each sensitized site on a solid support is potentially capable of attaching multiple anchor primers. Thus, each anchor pad may include one or more anchor primers. It is preferable to maximize the number of pads that have only a single productive reaction center (*e.g.*, the number of pads that, after the extension reaction, have only a single sequence extended from the anchor primer). This can be accomplished by techniques which include, but are not limited to: *(i)* varying the dilution of biotinylated anchor primers that are washed

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over the surface; (*ii*) varying the incubation time that the biotinylated primers are in contact with the avidin surface; (*iii*) varying the concentration of open- or closed-circular template so that, on average, only one primer on each pad is extended to generate the sequencing template; or (*iv*) reducing the size of the anchor pad to approach single-molecule dimensions (< 1 μm) such that binding of one anchor inhibits or blocks the binding of another anchor (*e.g.* by photoactivation of a small spot); or (*v*) reducing the size of the anchor pad such that binding of one circular template inhibits or blocks the binding of a second circular template.

In some embodiments, each individual pad contains just one linked anchor primer. Pads having only one anchor primer can be made by performing limiting dilutions of a selected anchor primer on to the solid support such that, on average, only one anchor primer is deposited on each pad. The concentration of anchor primer to be applied to a pad can be calculated utilizing, for example, a Poisson distribution model.

In order to maximize the number of reaction pads that contain a single anchor primer, a series of dilution experiments are performed in which a range of anchor primer concentrations or circular template concentrations are varied. For highly dilute concentrations of primers, primers and circular templates binding to the same pad will be independent of each other, and a Poisson distribution will characterize the number of anchor primers extended on any one pad. Although there will be variability in the number of primers that are actually extended, a maximum of 37% of the pads will have a single extended anchor primer (the number of pads with a single anchor oligonucleotide).

In other embodiments multiple anchor primers are attached to any one individual pad in an array. Limiting dilutions of a plurality of circular nucleic acid templates (described in more detail below) may be hybridized to the anchor primers so immobilized such that, on average, only one primer on each pad is hybridized to a nucleic acid template. Library concentrations to be used may be calculated utilizing, for example, limiting dilutions and a Poisson distribution model.

The nucleic acid templates that can be sequenced according to the invention, *e.g.*, a nucleic acid library, in general can include open circular or closed circular nucleic acid molecules. A "closed circle" is a covalently closed circular nucleic acid molecule, *e.g.*, a circular DNA or RNA molecule. An "open circle" is a linear single-stranded nucleic acid molecule having a 5' phosphate group and a 3' hydroxyl group. In one embodiment, the single stranded nucleic acid contains at least 100 copies of nucleic acid sequence, each copy

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covalently linked end to end. In some embodiments, the open circle is formed *in situ* from a linear double-stranded nucleic acid molecule. The ends of a given open circle nucleic acid molecule can be ligated by DNA ligase. Sequences at the 5' and 3' ends of the open circle molecule are complementary to two regions of adjacent nucleotides in a second nucleic acid molecule, *e.g.*, an adapter region of an anchor primer, or to two regions that are nearly adjoining in a second DNA molecule. Thus, the ends of the open-circle molecule can be ligated using DNA ligase, or extended by DNA polymerase in a gap-filling reaction. Open circles are described in detail in Lizardi, U.S. Pat. No. 5,854,033. An open circle can be converted to a closed circle in the presence of a DNA ligase (for DNA) or RNA ligase following, *e.g.*, annealing of the open circle to an anchor primer.

If desired, nucleic acid templates can be provided as padlock probes. Padlock probes are linear oligonucleotides that include target-complementary sequences located at each end, and which are separated by a linker sequence. The linkers can be ligated to ends of members of a library of nucleic acid sequences that have been, *e.g.*, physically sheared or digested with restriction endonucleases. Upon hybridization to a target-sequence, the 5'- and 3'-terminal regions of these linear oligonucleotides are brought in juxtaposition. This juxtaposition allows the two probe segments (if properly hybridized) to be covalently-bound by enzymatic ligation (*e.g.*, with T4 DNA ligase), thus converting the probes to circularly-closed molecules which are catenated to the specific target sequences (see *e.g.*, Nilsson, *et al.*, 1994. *Science* 265: 2085-2088). The resulting probes are suitable for the simultaneous analysis of many gene sequences both due to their specificity and selectivity for gene sequence variants (see *e.g.*, Lizardi, *et al.*, 1998. *Nat. Genet.* 19: 225-232; Nilsson, *et al.*, 1997. *Nat. Genet.* 16: 252-255) and due to the fact that the resulting reaction products remain localized to the specific target sequences. Moreover, intramolecular ligation of many different probes is expected to be less susceptible to non-specific cross-reactivity than multiplex PCR-based methodologies where non-cognate pairs of primers can give rise to irrelevant amplification products (see *e.g.*, Landegren and Nilsson, 1997. *Ann. Med.* 29: 585-590).

A starting library can be constructed comprising either single-stranded or double-stranded nucleic acid molecules, provided that the nucleic acid sequence includes a region that, if present in the library, is available for annealing, or can be made available for annealing, to an anchor primer sequence. For example, when used as a template for rolling

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circle amplification, a region of a double-stranded template needs to be at least transiently single-stranded in order to act as a template for extension of the anchor primer.

Library templates can include multiple elements, including, but not limited to, one or more regions that are complementary to the anchor primer. For example, the template libraries may include a region complementary to a sequencing primer, a control nucleotide region, and an insert sequence comprised of the sequencing template to be subsequently characterized. As is explained in more detail below, the control nucleotide region is used to calibrate the relationship between the amount of byproduct and the number of nucleotides incorporated. As utilized herein the term "complement" refers to nucleotide sequences that are able to hybridize to a specific nucleotide sequence to form a matched duplex.

In one embodiment, a library template includes: (*i*) two distinct regions that are complementary to the anchor primer, (*ii*) one region homologous to the sequencing primer, (*iii*) one optional control nucleotide region, (*iv*) an insert sequence of, *e.g.*, 30-500, 50-200, or 60-100 nucleotides, that is to be sequenced. The template can, of course, include two, three, or all four of these features.

The template nucleic acid can be constructed from any source of nucleic acid, *e.g.*, any cell, tissue, or organism, and can be generated by any art-recognized method. Suitable methods include, *e.g.*, sonication of genomic DNA and digestion with one or more restriction endonucleases (RE) to generate fragments of a desired range of lengths from an initial population of nucleic acid molecules. Preferably, one or more of the restriction enzymes have distinct four-base recognition sequences. Examples of such enzymes include, *e.g.*, Sau3A1, MspI, and TaqI. Preferably, the enzymes are used in conjunction with anchor primers having regions containing recognition sequences for the corresponding restriction enzymes. In some embodiments, one or both of the adapter regions of the anchor primers contain additional sequences adjoining known restriction enzyme recognition sequences, thereby allowing for capture or annealing to the anchor primer of specific restriction fragments of interest to the anchor primer. In other embodiments, the restriction enzyme is used with a type IIS restriction enzyme.

Alternatively, template libraries can be made by generating a complementary DNA (cDNA) library from RNA, *e.g.*, messenger RNA (mRNA). The cDNA library can, if desired, be further processed with restriction endonucleases to obtain a 3' end characteristic of a specific RNA, internal fragments, or fragments including the 3' end of the isolated RNA.

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Adapter regions in the anchor primer may be complementary to a sequence of interest that is thought to occur in the template library, *e.g.*, a known or suspected sequence polymorphism within a fragment generated by endonuclease digestion.

In one embodiment, an indexing oligonucleotide can be attached to members of a template library to allow for subsequent correlation of a template nucleic acid with a population of nucleic acids from which the template nucleic acid is derived. For example, one or more samples of a starting DNA population can be fragmented separately using any of the previously disclosed methods (*e.g.*, restriction digestion, sonication). An indexing oligonucleotide sequence specific for each sample is attached to, *e.g.*, ligated to, the termini of members of the fragmented population. The indexing oligonucleotide can act as a region for circularization, amplification and, optionally, sequencing, which permits it to be used to index, or code, a nucleic acid so as to identify the starting sample from which it is derived.

Distinct template libraries made with a plurality of distinguishable indexing primers can be mixed together for subsequent reactions. Determining the sequence of the member of the library allows for the identification of a sequence corresponding to the indexing oligonucleotide. Based on this information, the origin of any given fragment can be inferred.

Libraries of nucleic acids are annealed to anchor primer sequences using recognized techniques (see, *e.g.*, Hatch, *et al.*, 1999. *Genet. Anal. Biomol. Engineer.* 15: 35-40; Kool, U.S. Patent No. 5,714, 320 and Lizardi, U.S. Patent No. 5,854,033). In general, any procedure for annealing the anchor primers to the template nucleic acid sequences is suitable as long as it results in formation of specific, *i.e.*, perfect or nearly perfect, complementarity between the adapter region or regions in the anchor primer sequence and a sequence present in the template library.

A number of *in vitro* nucleic acid amplification techniques may be utilized to extend the anchor primer sequence. The size of the amplified DNA preferably is smaller than the size of the anchor pad and also smaller than the distance between anchor pads.

The amplification is typically performed in the presence of a polymerase, *e.g.*, a DNA or RNA-directed DNA polymerase, and one, two, three, or four types of nucleotide triphosphates, and, optionally, auxiliary binding proteins. In general, any polymerase capable of extending a primed 3'-OH group can be used a long as it lacks a 3' to 5' exonuclease activity. Suitable polymerases include, *e.g.*, the DNA polymerases from *Bacillus stearothermophilus*, *Thermus acquaticus*, *Pyrococcus furiosis*, *Thermococcus litoralis*, and

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Thermus thermophilus, bacteriophage T4 and T7, and the *E. coli* DNA polymerase I Klenow fragment. Suitable RNA-directed DNA polymerases include, *e.g.*, the reverse transcriptase from the Avian Myeloblastosis Virus, the reverse transcriptase from the Moloney Murine Leukemia Virus, and the reverse transcriptase from the Human Immunodeficiency Virus-I.

A number of *in vitro* nucleic acid amplification techniques have been described. These amplification methodologies may be differentiated into those methods: (*i*) which require temperature cycling - polymerase chain reaction (PCR) (see *e.g.*, Saiki, *et al.*, 1995. *Science* 230: 1350-1354), ligase chain reaction (see *e.g.*, Barany, 1991. *Proc. Natl. Acad. Sci. USA* 88: 189-193; Barringer, *et al.*, 1990. *Gene* 89: 117-122) and transcription-based amplification (see *e.g.*, Kwoh, *et al.*, 1989. *Proc. Natl. Acad. Sci. USA* 86: 1173-1177) and (*ii*) isothermal amplification systems - self-sustaining, sequence replication (see *e.g.*, Guatelli, *et al.*, 1990. *Proc. Natl. Acad. Sci. USA* 87: 1874-1878); the Qβ replicase system (see *e.g.*, Lizardi, *et al.*, 1988. *BioTechnology* 6: 1197-1202); strand displacement amplification Nucleic Acids Res. 1992 Apr 11;20(7):1691-6.; and the methods described in PNAS 1992 Jan 1;89(1):392-6; and NASBA J Virol Methods. 1991 Dec;35(3):273-86.

Isothermal amplification also includes rolling circle-based amplification (RCA). RCA is discussed in, *e.g.*, Kool, U.S. Patent No. 5,714,320 and Lizardi, U.S. Patent No. 5,854,033; Hatch, *et al.*, 1999. *Genet. Anal. Biomol. Engineer.* 15: 35-40. The result of the RCA is a single DNA strand extended from the 3' terminus of the anchor primer (and thus is linked to the solid support matrix) and including a concatamer containing multiple copies of the circular template annealed to a primer sequence. Typically, 1,000 to 10,000 or more copies of circular templates, each having a size of, *e.g.*, approximately 30-500, 50-200, or 60-100 nucleotides size range, can be obtained with RCA.

In vivo, RCR is utilized in several biological systems. For example, the genome of several bacteriophage are single-stranded, circular DNA. During replication, the circular DNA is initially converted to a duplex form, which is then replicated by the aforementioned rolling-circle replication mechanism. The displaced terminus generates a series of genomic units that can be cleaved and inserted into the phage particles. Additionally, the displaced single-strand of a rolling-circle can be converted to duplex DNA by synthesis of a complementary DNA strand. This synthesis can be used to generate the concatemeric duplex molecules required for the maturation of certain phage DNAs. For example, this provides the principle pathway by which λ bacteriophage matures. RCR is also used *in vivo* to generate

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amplified rDNA in *Xenopus* oocytes, and this fact may help explain why the amplified rDNA is comprised of a large number of identical repeating units. In this case, a single genomic repeating unit is converted into a rolling-circle. The displaced terminus is then converted into duplex DNA which is subsequently cleaved from the circle so that the two termini can be ligated together so as to generate the amplified circle of rDNA.

Through the use of the RCA reaction, a strand may be generated which represents many tandem copies of the complement to the circularized molecule. For example, RCA has recently been utilized to obtain an isothermal cascade amplification reaction of circularized padlock probes *in vitro* in order to detect single-copy genes in human genomic DNA samples (see Lizardi, *et al.,* 1998. *Nat. Genet.* 19: 225-232). In addition, RCA has also been utilized to detect single DNA molecules in a solid phase-based assay, although difficulties arose when this technique was applied to *in situ* hybridization (see Lizardi, *et al.,* 1998. *Nat. Genet.* 19: 225-232).

If desired, RCA can be performed at elevated temperatures, *e.g.*, at temperatures greater than 37° C, 42° C, 45° C, 50° C, 60° C, or 70° C. In addition, RCA can be performed initially at a lower temperature, *e.g.*, room temperature, and then shifted to an elevated temperature. Elevated temperature RCA is preferably performed with thermostable nucleic acid polymerases and with primers that can anneal stably and with specificity at elevated temperatures.

RCA can also be performed with non-naturally occurring oligonucleotides, *e.g.*, peptide nucleic acids. Further, RCA can be performed in the presence of auxiliary proteins such as single-stranded binding proteins.

The development of a method of amplifying short DNA molecules which have been immobilized to a solid support, termed RCA has been recently described in the literature (see *e.g.,* Hatch, *et al.,* 1999. *Genet. Anal. Biomol. Engineer.* 15: 35-40; Zhang, *et al.,* 1998. *Gene* 211: 277-85; Baner, *et al.,* 1998. *Nucl. Acids Res.* 26: 5073-5078; Liu, *et al.,* 1995. *J. Am. Chem. Soc.* 118: 1587-1594; Fire and Xu, 1995. *Proc. Natl. Acad. Sci. USA* 92: 4641-4645; Nilsson, *et al.,* 1994. *Science* 265: 2085-2088). RCA targets specific DNA sequences through hybridization and a DNA ligase reaction. The circular product is then subsequently used as a template in a rolling circle replication reaction.

RCA driven by DNA polymerase can replicate circularized oligonucleotide probes with either linear or geometric kinetics under isothermal conditions. In the presence of two

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primers (one hybridizing to the + strand, and the other, to the - strand of DNA), a complex pattern of DNA strand displacement ensues which possesses the ability to generate $1x10^9$ or more copies of each circle in a short period of time (*i.e.,* less-than 90 minutes), enabling the detection of single-point mutations within the human genome. Using a single primer, RCA generates hundreds of randomly-linked copies of a covalently closed circle in several minutes. If solid support matrix-associated, the DNA product remains bound at the site of synthesis, where it may be labeled, condensed, and imaged as a point light source. For example, linear oligonucleotide probes, which can generate RCA signals, have been bound covalently onto a glass surface. The color of the signal generated by these probes indicates the allele status of the target, depending upon the outcome of specific, target-directed ligation events. As RCA permits millions of individual probe molecules to be counted and sorted, it is particularly amenable for the analysis of rare somatic mutations. RCA also shows promise for the detection of padlock probes bound to single-copy genes in cytological preparations.

In addition, a solid-phase RCA methodology has also been developed to provide an effective method of detecting constituents within a solution. Initially, a recognition step is used to generate a complex h a circular template is bound to a surface. A polymerase enzyme is then used to amplify the bound complex. RCA uses small DNA probes that are amplified to provide an intense signal using detection methods, including the methods described in more detail below.

Other examples of isothermal amplification systems include, *e.g.*, (*i*) self-sustaining, sequence replication (see *e.g.,* Guatelli, *et al.,* 1990. *Proc. Natl. Acad. Sci. USA* 87: 1874-1878), (*ii*) the Qβ replicase system (see *e.g.,* Lizardi, *et al.,* 1988. *BioTechnology* 6: 1197-1202), and (*iii*) nucleic acid sequence-based amplification (NASBA™; see Kievits, *et al.,* 1991. *J. Virol. Methods* 35: 273-286).

Amplification of a nucleic acid template as described above results in multiple copies of a template nucleic acid sequence covalently linked to an anchor primer. In one embodiment, a region of the sequence product is determined by annealing a sequencing primer to a region of the template nucleic acid, and then contacting the sequencing primer with a DNA polymerase and a known nucleotide triphosphate, *i.e.*, dATP, dCTP, dGTP, dTTP, or an analog of one of these nucleotides. The sequence can be determined by detecting a sequence reaction byproduct, as is described below.

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The sequence primer can be any length or base composition, as long as it is capable of specifically annealing to a region of the amplified nucleic acid template. No particular structure for the sequencing primer is required so long as it is able to specifically prime a region on the amplified template nucleic acid. Preferably, the sequencing primer is complementary to a region of the template that is between the sequence to be characterized and the sequence hybridizable to the anchor primer. The sequencing primer is extended with the DNA polymerase to form a sequence product. The extension is performed in the presence of one or more types of nucleotide triphosphates, and if desired, auxiliary binding proteins.

The method comprises the steps of: (a) introducing the template nucleic acid polymer into a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added; (b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released; (c) separately recovering each of the feedstocks from the polymerization environment; and (d) measuring the amount of inorganic pyrophosphate by utilizing an ATP generating polypeptide-ATP converting polypeptide fusion protein in each of the recovered feedstocks to determine the identity of each nucleotide in the complementary polymer and thus the sequence of the template polymer.

The sequence primer can be any length or base composition, as long as it is capable of specifically annealing to a region of the amplified nucleic acid template. No particular structure is required for the sequencing primer so long as it is able to specifically prime a region on the amplified template nucleic acid. Preferably, the sequencing primer is complementary to a region of the template that is between the sequence to be characterized and the sequence hybridizable to the anchor primer. The sequencing primer is extended with the DNA polymerase to form a sequence product. The extension is performed in the presence of one or more types of nucleotide triphosphates, and if desired, auxiliary binding proteins.

This invention also includes a method wherein the amount of inorganic pyrophosphate is measured by (a) adding adenosine-5'-phosphosulfate to the feedstock; combining the recovered feedstock containing adenosine-5'-phosphosulfate with an ATP generating

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polypeptide-ATP converting polypeptide fusion protein such that any inorganic pyrophosphate in the recovered feedstock and the adenosine-5'-phosphosulfate will first react to the form ATP and sulfate and then react with luciferin in the presence of oxygen such that the ATP is consumed to produced AMP, inorganic pyrophosphate, carbon dioxide and light; and (b) measuring the amount of light produced. In a preferred embodiment, the template polymer and ATP generating polypeptide-ATP converting polypeptide fusion protein are immobilized on a solid support.

The invention also includes a method for determining the nucleic acid sequence in a template nucleic acid polymer, comprising: (a) introducing the template nucleic acid polymer into a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added; (b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released; (c) separately recovering each of the feedstocks from the polymerization environment; and (d) measuring the amount of PPi with a thermostable sulfurylase and a luciferase in each of the recovered feedstocks to determine the identity of each nucleotide in the complementary polymer and thus the sequence of the template polymer. In one embodiment, the thermostable sulfurylase and the luciferase are joined in a fusion protein. In another embodiment, the thermostable sulfurylase is joined to an affinity tag.

The invention further provides a method for sequencing a nucleic acid, the method comprising: (a) providing one or more nucleic acid anchor primers; (b) providing a plurality of single-stranded circular nucleic acid templates disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200; (c) annealing an effective amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex; (d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the

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circular nucleic acid template; (e) annealing an effective amount of a sequencing primer to one or more copies of said covalently linked complementary nucleic acid; (f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a sequencing reaction byproduct; and (g) identifying the sequencing reaction byproduct with the use of a thermostable sulfurylase and a luciferase, thereby determining the sequence of the nucleic acid. In one embodiment, the thermostable sulfurylase and the luciferase are joined in a fusion protein. In another embodiment, the thermostable sulfurylase is joined to an affinity tag.

Also included in the invention is a method for sequencing a nucleic acid, the method comprising: (a) providing at least one nucleic acid anchor primer; (b) providing a plurality of single-stranded circular nucleic acid templates in an array having at least 400,000 discrete reaction sites; (c) annealing a first amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex; (d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template; (e) annealing a second amount of a sequencing primer to one or more copies of the covalently linked complementary nucleic acid; (f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, when the predetermined nucleotide triphosphate is incorporated onto the 3' end of the sequencing primer, to yield a sequencing reaction byproduct; and (g) identifying the sequencing reaction byproduct with the use of a thermostable sulfurylase and a luciferase, thereby determining the sequence of the nucleic acid at each reaction site that contains a nucleic acid template. In one embodiment, the thermostable sulfurylase and the luciferase are joined in a fusion protein. In another embodiment, the thermostable sulfurylase is joined to an affinity tag.

The invention also includes a method of determining the base sequence of a plurality of nucleotides on an array, the method comprising: (a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, (b) adding an activated nucleotide 5'-triphosphate precursor of one known nitrogenous base to a reaction mixture in each reaction chamber, each reaction

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mixture comprising a template-directed nucleotide polymerase and a single-stranded polynucleotide template hybridized to a complementary oligonucleotide primer strand at least one nucleotide residue shorter than the templates to form at least one unpaired nucleotide residue in each template at the 3'-end of the primer strand, under reaction conditions which allow incorporation of the activated nucleoside 5'-triphosphate precursor onto the 3'-end of the primer strands, provided the nitrogenous base of the activated nucleoside 5'-triphosphate precursor is complementary to the nitrogenous base of the unpaired nucleotide residue of the templates; (c) detecting whether or not the nucleoside 5'-triphosphate precursor was incorporated into the primer strands through detection of a sequencing byproduct with a thermostable sulfurylase and luciferase, thus indicating that the unpaired nucleotide residue of the template has a nitrogenous base composition that is complementary to that of the incorporated nucleoside 5'-triphosphate precursor; and (d) sequentially repeating steps (b) and (c), wherein each sequential repetition adds and, detects the incorporation of one type of activated nucleoside 5'-triphosphate precursor of known nitrogenous base composition; and (e) determining the base sequence of the unpaired nucleotide residues of the template in each reaction chamber from the sequence of incorporation of said nucleoside precursors. In one embodiment, the thermostable sulfurylase and the luciferase are joined in a fusion protein. In another embodiment, the thermostable sulfurylase is joined to an affinity tag.

The invention will be further illustrated in the following non-limiting examples. There are several abbreviations which will be used in the following examples: FUS stands for fusion gene, S stands for sulfurylase, L stands for luciferase, TL stands for thermostable luciferase, X stands for XhoI, H stands for HindIII, N stands for NotI and B stands for BamHI. For example, FUS-L/S X F means a primer for the fusion gene, luciferase-sulfurylase Xho Forward and so forth. Primers 1 through 6 are for the L or TL to S fusions and primers 7 through 13 are for the S to L or TL fusions.

EXAMPLES

Example 1: Cloning Strategy for Obtaining the Bst Sulfurylase Gene

Gene specific primers, which incorporated restriction site linkers, were designed based on the sequence for a putative ATP sulfurylase from *Bacillus stearothermophilus* in

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ERGO, a curated database of genomic DNA made available on the World Wide Web by Integrated Genomics which included the *Bacillus stearothermophilus* Genome Sequencing Project at the University of Oklahoma (NSF Grant #EPS-9550478). The forward primer utilized was 5'-CCC TTC TGC AGC ATG AGC GTA AGC ATC CCG CAT GGC GGC ACA TTG-3' (SEQ ID NO: 7) and the reverse primer used was 5'-CCC GTA AGC TTT TAG CGC GCT GAC GGG GCG ACC GTT TCG CGT TCT TG-3' (SEQ ID NO:8). The reaction mix for PCR amplification contained 5.0 uL 10X polymerase buffer (Clontech, Cat. #8714), 2.0 uL 5 M betaine (Sigma, Cat. #B0300), 1.0 uL dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP), 0.8 uL Advantage 2 polymerase (Clontech, Cat. #8714), 0.2 uL Advantage-HF 2 polymerase (Clontech, Cat. #K1914), 10 pmol forward primer, 10 pmol reverse primer, 100 ng (or less) *Bst* genomic DNA (ATCC, Cat. #12980D), and enough distilled water to make total volume of 50 uL. As little as 1 ng *Bst* genomic DNA was sufficient to yield PCR product. The PCR amplification of Bst ATP sulfurylase gene from genomic DNA consisted of an initial step at 96°C for 3 min, then 35 cycles of 96°C for 15 sec, 60°C for 30 sec, 72°C for 6 min, a finishing step at 72°C for 10 min and finally 14°C until removal. The PCR product was cleaned using QIAquick PCR Purification Kit (QIAGEN).

Example 2: Cloning Strategy for Obtaining the Sulfurylase-Luciferase Fusion Protein

All chemicals were purchased from Sigma unless noted otherwise. Racemically pure D-luciferin was ordered from Pierce. The assay buffer for measuring ATP sulfurylase and luciferase activities contained *Taq* polymerase. A polymerase chain reaction (PCR)-mediated approach was utilized to link the open reading frames (ORFs) of luciferase and sulfurylase. The cloning strategy is outlined in Fig. 1. Briefly, it involved the amplification of luciferase and sulfurylase ORFs by PCR, using primers that contain convenient restriction sites (XhoI and HindIII) to clone the fusion gene into an expression vector, in-frame and, the design of a rare restriction site (Not I) at the junction of the two polypeptides so that other versions of luciferase, such as thermostable luciferase (TL), and sulfurylase can be conveniently swapped to obtain either sulfurlyase-luciferase (S-L) or luciferase-sulfurlyase (L-S) fusion proteins. A Not I site was used to fuse the variable heavy chain of antibodies to luciferase to generate a viable fusion protein. These primers were also designed in such a way that the primers that form part of the junction of the two ORFs contain sufficient overlapping regions of

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nucleotides. For example, the 5' end of FUS-L/S Not R contains deoxynucleotides in an anti-parallel orientation that encode the N-terminal 10 amino acids of yeast sulfurylase. Thus, a PCR product generated using this primer would anneal to the 5' end of yeast sulfurylase ORF and would generate the fusion protein, L-S.

The products in boxes were obtained by PCR as elaborated in Fig.2. As shown in Fig. 3, the PCR products were subjected to electrophoresis. The PCR products were then purified, digested with Xho I and Hind III and subcloned into Xho I/Hind III digested pRSETA-BCCP. pRSETA-BCCP is a derivative of pRSET A (Invitrogen) in which the sequence between *Nhe*I and *Bam*HI restriction sites has been replaced by the portion of the biotin carboxyl carrier protein (BCCP) gene from *E. coli* (GenBank accession #M80458) that codes for residues 87-165. The 87- amino acid BCCP domain was obtained by PCR and cloned into the NheI and Bam HI sites of pRSETA to obtain pRSETA-BCCP. The ligated fusion protein and pRSETA-BCCP were transformed into BL21DE3 and TOP10 cells. BL21DE3 cells yielded colonies for L-S and TOP10 cells yielded colonies for TL-S.

The following list of primers was used to construct the fusion proteins:

PRIMER NO	TITLE	NUCLEIC ACID SEQUENCE	SEQ ID NO
1	FUS-L/S X F	CCCC CTC GAG ATC CAA ATG GAA GAC GCC AAA AAC ATA AAG AAA GGC CC	9
2	FUS-TL/S X F	CCCC CTC GAG ATC CAA ATG GCT GAC AAA AAC ATC CTG TAT GGC CC	10
3	FUS-L/S Not R	TTG TAG AAT ACC ACC GTG AGG AGC AGG CAT AGC GGC CGC CAA TTT GGA CTT TCC GCC CTT CTT GGC C	11
4	FUS-TL/S Not R	TTG TAG AAT ACC ACC GTG AGG AGC AGG CAT AGC GGC CGC ACC GTT GGT GTG TTT CTC GAA CAT C	12
5	FUS-S-Not F	GCG GCC GCT ATG CCT GCT CCT CAC GGT GGT ATT CTA C	13
6	FUS-S-Hind III R	CCCC AAG CTT TTA AAA TAC AAA AAA GCC ATT GTC TTC CAA GAA TAG GAC	14
7	FUS-S/L B F	CCCC GGA TCC ATC CAA ATG CCT GCT CCT CAC GGT GGT ATT CTA CAA GAC	15
8	FUS-S/L R	GGGCCTTTCTTTATGTTTTTGGCGTCTTCCAT AGC GGC CGC AAA TAC AAA AAA GCC ATT GTC	16
9	FUS-L- F	GCG GCC GCT ATG GAA GAC GCC AAA AAC ATA AAG AAA GGC CC	17
10	FUS-L-N-R	CCCC CCA TGG TTA CAA TTT GGA CTT TCC GCC CTT CTT GGC C	18
11	FUS-S/TL R	GG GCC ATA CAG GAT GTT TTT GTC AGC CAT AGC GGC CGC AAA TAC AAA AAA GCC ATT GTC	19

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12	FUS-TL-F	GCG GCC GCT ATG GCT GAC AAA AAC ATC CTG TAT GGC CC	20
13	FUS-TL-H-R	CCCC AAG CTT CTA ACC GTT GGT GTG TTT CTC GAA CAT CTG ACG C	21

These primers were utilized to perform PCR. The following PCR condition was used.

PCR condition

96°C for 3:00; 96°C for 0:15; 76°C for 0:30; -1°C per cycle; 72°C for 6:00;
For 15 cycles; 96°C for 0:15; 60°C for 0:30; 72°C for 6:00;
For 29 cycles; 72°C for 10:00;
14°C forever

Example 3: Cloning of the His6-BCCP Bst ATP Sulfurylase Fusion Protein

The Bst-affinity tagged fusion construct is a derivative of pRSETA in which the NheI-XhoI fragment has been replaced by the BCCP domain and the ATP sulfurylase is inserted after the BCCP domain.

Briefly, the BstSulf PCR product, as described in Example 1, was double-digested with *Pst*I and *Hind*III, isolated on a 1% agarose/TAE gel, purified using QIAEXII (QIAGEN) and ligated into the large *Pst*I/*Hind*III fragment of pRSETA-BCCP using the Quick Ligation Kit from NEB according to manufacturer's instructions. As mentioned in Example 2, pRSETA-BCCP is a derivative of pRSET A (Invitrogen) in which the sequence between *Nhe*I and *Bam*HI restriction sites has been replaced by the portion of the biotin carboxyl carrier protein (BCCP) gene from *E. coli* (GenBank accession #M80458) that codes for residues 87-165. 2 uL ligation reaction was used to transform 50 uL TOP10 competent cells (Invitrogen) and plated on LB-Ap plates. Sequencing of plasmid insert from ten clones was used to determine the consensus sequence for the ATP sulfurylase gene from ATCC 12980.

The plasmid pRSETA-BCCP-BstSulf was transformed into the *E. coli* expression host BL21(DE3)pLysS (Novagen) and the induction expression of BstHBSulf was carried out according to the maufacturer's instructions. The cells were harvested and stored as frozen pellets. The pellets were lysed using BugBuster plus Benzonase according to manufacturer's instructions and protein was purified on a 20 mL column packed with Chelating Sepharose Fast Flow (Amersham, Cat. #17-0575-02) and charged with nickel (II). Protein was eluted

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using a 0-500 mM imidazole gradient. Analysis by SDS-PAGE showed a single band of the correct size.

Example 4: Binding enzymes to beads

The BCCP domain enables the *E. coli* to add a single biotin molecule onto a specific lysine residue. Hence these fusion proteins can be bound to solid supports that contain streptavidin. TL-S was successfully cloned into a TA vector. 25 µl of MPG-Streptavidin (CPG, Inc.) or Nickel-agarose (Qiagen) were taken in a 1.5 ml tube and placed on a magnet. The supernatant was removed and the beads were resuspended in 25 µg of His6-BCCP-sulfurylase and 75 µg of His6-BCCP-luciferase. To test the fusion protein, 100 µl of dialyzed fusion protein was bound to the 25 µl of beads. The beads were allowed to mix at room temperature for 1hr, washed with assay buffer (25mM Tricine (pH 7.8), 5mM MgAcetate, 1mM DTT, 1mM EDTA, and 1 mg/ml BSA) and assayed for enzyme activities with 1mM PPi, 4mM APS and 300 mM D-luciferin. With the nickel-agarose beads, the EDTA was omitted from the assay buffer.

As shown in Fig. 4, these fusion proteins displayed activity on both the NTA- Agarose and MPG-SA beads. S:L 1:3 represents sulfurylase and luciferase bound individually to beads in a 1:3 ratio. Ni-Ag and MPG-SA are nickel-agarose and MPG-Streptavidin beads, respectively. PL is Promega luciferase, which does not have a polyhistidine or a biotin tag on it and hence serves as a negative control. Fraction 19 contains the fusion protein and is active on both kinds of beads. This suggests that the fusion protein was synthesized with a poly-histidine tag and a biotin molecule on the BCCP domain of the fusion protein.

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What is claimed is:

1. A fusion protein comprising an ATP generating polypeptide bound to a polypeptide which converts ATP to an entity that is detectable.

2. The fusion protein of claim 1 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

3. The fusion protein of claim 2 wherein the ATP sulfurylase is a thermostable sulfurylase comprising the nucleotide sequence of SEQ ID NO:1.

4. The fusion protein of claim 3 wherein the nucleotide sequence encodes the polypeptide sequence of SEQ ID NO:2.

5. The fusion protein of claim 3 wherein the thermostable sulfurylase is active at room temperature.

6. The fusion protein of claim 2 wherein the ATP sulfurylase is from a thermophile.

7. The fusion protein of claim 6 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

8. The fusion protein of claim 1 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

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9. The fusion protein of claim 8 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

10. The fusion protein of claim 9 wherein the animal is selected from the group consisting of mammal, rodent, insect, worm, mollusk, reptile, bird and amphibian.

11. The fusion protein of claim 9 wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, *Brassica napus*, *Allium sativum*, *Amaranthus caudatus*, *Hevea brasiliensis*, *Hordeum vulgare*, *Lycopersicon esculentum*, *Nicotiana tabacum*, *Oryza sativum*, *Pisum sativum*, *Populus trichocarpa*, *Solanum tuberosum*, *Secale cereale*, *Sambucus nigra*, *Ulmus americana* or *Triticum aestivum*.

12. The fusion protein of claim 9 wherein the fungus is *Penicillum chrysogenum*, *Stachybotrys chartarum*, *Aspergillus fumigatus*, *Podospora anserina*, *Trichoderma reesei* and *Riftia pachyptila*.

13. The fusion protein of claim 9 wherein the yeast is *Saccharomyces cerevisiae*, *Candida tropicalis*, *Candida lypolitica*, *Candida utilis*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida spp.*, *Pichia spp.* and *Hansenula spp.*.

14. The fusion protein of claim 8 wherein the prokaryote is bacteria or archaea.

15. The fusion protein of claim 14 wherein the bacteria selected from the group consisting of *E. coli*, *B. subtilis*, *Streptococcus gordonii*, flavobacteria and green sulfur bacteria.

16. The fusion protein of claim 14 wherein the archaea is selected from the group consisting of *Sulfolobus*, *Thermococcus*, *Methanobacterium*, *Halococcus*, *Halobacterium* and *Methanococcus jannaschii*.

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17. The fusion protein of claim 1 wherein the detectable entity is selected from the group consisting of chemiluminescence, bioluminescence and fluorescence.

18. The fusion protein of claim 1 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

19. The fusion protein of claim 18 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

20. The fusion protein of claim 1 which further comprises an affinity tag.

21. The fusion protein of claim 20 wherein the affinity tag is selected from the group consisting of N-terminal poly-histidine, BCCP, protein A, glutathione S transferase, substance P and streptavidin binding peptide.

22. The fusion protein of claim 1 wherein the polypeptides are joined by a linker.

23. The fusion protein of claim 22 wherein the linker is an ala-ala-ala linker.

24. The fusion protein of claim 1 wherein the ATP generating polypeptide is N-terminal to the ATP converting polypeptide.

25. The fusion protein of claim 1 wherein the ATP converting polypeptide is N-terminal to the ATP generating polypeptide.

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26. An isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1, 3 and 5.

27. An isolated polypeptide comprising an amino acid sequence selected from the group consising of SEQ ID NOs:2, 4 and 6.

28. A fusion protein comprising a sulfurylase polypeptide bound to a luciferase polypeptide and at least one affinity tag.

29. The fusion protein of claim 28 wherein the fusion protein comprises the sequence of SEQ ID NO:4.

30. The fusion protein of claim 28 wherein the fusion protein is encoded by a nucleic acid comprising the sequence of SEQ ID NO:3.

31. A fusion protein comprising a thermostable sulfurylase bound to at least one affinity tag.

32. The fusion protein of claim 31 wherein the fusion protein comprises the sequence of SEQ ID NO: 6.

33. The fusion protein of claim 31 wherein the fusion protein is encoded by a nucleic acid comprising the sequence of SEQ ID NO:5.

34. A recombinant polynucleotide that comprises a coding sequence for a fusion protein having an ATP generating polypeptide sequence and an ATP converting polypeptide sequence.

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35. The recombinant polynucleotide sequence of claim 34 wherein the ATP generating polypeptide is ATP sulfurylase.

36. The recombinant polynucleotide sequence of claim 34 wherein the ATP converting polypeptide is luciferase.

37. The recombinant polynucleotide of claim 34 wherein the ATP generating polypeptide is N-terminal to the ATP converting polypeptide.

38. The recombinant polynucleotide of claim 34 wherein the ATP converting polypeptide is N-terminal to the ATP generating polypeptide.

39. An expression vector for expressing a fusion protein, said vector comprising a coding sequence for a fusion protein having: (i) a regulatory sequence, (ii) a first polypeptide sequence of an ATP generating polypeptide and (iii) a second polypeptide sequence that converts ATP to an entity which is detectable.

40. The expression vector of claim 39 wherein the vector further comprises an affinity tag.

41. The expression vector of claim 39 wherein the ATP generating polypeptide is ATP sulfurylase.

42. The expression vector of claim 39 wherein the ATP converting polypeptide is luciferase.

43. The expression vector of claim 39 wherein the regulatory element is an enhancer or a promoter.

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44. The expression vector of claim 43 wherein the promoter is a constitutive promoter or an inducible promoter.

45. A transformed host cell which contains the expression vector of claim 39.

46. The transformed host cell of claim 45 wherein the host cell is a eukaryotic cell.

47. The transformed host cell of claim 46 wherein the eukaryotic cell is human, rat or mouse.

48. The transformed host cell of claim 45 wherein the host cell is a prokaryotic cell.

49. The transformed host cell of claim 48 wherein the prokaryotic cell is bacteria.

50. A purified fusion protein expressed by cells transformed with an expression vector of claim 39.

51. The fusion protein of claim1 bound to a mobile support.

52. The fusion protein of claim 51 wherein the fusion protein is attached by a covalent or non-covalent interaction.

53. The fusion protein of claim 52 wherein the fusion protein is attached by a linkage selected from the group consisting of a metal, a CO^{2+}-hexahistidine complex, a Ni^{2+}-hexahistidine complex, a biotin binding protein, a glutathione S-transferase/glutathione

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complex, a monoclonal antibody/antigen complex, a maltose binding protein/maltose complex and pluronic coupling.

54. The fusion protein of claim 53 wherein the biotin binding protein is selected from the group consisting of NEUTRAVIDIN™ modified avidin, streptavidin and avidin.

55. The fusion protein of claim 51 wherein the mobile support is selected from the group consisting of a bead, optical fiber and glass surface.

56. The fusion protein of claim 55 wherein the bead is a nickel-agarose bead or a MPG-Streptavidin bead.

57. The fusion protein of claim 51 wherein the fusion protein is bound to the mobile support in a 1:3 ratio of protein to mobile support.

58. The fusion protein of claim 51 wherein the fusion protein is a sulfurylase-luciferase fusion protein.

59. A method for determining the nucleic acid sequence in a template nucleic acid polymer, comprising:

(a) introducing the template nucleic acid polymer into a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added;

(b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released;

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(c) separately recovering each of the feedstocks from the polymerization environment; and

(d) measuring the amount of PPi with an ATP generating polypeptide-ATP converting polypeptide fusion protein in each of the recovered feedstocks to determine the identity of each nucleotide in the complementary polymer and thus the sequence of the template polymer.

60. The method of claim 59 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

61. The method of claim 60 wherein the ATP sulfurylase is a thermostable sulfurylase.

62. The method of claim 60 wherein the ATP sulfurylase is from a thermophile.

63. The method of claim 62 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

64. The method of claim 59 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

65. The method of claim 64 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

66. The method of claim 59 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

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67. The method of claim 66 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

68. The method of claim 59 which further comprises an affinity tag.

69. A method according to claim 59, wherein the amount of inorganic pyrophosphate is measured by

(a) adding adenosine-5'-phosphosulfate to the feedstock;

(b) combining the recovered feedstock containing adenosine-5'-phosphosulfate with an ATP generating polypeptide-ATP converting polypeptide fusion protein such that any inorganic pyrophosphate in the recovered feedstock and the adenosine-5'-phosphosulfate will react to the form ATP and sulfate;

(c) combining the ATP, sulfate, and said fusion protein-containing feedstock with luciferin in the presence of oxygen such that the ATP is consumed to produced AMP, inorganic pyrophosphate, carbon dioxide and light; and

(d) measuring the amount of light produced.

70. The method of claim 69 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

71. The method of claim 70 wherein the ATP sulfurylase is a thermostable sulfurylase.

72. The method of claim 70 wherein the ATP sulfurylase is from a thermophile.

73. The method of claim 72 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

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74. The method of claim 69 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

75. The method of claim 74 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

76. The method of claim 69 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

77. The method of claim 76 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

78. The method of claim 69 which further comprises an affinity tag.

79. The method according to claim 59 wherein each feedstock comprises adenosine-5'-phosphosulfate and luciferin in addition to the selected nucleotide base, and the amount of inorganic pyrophosphate is determincd by reacting the inorganic pyrophosphate is determined by reacting the inorganic pyrophosphate-containing feedstock with an ATP generating polypeptide-ATP converting polypeptide fusion protein thereby producing light in an amount proportional to the amount of inorganic pyrophosphate, and measuring the amount of light produced.

80. The method of claim 79 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

81. The method of claim 80 wherein the ATP sulfurylase is a thermostable sulfurylase.

82. The method of claim 80 wherein the ATP sulfurylase is from a thermophile.

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83. The method of claim 82 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

84. The method of claim 79 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

85. The method of claim 84 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

86. The method of claim 79 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

87. The method of claim 86 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

88. The method of claim 79 which further comprises an affinity tag.

89. A method for sequencing a nucleic acid, the method comprising:

(a) providing one or more nucleic acid anchor primers;

(b) providing a plurality of single-stranded circular nucleic acid templates disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm;

(c) annealing an effective amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex;

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(d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template;

(e) annealing an effective amount of a sequencing primer to one or more copies of said covalently linked complementary nucleic acid;

(f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a sequencing reaction byproduct; and

(g) identifying the sequencing reaction byproduct with the use of an ATP generating polypeptide-ATP converting polypeptide fusion protein, thereby determining the sequence of the nucleic acid.

90. The method of claim 89 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

91. The method of claim 90 wherein the ATP sulfurylase is a thermostable sulfurylase.

92. The method of claim 90 wherein the ATP sulfurylase is from a thermophile.

93. The method of claim 92 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

94. The method of claim 89 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

95. The method of claim 94 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

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96. The method of claim 89 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

97. The method of claim 96 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

98. The method of claim 89 which further comprises an affinity tag.

99. A method for sequencing a nucleic acid, the method comprising:

(a) providing at least one nucleic acid anchor primer;

(b) providing a plurality of single-stranded circular nucleic acid templates in an array having at least 400,000 discrete reaction sites;

(c) annealing a first amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex;

(d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template;

(e) annealing a second amount of a sequencing primer to one or more copies of the covalently linked complementary nucleic acid;

(f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, when the predetermined nucleotide triphosphate is incorporated onto the 3' end of the sequencing primer, to yield a sequencing reaction byproduct; and

(g) identifying the sequencing reaction byproduct with the use of an ATP generating polypeptide-ATP converting polypeptide fusion protein, thereby determining the sequence of the nucleic acid at each reaction site that contains a nucleic acid template.

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100. The method of claim 99 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

101. The method of claim 100 wherein the ATP sulfurylase is a thermostable sulfurylase.

102. The method of claim 100 wherein the ATP sulfurylase is from a thermophile.

103. The method of claim 102 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

104. The method of claim 99 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

105. The method of claim 104 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

106. The method of claim 99 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

107. The method of claim 106 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

108. The method of claim 99 which further comprises an affinity tag.

109. A method of determining the base sequence of a plurality of nucleotides on an array, the method comprising:

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(a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm,

(b) adding an activated nucleotide 5'-triphosphate precursor of one known nitrogenous base to a reaction mixture in each reaction chamber, each reaction mixture comprising a template-directed nucleotide polymerase and a single-stranded polynucleotide template hybridized to a complementary oligonucleotide primer strand at least one nucleotide residue shorter than the templates to form at least one unpaired nucleotide residue in each template at the 3'-end of the primer strand, under reaction conditions which allow incorporation of the activated nucleoside 5'-triphosphate precursor onto the 3'-end of the primer strands, provided the nitrogenous base of the activated nucleoside 5'-triphosphate precursor is complementary to the nitrogenous base of the unpaired nucleotide residue of the templates;

(c) detecting whether or not the nucleoside 5'-triphosphate precursor was incorporated into the primer strands through detection of a sequencing byproduct with an ATP generating polypeptide-ATP converting polypeptide fusion protein, thus indicating that the unpaired nucleotide residue of the template has a nitrogenous base composition that is complementary to that of the incorporated nucleoside 5'-triphosphate precursor; and

(d) sequentially repeating steps (b) and (c), wherein each sequential repetition adds and, detects the incorporation of one type of activated nucleoside 5'-triphosphate precursor of known nitrogenous base composition; and

(e) determining the base sequence of the unpaired nucleotide residues of the template in each reaction chamber from the sequence of incorporation of said nucleoside precursors.

110. The method of claim 109 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

111. The method of claim 110 wherein the ATP sulfurylase is a thermostable sulfurylase.

112. The method of claim 110 wherein the ATP sulfurylase is from a thermophile.

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113. The method of claim 112 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

114. The method of claim 109 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

115. The method of claim 114 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

116. The method of claim 109 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

117. The method of claim 116 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

118. The method of claim 109 which further comprises an affinity tag.

119. A method for determining the nucleic acid sequence in a template nucleic acid polymer, comprising:

(a) introducing a plurality of template nucleic acid polymers into a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, each reaction chamber having a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added;

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(b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released;

(c) detecting the formation of inorganic pyrophosphate with an ATP generating polypeptide-ATP converting polypeptide fusion protein to determine the identity of each nucleotide in the complementary polymer and thus the sequence of the template polymer.

120. The method of claim 119 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

121. The method of claim 120 wherein the ATP sulfurylase is a thermostable sulfurylase.

122. The method of claim 120 wherein the ATP sulfurylase is from a thermophile.

123. The method of claim 122 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

124. The method of claim 119 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

125. The method of claim 124 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

126. The method of claim 119 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

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127. The method of claim 126 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

128. The method of claim 119 which further comprises an affinity tag.

129. A method of identifying the base in a target position in a DNA sequence of sample DNA, wherein:

(a) sample DNA is disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, said DNA being rendered single stranded either before or after being disposed in the reaction chambers,

(b) an extension primer is provided which hybridizes to said immobilized single-stranded DNA at a position immediately adjacent to said target position;

(c) said immobilized single-stranded DNA is subjected to a polymerase reaction in the presence of a predetermined nucleotide triphosphate, wherein if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer then a sequencing reaction byproduct is formed; and

(d) identifying the sequencing reaction byproduct with an ATP generating polypeptide-ATP converting polypeptide fusion protein, thereby determining the nucleotide complementary to the base at said target position.

130. The method of claim 129 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

131. The method of claim 130 wherein the ATP sulfurylase is a thermostable sulfurylase.

132. The method of claim 130 wherein the ATP sulfurylase is from a thermophile.

133. The method of claim 132 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus*

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caldolyticus, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

134. The method of claim 129 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

135. The method of claim 134 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

136. The method of claim 129 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

137. The method of claim 136 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

138. The method of claim 129 which further comprises an affinity tag.

139. A method of identifying a base at a target position in a sample DNA sequence comprising:

(a) providing sample DNA disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm, said DNA being rendered single stranded either before or after being disposed in the reaction chambers;

(b) providing an extension primer which hybridizes to the sample DNA immediately adjacent to the target position;

(c) subjecting the sample DNA sequence and the extension primer to a polymerase reaction in the presence of a nucleotide triphosphate whereby the nucleotide triphosphate will only become incorporated and release pyrophosphate (PPi) if it is complementary to the base

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in the target position, said nucleotide triphosphate being added either to separate aliquots of sample-primer mixture or successively to the same sample-primer mixture; and

(d) detecting the release of PPi with an ATP generating polypeptide-ATP converting polypeptide fusion protein to indicate which nucleotide is incorporated.

140. The method of claim 139 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

141. The method of claim 140 wherein the ATP sulfurylase is a thermostable sulfurylase.

142. The method of claim 140 wherein the ATP sulfurylase is from a thermophile.

143. The method of claim 142 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

144. The method of claim 139 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

145. The method of claim 144 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

146. The method of claim 139 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

147. The method of claim 146 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

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148. The method of claim 139 which further comprises an affinity tag.

149. A method of identifying a base at a target position in a single-stranded sample DNA sequence, the method comprising:
(a) providing an extension primer which hybridizes to sample DNA immediately adjacent to the target position, said sample DNA disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 um, said DNA being rendered single stranded either before or after being disposed in the reaction chambers;
(b) subjecting the sample DNA and extension primer to a polymerase reaction in the presence of a predetermined deoxynucleotide or dideoxynucleotide whereby the deoxynucleotide or dideoxynucleotide will only become incorporated and release pyrophosphate (PPi) if it is complementary to the base in the target position, said predetermined deoxynucleotides or dideoxynucleotides being added either to separate aliquots of sample-primer mixture or successively to the same sample-primer mixture,
(c) detecting any release of PPi with an ATP generating polypeptide-ATP converting polypeptide fusion protein to indicate which deoxynucleotide or dideoxynucleotide is incorporated;
characterized in that, the PPi-detection enzyme(s) are included in the polymerase reaction step and in that in place of deoxy- or dideoxy adenosine triphosphate (ATP) a dATP or ddATP analogue is used which is capable of acting as a substrate for a polymerase but incapable of acting as a substrate for a said PPi—detection enzyme.

150. The method of claim 149 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

151. The method of claim 150 wherein the ATP sulfurylase is a thermostable sulfurylase.

152. The method of claim 150 wherein the ATP sulfurylase is from a thermophile.

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153. The method of claim 152 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

154. The fusion protein of claim 149 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

155. The method of claim 154 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

156. The method of claim 149 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

157. The method of claim 156 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

158. The method of claim 149 which further comprises an affinity tag.

159. A method of determining the base sequence of a plurality of nucleotides on an array, the method comprising:

(a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm,

(b) converting PPi into light with a an ATP generating polypeptide-ATP converting polypeptide fusion protein;

(c) detecting the light level emitted from a plurality of reaction sites on respective portions of an optically sensitive device;

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(d) converting the light impinging upon each of said portions of said optically sensitive device into an electrical signal which is distinguishable from the signals from all of said other regions;

(e) determining a light intensity for each of said discrete regions from the corresponding electrical signal;

(f) recording the variations of said electrical signals with time.

160. The method of claim 159 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

161. The method of claim 160 wherein the ATP sulfurylase is a thermostable sulfurylase.

162. The method of claim 160 wherein the ATP sulfurylase is from a thermophile.

163. The method of claim 162 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

164. The method of claim 159 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

165. The method of claim 164 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

166. The method of claim 159 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

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167. The method of claim 166 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

168. The method of claim 159 which further comprises an affinity tag.

169. Method for sequencing a nucleic acid, the method comprising:

(a) providing one or more nucleic acid anchor primers;

(b) providing a plurality of single-stranded circular nucleic acid templates disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm;

(c) converting PPi into a detectable entity with the use of an ATP generating polypeptide-ATP converting polypeptide fusion protein;

(d) detecting the light level emitted from a plurality of reaction sites on respective portions of an optically sensitive device;

(e) converting the light impinging upon each of said portions of said optically sensitive device into an electrical signal which is distinguishable from the signals from all of said other regions;

(f) determining a light intensity for each of said discrete regions from the corresponding electrical signal;

(g) recording the variations of said electrical signals with time.

170. The method of claim 169 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

171. The method of claim 170 wherein the ATP sulfurylase is a thermostable sulfurylase.

172. The method of claim 170 wherein the ATP sulfurylase is from a thermophile.

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173. The method of claim 172 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

174. The method of claim 169 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

175. The method of claim 174 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

176. The method of claim 169 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

177. The method of claim 176 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

178. The method of claim 169 which further comprises an affinity tag.

179. A method for sequencing a nucleic acid, the method comprising:

(a) providing at least one nucleic acid anchor primer;

(b) providing a plurality of single-stranded circular nucleic acid templates in an array having at least 400,000 discrete reaction sites;

(c) converting PPi into a detectable entity with an ATP generating polypeptide-ATP converting polypeptide fusion protein;

(d) detecting the light level emitted from a plurality of reaction sites on respective portions of an optically sensitive device;

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(e) converting the light impinging upon each of said portions of said optically sensitive device into an electrical signal which is distinguishable from the signals from all of said other regions;

(f) determining a light intensity for each of said discrete regions from the corresponding electrical signal;

(g) recording the variations of said electrical signals with time.

180. The method of claim 179 wherein the ATP generating polypeptide is selected from the group consisting of ATP sulfurylase, hydrolase and ATP synthase.

181. The method of claim 180 wherein the ATP sulfurylase is a thermostable sulfurylase.

182. The method of claim 180 wherein the ATP sulfurylase is from a thermophile.

183. The method of claim 182 wherein the thermophile is a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

184. The method of claim 179 wherein the ATP generating polypeptide and ATP converting polypeptide are from a eukaryote or a prokaryote.

185. The method of claim 184 wherein the eukaryote is selected from the group consisting of animal, plant, fungus and yeast.

186. The method of claim 179 wherein the ATP converting polypeptide is selected from the group consisting of luciferase, ecto-nucleoside diphosphate kinase and ATPase.

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187. The method of claim 186 wherein the luciferase is selected from the group consisting of *Photinus pyralis*, *Pyroplorus plagiophihalamus* (*Coleoptera*), *Luciola cruciata* and *Luciola lateralis*.

188. The method of claim 179 which further comprises an affinity tag.

189. A kit comprising a sulfurylase-luciferase fusion protein expression vector as claimed in claim 39.

190. An isolated polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) a mature form of an amino acid sequence of SEQ ID NO: 2;

(b) a variant of a mature form of an amino acid sequence of SEQ ID NO: 2;

(c) an amino acid sequence of SEQ ID NO: 2;

(d) a variant of an amino acid sequence of SEQ ID NO: 2, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 4% of amino acid residues from said amino acid sequence; and

(e) an amino acid sequence of (a), (b), (c) or (d) further containing one or more conservative amino acid substitutions.

191. The polypeptide of claim 190 wherein said polypeptide comprises the amino acid sequence of a naturally-occurring allelic variant of an amino acid sequence of SEQ ID NO: 2.

192. The polypeptide of claim 190 wherein the amino acid sequence of said variant comprises one or more conservative amino acid substitution.

193. An isolated nucleic acid molecule comprising a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) a mature form of an amino acid sequence of SEQ ID NO: 2;

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(b) a variant of a mature form of an amino acid sequence of SEQ ID NO: 2, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 4% of the amino acid residues from the amino acid sequence of said mature form;

(c) an amino acid sequence of SEQ ID NO: 2;

(d) a variant of an amino acid sequence of SEQ ID NO: 2, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 4% of amino acid residues from said amino acid sequence;

(e) a nucleic acid fragment encoding at least a portion of a polypeptide comprising an amino acid sequence of SEQ ID NO: 2, or a variant of said polypeptide, wherein one or more amino acid residues in said variant differs from the amino acid sequence of said mature form, provided that said variant differs in no more than 4% of amino acid residues from said amino acid sequence; and

(f) a nucleic acid molecule comprising the complement of (a), (b), (c), (d) or (e).

194. The nucleic acid molecule of claim 193 wherein the nucleic acid molecule comprises the nucleotide sequence of a naturally-occurring allelic nucleic acid variant.

195. The nucleic acid molecule of claim 193 wherein the nucleic acid molecule encodes a polypeptide comprising the amino acid sequence of a naturally-occurring polypeptide variant.

196. The nucleic acid molecule of claim 193 wherein the nucleic acid molecule comprises nucleotide sequence selected from the group consisting of:

(a) a first nucleotide sequence comprising a coding sequence differing by one or more nucleotide sequences from a coding sequence encoding said amino acid sequence, provided that no more than 11% of the nucleotides in the coding sequence in said first nucleotide sequence differ from said coding sequence;

(b) an isolated second polynucleotide that is a complement of the first polynucleotide; and

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(c) a nucleic acid fragment of (a) or (b).

197. A vector comprising the nucleic acid molecule of claim 196.

198. The vector of claim 197, further comprising a promoter operably-linked to said nucleic acid molecule.

199. A cell comprising the vector of claim 197.

200. An antibody that binds immunospecifically to the polypeptide of claim 190.

201. A method for determining the nucleic acid sequence in a template nucleic acid polymer, comprising:

(a) introducing the template nucleic acid polymer into a polymerization environment in which the nucleic acid polymer will act as a template polymer for the synthesis of a complementary nucleic acid polymer when nucleotides are added;

(b) successively providing to the polymerization environment a series of feedstocks, each feedstock comprising a nucleotide selected from among the nucleotides from which the complementary nucleic acid polymer will be formed, such that if the nucleotide in the feedstock is complementary to the next nucleotide in the template polymer to be sequenced said nucleotide will be incorporated into the complementary polymer and inorganic pyrophosphate will be released;

(c) separately recovering each of the feedstocks from the polymerization environment; and

(d) measuring the amount of PPi with a thermostable sulfurylase and a luciferase in each of the recovered feedstocks to determine the identity of each nucleotide in the complementary polymer and thus the sequence of the template polymer.

202. The method of claim 201 wherein said thermostable sulfurylase comprises the amino acid sequence of a naturally-occurring allelic variant of an amino acid sequence of SEQ ID NO: 2.

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203. The method of claim 201 wherein the thermostable sulfurylase is derived from a thermophilic bacteria selected from the group consisting of *Bacillus stearothermophilus*, *Thermus thermophilus*, *Bacillus caldolyticus*, *Bacillus subtilis*, *Bacillus thermoleovorans*, *Pyrococcus furiosus*, *Sulfolobus acidocaldarius*, *Rhodothermus obamensis*, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

204. The method of claim 201 wherein the thermostable sulfurylase and the luciferase are joined in a fusion protein.

205. The method of claim 201 wherein the thermostable sulfurylase is joined to an affinity tag.

206. A method for sequencing a nucleic acid, the method comprising:

(a) providing one or more nucleic acid anchor primers;

(b) providing a plurality of single-stranded circular nucleic acid templates disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200;

(c) annealing an effective amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex;

(d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template;

(e) annealing an effective amount of a sequencing primer to one or more copies of said covalently linked complementary nucleic acid;

(f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, if the predetermined nucleotide triphosphate is incorporated onto the 3' end of said sequencing primer, a sequencing reaction byproduct; and

(g) identifying the sequencing reaction byproduct with the use of a thermostable sulfurylase and a luciferase, thereby determining the sequence of the nucleic acid.

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207. The method of claim 206 wherein said thermostable sulfurylase comprises the amino acid sequence of a naturally-occurring allelic variant of an amino acid sequence of SEQ ID NO: 2.

208. The method of claim 206 wherein the thermostable sulfurylase is derived from a thermophilic bacteria selected from the group consisting of Bacillus stearothermophilus, Thermus thermophilus, Bacillus caldolyticus, Bacillus subtilis, Bacillus thermoleovorans, Pyrococcus furiosus, Sulfolobus acidocaldarius, Rhodothermus obamensis, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

209. The method of claim 206 wherein the thermostable sulfurylase and the luciferase are joined in a fusion protein.

210. The method of claim 206 wherein the thermostable sulfurylase is joined to an affinity tag.

211. A method for sequencing a nucleic acid, the method comprising:

(a) providing at least one nucleic acid anchor primer;

(b) providing a plurality of single-stranded circular nucleic acid templates in an array having at least 400,000 discrete reaction sites;

(c) annealing a first amount of the nucleic acid anchor primer to at least one of the single-stranded circular templates to yield a primed anchor primer-circular template complex;

(d) combining the primed anchor primer-circular template complex with a polymerase to form an extended anchor primer covalently linked to multiple copies of a nucleic acid complementary to the circular nucleic acid template;

(e) annealing a second amount of a sequencing primer to one or more copies of the covalently linked complementary nucleic acid;

(f) extending the sequencing primer with a polymerase and a predetermined nucleotide triphosphate to yield a sequencing product and, when the predetermined nucleotide triphosphate is incorporated onto the 3' end of the sequencing primer, to yield a sequencing

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reaction byproduct; and

(g) identifying the sequencing reaction byproduct with the use of a thermostable sulfurylase and a luciferase, thereby determining the sequence of the nucleic acid at each reaction site that contains a nucleic acid template.

212. The method of claim 211 wherein said thermostable sulfurylase comprises the amino acid sequence of a naturally-occurring allelic variant of an amino acid sequence of SEQ ID NO: 2.

213. The method of claim 211 wherein the thermostable sulfurylase is derived from a thermophilic bacteria selected from the group consisting of Bacillus stearothermophilus, Thermus thermophilus, Bacillus caldolyticus, Bacillus subtilis, Bacillus thermoleovorans, Pyrococcus furiosus, Sulfolobus acidocaldarius, Rhodothermus obamensis, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

214. The method of claim 211 wherein the thermostable sulfurylase and the luciferase are joined in a fusion protein.

215. The method of claim 211 wherein the thermostable sulfurylase is joined to an affinity tag.

216. A method of determining the base sequence of a plurality of nucleotides on an array, the method comprising:

(a) providing a plurality of sample DNAs, each disposed within a plurality of cavities on a planar surface, each cavity forming an analyte reaction chamber, wherein the reaction chambers have a center to center spacing of between 5 to 200 μm,

(b) adding an activated nucleotide 5'-triphosphate precursor of one known nitrogenous base to a reaction mixture in each reaction chamber, each reaction mixture comprising a template-directed nucleotide polymerase and a single-stranded polynucleotide template hybridized to a complementary oligonucleotide primer strand at least one nucleotide residue shorter than the templates to form at least one unpaired nucleotide residue in each

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template at the 3'-end of the primer strand, under reaction conditions which allow incorporation of the activated nucleoside 5'-triphosphate precursor onto the 3'-end of the primer strands, provided the nitrogenous base of the activated nucleoside 5'-triphosphate precursor is complementary to the nitrogenous base of the unpaired nucleotide residue of the templates;

(c) detecting whether or not the nucleoside 5'-triphosphate precursor was incorporated into the primer strands through detection of a sequencing byproduct with a thermostable sulfurylase and luciferase, thus indicating that the unpaired nucleotide residue of the template has a nitrogenous base composition that is complementary to that of the incorporated nucleoside 5'-triphosphate precursor; and

(d) sequentially repeating steps (b) and (c), wherein each sequential repetition adds and, detects the incorporation of one type of activated nucleoside 5'-triphosphate precursor of known nitrogenous base composition; and

(e) determining the base sequence of the unpaired nucleotide residues of the template in each reaction chamber from the sequence of incorporation of said nucleoside precursors.

217. The method of claim 216 wherein said thermostable sulfurylase comprises the amino acid sequence of a naturally-occurring allelic variant of an amino acid sequence of SEQ ID NO: 2.

218. The method of claim 216 wherein the thermostable sulfurylase is derived from a thermophilic bacteria selected from the group consisting of Bacillus stearothermophilus, Thermus thermophilus, Bacillus caldolyticus, Bacillus subtilis, Bacillus thermoleovorans, Pyrococcus furiosus, Sulfolobus acidocaldarius, Rhodothermus obamensis, *Aquifex aeolicus*, *Archaeoglobus fulgidus*, *Aeropyrum pernix*, *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Penicillium chrysogenum*, *Sulfolobus solfataricus* and *Thermomonospora fusca*.

219. The method of claim 216 wherein the thermostable sulfurylase and the luciferase are joined in a fusion protein.

220. The method of claim 216 wherein the thermostable sulfurylase is joined to an affinity tag.

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FIGURE 1

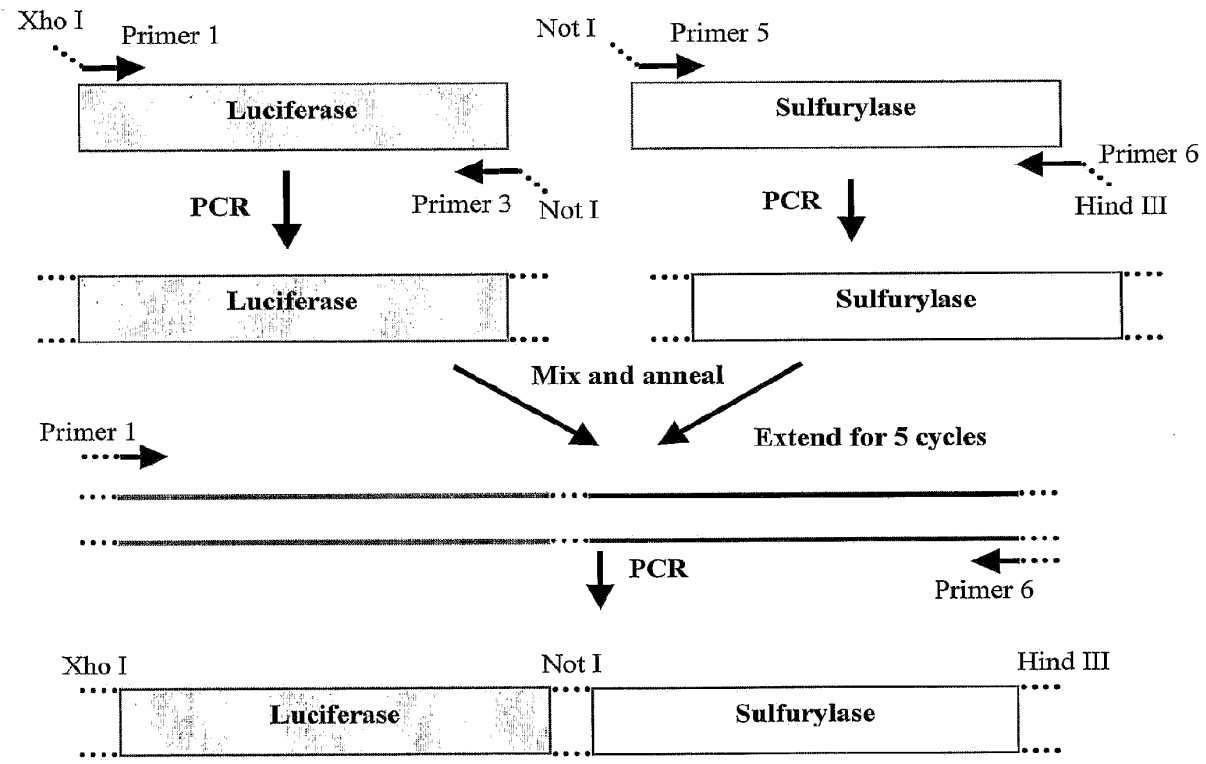
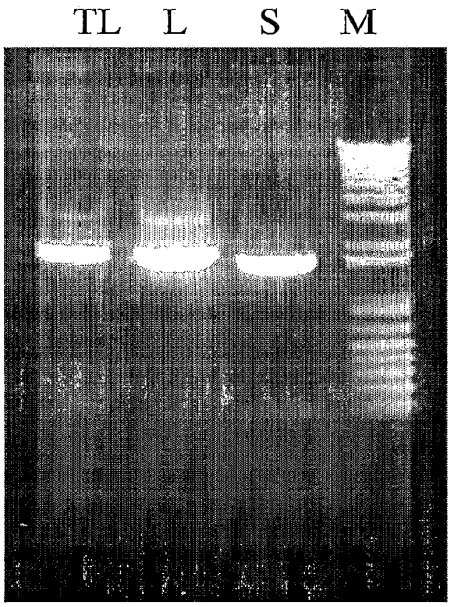
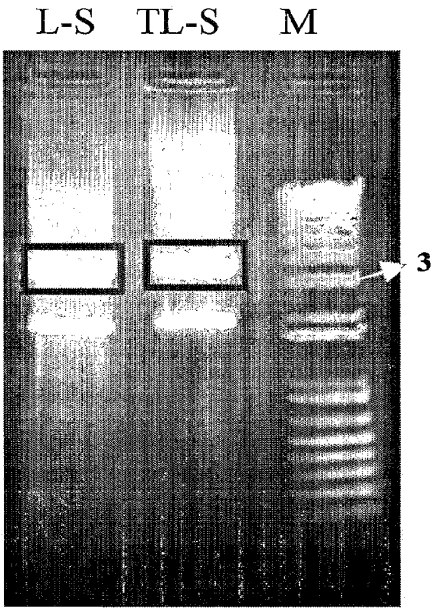

Xho I
Primer 1
Not I
Primer 5
Luciferase
Sulfurylase
Primer 6
PCR
Primer 3
Not I
PCR
Hind III
Luciferase
Sulfurylase
Mix and anneal
Primer 1
Extend for 5 cycles
PCR
Primer 6
Xho I
Not I
Hind III
Luciferase
Sulfurylase

Figure 2

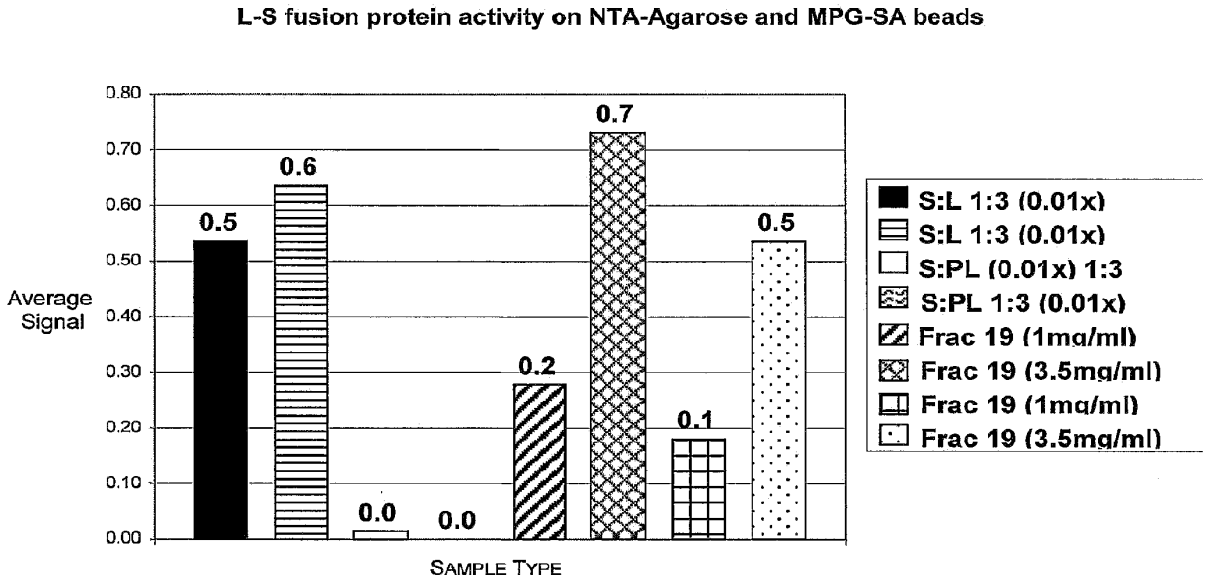


2A



2B

FIGURE 3


L-S fusion protein activity on NTA-Agarose and MPG-SA beads
Average Signal
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.5
0.6
0.0
0.0
0.2
0.7
0.1
0.5
SAMPLE TYPE
S:L 1:3 (0.01x)
S:L 1:3 (0.01x)
S:PL (0.01x) 1:3
S:PL 1:3 (0.01x)
Frac 19 (1mg/ml)
Frac 19 (3.5mg/ml)
Frac 19 (1mg/ml)
Frac 19 (3.5mg/ml)

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Application Number:	14111326
International Application Number:	
Confirmation Number:	3274
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First Named Inventor/Applicant Name:	David Wilson
Customer Number:	23628
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	Chang_et_al_J_Virol_Methods_2013_v188_p153.pdf	696474 c29308af159840bca7a681916edadc9dd0a6f780	no	8

Warnings:	
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(54) Title: RAPID AND SENSITIVE DETECTION OF CELLS AND VIRUSES

(57) Abstract: The invention provides efficient methods for rapidly and sensitively identifying cellular and viral targets in medical, industrial, and environmental samples. The invention labels targets and then detects them using large area imaging. Diagnostic tests based on the invention can be rapid, ultrasensitive, quantitative, multiplexed, and automated. The tests minimize sample preparation and do not require nucleic acid amplification or cell culture. A broad range of cells and viruses can be detected by the tests. Tests based on the invention can deliver the high level sensitivity of nucleic acid amplification tests, the user-friendliness, and speed of immunoassays, as well as the cost effectiveness and quantification offered by microbiological tests. The invention embodies the best attributes of the current diagnostic technologies, while addressing gaps in the diagnostic repertoire.

Rapid and sensitive detection of cells and viruses

Background of the Invention

The invention relates to the identification of cells and viruses in medical, industrial, or environmental samples.

Overview

Detecting, enumerating, and identifying low levels of specific cells and viruses are a cornerstone of routine medical, industrial, and environmental diagnostics. For example, samples are analyzed to detect infectious agents, cancer cells, food pathogens, microbial contaminants of pharmaceutical and cosmetic products, and microbes in water and the environment.

For simplicity, the discussion that follows focuses on routine medical diagnostics; similar methods are used for industrial and environmental applications.

Microbiological methods

Microbial culture allows simple visual detection of microbes, (*e.g.,* bacteria, viruses, and fungi) by exploiting their propensity to reproduce rapidly in large numbers. For example, a individual bacterial cell, which is much too small to see by eye (about one millionth of a meter), when placed in nutrient broth, can cause the broth to become visibly cloudy in less than 24 hours.

A related microbial culture technique, called microbial enumeration or colony counting, quantifies the number of microbial cells in a sample. The microbial enumeration method, which is based on *in situ* microbial replication, generally yields one *visually detectable* "colony" for each microbial cell in the sample. Thus, counting the visible colonies allows microbiologists to determine the number of microbial cells in a sample accurately. To perform microbial enumeration, bacterial cells can be dispersed on the surface of nutrient agar in petri dishes ("agar plates") and incubated under conditions that permit *in situ* bacterial replication. The individual, *visually undetectable,* microbe replicates repeatedly to create a large number of identical daughter microbes at the physical site where the progenitor microbial cell was deposited. The daughter cells remain co-localized (essentially contiguous) with the original cell, so that the cohort of daughter cells (which may grow to tens or hundreds of millions of cells) eventually form a visible colony on the plate.

Microbial culture is a remarkably successful method, as evidenced by the fact that even after more than a century the method still dominates medical microbiology and quality control testing in industrial microbiology (*e.g.,* pharmaceutical, food, and beverage manufacturing). The method is inexpensive, relatively simple, and ultra-sensitive. The sensitivity of microbial culture can be seen in the common test for foodborne pathogens

in ground beef. A individual microscopic bacterial pathogen cell can be detected in 25 grams of ground beef using microbial culture. Another advantage of microbial culture is its ability to detect a large range of microbes of medical and industrial significance.

Some viruses can be grown in culture. Viral culture has been especially useful for fast growing bacteriaphage (viruses that infect bacteria) in research applications. Viral culture is sometimes used to diagnose clinical infections although methods such as nucleic acid amplification are increasingly used instead.

Traditional microbial culture is slow — it takes time to generate the number of cells or viruses required for visual detection. The long growth period required for microbial culture is a significant problem in both healthcare and industry. For example, because it requires days to culture and identify the microbe causing a patient's blood infection, a patient with a fungal blood infection could die before anti-fungal therapy is even begun. Some infectious agents, such as the bacterium that causes tuberculosis, generally require weeks to grow in culture. The long time required for detecting *M. tuberculosis* can result in a patient with tuberculosis infecting many others with the highly contagious disease or the costly quarantine of patients who do not have tuberculosis. In the manufacture of food, long testing cycles can increase food spoilage. Slow microbial culture also adversely impacts the production of biopharmaceuticals and vaccines.

A number of microbial culture methods for more rapid microbial enumeration have been developed. One rapid method deposits bacterial cells on microscope slides coated with nutrient medium. Using microscopic examination, microbial growth can be detected much earlier than with the naked eye, since microscopes can detect microcolonies resulting from a small number of cell divisions. A commercialized system, the Colifast Rapid Microcolony Counter (Colifast), can detect small fluorescently labeled colonies of coliform bacteria hours before they can be seen by eye. The Colifast system achieves enhanced detection by using a fluorogenic compound (a substance that is not fluorescent until metabolized by coliform bacteria) is included in the nutrient agar media. A system for rapid enumeration of microbial colonies using bioluminescent labeling has recently been commercialized. The MicroStar system (Millipore) uses the cellular ATP in microcolonies to generate light via the action of applied luciferase enzyme and substrates. The method reduces time to detection substantially. The MicroStar imaging system has also been used in conjunction with labeled probes to identify specific bacteria (Stender, H., *et al.* (2001). J Microbiol Methods 46: 69-75).

Immunological methods

Immunological tests, or immunoassays, are frequently used to identify specific cells and viruses in medical diagnostics. Immunoassays can detect the specific binding of antibodies to sites on the molecular components of targets and viruses. Serological tests are immunological assays that, rather than testing directly for antigens, test for a host immunological response to previous exposure to the antigen – for example they can test for the presence of host antibodies to particular cells and viruses. Numerous immunoassay systems are available for ranging from large automated central lab systems to over-the-counter pregnancy tests. The tests cover a broad range of formats including agglutination assays, precipitin assays, enzyme-linked immunoassays, direct fluorescence assays, immuno-histological tests, complement-fixation assays, serological tests, immuno-electrophoretic assays, and rapid "strip" tests (*e.g.*, lateral flow and flow through tests). Immunological tests can be extremely simple and rapid. Thus, many of the most desirable tests, those that can be conducted in a physician's office or at home by the patient, are immunological tests.

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Genetic methods

Genetic methods are general and powerful tools for detecting and identifying nucleic acid molecules from cells and viruses. Revolutionary new methods for ultra-sensitively detecting and distinguishing the nucleic acid content of cells and viruses based on nucleic acid amplification have recently been developed. For example, commercial tests can detect the nucleic acids from a small number of sub-microscopic HIV virus particles (*e.g.*, 50 particles/ml). Amplification technologies include the polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), Transcription Mediated Amplification (TMA), and rolling circle amplification (RCA). Amplification methods can deliver impressive analytical sensitivity and can be moderately rapid, for example, the Smart Cycler (Cepheid) can deliver results in an hour (Belanger, S. D., *et al.* (2002). Journal of Clinical Microbiology 40: 1436-40).

Microscopic imaging methods

Microscopic imaging is one of the most common methods for detecting cells and viruses. Targets can be visualized microscopically by labeling with stains, antibodies, or nucleic acid probes, for example. The sensitivity of microscopic analysis can be enhance using methods such as catalyzed reporter deposition (CARD), which has been used to detect single copy level viral genomes in human cells (Huang, C.C., *et al.*, Modern Pathology 11: 971-7, 1998). Other methods for increasing the sensitivity of *in situ* hybridization rely on *in situ* amplification or replication, or signal amplification using, for example, branched DNA or dendrimer technology (*e.g.*, Orentas, R.J., *et al.*, Journal of Virological Methods 77: 153-63, 1999).

Multiple fluorescent labels can be used to detect several pathogens at once using *in situ* analysis. For example, antibodies to different category-specific antigens can be labeled with different fluorescent tags. Combinatorial labeling strategies have been also used in conjunction with microscopy to identify several bacterial pathogens simultaneously (Amann, R., *et al.*, Journal Of Bacteriology 178: 3496-500, 1996; U.S. Patent No. 6,007,994).

Several commercial systems have been developed that achieve high throughput *in situ* microscopic analysis of microscopic targets (*e.g.*, WO 98/22618 and WO 93/21511). Typically, microbes or cells are deposited on a slide or on the bottom of the wells of a multiwell plate. Labeled targets in the wells are then imaged microscopically.

Non-microscopic imaging methods

Non-microscopic imaging allows larger areas to be surveyed for the presence of cells. For example, researchers at Hamamatsu Corporation (Masuko, M., *et al.*, FEMS Microbiol Lett 67: 231-8, 1991; Masuko, M., *et al.*, FEMS Microbiol Lett 65: 287-90, 1991; Yasui, T., *et al.*, Appl Environ Microbiol 63: 4528-33, 1997) developed a system that can image individual bacterial cells without magnification. The system uses an ultrasensitive photon-counting CCD camera coupled to a fiber optic system, image intensifier, and image-processor. The Elisa spot test method is a specialized technique for enumerating single cells that produce a particular antibody (or other abundantly secreted product; Logtenberg, T., *et al.*, Immunol Lett 9: 343-7, 1985). The method's sensitivity derives from the fact that large numbers of targets (the secreted protein molecules) are localized around the secreting cell.

Flow cytometric methods

Flow cytometry is an important tool for characterizing cells in clinical diagnostic laboratories. Flow cytometric methods are used for quantitatively detecting particular

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cell types on the basis of their physical properties and their ability to bind labeled probes (*e.g.,* stains, antibodies, or nucleic acids). Individual cells or particles are forced to flow through a narrow channel, one at a time, past a laser beam. Fluorescence emission and size/shape information is gathered by analyzing the spectrum and light scattering caused by the organism/particle. Thousands of individual cells or particles can be analyzed per minute. For example, flow cytometry is used to quantify the population sizes of classes of lymphocytes in patients with AIDS. A highly multiplexed flow cytometric method that can detect and identify non-cellular molecules such as proteins or nucleic acids has been commercialized by Luminex (U.S. U.S. Patent No. 5,981,180).

Laser scanning methods

Laser scanning is a powerful and sensitive method for detecting cells and viruses deposited on surfaces. Typically, a microscopic laser beam (*e.g.*, 20 µm in diameter) is traced over a two dimensional sample containing fluorescently labeled targets on a solid surface in a large number of successive linear passes, each slightly offset from the previous, so that the entire sample area is eventually scanned. When a fluorescently labeled target falls under the beam, a flash of emitted fluorescence is detected by a detection device such as a photomultiplier. The number and position of the microscopic targets can be obtained by laser microbeam scanning.

A number of systems for laser scanning have been developed. The ScanRDI® system (Chemunex) uses a non-specific fluorescent dye to label microbial cells on a filter (U.S. Patent No. 5,663,057; Mignon-Godefroy, K., *et al.*, Cytometry 27: 336-44, 1997). The fluorimetric microvolume assay technology (FMAT; PE Biosystems and Biometric Imaging; Miraglia, S., *et al.*, J Biomol Screen 4: 193-204, 1999) has also been used to detect cells in microtiter wells. A sophisticated laser scanning system has been developed and commercialized by CompuCyte Corporation (Kamentsky, L., 2001, *Laser Scanning Cytometry*. In Cytometry, Z. Darzynkiewicz, H. Crissman and J. Robinsnon, eds.Methods in Cell Biology Vol. 63, Part A, 3rd ed, Series Eds. L. Wilson and P. Matsudaira. (San Diego: Academic Press)). A microbeam laser scanning system that detects individual microscopic targets in a liquid sample (*e.g.*, whole blood) has been developed by Immunicon Corporation and collaborators at Twente University (Tibbe, A. G., *et al.*, Nat Biotechnol 17: 1210-3, 1999).

Biochemical, chemical, and physical methods

Other technologies for sensitive detection of the cells and viruses, or their molecular components, molecular components of cells and viruses include flow cytometry, mass spectroscopy, biosensors, absorbance spectroscopy, fluorescence polarization, fluorescence fluctuation spectroscopy, electrophoresis, chromatography, among many others.

Biosensor technologies also hold promise for sensitive detection of cells and viruses. Biosensors use physical methods to convert a biological event, for example binding of an antibody to an antigen, to a detectable signal. One popular biosensor used for molecular detection uses surface plasmon resonance (Mullett, W. M., *et al.* (2000). Methods 22: 77-91). Thermo BioStar's optical immunoassay (Schultze, D., *et al.* (2001). Eur J Clin Microbiol Infect Dis 20: 280-3) uses the principle of optical interference to detect binding of antigens to antibodies. The BARC biosensor technology uses magnetoresistive detection (as used for hard disk storage) of analytes tagged with single magnetic microparticles (Edelstein, R. L., *et al.*, Biosens Bioelectron 14: 805-13, 2000).

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Unmet needs for detection of cells and viruses

Although numerous disparate and powerful methods for routine detection of low levels of cells and viruses have been commercialized there are still gaps in the testing repertoire. In particular, there is a need for tests to detect very low levels of cells and viruses that are rapid, do not require laboratory growth, are user-friendly, and are cost-effective.

Background of the Invention

The invention provides efficient methods for rapidly and sensitively identifying cellular and viral targets in medical, industrial, and environmental samples. The invention labels targets and then detects them using large area imaging. Diagnostic tests based on the invention can be rapid, ultrasensitive, quantitative, multiplexed, and automated. The tests minimize sample preparation and do not require nucleic acid amplification or cell culture. A broad range of cells and viruses can be detected by the tests. Tests based on the invention can deliver the high level sensitivity of nucleic acid amplification tests, the user-friendliness, and speed of immunoassays, as well as the cost effectiveness and quantification offered by microbiological tests. The invention embodies the best attributes of the current diagnostic technologies, while addressing gaps in the diagnostic repertoire.

The ability of the invention to detect low levels of targets and viruses rapidly and cost-effectively results from the advantages of combining high intensity labeling, formats that facilitate rapid reaction kinetics, and large area imaging based using either instrumentation made from off-the-shelf commercial components or no instrumentation at all. Table 1 lists some of some of the advantages of the invention.

Selected advantages of the invention
• Rapid results
• Ultra-sensitive
• Easy-to-use
• Cost-effective
• Multiple targets analyzed simultaneously
• Scans for all types of cells and viruses
• Analyzes large or small volumes
• Automated quantitative analysis
• Minimal sample preparation

Table 1

The invention detects low levels of cells and viruses by specifically labeling them to generate high-intensity signals. A variety of signal generating complexes can be used including fluorescently dyed, light-scattering, quantum dot, phosphor, and enzyme-coated particles. These particles, in turn, can generate a variety of types of signals including fluorescent, chemiluminescent, and colorimetric. Similarly, a variety of molecules can be used for achieving specific binding of the signal generating labels including antibodies, nucleic acids, ligands, and aptamers.

Detecting small numbers of targets and viruses in large volumes is a requirement when testing some clinical, environmental, and manufacturing samples. The invention's ability to survey large samples for low levels of targets rests, in part, on its ability detect individual cells and viruses without magnification. Non-magnified detection allows a large area to be surveyed for small numbers of the particles in a single image. Imaging a large area, in turn, is a key to the invention's ability to analyze large sample volumes

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efficiently. Detecting labeled particles in large volumes using high power microscopy or microfluidics can require challenging concentration steps or analysis of thousands of images. Methods that scan for small numbers of particles using microscopic beams become very time consuming and expensive when applied to large areas.

Enumerating individual microscopic labels adds robustness to the results of tests based on the invention. In contrast to many large area imaging methods for analyzing cellular and viral targets, the invention generally compares individual signals directly to small neighboring regions. This comparison improves the signal to background ratio for samples containing few labeled targets compared to methods that integrate the total signal and background in a large area.

Another advantage of detecting individual cells or viruses is that the sensitivity of the tests can be increased without sacrificing speed by increasing the sample volume with a proportionate increase in the size of the detection zone.

Enumerating individual signals in a large area image also decreases the chance of false positive results. (False positive results are positive test results that occur when the actual target is not present). This enumerating method is an advantage compared with methods that detect a single integrated signal, such as methods that measure the total amount of a molecule (*e.g.,* ATP, antigens, or nucleic acids) in the sample. Any artifact that causes a signal can generate a false positive when using methods that rely on signal integration. Consider a sample that contains 482 positive signals, each of which generates 100 fluorescent units. The result of an integrative method is a single number (48,200 fluorescent units). Artifacts that generate a similar number of fluorescent units, for example, a large fluorescent dust particle may be indistinguishable. In contrast, the present invention can easily distinguish between a single large fluorescent dust particle and 482 positive signals.

Tests constructed using the invention can detect targets and viruses over a broad range of concentrations, from very low levels to high levels. This property of the invention, its large dynamic range, allows users to forgo the sample preparation steps (*e.g.,* multiple dilutions) that are often required by technologies that have small dynamic ranges.

Tests based on the invention can exploit a variety of useful formats ranging from single-use strip tests that are simple, non-instrumented, and cost-effective, to sophisticated automated benchtop systems.

Various detection methods can be used by the invention including visual, film-based, and electronic detection. The range of detection methods is an advantage for addressing a broad spectrum of diagnostic problems and testing venues.

Other features and advantages of the present invention will be apparent from the following description and the claims.

By **target** is meant a cell or virus that is potentially present in a sample and whose presence is tested for by the invention.

By **category** of target is meant multiple targets that have in common one or more features and that are considered identical for the purposes of a test constructed using the invention. For example, for a test designed to detect all HIV viruses, the category would simply be HIV. Such a test would detect all HIV viruses, without differentiating the HIV-1 and HIV-2 variants. In this case, the category of the target includes both HIV-1 and HIV-2. The goal of another test might be to distinguish HIV-1 from HIV-2. In this case, each type of HIV would be considered a different category.

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By **non-overlapping categories** of targets is meant categories of targets whose union is the null set. the category of all *E. coli* bacteria, the category of all bacteria in the genus *Pseudomonas*, the category of all fungi, and the category of all HIV viruses are non-overlapping categories. That is, no member of any of the categories is a member of any of the other sets. In contrast, the category of HIV-1 viruses and the category of HIV viruses are *overlapping* categories of targets, since each member of the HIV-1 category is also a member of the category of all HIV viruses.

Tests that detect and identify multiple categories generally detect multiple non-overlapping categories of targets. For example, consider a test designed to identify HIV, HCV, and HBV viruses in blood. Such a test would differentiate three non-overlapping categories of targets, one for each of the three types of viruses. Note that each type of virus is in only one of the three categories (*e.g.*, there is no HIV virus that is in the HCV category).

By the **categorical complexity** of a test is meant the number of non-overlapping categories of targets that are detected in the test.

By a **category-specific binding site** is meant a site on a target that *specifically binds* to a category-binding molecule under *specific-binding conditions* (see definitions of terms in italics) and that distinguishes targets that are members of a particular category to be identified in a test from targets that are not members of that category but might also be present in the test sample. That is, the site is present typically on all members of one category, and typically not on any members of non-overlapping categories. Category-specific binding sites *specifically bind* to *category-specific binding molecules*.

A category-specific binding site is always physically associated with the target. Consider an ELISA spot assay in which a single antibody secreting hybridoma cell is identified by detecting a large number of antibody proteins that bind fluorescent antigen and that are immobilized near the secreting cell of origin. In this case, the secreted antibody molecules, which contain binding sites for the antigen, are not physically associated with the hybridoma cell. Therefore the antigen binding sites on the free antibodies are not category-specific binding sites.

If a test scans a sample for a category of targets that constitutes a taxonomic group, a category-specific binding site is one that is present in essentially all members of that taxonomic group, but is not present in essentially all members of other taxonomic groups that might be present in the test sample. An example is a site on an HIV membrane protein that binds to a particular monoclonal antibody.

Alternatively, a test might scan a sample for category-specific binding sites that are shared by members of different taxonomic groups. Examples of this type of category-specific binding sites include various macromolecules (*e.g.*, DNA) and genes, mRNAs, and proteins that confer antibiotic resistance, confer virulence, or indicate viability. A category-specific binding site is often a part of a larger molecule or complex. For example, a category-specific genomic sequence can be used as a category-specific binding site in a test. Such a category-specific binding site is part of a much larger genome that may contain: (*1*) sections that are not category-specific; (*2*) sections that are category-specific binding sites but for which the test does not scan; and (*3*) other sections that are distinct category-specific sequences for which the test does scan.

Binding sites that are present, e.g., in 80%, 90%, 95%, or more than 99% of the target that are members of a category but that are absent, e.g., in 80%, 90%, 95%, or more than 99% of the targets that are members of all other categories of the same class, are considered category-specific binding sites. Note that a category-specific binding site can be trivially or exceptionally absent from a target that is a member of the category.

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Similarly, a category-specific binding site can be trivially or exceptionally present in a target that is not a member of a category. For example, consider a protein site that occurs in essentially all *E. coli* bacteria but in no other bacterial species. If, as might be the case in less than one cell out of millions of bacteria, a mutation causes the protein not to be produced, the marker will not be present in that strain of *E. coli*. However, this protein site is still considered a category-specific binding site. Alternatively, the gene for the same protein is transferred to a strain of a different species of bacteria by recombinant DNA technology or by natural means (*e.g.*, by viral transduction). In this case, a bacterial strain that is not a member of the category *E. coli* would express what would still be considered an *E. coli*-specific binding site.

By **category-binding molecule** is meant a molecule or molecular complex that *specifically binds* to a category-specific binding site. Examples of category-binding molecules are nucleic acid probes that hybridize to genomic DNA; nucleic acid aptamers that have been selected or "evolved" *in vitro* to bind specifically to sites on proteins; antibodies that bind to cellular antigens or serum proteins; and ligands such as epidermal growth factor or biotin that bind specifically to hormone receptors or to binding molecules, such as avidin. Two category-binding molecules are said to be distinct if they bind to distinct and non-overlapping category-specific binding sites. Category-binding molecules may be referred to according to their molecular composition, *e.g.*, a category binding oligonucleotide, probe, antibody, ligand, *etc.*

By **capture molecule** is meant a category-binding molecule that is stably bound to a surface, membrane, or other matrix that is not a particle.

By a category-binding molecule that **specifically binds** to a category of targets is meant a category-binding molecule that binds under defined *binding conditions* to essentially all targets that are members of a category scanned for by a test, but to essentially no other species that are not members of the category but that are likely to be present in the sample. The number of category-binding molecules that are bound by targets in a category scanned for as compared to the number bound by targets not in such a category, are, e.g., two-fold, five-fold, ten-fold, or greater than fifty-fold greater.

By **binding conditions** is meant the conditions used in a test to achieve specific binding of category-binding molecules to category-specific binding sites. For example, when the category-binding molecules are category-specific DNA probes, the binding conditions for a particular test might be stringent DNA hybridization conditions. The appropriate stringent DNA hybridization conditions depend on the nature of the probes, as is well known by those familiar with the art. For example, for typical DNA probes of length greater than 500 bases, an appropriate binding condition (usually referred to as a "washing condition" in the hybridization vernacular) is 65°C at 0.2X SSC. For binding an antibody to an antigen, typical binding conditions are room temperature in PBS-TB.

By a **family** of category-binding molecules is meant a set of category-binding molecules that specifically bind to a particular category of targets.

A polyclonal antibody preparation raised to Hepatitis C virus constitutes a family of category-binding molecules since it comprises multiple distinct antibodies that bind specifically to the same category of target – HCV, in this case. Polyclonal antibodies generally constitute families of category-binding molecules since they generally include multiple distinct category-binding molecules that bind to the same category of target. Note that, unless affinity purification is used, polyclonal antibody preparations typically also contain antibodies that *do not* bind to the chosen category of target and may contain antibodies that bind to other categories because the antibody repertoire of an animal is determined by the animal's infection history. Therefore, polyclonal antibodies are preferably purified by affinity methods.

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Category-binding molecules in a family might bind to some targets in the category but not to others. For example, consider HIV-1-specific antibodies that do not cross-react with HIV-2, and HIV-2-specific antibodies that do not cross-react with HIV-1. If HIV is to be a detected as a category in a test without differentiating between HIV-1 or HIV-2, a mixture of the two types of antibodies could be labeled with signaling moieties with the same signal signature. The same signal is obtained whether HIV-1 or HIV-2 is present when this family of category-binding molecules, which is a mixture of the two antibody preparations, is used in a test. (Note that if antibodies are used to capture the HIV targets at a site in the detection zone in this example, a mixture of anti-HIV-1 and anti-HIV-2 capture antibodies is used at the site).

Another example of a family of category-binding molecules is a set of 80 category-specific genomic DNA sequences that occur in all *E. coli* O157:H7 strains, but that do not occur in members of other groups of bacteria. This family of category-binding molecules can hybridize as a group to suitably prepared *E. coli* O157:H7 cells, but does not hybridize to other categories of cells. Families can include different types of category-binding molecules. For example, a monoclonal antibody that specifically binds to the O157 antigen and one that binds to the *intimin* protein (a virulence factor) could also be included in the above family of category-binding molecules. A family of category-binding molecules can comprise any number of category-binding molecules (*i.e.*, one or more).

By ***non-overlapping families*** of category-binding molecules or is meant families of category-binding molecules in which each family binds specifically to one, and only one, category in a set of non-overlapping categories. That is, a set of non-overlapping families of category-binding molecules map to a congruent set of non-overlapping categories. For example, in a test that scans the 4 USP objectionable organisms *E. coli*, *Salmonella*, *Pseudomonas* spp., and *Staphylococcus aureus*) there are four non-overlapping categories. Such a test might incorporate four different non-cross-reacting polyclonal antibodies, each specific for one of the test categories. Thus, the test comprises four non-overlapping families of category-binding molecules. The non-overlapping families of category-binding molecules in a test are called an ensemble of category-binding molecules (see definition below).

By an **ensemble of category-binding molecules** is meant a set of one or more non-overlapping families of category-binding molecules that are combined in a mixture for a particular test. Tests that scan for multiple non-overlapping categories of targets comprise one family of category-binding molecules per category. The entire set of category-binding molecules, that comprise these families, is referred to as an ensemble. For example, consider a test that scans for the presence of five types of upper respiratory viruses (RSV, influenza A, influenza B, parainfluenza, and adenovirus) using five virus-specific monoclonal antibodies. The five monoclonal antibodies constitute five non-overlapping families of category-binding molecules. The combined set of antibodies is an ensemble of category-binding molecules.

By the **category-binding molecule complexity** of an ensemble is meant the number of distinct category-binding molecules or moieties in an ensemble. For example, if an ensemble of category-binding molecules consisted of 234 oligonucleotide probes, the category-binding molecule complexity of the ensemble would be 234.

By the **family complexity** of an ensemble is meant the number of non-overlapping families of category-binding molecules in an ensemble. The family complexity is the same as the minimum number of targets required to bind a category-binding molecule from each of the families in an ensemble. The family complexity of a test corresponds to the categorical complexity of a test – *i.e.*, the number of distinct categories for which the

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sample is scanned. Consider an ensemble of DNA probes consisting of three families of probes. One family consists of a set of 12 *E. coli* category binding DNA sequences, another family consists of a set of 10 rotavirus category binding DNA sequences, and another family consists of a set of 15 Giardia category binding DNA sequences. The family complexity of this probe ensemble is three since the genomes of no fewer than three types of targets (*E. coli*, rotovirus, and Giardia) are required to bind to all of the probes in the ensemble.

By **signal element** is meant a molecule or particle that directly generates a detectable signal. The phrase "directly generates" refers to the fact that signal elements are the immediate source or critical modulator of the detectable signal. Thus, if the signal is photons that arise from a fluorophore, the fluorophore is the immediate source of the photons and, therefore, is a signal element. If the signal is photons scattered by an RLS particle, the RLS particle is a signal element. Alternatively, if the signal is the light transmitted or scattered from a chromogenic precipitated product of the enzyme horseradish peroxidase, the chromogenic product is the signal element.

A characteristic of a signal element is that such an element cannot be divided into parts such that each part generates a signal that is comparable (in character, not necessarily in intensity) to the whole. Thus, a 2 nM diameter quantum dot is a signal element, as dividing it changes the character (emission spectrum) of the resulting nanocrystals. A 5 µm particle impregnated with a fluorescent dye such as fluorescein, is not a signaling element, since it could be divided into parts such that each part has signaling characteristics comparable to the intact particle. The molecule fluorescein, in contrast, is a signaling element. The detectable products of signal generating enzymes (*e.g.*, luciferase, alkaline phosphatase, horseradish peroxidase) are also considered signal elements. Such signal elements (or their precursors when there is a chemical conversion of a precursor to a signal element) may be diffusible substances, insoluble products, and/or unstable intermediates. For example, the enzyme alkaline phosphatase converts the chemiluminescent substrate CDP-Star (NEN; catalog number NEL-601) to an activated product, which is a photon-emitting *signal element*.

By **signaling moiety** is meant a molecule, particle, or substance comprising or producing (in the case of enzymes) one or more signal elements and that is or can be conjugated to a category-binding molecule. The signaling moiety can be attached to the category-binding molecule either covalently or non-covalently and either directly or indirectly (*e.g.*, via one or more adaptor or "chemical linker" moieties or by both moieties being conjugated to the same particle). Examples of signaling moieties include carboxylated quantum dots; a fluorophore such as Texas Red that is modified for binding to a nucleic acid probe or an antibody probe; streptavidin-coated fluorescent polystyrene particles (which can be conjugated to biotinylated category-specific binding proteins); a rolling-circle replication product containing repeated nucleic acid sequences each of which can hybridized to several oligonucleotides tailed with fluorescently modified nucleotides and which contains a category-specific binding oligonucleotide at the 5' end. A signaling moiety can comprise physically distinct elements. For example, in some cases the signaling moiety is an enzyme (*e.g.*, alkaline phosphatase) that is conjugated to a category-binding molecule (an antibody, for example). Signal is generated when a substrate of alkaline phosphatase (*e.g.*, CDP-Star, or BM purple from NEN and Roche, respectively) is converted to products that are signal elements (*e.g.*, an unstable intermediate that emits a photon, or a precipitable chromogenic product). It is not unusual for the category-binding molecules, enzymatic signaling moieties, and substrate to be applied to the reaction at distinct times.

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By **signaling moiety complex** is meant a physical entity that comprises more than one signaling moiety and more than one category-binding molecule. The physical association of the signaling moieties and category-binding molecules in a signaling moiety complex must be stable (*e.g.*, the signaling moieties and category-binding molecules should have mean half-lives of association with the complex of at least one day in PBS at 4°C). As an example of a signaling moiety complex, consider a polystyrene microparticle that is coated with thousands of molecules of two types: a target-specific antibody and alkaline phosphatase. Such a signaling moiety complex binds to the target via the conjugated antibody category-binding molecule. When incubated with a chromogenic alkaline phosphatase substrate (the signal element; *e.g.*, BM purple, Roche), a colored spot can be generated which can be detected by eye. Alternatively, the same signaling moiety complex, when incubated with either a chemiluminescent or a fluorescent alkaline phosphatase substrate, generates either a chemiluminescent or fluorescent signal. Further examples of signaling moiety complexes include: nanogold particles conjugated to fluorescein-labeled antibodies, and latex particles conjugated to both oligonucleotide category-binding molecules and acridinium esters that chemiluminescence upon addition of hydrogen peroxide.

By **particle** is meant an object or matrix which is less than 50 microns in size. The size of a population or batch of particles is defined as the mean measurement of the longest pair of orthogonal dimensions for a sample of the particles. The longest pair of orthogonal dimensions is the pair of orthogonal dimensions of a particle the sum of the lengths of which is the maximum for all such sums for the particle. If a sample of two particles has a longest pair of orthogonal dimensions of 1 micron X 2 micron and 2 micron X 3 micron, respectively, the mean measurement of the longest pair of orthogonal dimensions is 2 microns [(1+2+2+3)/4 = 2 microns]. The mean measurement of the longest pair of orthogonal dimensions for a sample of particles is, e.g., less than 50 microns, less than 20 microns, or less than 5 microns.

Many particles have some characteristics of a solid. However, molecular scaffolds or complexes, which may not be rigid, are also defined as particles. For example, dendrimers or other branching molecular structures are considered to be particles. Similarly, liposomes, are another type of particle. Particles can be dyed with or conjugated to signal elements. Particles are often referred to with terms that reflect their dimensions or geometries. For example, the terms **nanosphere, nanoparticle,** or **nanobead** are used to refer to particles that measures less than 1 micron along any given axis. Similarly, the terms **microsphere, microparticle,** or **microbead** are used to refer to particles that measure less than one millimeter along any given axis. Examples of particles include latex particles, polyacrylamide particles, magnetite microparticles, ferrofluids (magnetic nanoparticles), quantum dots, *etc.*

By **labeling particle** is meant a particle that can specifically bind to targets and generate a signal. Labeling particles are conjugated or stably associated with both signaling moieties and to category-binding molecules.

By target:**labeling particle complex** is meant a labeling particle to which one or more targets are specifically bound.

By target:**label complex** is meant a target that is specifically bound to one or more category-binding molecule and associated with one or more signaling moieties.

By **labeling ratio** is meant the ratio of targets to labeling particles during the contacting step. For example, if $1X10^7$ labeling particles are contacted with a sample containing $1X10^6$ targets the labeling ratio is 10.

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By **signal character** of a signal element or signal moiety is meant the aspect or aspects of a signal generated by the signal element signaling moiety that is useful for distinguishing it from other signal elements or signaling moieties. For example, the signal character of a signaling moiety labeled with fluorescein and rhodamine is *fluorescence*. The character of a radio transponder is *radio frequency.* Examples of photonic signaling character are fluorescence, light scattering, phosphorescence, reflectance, absorbance, chemiluminescence, and bioluminescence. All but the latter two examples of photonic signaling character depend on external illumination (*e.g.*, a white light source, a laser light source, or daylight). In contrast, chemiluminescence and bioluminescence are signaling characters that are independent of external light sources.

By the **class** of a signal element or signaling moiety is meant the distinct quality of the signal that is useful for distinguishing it from other signal elements or signaling moieties. For example, a liposome that is labeled with red dye is distinguished from differently colored liposomes. The color red is its class. For a micro-transmitter that broadcasts a particular radio-frequency signal, the quality of the radio-frequency signal that differentiates the micro-transmitter from other micro-transmitters constitutes the signal element class.

By **signal signature** is meant the distinctive signaling quality of the combination of signaling moieties that bind to a category of targets in a test. A target that is bound to four types of antibodies, one of which is conjugated to a fluorescein molecule, and three of which are conjugated with rhodamine molecules has a signal signature that is described by the combined weighted absorbance and emission spectra of fluorescein and rhodamine.

By **signal complexity** of a test or an ensemble of labeled category-binding molecules is meant the number of categories of targets that can be distinctly labeled in the test or by binding to the ensemble. Alternatively, the signal complexity is defined as the number of distinct *signal signatures* that would be expected to occur if a member of each category of target were present. For some tests, the signal complexity of an ensemble of category-binding molecules is the same as the number of categories for which the test scans. Other tests, which scan for many categories, may only have a signal complexity of one.

By **selection force** is meant a force that is used to capture, isolate, move, or sequester targets. Examples of selection forces include gravity, magnetism, electrical potential, centrifugal force, centripetal force, buoyant density, and pressure. Targets can be mobilized by a selection force acting on the target alone. Alternatively, selection forces can act specifically on targets that are associated with selection moieties (see definition below).

Examples of the application of selection forces to mobilize targets include centrifugation of targets; magnetic selection of targets bound to magnetic particles; gravitational sedimentation of targets labeled with metallic particles; and deposition of targets on a porous membrane by vacuum filtration. Further instances of the use of selection forces are included in the examples below.

By **selection moiety** is meant an atom, molecule, particle, or other entity that can be conjugated to a category-binding molecule and that confers on the category-binding molecule the ability to be selectively captured, isolated, moved, or sequestered by a selection force. When a category-binding molecule:selective moiety complex is specifically bound to a target, the target can also generally be selectively captured, isolated, moved, or sequestered by the selection force. Selective, in the sense used here, refers to the preferential conferring of susceptibility to mobilization by the selection

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force on selection moieties and associated entities over entities not associated with selection moieties.

Paramagnetic particles and ferritin are examples of selection moieties. A dense silica particle that sinks in solution is another type of selection moiety. Such particles, when coated with category-binding molecules and bound to a target will cause the target to sink in aqueous solution, thus enabling separation of the bound target from other sample unbound constituents.

By **selective character** is meant the aspect or aspects of a selection moiety that is useful for capturing, selecting, or moving the selection moiety. For example, the selective character of a paramagnetic particle is *magnetism*. The selective character of a silica particle that rapidly sinks in aqueous solution is *mass*.

By a **roughly planar** surface or substrate is meant a surface that can be aligned in parallel to an imaginary plane such that when the distance is measured from points in any 1 mm × 1 mm square on the surface to the closest points on the imaginary plane, the absolute value of the mean distance is less than 50 micrometers.

By **detection surface** is meant the surface of a roughly planar substrate onto which targets are deposited. In embodiments using photonic signaling character, if the detection surface is optically clear, detection can be effected via either face of the detection surface. If the detection surface is opaque, detection is effected via the face of the detection surface on which the targets are deposited.

By **detection area** is meant the area of the detection surface or detection zone that is simultaneously analyzed by the invention. The detection area is typically greater than 1 mm, e.g., greater than 5 mm, greater than 10 mm, or greater than 15 mm in its longest linear dimension. For example, the section of a glass slide that is simultaneously imaged by an optical device that includes a collection lens and a CCD chip might measure 0.8 cm X 0.5 cm. The detection area is then 0.4 cm^2.

By **detection zone** is meant the volume in which targets can be detected. The detection zone has the same dimensions as the detection area but has a depth corresponding to the depth in which signaling moieties can be detected and identified. The depth of the detection zone is therefore dependent on the threshold criteria used to score for positive signal. When optical detection is used, the depth of the detection zone is dependent on the optical depth of field.

By the **longest dimension** of the detection area is meant the line of maximum length that can be drawn between two points on the perimeter of the detection area. For example, if the detection area is a rectangle measuring 0.3 cm X 0.4 cm, the longest dimension of the detection area is the diagonal, 0.5 cm. If the detection area is an ellipse with semi-major axis of length 7 mm and semi-minor axis of length 2.5 mm, the longest dimension of the detection area is 14 mm.

By **large area detection or large area imaging** is meant a method for detecting microscopic targets in which the detection area (the area that is simultaneously analyzed by the detection device) is much larger than the target. The detection area for large area detection has at least one linear dimension that is ≥ 1 mm. In contrast, the microscopic targets are substantially smaller, typically measuring less than 50 μm in at least two orthogonal dimensions. Examples of large area detection include imaging a 9mm diameter detection area with a CCD camera; imaging a 2 cm X 1 cm rectangle by scanning with a CCD line scanner that has a long dimension of 1 cm; imaging a 4cm X 4cm filter containing microbial targets using direct exposure on photographic film; and

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visual detection of colored spots corresponding to microscopic targets on a 1cm X 3cm test area in a rapid lateral flow strip test.

Several technologies scan samples for microscopic targets but do not exploit large area detection. Examples include: flow cytometry; solid phase laser microbeam scanning cytometry; liquid phase scanning (as in Tibbe, et al., Nat Biotechnol 17: 1210-3, 1999); and examining/imaging multiple high power microscopic fields on a slide.

By **conjugated** or **stably associated** is meant a physical association between two entities in which the mean half-life of association is least one day in PBS at 4°C. Consider, for example, the complex case of passive protein adsorption to polystyrene particles. There are several different classes of adsorbed proteins. Some proteins are stably associated to the surface with half-lives of many months. Other proteins, such as those that are loosely bound on the outer layer of adsorbed protein, may not be stably associated with the particles and can leach out within hours.

By **image intensifier** or **image tube** is meant a device that amplifies a photonic signal, as defined in the glossary of Inoué, Shinya, *et al.*, *Video microscopy: the fundamentals* (Plenum Press, New York, 1997; p. 665): "A device coupled (by fiber optics or lenses) to a video camera tube to increase sensitivity. The intensifier is a vacuum tube with a photocathode on the front end that emits electrons according to the image focused upon it, an electron lens and/or microchannel plate(s) that focuses the electrons onto a phosphor at the back end, and a high voltage accelerator that increases the energy of the electrons. Can be single or multiple stage." A variety of such image intensifiers is described in detail in Chapter 8 of the same reference.

By **simultaneously detecting** targets in a section of the detection area is meant detection of the signal from a section of a roughly planar detection surface in one step. Large area imaging of targets in a detection area using a CCD chip, visual detection, or photodiode-based signal integration are examples of simultaneous detection.

By targets in the **stationary phase** is meant targets that are non-mobile. For example, targets fixed on glass slides are in the stationary phase. Targets that are captured by category-binding molecules in fixed positions on the bottom of the well of a microtiter dish are in the stationary phase. Even if such targets are not affixed to a surface, and might be moved by hydrodynamic or other forces, targets are considered to be in the stationary phase if, during detection/imaging, successive images taken with intervals of more than 10 seconds detect essentially the same targets in essentially the same relative positions. Targets in flow cytometry applications are *not* in the stationary phase. However, targets captured by antibodies bound to the solid-phase test zone of a lateral flow test *are* in the stationary phase.

By **homogenous assay** or **homogenous immunoassay** is meant an assay or immunoassay in which the reactants are not physically removed from the products of the completed assay.

By **identification** is meant determining the category or categories of which a target is a member. For example, consider a lateral flow test that scans for several categories of targets, each of which is potentially present in a sample. A target belonging to a particular category is captured at the region of the membrane to which the corresponding category-specific antibodies are bound. Since it is known which membrane zones contain which capture antibodies, targets are identified by the zone in which capture occurs.

By **sample** is meant material that is scanned by the invention for the presence of targets.

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By **direct visual detection** is meant visual detection without the aid of instrumentation other than wearable corrective lenses. For example, direct visual detection can be used to detect the reddish reflective signal of nanogold particles in some rapid lateral flow tests.

By **photoelectric detector** is meant a man-made device or instrument that transduces photonic signals into electric signals. Examples of photoelectric detectors include CCD detectors, photomultiplier tube detectors, and photodiode detectors, e.g., avalanche photodiodes.

By **encircled energy** or **ensquared energy** is meant the percentage of photons from an infinitely small light source that are captured on a pixel of a photodector array.

By **thermal radiation** is meant black body radiation.

By **cellular autofluorescence** or **autofluorescence** is meant the fluorescence exhibited by cells due to the fluorescence of natural intrinsic cellular constituents, such as NADH and oxidized flavoproteins. Cells expressing fluorescence due to recombinant fluorescent proteins such as green fluorescent protein are not considered to be autofluorescent.

By **illuminating** is meant irradiating with electromagnetic radiation. Electromagnetic radiation of various wavelengths can be used to illuminate. It includes, for example, radiation with wavelengths in the X-ray, UV, visible, or infrared regions of the spectrum. Note that illuminating radiation is not necessarily in the visible range.

By signal elements or signaling moieties with **photonic signaling character** is meant signal elements or signaling moieties that are detectable through the emission, reflection, scattering, refraction, absorption, capture, or redirection of photons, or any other modulation or combination of photon behavior. Some examples of signal elements or signaling moieties that have photonic signaling character include: the fluorophore Texas Red (fluorescent signaling character); CDP-Star (chemiluminescent signaling character); luciferase (bioluminescent signaling character); resonance light scattering particles (light scattering signaling character); BM purple (light absorption or chromogenic signaling character); and up-converting phosphors (absorption of two long wavelength photons and emission of one shorter wavelength photon).

By '**number**'**X** '**solution name**' is meant an aqueous solution comprising the constituents of the solution at **number** times the concentration of the solution (except for water). For example, 10X EE contains 10 mM EDTA/100 mM EPPS (EE, or 1X EE, contains 1 mM EDTA/10 mM EPPS).

EE is a solution that is 1 mM EDTA/10 mM EPPS. Before mixing them together, the conjugate acids of both components are brought to pH 8.0 with NaOH

HYB is a solution used for hybridization containing: 1M NaCl, 50 mM EPPS pH 8.0, 2% blocking reagent (Boehringer Mannheim); 0.5% v/v Tween, 20 µg/ml yeast tRNA (Sigma).

UBB (universal binding buffer) is a solution useful for binding mixtures of various types of category-binding molecules (such as antibodies and nucleic acids) containing: 250 mM NaCl, 50 mM EPPS pH 8.0, 2% blocking reagent (Boehringer Mannheim); 0.5% v/v Tween, 20 µg/ml yeast tRNA (Sigma).

BB (blocking buffer) contains 100 mM EPPS pH 8.0/150 mM NaCl/2% blocking reagent (Boehringer Mannheim).

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PB is 0.1 M sodium phosphate buffer pH 7.4.

PBS is a phosphate-buffered saline solution containing: 120 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer (sodium salt) pH 7.4.

PBS-B is 0.1% BSA (IgG Free; Sigma Cat. No. A-7638) in PBS.

PBS-T is 0.05% Triton X-100 (Sigma Cat. No. X-100) in PBS

PBS-TB is PBS/0.1%BSA/0.05% Triton X-100

PBT is PBS/0.1% BSA (IgG Free; Sigma Cat. No. A-7638)/.05% Tween-20 (Sigma Cat. No X-100)

LB is Luria Broth for growing bacteria and is made as described previously (Ausubel 1987, *supra*).

SSC is 150 mM NaCl/15 mM Na_3citrate adjusted to pH 7.0 with HCl.

MES (2-[N-Morpholino]ethanesulfonic acid)

MESB is 0.05M MES (2-[N-Morpholino]ethanesulfonic acid), pH 6.1

EDAC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

Ligation buffer is10 mM $MgCl_2$/50 mM Tris-HCl/10 mM dithiothreitol/1 mM ATP/25 μg/μl bovine serum albumin.

Prelysis solution: The solution is prepared according to Graves, L, *et al.* (1993). *Universal Bacterial DNA isolation procedure.* In Diagnostic molecular microbiology: principles and applications, D. Persing, T. Smith, F. Tenover and T. White, eds. (Washington, D.C.: American Society for Microbiology). The solution, which is freshly prepared and maintained on ice, contains: 0.25 ml of 2M Tris (pH 7.0), 3.1 ml of pancreatic lipase (prepared by dissolving 6.1 mg of pancreatic lipase (Sigma) in 59.8 ml of water and adding 1.2 ml of 0.1M $CaCl_2$; stored at -20°C in 3.1ml aliquots), 0.3 ml of 1% sodium taurocholate (Difco), 0.5 ml of 0.1M $CaCl_2$, 5.25 ml of sucrose, and 0.05 g of lysozyme.

Standard PCR protocol: All PCR reactions (unless otherwise noted) are carried out in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl pH 8.3, 250 μM (each) dNTP, and 10 μM (each) primer, in a Perkin-Elmer Geneamp 9700 thermocycler, for 30 cycles followed by incubation for 10 min at 72°C. A cycle is composed of three steps: 94°C, 30 sec; 55°, 30 sec; 72°C 90 sec.

AP: Alkaline phosphatase

BAL: Bronchoalveolar lavage

BSA: Bovine Serum Albumin

CCD: Charged coupled device

CFTR: Cystic fibrosis transmembrane conductance regulator

cfu: Colony forming unit (a measure of bacterial concentration that corresponds to the number of viable bacterial cells)

CMV: Cytomegalovirus

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FITC: Fluorescein isothiocyanate

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HIV: Human Immunodeficiency virus

pfu: Plaque forming unit (a measure of virus concentration that corresponds to the number of infectious virus particles)

PNA: Peptide nucleic acid

RSV: Respiratory syncytial virus

$TCID_{50}$: Tissue culture infectious dose at which 50% of flasks demonstrate infection

Oligonucleotide sequences are presented in the 5' to 3' orientation when written as text, unless otherwise noted.

Unless otherwise noted, microbiological strains described in the specifications are obtained from the American Type Culture Collection (ATCC), Manassas, VA.

Brief Description of the Drawings

Figure 1. **The principle of non-magnified large-area imaging of individual signal generating particles with a CCD array.**

The figure shows the principle of imaging individual targets using a CCD array detector.

Figure 2. **Detecting *M. tuberculosis* using non-magnified large area imaging.**

The figure shows a test based on the invention that scans a sputum sample for the presence of *M. tuberculosis*, the bacterial pathogen that causes tuberculosis. After heat-fixing a sputum sample to a microscope slide, the sample is incubated with labeled antibodies that specifically bind to *M. tuberculosis* (anti-MTB Ab). The unbound antibodies are removed leaving only the antibodies that are bound to *M. tuberculosis* bacteria. The antibodies are labeled with a fluorophore, so that when illuminated with one color of light (the excitation spectrum), they emit a second color (the emission spectrum). Illuminating the slide with the excitation spectrum causes the antibodies, which are localized at the sites where bacteria are fixed to the slide, to emit light in the emission spectrum. The emitted light is focused onto the array of pixels on a CCD chip. Pixels lying beneath a fluorescent bacterium are illuminated and transmit an electronic signal to a computer where it is preserved as an image. Software analyzes the image and the user interface reports the number of bacteria found in the scan.

Figure 3. **A CCD imaging device for large area imaging.**

The CCD-based imager depicted in the figure was used to collect much of the data described in the examples (see also Step 6 of Detailed Description section). Excitation light is provided by introducing light from a high intensity white light source (1000 Watt Xenon arc lamp, Model A-6000, Photon Technology Incorporated, Monmouth Junction, NJ) into a

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liquid light-guide (5 mm core diameter, Model 380, Photon Technology Incorporated, Monmouth Junction, NJ). The liquid light-guide carries the light to an excitation filter-wheel (BioPoint FW, Ludl Electronics, Hawthorne, NY) and directs the filtered beam (typically 9 mm in diameter) onto the detection surface containing the labeled targets. The apparatus can detect labeled targets on various detection surfaces (*e.g.*, porous membranes, microscope slides, microtiter dishes, coverslips, and tubes with flat, optically clear, bottoms). The incident light strikes the detection surface inducing fluorescence in the signaling moieties that are bound to targets via category-binding molecules and that are deposited on the optically clear surface. A portion of the emitted fluorescent light is collected by a high-collection efficiency lens system and transmitted through an emission filter-wheel (BioPoint FW, Ludl Electronics) to a CCD Camera (Orca II, Hamamatsu, Bridgewater, NJ).

Figure 4. **A CCD imaging system for non-magnified large area imaging.**

The figure shows a CCD imager with an angular illumination configuration in which light is introduced onto the detection surface (shown here as the bottom of a well of a microtiter plate) at an angle from the side of the collection optics. The angle is chosen to optimize collection efficiency and to avoid obstruction of the incident beam by the collection lens. The advantage of this configuration is that reflections from the bottom surface of the sample holder are not collected by the collection lens and therefore do not contribute to the fluorescence background noise.

Figure 5. **Detecting different pathogens using combinatorial labeling.**

Combinatorial labeling using multiple signaling moieties (labels) can be used to achieve high signal complexity (*i.e.*, a large number of distinct signal signatures; see also discussion in Step 2 in the Detailed Description section). Each of the three pathogens represented in the diagram are labeled with a distinct combination of quantum dots. Thus, each pathogen emits the distinct coded signal (shown as a colored bar code to the right of the pathogen). The category-binding molecules shown are pathogen-specific oligonucleotide probes that hybridize to genomic DNA. Each probe is conjugated to a fluorescent quantum dot. Many types of category-binding molecules (e.g., antibodies, ligands, PNA probes, *etc.*) can be used for combinatorial labeling as can other types of signaling moieties (*e.g.*, fluorophores, fluorescent particles, RLS light-scattering particles, *etc.*).

Figure 6. **Using tag complements to conjugate signal moieties.**

The illustration depicts a method for indirectly labeling a category-specific oligonucleotide category-binding molecule. A bipartite oligonucleotide is synthesized comprising a category-specific binding moiety and a tag sequence moiety. A signaling moiety is conjugated to a second oligonucleotide, called a tag complement, which is complementary to the tag sequence. Using this system for indirect labeling, families of category-specific oligonucleotides with tags can easily be synthesized encoded with a combination of signaling moieties.

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Figure 7. Large area imaging of individual bacteria labeled with a fluorescent DNA-binding stain

The figure shows detection of individual fluorescently stained bacterial cells on a porous membrane support. Cells were stained with the nucleic acid stain Sybr Green I, and filtered through a black polycarbonate membrane. The fluorescent signal was imaged with the CCD based non-magnifying large area imager, with an FITC optical filter set. The left panel shows the fluorescent image from about 100 E. coli cells. The number of spots correlates with the number of cells added to the membrane. The right panel shows a negative control where no cells were added to the membrane

Figure 8. Large area imaging of individual bacterial cells labeled with various category-binding molecules and signaling moieties.

The figure shows the results of experiments in which *E. coli* cells on glass coverslips were labeled with four types of signaling moieties (Syber Green I, Example 2); fluorophore-labeled oligonucleotide probes, Example 3; fluorophore-labeled PNA probes, Example 4; fluorophore-labeled antibodies, Example 5). Individual cells were detected by non-magnified large area imaging using the apparatus shown in Figure 3. Dilutions of the labeled cells were spotted onto coverslips coated with poly-l-lysine. After imaging the labeled cells using the CCD imager, the imaged objects were confirmed to comprise single cells or occasional groups of several cells by fluorescence microscopy. The upper panels of images show labeled cells as detected by CCD imaging. Bright spots correspond to labeled cells. The lower panels show the same labeled cells as viewed in a fluorescent microscope (1000X magnification).

Figure 9. Detecting individual viable bacterial cells using non-magnified large area imaging

The figure shows that the invention can detect live bacteria using a fluorogenic viability stain. Live *E. coli* cells (left panel) were detected as bright fluorescent spots, while dead *E. coli* cells (right panel) do not have a detectable fluorescent signal. Approximately 400 live *E. coli* cells are present in the imaged area of the left panel, and approximately 400 dead *E. coli* are in the right panel.

Figure 10. Large area non-magnified imaging of individual bacteria labeled with highly fluorescent particles

The figure shows the results of an experiment (Example 7) in which pathogenic *E. coli* cells were bound to the optically clear surface of a microtiter dish well, labeled with fluorescent particles, and imaged using non-magnified large area imaging. *E. coli O157* cells (stained with Syber Green I) were fixed to the bottom of a 96-well plate using heat, and labeled by binding to fluorescent particles coated with anti-*E. coli O157* antibodies. The left panel shows the cell:particle complexes as seen in the CCD imager. The middle panel shows the same sample as seen by low power fluorescence microscopy (50X magnification). The stained *E. coli O157* cells are surrounded by numerous fluorescent particles as shown by high power fluorescence microscopy (right panels;1000X magnification).

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Figure 11. Homogenous immunoassay for a virus (HCV) using liquid-phase magnetic selection of fluorescent particle:virus:magnetic particle complexes followed by non-magnified large area imaging.

The figure diagrams a useful method for scanning for microscopic targets – in this case Hepatitis C virus (HCV) – in a liquid-phase sample. Magnetic and fluorescent particles, coated with antibodies that bind specifically to the virus, are mixed in a well of a microtiter dish with a sample that potentially contains the virus. Viruses in the sample then bind to the particles. Some of the viruses bind to a magnetic particle and to a fluorescent particle as represented in the magnified projection in the figure. This magnetic particle:virus:fluorescent particle complex has the properties of being selectable by magnetic force *and* of being highly fluorescent. The microtiter dish is placed on a magnet so that the magnetic particles, and HCV viruses and fluorescent particles associated with the magnetic particles, are drawn down to the optically clear bottom surface of the well. Using a CCD imaging apparatus (like the one shown in Figure 3), the bottom of the well is imaged. The virus complexes appear as bright spots in the image due to the highly fluorescent particles that are bound to the viruses. Software analysis of the image quantifies the number of complexes on the detection surface and integrates the total intensity of signal derived from the labeled complexes (see Example 8 and Figure 12 for representative results of the homogenous immunoassay method).

Figure 12. An homogenous immunoassay that detects individual bacteria using non-magnified large area imaging.

The figure shows the results of immunoassays that sensitively detect *E. coli* by non-magnified large area imaging described in Example 8. Magnetic and fluorescent particles coated with category-specific antibodies were first bound to the bacterial cells, the complexes were then drawn to the bottom surface of the optically clear well (the detection area) using magnetic force, and the complexes were detected using non-magnified large area CCD-based imaging. The figure shows the comparison of the results of the homogenous immunoassay in blood (bottom panel) and the non-homogenous (washed) immunoassay in both blood (top panel) and in buffer (middle panel). The simplest format, the homogenous immunoassay, effectively detected cells with excellent signal to background ratios (see quantification of the homogenous assay in the bottom right panels of the figure).

The two leftmost panels show the images obtained using non-magnified, large area CCD-based imaging. All three assays gave strong signals when *E. coli* were present (left panel) but low background signals when *E. coli* were not added. Further confirmation that the signals obtained using the CCD camera corresponded to *E. coli* cells coated with particles were obtained using high power fluorescence microscopic analysis (1000X; top two rows, rightmost two columns). Images are shown of typical complexes containing *E. coli* cells, magnetic particles, and fluorescent particles. Two images of each complex were made using two filter sets: one for visualizing DNA fluorescence (Syber Green I; second panel from right) and one for visualizing the fluorescent particles (rightmost panel). The figure shows that the complexes consist of an *E. coli* cell surrounded by magnetic and fluorescent particles.

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Quantitative analysis of the images was carried out by software that counts the number of fluorescent objects (Figure 12, bottom panel, left bar graph) and that integrates the intensity of all of the objects (Figure 12, bottom panel, right bar graph). The data shown represent the analysis of six samples: three samples that contained 1000 *E. coli* cells, and three samples containing no cells. In both measurements, the sample containing *E. coli* (1000 cells) scores significantly higher than the sample containing no *E. coli*. The height of the bars indicates the average values for the three samples. Error bars indicate three standard deviations around the mean values of the three samples.

For further details and discussion refer to Example 8.

Figure 13. Large Area Imaging of Acid Fast Stained *Mycobacteria* in "simulated sputum"

The figure shows the results of the test described in Example 9 in which a strain of mycobacteria that is closely related to the pathogen that causes tuberculosis was detected using the acid-fast bacillus (AFB) staining method combined with non-magnified large area imaging. Commercial slides containing either mycobacteria cells (*Mycobacteria scrofulaceum*; left panel: 1A, 2A) or *E. coli* (right panel: 1B, 2B) in "simulated sputum" were stained with auramine-rhodamine and visualized using non-magnified, large-area, CCD-based imaging (top panel: 1A, 1B). Numerous objects were detected in the sample containing mycobacteria cells compared to a much lower number of background spots in the sample containing *E. coli*. Fluorescence microscopic analysis (400X; bottom panel: 2A, 2B) confirmed that the signals captured with the CCD camera without magnification correspond to single mycobacteria cells.

Figure 14. Rapid antimicrobial susceptibility testing using large area imaging.

The figure shows the results of an experiment designed to determine the minimal inhibitory concentration (MIC) of an antibiotic on the growth of *E. coli* by using non-magnified large area imaging (Example 10). Duplicate aliquots of the cells at various time points were cultured on LB agar and deposited on the optically clear surface of a 96 well microtiter dish well for imaging using a CCD imager (Figure 3). Growth (displayed qualitatively in the figure with +/- symbols) was measured by colony counts on LB agar plates (not shown in figure) and by object and intensity measurements of the images captured by the CCD camera. The 18 hr culture time point corresponds to the NCCLS standard protocol. Also shown are four hour culture results and four hour CCD imaging results. Comparison of the antibiotic concentration dependence of the growth of the resistant and sensitive strains, as detected by culture and CCD imaging, shows that the two approaches are comparable.

Figure 15. Large area detection of individual fluorescently labeled *Candida albicans* cells that have been magnetically selected.

The figure shows the results of the experiment described in Example 11. *C. albicans* cells were pre-labeled with a nucleic acid stain (YOYO-1) and a rabbit anti-Candida polyclonal antibody. Labeled cells were added to magnetic particles coated with goat anti-rabbit IgG antibodies in the wells of a glass-bottomed microtiter tray. Cells were caused to align evenly over the bottom of the well by application of a magnetic field. The

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cells were detected by imaging the entire bottom surface of the well using the CCD imager and appropriate excitation and emission filters for the YOYO-1 fluorescent dye (FITC set, 480/40 nm excitation, 535/50 nm emission).

Non-magnified large area CCD image of a microtiter tray well containing *C. albicans* cells stained with YOYO-1 as described. Fluorescent signals (white spots in figure) were shown to correspond to single or small groups of *C. albicans* cells by examining the microtiter well in a fluorescent microscope under high power magnification (1000X).

Non-magnified large area CCD image of a microtiter tray well with no *C. albicans* cells added (Negative control).

Figure 16. Large area detection of individual *Candida albicans* cells specifically bound to fluorescent and paramagnetic polystyrene particles..

The figure shows the results of the experiment described in Example 12. Cells were added to a mixture of magnetic and red fluorescent particles coated with anti-*Candida* antibodies in a microtiter dish. Cells bound to magnets were separated from unbound particles by application of a magnetic field and fluidic washing. The cell-fluorescent particle complexes were detected by imaging the entire bottom surface of the well using the CCD imager (A and B) or fluorescent microscopy (C-E) and appropriate excitation and emission filters for the fluorescent particles.

A. Non-magnified large area CCD image of microtiter well containing *C. albicans* cells labeled with multiple red fluorescent particles.

B. Non-magnified large area CCD image of microtiter well with no *C. albicans* cells added (negative control).

C. Magnified fluorescent microscopic image (100X magnification) of an area of the same well imaged in **A.** Single or small clumps of *C. albicans* cells (green stain) are surrounded by multiple red fluorescent particles.

D. Magnified fluorescent microscopic image (100X magnification) of an area of the same well imaged in **B** (negative control with no cells added).

E. and F. High power magnification (1000X) fluorescent microscopic image of an area of the same sample that is shown in **A.** *C. albicans* cells (approximately 5 μm diameter, green stain) are surrounded by multiple 1 μm diameter red fluorescent particles. Magnetic particles are not visible.

Figure 17. Large area detection of individual Candida albicans specifically bound to fluorescent antibodies and magnetic particles.

The figure shows the results of the experiment described in Example 13. *C. albicans* cells were mixed with magnetic particles coated with anti-*C. albicans* or anti-*E. coli* antibodies in wells of a glass-bottomed microtiter tray. After 30 minutes, a fluorophore-labeled anti-*C. albicans* or anti-*E. coli* antibody was added. Magnetic complexes were washed and then caused to align evenly over the bottom of the well by application of a magnetic field. Cells were detected by imaging the entire bottom surface

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of the well using the CCD imager of the present invention and appropriate excitation and emission filters for the fluorophore.

A. Non-magnified large area CCD image of reaction well containing *Candida albicans* cells labeled with fluorescent anti-*C. albicans* antibody.

B. Non-magnified large area CCD image of reaction well with no cells added (Negative control).

C. Non-magnified large area CCD image of reaction well containing *E. coli* cells labeled with fluorescent anti-*E. coli* antibody.

D. Non-magnified large area CCD image of reaction well with no cells added (Negative control).

Note: Punctate fluorescent signals were shown to correspond to single or small groups of cells by staining with a red fluorescent nucleic acid-binding dye (YOYO-3) and examining the microtiter well in a fluorescent microscope under high power magnification (1200X)

Figure 18. Non-magnified large area detection of individual chemiluminescent yeast cells using a CCD camera.

The figure shows the results of the experiment described in Example 14. *C. albicans* cells were labeled with FITC-conjugated anti-*C. albicans* antibodies, which were, in turn, bound to anti-fluorescein antibody:alkaline phosphatase conjugates. Dilutions of the labeled cells were spotted onto slides coated with nylon membrane, and the chemiluminescent substrate CDP-Star was added. The cells were imaged without magnification in the CCD Imager. Positions of single cells were confirmed by fluorescence microscopy.

From left to right, panels represent approximately 200, 60, and 20 individual *C. albicans* cells. Many individual cells are imaged in the middle and right panels. Larger brighter spots represent two or more cells that are close together.

Figure 19. Non-magnified large area detection of individual chemiluminescent yeast cells using direct exposure of instant film.

The figure shows the results of the experiment described in Example 15. *C. albicans* cells were labeled with FITC-conjugated anti-*C. albicans* antibodies, which were, in turn, bound to anti-fluorescein antibody:alkaline phosphatase conjugates. Dilutions of the labeled cells were spotted onto a nylon membrane and the chemiluminescent substrate CDP-Star was added. The nylon membrane was loaded into a Spot Light camera (Boston Probes) and imaged with ASA2000 Polaroid Film.

From left to right, panels represent approximately 200, 60, and 20 individual *C. albicans* cells, as in Figure 15. Given the number of cells spotted in each panel, it can be concluded that many of the spots represent individual cells, especially in the right most panel.

Figure 20. Detection of organisms involved in lower respiratory tract infections using non-magnified large area imaging

The figure shows the results of an image captured using non-magnified large area imaging that sensitively detects the signal generated from fluorescently labeled lower respiratory tract organisms described in

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Example 16. The figure shows the signal generated using non-magnified large area imaging (two left most panels) from various lower respiratory tract pathogens from top to bottom: *Chlamydia pneumoniae, Mycoplasma pneumonia* and *Legionella pneumophila.* The two right most panels show the images obtained using fluorescent microscopy.

Figure 21. A mulitiplexed direct fluorescence immunoassay that simultaneously scans a sample for 3 disparate microbes using non-magnified large area imaging

The figure shows the results of the experiment described in Example 17. Three samples, containing three different microbes (*E. coli*, *C. albicans*, and *S. pyogenes*) were fixed to a glass coverslip and allowed to incubate with an ensemble of category-specific (in this case, species-specific) antibodies. Each family of antibodies in the ensemble was conjugated to a different fluorophore (*E. coli* = green, *C. albicans* = blue, *S. pyogenes* = red). After washing away the unbound antibodies, images of the samples were captured using three different filter sets (green, blue, and red channels). For all three samples, the strongest signal was obtained in the channel that corresponds to the labeled antibody that binds to the microbe in the sample.

Figure 22. Solid phase capture assay for Adenovirus

The figure shows the results of a test that scans a sample for adenovirus using antibodies bound to the solid-phase to capture the virus (Example 18). The wells of a microtiter plate were coated with anti-adenovirus antibodies. Fixed adenovirus samples or fixed RSV samples were added to the wells along with anti-adenovirus coated fluorescent particles. After incubation for an hour, all unbound particles were washed away and the remaining bound particles were visualized without magnification in the CCD Imager.

The negative control (RSV panel) shows very few particles remain in the well. In the adenovirus panel, thousands of fluorescent particles have been captured in the well due to the interactions of the anti-adenovirus coated wells, the adenovirus, and the anti-adenovirus coated particles.

Figure 23. Solid phase capture of a virus in blood

The figure shows the results of a test that was used to scan a blood sample for the adenovirus using antibodies bound to the solid-phase to capture the virus (Example 19). The wells of a microtiter plate were coated with anti-adenovirus antibodies. Fixed adenovirus samples or fixed RSV samples were added to the wells in 50% mouse blood. After an hour incubation all unbound particles were washed away and anti-adenovirus coated fluorescent particles were added to the wells. After washing, the bound particles were visualized without magnification in the CCD Imager.

The negative control (RSV panel) shows very few particles remain in the well. In the adenovirus panel, thousands of fluorescent particles have been captured in the well due to the interactions of the anti-adenovirus coated wells, the adenovirus, and the anti-adenovirus coated particles. The presence of blood during the adenovirus capture phase did not interfere with the assay.

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Figure 24. Liquid phase assay for Adenovirus

The figure shows the results of a test that uses a "dual particle" test format to detect adenovirus (Example 20 see also diagram in Figure 11). Fixed adenovirus or fixed RSV were mixed with anti-adenovirus coated magnetic and fluorescent particles. After incubating four hours, the magnetic particles and any bound particles were separated from the other materials, transferred to a microtiter plate, and visualized without magnification in the CCD Imager.

The adenovirus panel shows thousands of anti-adenovirus coated fluorescent particles captured by the magnetic particles due to their interaction with adenovirus. Very few fluorescent particles have been captured in the RSV panel.

Figure 25. Multiplex large area imaging immunoassay that simultaneously scans for a bacterium and a virus

The figure shows the results of an experiment using a test that simultaneously scans a sample for the presence of *E. coli* and adenovirus (Example 22). Anti-adenovirus coated red fluorescent particles and magnetic particles and anti-*E. coli* coated green fluorescent particles and magnetic particles were mixed in the presence of fixed adenovirus, fixed *E. coli*, both or neither. After an hour of incubation, the magnetic particles and any bound particles were separated from the other materials, transferred to a microtiter plate, and visualized without magnification in the CCD Imager.

Top row: Only adenovirus was added to the particle mixture. Most of the signal can be seen with the Texas Red filter set, which visualizes the red fluorescent anti-adenovirus particles.

Second row: Only *E. coli* was added to the particle mixture. Most of the signal can be seen with the FITC filter set, which visualizes the green fluorescent anti-*E. coli* particles.

Third row: Both adenovirus and *E. coli* were added to the mixture. Signal can be seen with both the Texas Red and FITC filter sets.

Figure 26. Filter flow-through assay for detecting single bacteria using non-magnified large area imaging.

The figure shows the results of a rapid "flow-through" assay that detects single dispersed bacteria in a liquid sample (Example 23). Two nitrocellulose membranes were soaked in anti-*E. coli O157* antibodies and allowed to dry. One filter was used to collect *E. coli* cells that had been pre-incubated with fluorescent *E. coli*-specific particles ("*E. coli*"; these particles had been coated with *E. coli* O157:H7-specific antibodies. A sample that contained the *E. coli*-specific particles but no *E. coli* cells was passed over the other filter ("No *E. coli*"). Unbound particles were removed by washing the filters. A CCD imaging device was used to detect the fluorescent signal (top panels) using non-magnified large area imaging. Confirmatory microscopic images of the two filters are shown in the middle (50X magnification) and bottom (100X magnification) panels.

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Figure 27. Quantification of bacteria using large area imaging of cells stained with a fluorogenic esterase substrate

Objective: In many applications it is useful to have a large dynamic range for quantifying live bacterial cells. An ideal system would be able to accurately count from zero or one bacterial cell up to millions or tens of millions, thus eliminating the serial dilutions and their inherent lack of precision that are necessary for traditional microbiological plating methods. In this example we show how staining live cells with fluorogenic substrates, coupled with CCD-based, non-magnified large area imaging can be used to quantify cells over at least 5 orders of magnitude.

Experimental Methods: *E. coli* ATCC 8739 cells were grown and processed as described in Example Y (Cell Direct example). Serial 10-fold dilutions of the cells were made in PBS and filtered in duplicate samples through black polyester membranes (Chemunex cat. # 200-C2010-01) mounted on absorbent pads (Chemunex cat. # 200-C3012-02) in a Millipore 1225 manifold and stained as described in Example Y (Cell Direct Example). In addition, 10 μλ of the 10-5 dilution was plated in triplicate on TSA (BD catalogue no. 236950) and grown at 37°C overnight to get a cell titer. The fluorescent signals on the polyester membranes were captured using a CCD Imager (described in step 6 above; Figure 3) with an FITC optical filter set (Chroma/excitation 470/40 nm, emission 522/40 nm). Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager. The signal generated from each filter was defined as the sum of the pixel intensities of all objects (where objects are defined in this particular example as containing pixel intensities from 350-65301). This method of defining signal eliminates the background from regions of the filter that do not contain any stained cells, but does not mask or undercount intensities from overlapping objects. (Since the cells are small and fairly transparent, signals are additive as long as the layer of cells is thin.)

Results: As shown in Figure 28, the signal generated from this method is linear over at least 5 orders of magnitude, which is considered a large dynamic range for this test.

Variations. At low object numbers, accurate quantification can be achieved by counting individual objects rather than using the sum of their pixel intensities, since the objects will be unlikely to overlap. This can extend accurate counting down to one or zero cells, especially if multiple stains and multiple excitation and/or emission wavelengths are used to determine which objects represent viable cells. In addition, more sophisticated object finding algorithms can be employed to take into account local background intensities and variations in illumination.

Detecting individual stained bacteria on a filter using non-magnified large area imaging.

The figure shows that non-magnified large area imaging using a CCD camera detects individual bacterial cells that have been deposited on a membrane (Example 24). *E. coli* cells stained with Syber Green I were filtered through a black polycarbonate filter. The filter was then imaged using a CCD imager (top panel). The bottom panels show fluorescent microscopic confirmation that the luminescent objects in the CCD images are indeed Syber green stained *E. coli* (1000X).

Figure 28. The dynamic range of a test based on the invention

The figure shows the large dynamic range possible when using the invention. *E. coli* cells were diluted from a few million cells to a few hundred, filtered, and stained with fluorogenic esterase substrates. The

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fluorescent images generated by CCD-based non-magnified large area imaging were analyzed and their signals plotted vs. cell count. The signal is linear over at least 5 orders of magnitude.

Figure 29. Non-instrumented detection of small numbers of bacterial cells without magnification

The figure demonstrates use of a dually coated (alkaline phosphatase/anti-*E. coli* antibody) particle in a liquid capture assay that detects single *E. coli* cells (described in Example 26). The images in each of the three columns of panels were obtained using a different detection method. The upper row of panels shows the assay as applied to a sample containing 100 cells. The bottom row of panels shows the results when the sample contained no cells. Each of the panels labeled "100 cells" has significantly more spots than the corresponding "no cells" control. For chemiluminescent signals, the spots are white on a black background, while for chromogenic signals the spots appear black on a white background. Note that the latter spots were also apparent by inspection of the membranes with the naked eye, without the use of magnification. Thus, the results of this example demonstrate that non-magnified large area imaging using bifunctional beads can be a powerful tool for sensitive detection even without complex instrumentation.

Figure 30. A rapid homogenous immunoassay for Chlamydia trachomatis and Neisseria gonorrhoeae; part 1: detecting the presence of *Chlamydia trachomatis*

The figure diagrams the homogenous immunoassay described in Example 31 that scans for the common sexually transmitted disease pathogens *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. The figure shows how the immunoassay detects the presence of *Chlamydia trachomatis* in a sample. To understand how the test differentiates between the two pathogens refer to Figure 31. The format of the assay corresponds to the one diagrammed in Figure 11. Experimental details are similar to the multiplexed assay used to detect a bacteria and a virus in Example 22 (Figure 25).

Figure 31. A rapid homogenous immunoassay for Chlamydia trachomatis and Neisseria gonorrhoeae; part 2: the principle of multiplex detection

The figure, a continuation of Figure 30, diagrams the homogenous immunoassay described in Example 31, which scans for the common sexually transmitted disease pathogens *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. Detection of red particles indicates *Chlamydia trachomatis* infection, while detection of green particles indicates *Neisseria gonorrhoeae* infection.

The format of the assay corresponds to the one diagrammed in Figure 11. Experimental details are similar to the multiplexed assay used to detect a bacteria and a virus in Example 22 (Figure 25).

Figure 32. A multiplexed homogenous immunoassay with high signal complexity using non-magnified large area imaging.

The figure shows the scheme for a highly multiplexed homogenous immunoassay. The principle of the assay is the same as the one diagrammed in Figure 30 except, that rather than two pairs of analyte-

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specific fluorescent particles, this assay incorporates many analyte-specific particle pairs to scan for many analytes simultaneously. Methods for achieving the high signal complexity (*i.e.*, the large number of distinct signals) required to identify numerous categories of targets are discussed in Step 6 of the Detailed Description section.

Figure 33. An immunoassay that scans in parallel for numerous lower respiratory pathogens.

The figure diagrams the immunoassay described in Example 33. A lower respiratory sample (*e.g.*, sputum or BAL) is fixed to a microscope slide. After affixing a multiwell template to the slide, a different family of pathogen-specific biotinylated antibodies is added to each well (*e.g.*, anti-*S. pneumoniae or* anti-*legionella* antibodies are added to separate wells). Unbound antibodies are washed away and fluorescent particles coated with streptavidin are bound to pathogens that have bound to biotinylated antibodies. The location of sectors where fluorescent particles have bound indicates the identity of the pathogen since such sectors corresponds to wells that contained antibodies to a known pathogen. The particle-containing sectors are imaged using CCD detection

Figure 34. Multiplexed identification of urinary tract infections without culture

This figure diagrams the test described in Example 34. Capture antibodies for different urinary tract pathogens are affixed in parallel stripes on a glass slide. The slide is immersed in a urine solution that has been treated with a fluorescent stain that binds to the nucleic acids in bacterial cells. Bacterial pathogens, if present, adhere to the corresponding line of capture antibodies on the slide, and are imaged using non-magnified large area imaging.

Figure 35. Rapid, multiplexed, identification of blood-borne viruses: HIV, HCV, HBV, and CMV

This figure diagrams the test described in Example 35. Adsorbed to the optically clear bottom of a microtiter dish are four distinct spots containing 4 different capture antibodies that are specific for four different viruses (HIV, HBV, HCV, and CMV). Blood (containing HIV in the figure) is added to the well and viruses are allowed to bind to the capture antibodies. After removing the blood, a mixture of four types of virus-specific fluorescent particles is added to the well. Each type of particle is dyed with a fluorophore with distinct spectral characteristics (signal signature) and is coated with a different viral-specific antibody. Virus particles, which are trapped on the surface of the well, bind to the corresponding fluorescent particles that are detected and quantified by CCD-based non-magnified large area imaging. Both the position and signal signature of the bound particles identify the virus in the blood.

Figure 36. Ultra-sensitive lateral flow test for Influenza A virus using non-magnified large area imaging

The figure shows the results of lateral flow tests for Influenza A virus labeled with fluorescent labeling particles and analyzed using non-magnified large area imaging. The figure shows images of the capture and control lines from test strips onto which were applied samples containing (from left to right) 0, 10^5, 10^6,10^7 and 10^8 virion/ ml. The

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fluorescent signal increases with increasing concentration of Influenza A virions. The data shown shows the test can detect concentrations at least as low as 10^5 virions/ ml. This experiment therefore demonstrates the sensitivity of lateral flow tests based on the invention.

Figure 37. A rapid lateral flow test for M. tuberculosis using visual detection

This figure diagrams the test described in Example 36. The top panel shows a side view of the lateral flow test configuration. The test zone contains a line of anti-*M. tuberculosis* capture antibodies that are bound the bibulous membrane. The positive control zone contains *M. tuberculosis* bacteria bound to the membrane. A sample (containing *M. tuberculosis* in the figure) is applied to the sample pad. Capillary flow draws the pathogen cells along the membrane so that they encounter and are trapped by the capture antibodies in the test zone. Particles, conjugated to anti-*M. tuberculosis* antibodies and alkaline phosphatase, are applied to the sample pad. In the course of migrating towards the absorbent pad via capillary flow, the conjugates bind to captured *M. tuberculosis* cells in the test zone and to the filter-bound *M. tuberculosis* in the control zone. These bound conjugates are directly visualized after chromogenic staining with an alkaline phosphatase substrate.

Figure 38. A rapid lateral flow test for detecting numerous disparate biowarfare agents using non-magnified large area imaging

This figure diagrams the test described in Example 38. The strategy is similar to the one described in the legend of Figure 37 with the following exceptions. This test scans a sample for numerous disparate biowarfare agents including bacteria, viruses, and toxins. (Note, that for simplicity, the figure does not depict all of the targets, conjugates, and capture antibodies in the test described in Example 38.) In this example, the sample encounters and mobilizes the conjugates in a conjugate pad. Finally, this test uses a chemiluminescent substrate of alkaline phosphatase and CCD imaging to detect specifically bound conjugate particles.

Figure 39. Using genomic subtraction to isolate *S. pneumoniae* category-specific sequences.

This figure diagrams the isolation of *S. pneumoniae*-specific sequences as described in Example 39. Figure 39A shows the phylogenetic relationship between *S. pneumoniae* and its closest relatives (Kawamura, et al., International Journal Of Systematic Bacteriology 45: 406-8, 1995). The strategy for isolating category-specific sequences can be broken down into two steps: a genomic subtraction step (Figure 39B) and a screening step (Figure 39C). First, genomic subtraction (Straus, 1995, supra) is carried out using the DNA from the pathogenic strain (in this case *S. pneumoniae*) as the "+" genomic difference sample (Figure 39B). The "–" genomic difference sample is constructed by pooling several of the closest related strains (strains that do not commonly cause pneumonia). The resulting subtraction products are fragments that hybridize to the pathogen's genome but not to the genomes of the closely related species. After cloning the subtraction products, the subset that are category-specific are isolated (Figure 39C). These are DNA fragments that hybridize to all *S. pneumoniae* strains but do not hybridize to any strains in other groups. Probes are chosen that hybridize to all *S.*

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pneumoniae strains tested but to no strains of the genus *Streptococcus* that are in other species. For details, see Example 39.

Figure 40. A comprehensive test for respiratory pathogens using nucleic acid probes.

The figure diagrams the test described in Example 39. An ensemble of pathogen-specific probes is hybridized to a slide comprising two sectors: one containing a respiratory sample and one containing an array of spots each of which includes control respiratory pathogens in a different category. Each family of probes in the ensemble is labeled with a distinct signal signature. Comparison of the signal signatures of pathogens in the clinical sample to the signal signatures in the control spots identifies the pathogen in the clinical sample. The number and intensities of objects found by the image analysis software quantifies the number of pathogens in the sample. For details, see Example 39.

Figure 41. Indirect combinatorial labeling of category-specific sequences.

The figure shows the method used to achieve high signal complexity in Example 40. Each family of category-specific oligonucleotide category-binding molecules is labeled with a particular signal signature using a scheme analogous to the one shown in Table 5. Thus, as shown in the figure, *S. pneumoniae* probes are indirectly labeled with either violet or blue quantum dots while *S. aureus* probes are labeled with either yellow or green quantum dots. For example, probes that are to be labeled with violet quantum dots are synthesized with two adjacent moieties: a category-specific oligonucleotide and a "violet tag." The violet tag sequence can hybridize to a "violet tag complement" that is conjugated to a violet quantum dot.

Figure 42. CNS strip test.

This figure diagrams the test described in Example 41.

Figure 43. Bipartite PCR primer for amplifying genomic difference sequences pinpointed by virtual subtraction (Example 43).

The figure shows the PCR primers that are used in Example 43 for amplifying regions (~200– 500 bp) that occur in *Chlamydia trachomatis*, but not in *Chlamydia pneumoniae*. The PCR primers are designed with a bipartite structure. The "left primer" has the XhoL primer sequence (5'-GGG CCC CCC CTC GAT C-3') linked to a 20 bp primer corresponding to the "left" end of an insertion/deletion region in *Chlamydia trachomatis* (5'-XhoL-*Chlamydia trachomatis* left primer-3'). The "right primer" has the XhoR primer sequence (5'-ATC GAT ACC GTC GAC CTC-3') linked to a 20 bp primer corresponding to the "right" end of an insertion/deletion region in *Chlamydia trachomatis* (5'-XhoR-*Chlamydia trachomatis* right primer-3').

Detailed description of the invention

Overview of the invention. The invention rapidly and cost-effectively scans a minimally processed sample for small numbers of microscopic and submicroscopic targets of many types (*e.g.*, cells, viruses, or protein molecules). The powerful features offered by the invention arise from a novel diagnostic approach that combines modern

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high-intensity labeling, low-cost imaging technology, and non-magnified large-area detection.

To understand how the invention detects small numbers of microscopic targets in a relatively large sample, it is helpful to first examine a specific embodiment. Figure 1 shows a simple schematic of one embodiment of the invention that detects small numbers of bacteria spread out over a large area, in this case a part of a microscope slide. The bacteria are labeled so that they emit light, as will be described shortly. The approach that is used to detect the "glowing" bacteria, which is used in many of the examples below, uses a digital camera (CCD chip) to capture an image of an area of a sample that is much larger than the individual bacteria. The CCD chip has an array of photosensitive pixel elements. Light from a "glowing" bacterium at one position in the sample impinges on pixel elements at the corresponding position of the CCD chip. The end-user receives information about the number of bacteria in the sample from a computer that processes the image data (*i.e.*, the number and intensities of the illuminated pixels) acquired from the CCD chip.

Next, consider a specific application of the embodiment (Figure 1) that scans a respiratory sample for the presence of the important bacterial pathogen, *Mycobacterium tuberculosis*. In this test, depicted in Figure 2, the bacteria are labeled with a fluorescent chemical, a fluorophore, so that they emit light of a specific color (the emission spectrum) when illuminated by a light of a different color (the excitation spectrum). To develop reagents for labeling the bacteria, a category-binding molecule is first chosen. An ideal category-binding molecule would be a monoclonal antibody that binds to *M. tuberculosis* but to no other type of bacterium or substance that might be present in a respiratory sample. The antibody might, for example, bind specifically to a particular molecular site, a "category-specific binding site," that is found only on the surface of the *M. tuberculosis* bacterium. Next, the *M. tuberculosis*-specific category-binding molecule is labeled by conjugation to a fluorophore, *e.g.*, fluorescein. The fluorescein molecule is called the "signaling moiety," as it provides the signal to be detected in the test. Having prepared the category-binding molecule (the anti-*M. tuberculosis* antibody) labeled with the signaling moiety (fluorescein), diagnostic tests for *M. tuberculosis* can be performed.

Figure 2 illustrates how the test determines whether a patient is infected with *M. tuberculosis*. A sputum sample is spread on and affixed to a glass slide. After incubating the slide with the fluorophore-labeled antibodies, the unbound antibodies are removed from the sample by washing. Illuminating the sample with light in the excitation range of fluorescein elicits fluorescence emission by the fluorescein-tagged antibodies coating the surface of *M. tuberculosis* cells that are present in the sample. The fluorescent image of the cells is collected, focusing the emitted light on the surface of a CCD chip without magnification. Thus, a large area of the sample smear, typically about 1 to 2 cm^2, is scanned concertedly for *M. tuberculosis* cells. Fluorescent light from each cell impinges on a small cluster of pixels causing a localized electronic signal that is relayed from the CCD chip to a computer where it can be stored in the form of an image file. Image analysis software enumerates the *M. tuberculosis* in the sample by counting the number of pixel clusters responding to light from cells and by integrating the total photonic intensity of the responding pixels.

The invention can be used to construct tests using a number of types of formats, labeling methods, category-binding molecules, and detection methods. However, the tests have several key features in common. The steps and processes that are common to many embodiments of the invention are described below.

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The general configuration of applications of the invention consists of the following steps:

Step 1: Formulating the test question, choosing category-binding molecules for detecting targets

Step 2: Choosing and preparing signaling moieties

Step 3: Preparing the biological sample

Step 4: Binding targets in the sample to category-binding molecules and signaling moieties

Step 5: Selecting targets and bound category-binding molecules

Step 6: Identifying and quantifying targets present in the sample by detecting the signaling moieties bound to targets

Step 1: Formulating the test question, choosing category-binding molecules for detecting targets

Choosing the categories of targets to be detected by the test. Categories are defined functionally as a range of related targets that are to be considered identical, for the purposes of a test. Choosing the categories of targets that will be identified by a test is tantamount to defining the goals of the diagnostic test. Choosing the categories is also central to defining the molecular reagents, the category-binding molecules that will be used in the test.

When constructing a new test based on the invention, the first step is to decide which categories of targets the test should identify and distinguish. For example, to construct a test to identify the infectious agents that cause a patient's pneumonia, one selects categories of pathogens that commonly cause pneumonia, such as Respiratory Syncytial Virus and *Streptococcus pneumoniae*; or, to test for foodborne pathogens, one selects categories of bacteria that cause toxicity. The invention can identify a broad range of targets including multicellular organisms, bacteria, and viruses. Furthermore, targets from various categories (*e.g.*, bacteria and viruses) can be tested for in the same assay.

A significant use of the invention is in the identification of pathogens in a human bodily fluid sample, such as blood, urine, cerebrospinal fluid, sputum, or feces. (The method is also important as applied to other types of clinical samples including tissue samples.) Depending on the bodily source of sample and the symptoms of the patient, a decision is made as to the important types of organisms to be identified. For example, in the case of a sputum sample from a patient with lower respiratory symptoms, one can choose to detect viruses, bacteria, and fungi that are common causes of pneumonia.

The choice of categories depends on the diagnostic question that is addressed by a test. Thus, when two tests answer two different diagnostic questions, different categories may be chosen even if the tests both scan the same type of sample for the same targets. When considering a test for screening urine samples for urinary tract infections the operative diagnostic question is often: "Are there a significant number of bacterial cells in the urine?" For such a test, the test category might comprise all bacteria. That is, the test does not distinguish different species of bacteria from each other. So, for example, both *Escherichia coli* and *Enterococcus facacium* fall into the

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same "pan-bacterial" category for the purposes of this type of screening test. In contrast, the goal of a different type of urinary tract infection test is to identify the pathogen at the species level. For this type of test, each species of bacteria that commonly causes urinary tract infections is defined as a different category (*e.g.*, *Escherichia coli* and *Enterococcus facacium* would be separate test categories).

For some tests, multiple categories of organisms that span multiple kingdoms are chosen. It is frequently clinically important to determine which of a panel of disparate pathogens is the cause of a patient's symptoms. The invention addresses the critical unmet need for cost-effective efficacious tests that simultaneously scan a patient's sample for the presence of various viruses, bacteria, and fungi. The **categorical complexity** is a measure of the number of categories of targets for which a test scans. (see also the Definition section).

Category-specific binding sites and category-binding molecules. Different categories of targets are distinguished by their distinct molecular constituents. For example, consider the following set of disparate pathogens, each of which causes lower respiratory disease: Influenza A virus, RSV, *Haemophilus influenzae*, *Streptococcus pneumoniae,* and *M. tuberculosis*. Each pathogen category possesses molecular constituents that are characteristic of the category but that do not occur in members of other categories or in other components of respiratory samples. Tests based on the invention detect members of a particular category by scanning for the presence of category-specific molecular constituents.

To detect the presence of a category of targets, the invention deploys molecules that bind specifically to category-specific molecular constituents. The category-specific molecular constituents that occur on targets are called **category-specific binding sites** and the molecules that bind specifically to them are called **category-binding molecules**. Note that category-specific binding sites are a property of targets that are potentially present in the sample to be tested. In contrast, category-binding molecules are a reagent provided in the diagnostic test kit.

An advantage of the invention is that a broad spectrum of category-binding molecules can be used. This is important since different types of category-binding molecules are used to ask different types of diagnostic questions (*e.g.*, broad kingdom-level screening *vs.* narrow subspecies-level identification). Classes of category-binding molecules (also sometimes referred to as probes) comprise: nucleic acids (oligonucleotides, aptamers, cloned sequences, genomic DNA, RNA, etc.), chemical variants related to nucleic acids, such as peptide nucleic acids (PNA); antibodies; enzymes (which can bind target substrates); non-enzymatic proteins such as avidin (which binds the target molecule biotin); molecules that bind cellular constituents specifically (*e.g.*, phalloidin which binds actin or biotin which binds avidin); dyes and stains, such as propidium iodide, auramine-rhodamine, or SYTO 17); ligands (*e.g.*, epidermal growth factor, which binds specifically to the epidermal growth factor receptor); and polypeptide or nucleic acid binding reagents that have been selected using *in vitro* evolution techniques (*e.g.*, Zhang, *et al.*, Nat. Biotech. 18: 71-74, 2000).

Category-binding molecules can incorporate other functional domains or modifications. For example, category-binding molecules are often covalently or non-covalently associated with signaling moieties (*i.e.*, a labeling domain such as a fluorophore or a dyed microparticle) or selection moieties (*e.g.*, magnetic particles or solid surfaces). Alternatively, a category-binding molecule may be linked to an adaptor moiety that, in turn, facilitates linkage to another functional moiety. Sometimes the category-binding molecule has dual non-separable functions. For example, propidium iodide, a nucleic acid stain, can be used as a category-binding molecule (*e.g.*, the category-specific

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binding site might be the cellular nucleic acid in a yeast) while, at the same time, the bound dye functions as a signaling moiety (*i.e.*, it can fluoresce when bound to the category-specific binding site). Tests based on the invention can incorporate more than one class of category-binding molecule (*e.g.*, antibodies and nucleic acid stain, or antibodies and oligonucleotides).

The simplest tests incorporate a *single* type of category-binding molecule to scan for a *single* category of target. For example, a test for *M. tuberculosis* might use a monoclonal antibody that binds specifically to a category-specific binding site on the surface of *M. tuberculosis*. Alternatively, for example, when screening for urinary tract infections, the single category is "all cells" – or, if human cells are lysed, "all non-human cells" – and the single type of category-binding molecule could be a nucleic acid stain (*e.g.*, propidium iodide).

A ***family*** of category-binding molecules is a set of distinct category-binding molecules that bind to members of the same category of target. For example, a polyclonal antibody raised to Hepatitis C virus is a family of antibodies since it comprises multiple category-binding molecules that bind specifically to the same category of target – in this case HCV. Another example of a family of category-binding molecules is a set of 80 category-specific genomic DNA sequences that occur in all *E. coli* O157:H7 strains, but do not occur in members of other groups of bacteria. This family of category-binding molecules can hybridize as a group to suitably prepared *E. coli* O157:H7 cells, but does not hybridize to other types of cells.

To detect multiple categories of targets, a test includes one family of category-binding molecules for each category. A set of families of category-binding molecules is called an ***ensemble*** of category-binding molecules. For example, tests for pneumonia or tests for drugs of abuse, must distinguish numerous categories of targets from each other. One family of category-binding molecule is used for each category of target. For a pneumonia test, an ensemble of antibodies that react to category-specific antigens on the surface of microbes that cause pneumonia might be used. One family in this category-binding molecule ensemble might comprise polyclonal antibodies from the immunoglobulin fraction of antiserum raised in a rabbit host and directed against *Streptococcus pneumoniae*. Another family could comprise a recombinant antibody or a monoclonal antibody directed against a coat protein of adenovirus.

The number of distinct groups or categories of targets tested for by an ensemble, *i.e.*, the **categorical complexity**, is reflected by the number of families of category-binding molecules in the ensemble. The number of families in an ensemble can, in turn, be accurately defined by a quantity called the "minimum categorical derivation" of an ensemble. The **family complexity** is the minimum number of distinct targets required to bind members from each of the families of category-binding molecules in the test ensemble. For example, consider an ensemble of category-specific antibodies used to simultaneously test a sputum sample for the presence of *Mycobacterium tuberculosis*, *Legionella spp*, *Coccidoides immitus*, influenza virus, and Respiratory Syncytial Virus. The family complexity of the ensemble would be five, since a minimum of five targets, one from each pathogen category, would be required to bind to members of each family of category-binding molecules in the ensemble. The ability of the invention to identify a broad spectrum of targets in a single test is a consequence of its ability to scan a sample using an ensemble of category-binding molecules that has a large family complexity.

Category-binding molecules used in conjunction with the invention should be specific in that they should bind under assay conditions to the desired target (analyte) but not to other types of targets (meant to be distinguished by the assay) or to other possible

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constituents of the sample or test. Thus, in a test for upper respiratory bacterial infection, potential category-binding molecules are screened to eliminate those that cross react with normal (commensal) microbial constituents of the upper respiratory tract.

Representative methods for obtaining and characterizing category-binding molecules are included in the examples below.

Step 2: Choosing and preparing signaling moieties

Labeling category-binding molecules with signaling moieties. The invention's ability to detect individual microscopic targets without optical magnification or expensive instrumentation generally depends on specifically labeling the targets at high signal intensity. Labeling is achieved by specifically binding signaling moieties to the targets via an association with category-binding molecules. Note, however, that for certain applications of the invention that use intrinsic properties of the targets as signaling moieties, labeling is not required (*e.g.*, cellular autofluorescence).

The invention discriminates between categories of targets in two general ways. One method, called signal differentiation, labels each category-specific family of category-binding molecules with signaling moieties such that it has a unique *signal signature*. The ability to generate and detect large numbers of distinct signal signatures (*i.e.*, high *signal complexities*) enables construction of tests that scan for numerous categories of targets (*i.e.*, highly multiplexed tests). The other method for distinguishing between multiple categories of targets, geometric differentiation, relies on depositing different categories of targets in different regions of the detection area. Geometric differentiation, which can be independent of the signal signature of signaling moieties, is discussed below (Step 5).

The invention can exploit various types of *signal character* including: fluorescence, scattered light, light polarization, radio waves, particle size, magnetic field, chemiluminescence, and radioactivity. Examples of signaling moieties and detection schemes using various signal characters appear below. There can be multiple signal classes within a signal character. For example, if the signal character is fluorescence, various characteristic emission spectra comprise the signal classes (*e.g.*, red fluorescence, green fluorescence, and blue fluorescence). Alternatively, as another example, consider red fluorescent microparticles that are dyed with different concentrations of the same fluorophore. Fluorescence is again the signaling character; however in this case the different intensities of the particles constitute the classes of signal character, *i.e.*, fluorescence intensity is the quality of the signal character that differentiates one group of particles from another.

A great variety of signaling moieties can be used in conjunction with the invention as demonstrated in the examples below. Signaling moieties can comprise simple fluorophores, up-regulated phosphors, naturally fluorescent proteins (such as green fluorescent protein and its relatives), dyes, enzyme:substrate systems (generating substate:product color changes or chemiluminescence), fluorescent microparticles, light scattering particles, magnetic particles, or radio transmitting microdevices.

Attaining a high *signal complexity* is useful for developing tests that scan for numerous types of targets (*i.e.*, tests with high *categorical complexity*).

Achieving high signal complexity. The number of distinguishable labels (or signaling moieties) in a mixture is called the *signal complexity*. For highly multiplexed tests, it is sometimes advantageous to use signaling moieties with high signal complexity. Three

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general approaches that can be used with this invention to generate high signal complexity are: (*1*) distinct labeling, (*2*) combinatorial labeling, and (*3*) ratio labeling.

1. For distinct labeling, probes in different probe families are tagged with a single signaling moiety that can be readily detected in the presence of all other signaling moieties in the experiment. Thus far, it has been difficult to achieve distinct labeling at high signal complexities. This is because most labeling methods use optical signals (*e.g.*, chromogenic, fluorescent, chemiluminescent) or radioactive labeling. Due to the spectral bandwidth of optical signals and the limited range of signals detectable by current instruments, the resolvable signal complexity using optical signals is rather small. For example, due to spectral overlap, the resolution of dozens of fluorophores with distinct emission spectra is currently impossible.

2. Another way to achieve the high signal complexity used in the invention is to apply a combinatorial labeling approach. Combinatorial labeling is a technique for achieving high signal complexity using a relatively small number of distinct signaling moieties. With this approach, distinct *combinations* of signaling moieties are bound to different targets. The concept is illustrated in Figure 5 (see also Example 1). Currently, fluorophores are a favored class of signal moiety for molecular diagnostics. However, given the complications involved in analyzing multiple distinct fluorophores (arising in large part from overlap of the excitation and emission spectra), it is only currently practical to incorporate about seven or fewer conventional fluorophores. However, used in combination, seven fluorophores can be used to generate 127 distinct signals (N fluorophores generate $2^N - 1$ combinations). High signal complexity can also be achieved via combinatorial labeling using other types of signaling moieties. For example, particles impregnated with different dyes, particles that fall into different discrete size classes, or transponders emitting distinct radio signals could be used with this approach. Combinatorial labeling using fluorophores has recently been applied with success for human karyotyping (Speicher *et al* 1996, *supra*; Schröck *et al* 1996, *supra*). Instrumentation and software for analysis of combinatorial labeling experiments is commercially available.

3. High signal complexity can also be obtained using the ratio labeling approach (Fulton, *et al* 1997, *supra*). In ratio labeling, as in combinatorial labeling, many distinct types of signaling moieties are generated using a relatively small number of distinct signaling elements. However, in contrast to combinatorial labeling, the signaling moieties in ratio labeling are distinguished by the ratio of the signaling elements. For example, two fluorophores, X and Y, with different excitation/emission characteristics can be used to dye polystyrene particles. Different relative concentrations of the fluorophores ([X], [Y]) are applied to different sets of particles. For example, eight different concentrations of X and eight different concentrations of Y can be used to dye particles in all combinations (X_1Y_1, X_1Y_2, X_8Y_7, X_8Y_8) resulting in 64 classes of distinguishable particles. Ratio labeling simplifies instrumentation, as only a small number of signal types need be used. Signal elements, other than fluorophores and including non-optical signal elements, can also be used to generate high signal complexities using a ratio labeling approach.

Generating strong signals facilitates detection of individual microscopic targets. The level of signal intensity needed is, of course, dependent on the type of signal character (optical, particle size, etc.) and the detection method/instrumentation (see below).

Various approaches for labeling category-binding molecules can be used to achieve the required sensitivity. One method for optimizing the signal strength is to label target molecules with highly fluorescent signaling moieties. For example, quantum dots,

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fluorescently dyed nanospheres, and polymerized fluorophore molecules generate strong fluorescent signals. Incorporating numerous signal elements can increase the fluorescence intensity of a signaling moiety. For example, fluorescent nanospheres (~20 nm in diameter; Molecular Probes) can generate a signal equivalent to about 180 fluorescein molecules. Fluorescently dyed polystyrene microparticles (*e.g.*, 1 μm in diameter) can incorporate millions of fluorophore signaling elements. A method for incorporating multiple fluorophores in a signal moiety associates with a nucleic acid category-binding molecule is to incorporate fluorophore-dNTP conjugates during PCR amplification of a cloned category-specific sequence. Alternative methods for incorporating multiple fluorophores into nucleic acid category-binding molecules include approaches using: dendrimers, branched DNA, or rolling circle templates bound to multiple signal moieties, or tailing with numerous polymerized fluorophore labeled nucleotides. Conjugating category-binding molecules to multiple signaling moieties also increases signal intensity. For example, signal amplification can also be achieved by conjugating large numbers of signaling enzymes (*e.g.*, alkaline phosphatase or horseradish peroxidase) to a nanoparticle.

Another approach to obtain strong signals is to bind numerous labeled category-binding molecules to each target. This can be done by various means including: using multiple category-binding molecules (recognizing multiple category-specific binding sites in the same target) or by choosing category-binding molecules that bind to target molecules that are highly represented in a target. For example, a labeled microbe-specific polyclonal antibody can achieve high signal intensities by binding to numerous distinct epitopes on a microbial target (*e.g.*, see Example 11). Example 1 also describes using labeled category-binding molecules that bind to many distinct category-specific binding sites in each target organism. The strategy of choosing category-specific binding sites that are present in large numbers in each target has been frequently used previously. Examples of this strategy include the use of nucleic acid probes for ribosomal RNA (which depending on the target organism and cell type can be present in thousands of copies per cell). Similarly, some antigenic target molecules are present in hundreds or thousands of copies in each cell of a target organism. The invention can exploit both of these strategies. As another example, the large number of category-specific binding sites present in a bacterium yield strong signal intensity when using the nucleic acid-binding fluorescent dye Syber Green I as the category-binding molecule/signaling moiety (*e.g.*, see Examples).

Binding numerous signal moieties to a target not only increases signal strength, but it endows the invention with robustness since the chances are small of observing numerous clusters of a large number of signaling moieties with the expected composite signal signature in the absence of the target.

Conjugating signaling moieties to category-binding molecules. The invention can incorporate numerous types of signaling moieties which can be directly conjugated to category-binding molecules using various methods which are known by those familiar with the art (see, for example, Hermanson, G., *Bioconjugate Techniques* (Academic Press, 1996) and specific examples below). For example, antibody or oligonucleotide category-binding molecules can be directly conjugated to a fluorophore or a quantum dot signaling moiety. Alternatively, for example, antibodies or oligonucleotide category-binding molecules can be used to coat fluorescent microparticle-based or light-scattering nanoparticle-based signaling moieties. Signaling moieties can be indirectly conjugated to category-binding molecules. For example, as shown in Figure 6, a signal moiety can be conjugated directly to a tag complement that is then hybridized to a tag sequence, which is part of an oligonucleotide also containing a category-binding sequence. Alternatively, for example, avidin can be directly conjugated to multiple signal elements to constitute a signaling moiety. The labeled avidin molecule can then

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be bound to a biotinylated category-specific antibody. Signaling moieties can be conjugated to the category-binding molecules before, during, or after the binding steps. For example, in one embodiment of the invention, digoxygenin-labeled nucleic acid probes are used as the category-binding molecules. After binding the category-binding molecules to the category-specific binding sites in the targets in the sample, the unbound probes are washed away. Anti-digoxygenin antibody:alkaline-phosphatase conjugates (the signaling moieties) are then conjugated to the bound digoxygenin-labeled probes. An alkaline-phosphatase substrate (*e.g.*, the chemiluminescent substrate CDP-Star; NEN)) is then added to the bound alkaline-phosphatase to generate the signal.

Step 3: Preparing the biological sample.

Sample preparation. An important attribute of the invention is its compatibility with rapid and simple sample preparation protocols. This represents a major advantage over other sensitive diagnostic methods, such as nucleic acid amplification–based techniques, which require much more demanding sample preparation procedures to eliminate enzyme inhibitors.

Sample preparation can have several functions depending on the nature of the sample and the test format. In some cases, sample preparation concentrates targets and/or deposits them on a substrate. For example, a test for water-borne microbes might concentrate the microbes by filtration, depositing the cells on a paper filter for detection. Other samples are smeared onto a solid substrate (*e.g.*, a glass microscope slide).

Making category-specific binding sites on targets accessible to binding by category-binding molecules is an important function of the sample preparation. In some cases little or no treatment is necessary as, for example, when the category-specific binding site is an epitope on the surface of a microbe in an aqueous sample that is freely accessible to a category-specific antibody (*e.g.*, see Example 8). In other cases, sample preparation is required to make internal category-specific binding sites accessible. This is the case, for example, when category-specific DNA sequences are used to bind to category-specific binding sites on genomic DNA. Target cells must be made permeable to the probes and their genomic DNA must be denatured. When a large number of different types of targets are tested for in the same sample, the sample preparation must be effective for the entire spectrum of targets. Further specific examples of sample preparation methods are detailed in the examples below.

If an assay yields a negative result, it is important to know whether the sample is truly free of target organisms or whether the assay itself failed, i.e., whether or not the result is a false negative. To identify false negative results, one or more positive control targets can be added to the experimental sample. The positive control target contains category-specific binding sites that do not occur in the range of targets being tested. Category-binding molecules corresponding to the positive control targets are included with the other category-binding molecules used in the test. These targets will be detected in all assays, unless one or more of the assay steps is unsuccessful. Failure to detect a signal from a positive control thus can indicate a false negative result.

Step 4: Binding targets in the sample to category-binding molecules and signaling moieties.

In this step, category-binding molecules and associated signaling moieties are brought into contact with targets in the sample under conditions that facilitate specific binding. For example, an ensemble of category-specific nucleic acid sequences is hybridized to complementary target sequences in the sample in this step. Similarly, category-specific

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antigens in the sample are allowed to bind to the corresponding category-specific antibodies. There are several possible physical configurations for the binding step. For example, binding can be carried out in the liquid phase (*e.g.*, see Example 8) on a microscope slide (*e.g.*, see Examples) or on a nitrocellulose strip, using lateral flow chromatography (*e.g.*, see Example 38). The concentration of the category-binding molecules is optimized to achieve rapid binding kinetics. The chosen conditions for selecting for specific binding depend on the characteristics of the category-binding molecules and their interactions with target molecules. Specific conditions and procedures are described in the examples below.

Step 5: Selecting targets complexed with category-binding molecules and signaling moieties.

Physical separation of targets that are bound to signaling moieties from unbound signaling moieties enhances the subsequent detection process. A further purpose of the selection step is to deposit the targets in a detection zone (*e.g.*, an area in the focal plane of an imaging device). The complexes are generally bound to a solid phase while the unbound category-binding molecules and signaling moieties remain in a liquid phase. In some cases the unbound material is washed away. In other test formats (*e.g.*, homogenous liquid phase formats and membrane-based assays), washing is not necessary.

For assays in which the sample is fixed to a solid substrate before the binding step, the unbound category-binding molecules and signaling moieties are generally removed by washing. Representative examples below include those using *in situ* hybridization and immunocytochemical methods.

Other test formats are carried out in the liquid phase, for example in microtiter wells. In these examples the target/category-binding molecule/signaling moiety complexes are generally deposited on a surface after the binding step (see *e.g.*, Example 8). Methods for depositing the target complexes on a surface include centrifugation, filtration, gravitational settling, magnetic selection, or binding to surface bound category-binding molecules (*e.g.*, capture antibodies, as in Example 18). In some cases (*e.g.*, magnetic separation) a distinct moiety, the selection moiety is used. Magnetic microparticles coated with category-specific antibodies are an example of a selection moiety (see *e.g.*, Example 8). The unbound category-binding molecules and signaling moieties generally remain in the liquid phase and can be removed. If the detection procedure (*e.g.*, optical imaging) selectively analyzes the solid surface with a narrow depth of field, the unbound material (lying outside of the plane of focus) sometimes need not be removed (*e.g.*, see Example 8). Further examples of each of these and other methods are presented below.

Lateral-flow and flow-through formats are arguably the most successful test formats in point-of-care testing. These formats exploit the advantages of capillary flow in bibulous membranes. They generally select the target complex using surface-bound category-binding molecules. Unbound category-binding molecules and signaling moieties flow out of the capture zone by capillary action. Another important advantage of membrane-based assays is the ease of multiplexing by using geometric differentiation (see below).

Geometric differentiation for constructing multiplexed tests. Geometric differentiation is an important method when scanning for multiple categories of targets (*i.e.*, in multiplexed tests). Geometric differentiation has the advantage, when compared to high signal complexity multiplexed tests (see Step 2), of requiring only a single signal signature for multiplexed tests. In a typical immunoassay that uses geometric differentiation, different category-specific capture antibodies are deposited in distinct

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areas in the detection zone (*e.g.*, different stripes in a lateral flow test or different spots in a flow through or microtiter well-based test). Thus, different categories of targets are captured in different pre-determined areas of the capture zone. Other types of capturing moieties that are analogous to capture antibodies include antigens, ligands, and nucleic acids. Several examples of geometric differentiation appear below (e.g., see Example 38 and Example 34).

Step 6: Detecting, identifying, and quantifying target **organisms present in the sample by detecting the signaling moieties bound to** targets **in the sample**

Significant advantages of the invention, including its sensitivity and its ability to quantify targets, derive from the ability to use large area imaging to detect individual targets. Detection of the targets labeled with signaling moieties is effected once the complexes are localized in a detection zone. The detection process used depends on the type of signal character of the signaling moieties (*e.g.*, fluorescence, chemiluminescence, or light scattering). For some signal characters (*e.g.*, light scattering and fluorescence), the complexes in the detection zone must be illuminated by a light source. For others (*e.g.*, chemiluminescence, radio transmission, or magnetic fields), illumination is not required. Various detection modes can be used including CCD cameras, film, and direct visualization.

Non-microscopic large area imaging can greatly increase sensitivity. Detecting targets without microscopy is an important aspect of the invention, as it can in many cases achieve more rapid, efficient, and sensitive sample analysis than microscopic methods used in standard *in situ* analysis methods. The gain in sensitivity of non-microscopic, large area detection over microscopic detection arises from the ability to scan a much larger volume of the sample for target. For example, the invention can image an area on a microscopic slide that is several thousand times larger than the typical area imaged using microscopic imaging. The resulting increase in sensitivity is illustrated by considering a sputum sample that contains 10,000 bacteria per milliliter from a patient with tuberculosis. For *in situ* analysis, a portion of the sample (*e.g.*, about 10 µl containing about 100 *Mycobacterium tuberculosis* bacteria) is spread over an approximately 1 cm^2-sized area on a microscope slide. In this case, the invention, which images the entire 1 cm^2-sized field, simultaneously detects the 100 individual cells. In contrast, when using microscopic analysis, due to the small field size examined, there is a high probability that a single field will contain no bacteria at all. Thus, numerous (often hundreds of) fields must be examined carefully when using microscopic analysis. This is a cost-, labor-, and time- intensive process that is circumvented by tests based on the invention.

Detection of single targets is naturally quantitative. During the detection or imaging step, the invention can detect the individual targets in a defined volume of the sample. Quantification can be accomplished manually by counting individual cells in a photographic or digital image or automatically by software analysis in the case of digital images. Integrating signal intensity over the sample can also be used to quantify the targets. Signal integration is particularly useful with samples containing high concentrations of targets or physically linked targets (e.g., filamentous fungi). In these cases, resolving coincident signals may not always be possible. When it is appropriate to use them together (*e.g.*, when the levels of targets are low enough that dispersed targets can be distinguished), automated signal integration and particle counting provide a robust, and therefore preferred, quantification regime.

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Decoding the signatures of labeled probe families allows identification of numerous categories of targets. An important goal of this step is to identify the category of targets in the sample by determining the signature of labeled category-binding molecules that have adhered to the sample.

Imaging control standards provides identification references and indicates that the assay is functioning correctly. Robustness and reproducibility are greatly enhanced by the ability to easily incorporate internal standards and controls in the invention. For example, when scanning a lower respiratory sample for 24 pathogens that commonly cause pneumonia, a set of internal standards comprising each of the pathogens can be scanned in parallel (e.g., see Example 1). Besides offering a comprehensive and highly redundant check of all reagents and procedures, the internal standards provide a set of reference signals to which sample signals are compared for identification.

The CCD camera-based imager, shown in Figure 3, is a useful device for large area imaging using when fluorescence is used as the signal character. This device was used to collect the data for many of the examples below. Excitation light is provided by introducing light from a high intensity white light source (1000W Xenon arc lamp, Model A-6000, Photon Technology Incorporated, Monmouth Junction, NJ) into a liquid light-guide (5mm core diameter, Model 380, Photon Technology Incorporated, Monmouth Junction, NJ). The liquid light-guide carries the light to an excitation filter-wheel (BioPoint FW, Ludl Electronics, Hawthorne, NY) and directs the filtered beam (typically 9 mm in diameter) onto the detection surface containing the labeled targets. The device shown in Figure 3 can detect labeled targets on various detection surfaces (*e.g.*, microtiter wells, microscope slides, coverslips, and tubes with flat, optically clear, bottoms). The incident light strikes the detection surface inducing fluorescence in the signaling moieties that are bound to targets via category-binding molecules. A portion of the emitted fluorescent light is collected by a high-collection efficiency lens system and transmitted through an emission filter-wheel (BioPoint FW, Ludl Electronics) to a CCD Camera (Orca II, Hamamatsu, Bridgewater, NJ). The design and construction of the optical train is based on principles and practices known to workers familiar with the art.

Experiments in the examples below use the instrument diagrammed in Figure 3 equipped with an X-Y positioning Stage (BioPoint XY, Ludl Electronics) to move the sample vessel (*e.g.,* a microtiter plate) over the excitation and collection optics (the stage is not shown in the figure). Image-Pro and Image-Pro add-ins control all instrument components and image acquisition. Filter wheels are managed with the ScopePro add-in (Media Cybernetics, Baltimore MD), and the StagePro add-in (Media Cybernetics, Baltimore MD) handles stage positioning, while the camera control is via the Hamamatsu Orca II driver (Hamamatsu, Bridgewater, NJ). Image-Pro Plus is also used for Image-Processing and analysis as described below.

A low cost camera that captures a medium resolution image of the sample area can be used. For a typical infectious disease application, it is expected that each sample will have on the order of one target to 10,000 targets spread out over a 20x20 mm sample area. A single, non-filamentous, prokaryotic microbe is expected to appear as a point source less than 10 um in diameter. For routine tests carried out in cost conscious medical diagnostics laboratories, medium resolution cameras can be used. For example, inexpensive cameras with excellent sensitivity and noise characteristics (e.g, SenSys CCD from Roper Scientific) are readily available. These systems typically have resolutions of 500x500 or greater pixels. Imaging the 20mm square sample area with a resolution of 500x500 pixels provide 250,000 pixels, more than sufficient to discriminate the majority of the one target to 10,000 targets expected in a typical assay.

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The sensitivity of the imaging system can be increased by choosing a more sensitive camera (e.g., a camera cooled to a lower temperature, or a camera that uses a back-thinned chip). Alternatively, the detection sensitivity and resolution can be increased by implementing a line scanning system (e.g., BT Image Array; Hamamatsu). For line scanning, a linear CCD or photodiode array (e.g. 1x500 or 1x1000 pixels) is used to capture the image. The resolution in one dimension corresponds to the number of array elements, while the second dimension is generally captured by moving the sample slide perpendicularly under the linear array. Since there are fewer elements, similar sensitivity linear arrays are typically less expensive than area format CCD cameras.

Embodiments of the invention using white light illumination utilize spectral filters to provide an optimal excitation peak for each of the fluorophores. The white light spectrum is large, allowing a wide variety of fluorophores to be selected to eliminate emission spectrum overlaps. Typically spot sizes achievable with white light illuminators, e.g., 2 mm to 5 mm, are appropriate for large area imaging. Since filter changes are relatively simple, and can be automated, white light systems are very adaptable, allowing the same apparatus to be used for tests that use distinct sets of fluorophores.

The collection efficiency of the system shown in Figure 3 is maximized by incorporating a custom designed collection optic consisting of two components: a collection objective and a focusing element. The collection objective has high collection efficiency ($\geq$ f#/1.2) and outputs a relatively collimated beam. The focusing lens captures the light output from the collection objective and focuses it onto the detection surface of the CCD. The optics are designed in two parts to allow a filter wheel to be inserted in the path of the collection lens. For certain embodiments of the invention, *e.g.* for some embodiments that do not require filter changes, it may be desirable to include a tapered optical fiber bundle for achieving high collection efficiency. The fiberoptic bundle contains fibers that collect light proximally to the sample and channel the light directly to a CCD chip. Alternatively, the invention can detect signals very sensitively using direct proximal detection in which the sample is applied directly or in close proximity to the CCD chip (for highest sensitivity to the back of a back-thinned CCD chip).

In addition to the white-light, multi-spectral system described above, we have also developed a simpler single-color fluorescence imaging system for non-magnified large area imaging. In this system, excitation light is provided by a 532nm Frequency-Doubled Diode Laser (50mW, Model#BWT-50E, B&W Tek, Newark, DE). The system using angular illumination is shown in Figure 3.

Since the detection systems shown in Figure 3 are single color, filter wheels are not necessary. A single excitation filter removes harmonic spectra from the laser output (Model HQ532/10x, Chroma Technology, Brattleboro, VT) and a single emission filter (Model HQ620/60m, Chroma Technology, Brattleboro, VT) allows only the specific fluorescent signals to pass to the CCD camera. These systems also use a less-expensive CCD camera (Model KX-2E, Apogee CCD, Auburn, CA) than the one described previously, to capture images. The instruments shown in these figures can easily be adapted to multicolor analysis by incorporating multiple lasers and filter sets.

Means for depositing targets on the detection surface can be incorporated in the detection system. For example, a magnetic station can be integrated into the instrument. A magnet (Dexter Laboratories) can be configured to deposit magnetic beads onto the bottom face of a 96-well format microtiter dish. Using a automated stage, the microtiter dish is moved into position over the magnet for a period of time sufficient to deposit the magnetic beads and associated targets onto the bottom face of

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the wells (generally several minutes is sufficient). The microtiter dish is then moved off the magnetic station and the wells are imaged individually.

Other means for selection can also be incorporated in the system containing the detection instrumentation. For example, a filtration station can be used to deposit targets onto filters that can then be imaged automatically. Similarly, targets can be deposited on the detection surface by centrifugation in an onboard centrifuge. Gravity selection of targets labeled with dense particles simply requires incubation within the detection instrument.

Other modules can be interfaced with or incorporated in the instrument system that includes the detection device. An automation module for handling sample vessels such as microtiter dishes are one example.

The CCD cameras incorporated in the invention are generally cooled to a temperature between -5°C and –20°C, sufficient for integration times from ten seconds to about two minutes (depending on the camera sensitivity) with minimal camera noise build-up. Longer integration times generally give higher sensitivity by allowing the collection of the photons emitted from the fluorophores for an extended period. Long integration times are inappropriate for line scanning; however, there are back-thinned linear arrays available that have very high quantum efficiencies, increasing sensitivity.

The invention can also use interferometer-based spectral imaging for the detection and decoding of signals (Schrock, E., 1997, *supra*). Using this technique, light emitted or scattered by signaling moieties is split into two paths, passed thorough prisms (so that different wavelengths travel different distances), and allowed to recombine to create an interference pattern. Fourier analysis of the interference pattern generates a spectrograph for each point in the image.

Alternatively, photographic film can be used to inexpensively record images of the targets in a sample. When the signaling character is chemiluminescence, this approach is most easily implemented (e.g., see Example 15).

For embodiments of the invention that use imaging detectors, computer software identifies and quantifies the targets. In general the software: (1) corrects for illumination non-uniformity; (2) if necessary, corrects for fluorescence cross-talk through a deconvolution matrix; (3) if necessary, aligns images using registration marks imprinted on the substrate; (4) performs algorithms to distinguish targets from other signals; (5) assigns an identity to each imaged target in the sample; (6) calculates the total number of targets in each category; (7) images and records the sample bar code for sample identification; and (8) automatically saves the output data (including internal standard and sample data), images, and bar code to a database that can be queried via a web browser interface. Commercially available image analysis packages can be used to provide these functions. Software packages for multicolor image analysis can be used (e.g., Image-Pro, Media Cybernetics; MetaMorph, Universal Imaging, MatLab, The Mathworks).

Although the invention can enumerate individual dispersed targets, the targets may be physically linked (as in the case of filamentous fungi, for example) or may be overlapping, or coincident (as will occur frequently at high densities). Therefore, software analysis packages used with the invention preferably include modules that quantify targets by integrating the signal intensities for the signal signatures over the imaged area. The number of targets in the sample can often be determined by comparing the integrated signal intensity of the sample image to the average signal intensity of individual targets in the internal standard control. The software quantification module is preferably "tuned" for each type of target by comparing the output for

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numerous samples to "gold standard" methods. For example, samples containing a filamentous fungus are preferably quantified using a software module that has been calibrated by comparison to microscopic quantification. Or, for certain bacteria, quantification by microbiological culture or microscopic examination of stained bacteria can be used to calibrate the software. The examples below provide specific examples of software analysis of images obtained using the invention.

It is useful to outline here the software packages and methods that were used to analyze the fluorescence data collected in many of the examples that follow. The detection surface is imaged to determine the number of fluorescence-labeled complexes and/or the total fluorescent signal. The fluorescence was captured from the bottom of the assay container by a CCD camera and stored as a TIFF (Tagged Image File Format) image file that contains records of pixel locations and intensities. Three approaches were used to quantify the assay results. The total integrated signal of the imaged detection zone was determined by summing the fluorescent signal from all of the pixels. The integrated signal from the sample was compared to that of negative controls. Measuring the total integrated signal is especially useful for samples containing numerous targets. A second approach was to count the objects in the detection area. A third approach was to integrate the intensity of all of the pixels contained within the fluorescent objects (as opposed to summing the intensity of all of the pixels in the image). All image analysis was performed using Image-Pro v 4.0 (Media Cybernetics, Silver Springs, MD).

Obtaining the total integrated signal was achieved by initially defining an area on the image (the area of interest) that represents the bottom of the container. Image-Pro allows the area of interest to be converted into a single object and other Image-Pro tools permit the total signal of the pixels represented in this object to be summed. A similar image from an assay container to which no target was added was then analyzed in the same way and used as a negative control. The negative control values were subtracted from the values of target containing samples. This removed both assay and electronic noise.

The second and third quantification methods used Image-Pro's object-finding utility. This utility joins contiguous pixels that have a value (signal) above an automatic or user-defined threshold. This establishes a contour line around the perimeter of the object. The perimeter pixels and those inside are defined as the object, and summing these pixel values results in the object integration value. The analysis software was then used to automatically count all the objects in an area of interest that represents the bottom of the sample container and, in addition, can calculate the integrated signal intensity of all objects found.

Using the IPP Image-Pro macro language, the above utilities can be automated to allow batch processing of several images at one time. In addition, the data can be manipulated with other user-defined IPP scripts. For example, objects below or above a certain size (area) or intensity can be included or excluded, which can be a useful tool for dust exclusion.

Examples. The examples below provide technical details for implementing various embodiments of the invention for use in conjunction with a range of applications and are not intended to be limiting.

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Example 1. Large area imaging of individual bacteria labeled with a fluorescent DNA-binding stain

Overview: This example demonstrates the use of the invention to detect individual bacterial cells on a porous membrane without magnification. *E. coli* bacterial cell targets were labeled with a fluorescent nucleic acid stain, filtered through a membrane, and the fluorescent image captured with a CCD camera using large area imaging

Experimental Methods:. A culture of *Escherichia Coli* ATCC 8739 was grown in Trypticase Soy Broth (TSB, BD cat. #211822) for 18 hours at 37 C. A 1ml aliquot of cells was spun down in a microcentrifuge and resuspended in an equal volume of water. The cells were killed by heating the 1 ml aliquot to 100°C for 5 minutes. The nucleic acid stain Sybr Green I (Molecular Probes; catalogue no. S-7563) was added to the killed cells to a final concentration of "10x". (The Sybr Green I stock is defined as 10,000 X). The stained *E. coli* cells were serially diluted in water and filtered through 0.22 μm black polycarbonate filtration membranes (Osmonics catalogue no. K02BP04700) using a vacuum filtration device and a plastic funnel cup (Millipore Microfil V User Guide, PF07114, Rev A 3/00). Fluorescence was detected using the large area non-magnifying CCD Imager (described in step 6 above; Figure 3) with an FITC optical filter set (Chroma/excitation 470/40 nm, emission 522/40 nm). Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager.

Results: The left panel of

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Figure 7 shows individual *E. coli* cells stained with Sybr Green I and imaged using large area non-magnified imaging. The number of fluorescent signals, visible as white spots, correlates well with the expected number of about 100 *E. coli* cells, based on the dilution used and the area of the field of view. The right panel shows a negative control where "no cells" were filtered through the membrane and imaged in an identical manner.

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Example 2. Large area imaging of single bacteria stained with a DNA-binding fluorescent dye

Overview. In this example, large area imaging on a glass coverslip was used to detect single *E. coli* bacterial cell targets that were labeled with a fluorescent nucleic acid stain. The stained cells were illuminated with white light and imaged with a CCD camera.

Experimental design. A culture of *Escherichia Coli* MG1655 was grown in LB broth (BD; Sparks, MD) for 18 hours at 37 C. The cells were fixed by heating a 1 ml aliquot of an 18-hour culture to 95 C for five minutes in a heat block. The nucleic acid stain, Syber Green I, (Molecular probes; cat. num.S-7563) was added to the fixed culture to achieve a 1:1000 dilution of the stain stock. (1 µl Syber Green I stock (10,000 X) to 999 µl of heat fixed culture). The *E. coli* cells were stained for 10 minutes. Then the cells were spun down in a microcentrifuge at 11700 g for 10 minutes. The supernatant was discarded and the pellet of cells was resuspended in an equal volume of H_2O (type 1 quality). Stained cells were serially diluted to achieve concentrations ranging from of 10^8 to 10^4 cells per ml. Corning No. 1 1/2 cover slips were coated with Poly-L-lysine by submerging slides in a 1:10 dilution (5ml Poly-L-lysine in 45 ml type 1 H_2O) of Poly-L-lysine solution (Sigma Diagnostics; cat. num. P-8920). Once coated the cover slips were washed in H_2O (type 1 quality) and were allowed to air-dry. A 5 µl aliquot of diluted stained cells was pipetted onto a Poly-L-lysine cover slip and dried at room temp (about 20 min). Fluorescence was detected by imaging using a CCD Imager (described in step 6 above; Figure 3) with an FITC optical filter set (Chroma/excitation 470/40 nm, emission 522/40 nm). Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager. Positive signals detected on the Imager were confirmed to be *E. coli* by using an Axioplan II fluorescent microscope (Carl, Zeiss Inc., Thornwood, NY) equipped with the same filter set.

Results: Figure 8 shows that cells stained with Syber Green I were detected using large area non-magnified imaging. The fluorescence signals, visible as white spots, were shown to correspond to either single *E. coli* cells or grouped cells by using high magnification fluorescence microscopy (1000X;Figure 8).

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Example 3. Large area imaging of individual bacteria labeled by hybridization to fluorescently labeled oligonucleotide category-binding molecules

Overview. In this example, large area imaging was used to detect single *E. coli* bacterial cell targets that were labeled by hybridization of cellular rRNA to a fluorescently labeled oligonucleotide category-binding molecule. The cells were imaged as in Example 2.

Experimental procedure. *E. coli* cells were grown as in Example 2. Cells were fixed in a 2.5% solution of Formaldehyde in PBS for 30 minutes at room temperature. The cells were "washed" by centrifuging in a microcentrifuge for 10 min at 11,700 g followed by resuspending the pellet of cells in an equal volume of PBS. The cells were spun down as before, resuspended in 50% ethanol, placed at 20 C for 14 hours, and were washed as above with PBS. The cells were then spun down as before and resuspended in hybridization buffer (1 M NaCl/50 mM EPPS/0.5% Tween 20/0.4 µg/µl yeast t-RNA/2% blocking agent, where the blocking agent was100 mM Maleic acid pH 7.65/150 mM NaCl/10% Blocking Agent (Boehringer Mannheim)). The cells were then incubated at 49 C for 30 minutes (pre-hybridization step). FITC conjugated DNA ribosomal probe (1 µl; Synthetic genetics 5' FITC-GCTGCCTCCCGTAGGAGT) was added and the cells were incubated at 49 C for 1 hour (hybridization step). After hybridization, the cells were washed with wash buffer (PBS-TB) and incubated at 49 C for 10 min. The wash step was repeated twice more for a total of three washes. Labeled cells (5 µl) were deposited on a Poly-L-lysine coated cover slip (described in Example 2) and imaged using a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3). Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager.

Examination of the sample using a fluorescent microscope (1000X; Figure 8) was used to confirm that the spots in the non-magnified digital image correspond to *E. coli* cells.

Results. After probing *E. coli* cells with FITC conjugated *E. coli*-specific rRNA oligonucleotide probes, individual cells were readily seen (Figure 8) using the CCD imager. The fluorescence signals, seen as white spots, were shown to correspond to either single *E. coli* cells or grouped cells by using high magnification fluorescence microscopy (1000X; Figure 8).

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Example 4. Large area imaging of single bacteria labeled by hybridization to fluorescently labeled PNA category-binding molecules

Overview. In this example, large area imaging was used to detect single *E. coli* bacterial cell targets that were labeled by hybridization of cellular rRNA to a fluorescently labeled PNA category-binding molecule. The cells were imaged as in Example 2.

Experimental procedure. Cells were grown as in Example 2. The cells were prepared for hybridization and hybridized with PNA probes specific for *E. coli* as specified by the manufacturer in the PNA micro Dx Fish Reagent kit (Boston Probes; cat. num. KT11000). The labeled cells (5 µl) were spotted on a poly-L-lysine coated cover slip and imaged as in Example 2. Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager.

Results. Figure 8 shows that single bacterial cells tagged with fluorophore-labeled species-specific PNA probes were detected by large area CCD imaging without magnification. The white spots in the image obtained with the CCD were shown to correspond to either single *E. coli* cells or groups of several cells by analyzing the same sample using high magnification fluorescence microscopy (1000X; Figure 8).

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Example 5. Large area imaging of single bacteria labeled by binding to a fluorescently labeled antibody

Experimental procedure. Cells were grown as in Example 2. FITC labeled rabbit anti-*E. coli* polyclonal antibody (1:200 dilution; Biodesign; cat. num. C65110M) was added to dilutions of cells ranging from 10^5 to10^2 cells per ml and allowed to incubate for 1 hr at room temperature. The cells were then "washed" twice by spinning down the cells by centrifugation (11,700 g for 10 min) and resuspended in PBT. The washed cells were then spotted on Poly-L-lysine coated cover slips as in Example 2. CCD imaging and microscopic confirmation were performed as described in Example 2. Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager.

Results. Figure 8 shows that single bacterial cells tagged with fluorophore-labeled category-specific antibody probes were detected by large area CCD imaging without magnification. The white spots in the image obtained with the CCD were shown to correspond to either single *E. coli* cells or groups of several cells by analyzing the same sample using high magnification fluorescence microscopy (1000X; Figure 8).

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Example 6. Large area imaging of individual live bacteria stained with a fluorogenic esterase substrate

Overview and objectives: In this example, large area imaging was used to detect individual live *E. coli* bacterial cell targets that were stained with a combination of fluorogenic esterase substrates (signaling moieties). The substrates can diffuse through the cell membrane of intact living cells where they become both fluorescent and charged when acted upon by esterase enzymes found in metabolically active cells. These charged fluorescent products can no longer passively diffuse through cell membranes and become trapped in intact cells. This technique can be useful when it is important to distinguish live cells from dead cells, as only cells with active esterases and intact cell membranes will stain properly. In this example, either live or dead *E. coli* cells were filtered through a black polyester membrane, incubated with the fluorogenic substrates fluorescein diacetate (FDA) and carboxyfluorescein diacetate (CFDA) and imaged using non-magnified large area CCD imaging.

Experimental Methods: *E. coli* ATCC 8739 was grown overnight in Tryptic Soy Broth (TSB, BD cat. #211822), and washed once by centrifuging a volume of cells, removing the supernatant, and resuspending the pellet in an equal volume of PBS. Dead cells were prepared by heating a 1 ml aliquot of washed cells to 100°C for 20 minutes. Appropriate dilutions of both live and dead cells were made in PBS. Using a Millipore 1225 manifold, cells were filtered through black polyester membranes (Chemunex cat. # 200-C2010-01) mounted on absorbent pads (Chemunex cat. # 200-C3012-02). While still in the filtration manifold, the cells were overlayed with 500 µl filtered TSB and incubated for 60 min. at 30°C. The media was suctioned through the membrane, and replaced with 500 µl of a solution of 20 µM CFDA (Molecular Probes cat. # C-195), 10 µM FDA (Polysciences cat. no. 00615) in ChemSolB16 (Chemunex cat. # 200-R2023-02) and incubated at 37°C for 60 minutes. The reagents were suctioned through the membranes. The manifold was disassembled, polyester membranes were mounted on glass slides and allowed to dry. Fluorescent signal on the polyester membranes was captured using a CCD Imager (described in step 6 above; Figure 3) with an FITC optical filter set (Chroma/excitation 470/40 nm, emission 522/40 nm). Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager.

Results: Figure 9 shows that live *E. coli* cells (left panel) are detected as bright fluorescent spots, while dead *E. coli* cells (right panel) do not have a detectable fluorescent signal. The number of spots in the field of live cells is about 400 and correlates well with the expected number of cells based on the dilution of cells added to the filter and the area of the field of view.

Variations. Other dyes that distinguish live or dead cells can be used. For example, other fluorogenic substrates, or DNA stains that can or cannot cross intact cell membranes can be used instead of or in conjunction with FDA and CFDA. Multiple stains and dyes can be distinguished by using multiple excitation and emission wavelengths for fluorescence detection. The spectrum of fluorescence associated with an object can be use to determine whether a cell is counted as live or dead. In addition, fluorogenic substrates that are specific for the biochemical activity of a particular type of bacteria can be used to determine its presence. For example a fluorogenic β-galactosidase substrate can be cleaved to its fluorescent product by β-galactosidase, which is specific to coliforms.

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Example 7. Large area non-magnified imaging of individual bacteria labeled with highly fluorescent particles

Overview. In this example, large area imaging was used to detect individual *E. coli* bacterial cell targets that were labeled with highly fluorescent particles. The individual bacteria:particle complexes were illuminated with light of 3 distinct wavelengths and imaged with a CCD camera as in Example 2.

Experimental procedure. A culture of *Escherichia Coli* MG1655 was grown in LB broth (BD, Sparks, MD) for 18 hours at 37 C. *E. coli* cells were added to a microtiter plate with an optically clear bottom (Greiner America, Inc.; cat. num. 655896) to a final concentration of 10^5 cells per well (10^5 cells per 50 µl). The microtiter plate was then heated to 95 C on a heat block until the solution containing the cells had completely dried. The nucleic acid stain, Syber Green I, (Molecular probes; cat. num. S-7563) was added to the fixed culture to achieve a 1:1000 dilution of the stain stock. (1 µl Syber Green I stock (10,000 X) to 999 µl of heat fixed culture). After ten minutes of exposure to the stain, the cells were "washed" twice with water (200 µl for each wash, followed by aspiration). Undiluted sheep serum (Fitzgerald; cat. num. 88-NS55) was added to each well and incubated at room temperature for 30 minutes. The wells were aspirated and Rabbit anti-*E. coli* antibodies (Biodesign; cat. num. C65110M), diluted 1:500 in sheep serum, were then added to the appropriate wells and incubated for two hours at 37 C. The wells were then washed three times, twice with wash solution 1 (PBT) and once with wash solution 2 (PBS-B). Next, a biotin labeled Goat anti-rabbit IgG antibody (Jackson; cat. num. 111-005-003) diluted 1:500 in sheep serum was added to appropriate wells. The antibody was allowed to incubate for 2 hours at 37 C. The wells were washed twice with wash solution 1 and once with wash solution 2. AvidIn coated Texas Red Fluorescent particles (0.45µm; Spherotech; cat. num. VFP-0562-5) were added to all wells to a final concentration of 10^5 particles per well. The plate was incubated at room temperature overnight. The wells were then washed with solution 1 five times followed by two washes with water. Fluorescence was detected by imaging on a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) with FITC optical filter set (Chroma/excitation 470/40nm, emission 522/40 nm) for Syber Green I, Texas Red optical filter (Chroma excitation 560/55, emission 645/75) for the avidin coated Texas red particles and GFP Long Pass filter set (Chroma/ excitation 470/40, emission 500LP) for viewing both Syber Green stained cells and Texas Red Particles. Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager. Positive signals detected on the Imager were confirmed to be individual *E. coli* cells by using an Axioplan II fluorescent microscope (Carl, Zeiss Inc., Thornwood, NY) equipped with the same filter sets.

Results. Figure 10 shows detection of individual *E. coli* cells tagged with category-specific antibodies and fluorescent particle signaling moieties using large area, non-magnified, CCD-based imaging. The fluorescence signals, visible as white spots, were shown to correspond to either individual *E. coli* cells with multiple particles or groups of cells surrounded with particles by using high magnification fluorescence microscopy (1000X; Figure 10). These were avidin-coated particles. Cells were labeled with group-specific antibodies, then with anti-antibody-biotin, then avidin particles.

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Example 8. An homogenous immunoassay that detects individual bacteria using non-magnified large area imaging

Objective: This example demonstrates immunoassay methods for detecting individual bacterial cells, including a user-friendly homogenous immunoassay for detecting small numbers of pathogenic *E. coli* O157:H7 cells.

Figure 11 diagrams the homogenous assay approach used here and in subsequent examples. Homogenous immunoassays are favored in diagnostics because they are simple, fast, inexpensive, and easily automatable. (Homogenous, in the sense used here, means that binding of the antibody and analyte is detected after mixing the reagents and sample, but without physically removing – *e.g.*, by aspiration – the unreacted category binding molecules from the category binding molecule:target complexes). However, most homogenous immunoassays are insensitive (compared to methods like nucleic acid amplification), non-quantitative, and non-multiplexed (or very shallowly multiplexed). Here, in contrast, the invention is used to make a homogenous immunoassay that is sensitive, quantitative, and multiplexed.

In the example, a sample containing *E. coli* O157:H7 was mixed with magnetic and fluorescent particles, magnetically selected, and imaged using non-magnified large area CCD imaging. No washing steps were required.

Experimental Methods: Anti-*E. coli* magnetic particles were made by coupling magnetic particles with active tosyl- groups (Dynal, Oslo, Norway, Cat. No. 140.03) to polyclonal antibodies raised against *E. coli O157:H7* (BioTrace affinity purified; Kirkegaard & Perry Laboratories, Gaithersburg, MD, Cat. No. 01-95-90) *M*agnetic particles (30 mg/ml; 100 µl; Dynal, Oslo, Norway, Dynaparticles M-280 Tosylactivated Cat. No. 140.03) were washed in PB (three wash repetitions, 1 ml each) in a microcentrifuge tube (1.5 ml) using a magnetic separation of the particles followed by removal of the supernatant (all magnetic separations in this example, except where noted, were carried out using a device from Polysciences Inc.; cat. no. 8MB4111S). Particles were resuspended in PB (70 µl). Affinity purified polyclonal antibody raised against *Escherichia coli* 0157:H7 (60 µg; Kirkegaard & Perry Laboratories, Gaithersburg, MD, Cat. No. 01-95-90) was purified by passing the solution obtained from the manufacturer through a centrifugal filtration device (Millipore Microcon Model No. YM-30; nominal molecular weight limit 30,000 Daltons) according to the manufacturer's instructions. The antibody, which was collected on the filter, was resuspended in PB (30 µl;). Antibody (30 µl) was combined with magnetic particles (70 µl) in a microcentrifuge tube (1.5 ml) and vortexed briefly. The reaction was incubated at 37°C for 20 minutes using rotation (about 30 rpm unless otherwise noted). After 20 minutes BSA (IgG Free) was added to a final concentration of 0.1% and incubated overnight at 37°C with rotation. The magnetic particles were washed twice (1 ml each; using magnetic separation) with PBS-B. The magnetic particles were resuspended in buffer (0.2M Tris pH 8.5 supplemented with 0.1% (w/v) BSA (IgG free)) and incubated for 4 hours at 37°C with rotation. Finally, the magnetic particles were washed twice (in PBS-B using magnetic separation) and resuspended (the final concentration was 1% solids in PBS-B).

Anti-*E. coli* fluorescent particles were made by coating fluorescent particles (Fluospheres; Molecular Probes, Sulfate Microspheres, 1 µm, Red fluorescent (580/605) Cat No. F-8851) by passive adsorption of the same antibody used to coat the magnetic particles. Rabbit anti-*Candida albicans* antibody (1.25 nmol type A polyclonal purified IgG; Biodesign Cat. No. B65411R, Lot No. 3B03200) antibody was purified as described above. To passively adsorb antibodies to surface sulfate groups, particles (62.5 µl; 2% solids; Molecular Probes Cat. No. F8851, 1um, red fluorescent (580/605),) were washed by repeatedly (3 repetitions) by centrifugation (5 min; 10,200 x g; Eppendorf

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Centrifuge Model 5417C, Eppendorf Swinging Bucket Rotor Model A-12-11) and resuspension of the particle pellet (1ml PBS/.15 M NaCl). The particle pellet was resuspended in PBS (125 µl, for a concentration of 1% solids) followed by dropwise addition of purified antibody (1.25 nmol for a ratio of 1 nmol antibody /mg particles) with vortexing. The suspension was incubated with rotation, for 2 hours at 25°C followed by overnight incubation at 4°C. Particles were washed (3 repetitions as above, but with resuspension after the centrifugations in PBS-TB), resuspended in PBS-TB (200 µl), and incubated (30 minutes, 25°C) with rotation. Particles were washed twice as above and resuspended in PBS-TB (125 µl for a concentration of 1% solids).

Following particle preparation, an inoculum (200 µl of an overnight culture) of *E. coli* O157:H7 (Strain DEC 3B, Dr. Tom Whittam, Pennsylvania State University) was cultured (200 ml LB; 4 hr; 250 rpm, 37°C) and titrated by plating serial dilutions as follows. The culture was immediately placed on ice to prevent further growth. A 10 ml aliquot was fixed by addition of formalin (final concentration = 2.5%) directly into the media containing the bacteria. Serial dilutions of aliquots (100 µl) of the culture were made (1:10 in PBS) and an aliquot of each dilution was plated to determine the concentration of bacteria in the stock solution of fixed bacteria. This stock solution was diluted to achieve a concentration of $1x10^6$ bacteria/ml. The stock solution (1 ml) was washed by centrifugation (950 X *g*) and resuspension (1ml; PBS) followed by resuspension of the bacterial pellet in PBS (1 ml).

A mixture of the anti-*E. coli* magnetic and fluorescent particles ($1x10^6$ of each type per 10 µl) was sonicated (1 min; setting 8; Fisher Scientific 550 Sonic Dismembrator). Wells of a 96-well glass bottom plate (Whatman Inc., Clifton, NJ, Cat. No. 7706-2370). were treated with Gel Slick (Biowhittaker Molecular Applications, Cat. No. 50640) for 1 minute. The gel slick was removed by aspiration and washed three times by the addition of sterile water to each well followed by aspiration. The wells were allowed to dry for 5 minutes before the sample was added. To the wells were added buffer (PBS-TB; 115 µl in each of 6 wells) or blood (115 µl in each of 6 wells), followed by the addition of the sonicated particle slurry (10 µl in each well). Fixed *E. coli* O157:H7 cells ($1x10^3$ in 10 µl) were added to three of the wells that contained buffer and three of the wells that contained blood. The wells were sealed using a storage mat (Corning COSTAR, Cat. No. 3080). The samples were incubated with rotation (30 rpm; Dynal Sample Mixer, Oslo, Norway, Cat. No. 947.01) for 1 hour at room temperature. The magnetic particles were captured on the detection surface at the bottom of the wells by using a magnetic separation device (Dexter Magnetics, Silver Spring, MD, LifeSep, Cat. No. 2501008).

To test the efficacy of a homogenous immunoassay to detect individual bacterial cells in blood, the immunoassays performed in mouse blood were imaged using the CCD imager shown in Figure 3A. Images of the detection area, *i.e.*, the optically clear bottom of the microtiter wells, were captured (20 msec exposures) using a Texas Red filter set (excitation 560/55, emission 645/75) and Image-Pro Plus digital imaging software (Media Cybernetics, Silver Spring, MD). Next, to test the efficacy of immunoassays incorporating wash steps, all of the samples (*i.e.*, those containing buffer and those containing blood that were previously imaged) were washed with buffer (PBS-TB; 200 µl; 3 washes achieved by pipetting up and down 10 times). During the washing steps, a magnetic separation device (Polysciences, Inc., Warrington, PA, Cat. No. 8MB4111S) was used to capture and secure the magnetic particles, and complexes containing magnetic particles, to the surface of the wells. Each wash was followed by magnetic selection (5 min) and aspiration of the supernatant. The samples were resuspended in PBS-TB (200 µl) containing the fluorescent DNA intercalating dye YOYO-1 (10 nm; Molecular Probes, Cat. No. Y-3601) and magnetically selected (using the device by Dexter Magnetics). The 96-well plate was imaged using the CCD imager as described above. Following CCD imaging the plate was inverted and examined using fluorescence microscopy (Ax-

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ioplan II fluorescent microscope, Carl, Zeiss Inc., Thornwood, NY). The images (in triplicate; 20 msec exposures) obtained in the homogenous assay in blood (*i.e.*, the images obtained before the washing steps) were quantified using software analysis. A macro (Image-Pro Plus) was used to circumscribe a region of interest containing the detection area contained within the perimeter of each well. Object counting and object integrated intensity within the circumscribed area was performed using a series of Visual Basic scripts to analyze the Image-Pro Plus analysis data (as described in Step 6 of the Detailed Description section). Two bar-graphs representing the analysis for the homogenous assay in blood were plotted (Figure 12; Microsoft Excel; Microsoft Corp.). Identical threshold value settings were used for the samples that were compared in each experiment.

Results: Figure 12 shows the results of immunoassays that sensitively detect *E. coli* by non-magnified large area imaging. Magnetic and fluorescent particles coated with category-specific antibodies were first bound to the bacterial cells, the complexes were then drawn to the bottom surface of the optically clear well (the detection area) using magnetic force, and the complexes were detected using non-magnified large area CCD-based imaging. Figure 12 compares the results of the homogenous immunoassay in blood (bottom panel) and the non-homogenous (washed) immunoassay in both blood (top panel) and in buffer (middle panel). The simplest format, the homogenous immunoassay, effectively detected cells with excellent signal to background ratios (see quantification of the homogenous assay in the figure (bar graphs in bottom right panels).

The two leftmost panels show the images obtained using non-magnified, large area CCD-based imaging. All three assays gave strong signals when *E. coli* were present (left panel) but low background signals when *E. coli* were not added. Further confirmation that the signals obtained using the CCD camera corresponded to *E. coli* cells coated with particles were obtained using high power fluorescence microscopic analysis (1000X; top two rows, rightmost two columns). Images are shown of typical complexes containing *E. coli* cells, magnetic particles, and fluorescent particles. Two images of each complex were made using two filter sets: one for visualizing DNA fluorescence (Syber Green I; second panel from right) and one for visualizing the fluorescent particles (rightlmost panel). The figure shows that the complexes consist of an *E. coli* cell surrounded by magnetic and fluorescent particles.

The percentage of *E. coli* cells selected by the immunoassay is consistently greater than 95%, as shown by experiments measuring the unselected material and microscopic comparisons of the sample before and after the immunoassay (not shown here). Microscopic surveys showed that the selected complexes containing *E. coli* cells were associated with an average of 3.33 fluorescent particles per cell. (n=10; minimum = 2; maximum =10).

Quantitative analysis of the images was carried out by software that counts the number of fluorescent objects (Figure 12, bottom panel, left bar graph) and that integrates the intensity of all of the objects (Figure 12, bottom panel, right bar graph). In both measurements, the sample containing *E. coli* (1000 cells) scores significantly higher than the sample containing no *E. coli*. Note, that the total objects found in the software analysis (1.5×10^4 objects) is about 15 times greater than the number of cells in the sample (1000). This observation is consistent with the microscopic analysis of the complexes described in the following paragraph. By software analysis, the sample containing *E. coli* had 75 times more objects and about 400 times the integrated intensity compared to the sample without *E. coli*. Both analyses show that the signal obtained with 1×10^3 *E. coli* cells in a sample more than three standard deviations above the signal generated from the no cell control.

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Although complex formation is strongly dependent on the presence of *E. coli* cells in the immunoassay (as can be seen by comparing the two leftmost columns of the figure), microscopic examination reveals that many of the complexes lack *E. coli* cells. This is a consistent feature of the *E. coli* immunoassay. It is possible that the formation of cell-less complexes is due to the presence of non-cellular structures, such as fragments of *E. coli* flagella that are not visible in the microscope. Alternatively, it is possible that large particle:cell complexes break apart during the assay yielding some fragments with cells and some without cells. Besides complexes containing a single cell and complexes lacking cells, microscopic analysis revealed that some of the complexes comprised more than one cell.

Based on these results, the simple, rapid, and user-friendly homogenous immunoassay, combined with non-magnified large area imaging detects and identifies low numbers of bacteria in blood.

Variations. As shown in subsequent examples, the methods of this example can be applied to detect other targets in numerous sample types (biological and non-biological). For example, by using the appropriate antibodies to coat particles, the methods described in the example can be applied to detecting specific viruses (*e.g.*, HIV), bacteria, fungi, parasites, proteins, small molecules, or human cells (such as cancer cells or infected cells).

Alternative formats can also be used in conjunction with the methods of the example. By appropriate re-configuration of the magnet and optics modules described for the instrument described in this example, a variety of containers can be accommodated. For example, samples of larger or smaller volume can be used in other types of vessels (*e.g.*, test tubes with optically clear bottoms and/or sides).

Various methods of labeling category-binding molecules are also possible. Particles of various sizes and with various signaling properties can be used. Fluorescent particles can be used that contain one or more fluorescent dyes with various spectral properties or that contain fluorescent cascades (*e.g.*, Transfluorospheres from Molecular Probes). Other types of fluorescent particles, such as quantum dots can also be used. Particles that scatter light (*e.g.*, RLS, PRP, or nanogold particles) can be substituted for the fluorescent particles in this example. Particles labeled with visible dyes (*e.g.*, dyed polystyrene particles or dye-containing liposomes) can be used. Alternatively, antibodies, or other types of category-binding molecules, can be directly labeled with a fluorophore (or other signaling moiety). Such directly labeled antibodies can coat the targets making them detectable. Similarly, category-binding molecules can be directly labeled with selection moieties. For example, antibodies can be coupled to ferritin, a magnetic selection moiety.

Staining of the target can also be used for detection instead of fluorescent particles. In this case, the specificity is conferred by the selection moiety, the category-specific magnetic particles. For example, nucleic acid stain (*e.g.*, propidium iodide, STYO 17, or DAPI) can be used to non-specifically label targets. Targets that bind to pathogen-specific magnetic particles are selectively brought to the bottom of the sample well and visualized by virtue of the non-specific stain. Category-specific staining procedures can also be used (*e.g.*, see Example 9).

Alternative methods for coupling antibodies to particles are also possible. Such methods are known to those familiar with the art and are detailed in numerous references (*e.g.*, Hermanson, *Bioconjugate Techniques* (Academic Press, San Diego, CA, 1996); and Edwards, ed. (1999). *Immunodiagnostics: A Practical Approach.* Oxford: Oxford University Press).

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Other forms of antibodies can also be used (*e.g.*, Fab, Fab', Fv). Different category-specific antibodies can be used in combination. For example, in the application above, antibodies for different *M. tuberculosis* antigens can be bound to separate yellow-green fluorescent particles and then combined. Alternatively, different antibodies can be bound to the same particle. Other types of category-binding molecules (*e.g.*, lectins, polypeptides, or ligands) can be substituted for antibodies.

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Example 9. Large Area Imaging of Acid Fast Stained *Mycobacteria* in "simulated sputum"

Background: Tuberculosis caused more than 3 million deaths in 1995, about 3 times the number of AIDS/HIV deaths in that year. About 25% of avoidable deaths in the developing world are caused by *Mycobacterium tuberculosis* infections. Unfortunately, diagnosing *Mycobacterium tuberculosis* infections is problematic. Consequently, there is a great worldwide unmet need for a diagnostic test that is inexpensive, fast, simple, and rapid. The acid-fast bacillus (AFB) testing method that predominates in the developing world, where the majority of tuberculosis cases occur, is insensitive and thus misses a large fraction of infected patients. As a result, untreated tuberculosis patients with false negative test results continue to transmit this very contagious disease in the community. The AFB test is also time consuming and laborious, as many microscopic fields must be examined carefully for the presence of stained cells. Culture, the testing gold standard, is extremely slow – it generally takes *weeks* to grow colonies of *Mycobacterium tuberculosis* in the laboratory. New amplification-based molecular tests are rapid and sensitive, but high expense inhibits adoption of the tests even in the wealthiest nations.

Objective: In this example, the invention was used to construct a rapid and sensitive fluorescent acid-fast bacillus test. The test achieves its sensitivity and labor savings by imaging (in less than 1 second) a much larger area than would be examined in the microscopy-based version of the test.

Experimental Methods: Prepared slides (Remel; Cat. No. 40-146) containing a smear of "simulated sputum" and mycobacteria (*Mycobacteria scrofulaceum*) that are very similar to the agent that causes tuberculosis were stained with auramine O and auramine-rhodamine. Each slide contained a positive (*M. scrofulaceum*) and a negative control (*E. coli*) in a simulated sputum smear. Slides were prepared using either the TB Auramine-Rhodamine Stain (Remel Cat. No. 40090) or the TB Auramine O Staining Kit (Remel Cat. No. 40086). Slides were passed briefly four times through a flame. The slides were flooded with either Auramine O Stain or Auramine-Rhodamine Stain and incubated at room temperature for 15 minutes followed by 3 rinses in deionized water. Slides were decolorized with TB decolorizer (Remel Cat. No. 40-107) for 2 minutes followed by 3 rinses in deionized water. Slides were flooded with potassium permanganate counterstain (Cat. No. 40-092) for 3 minutes followed by 3 rinses with deionized water. The slides were then air dried. Auramine-Rhodamine stained slides were imaged using fluorescence microscopy (Axioplan II fluorescent microscope; Carl, Zeiss Inc., Thornwood, NY; Cy3 channel: excitation 546/11, emission 567/15, 400X magnification; 500msec exposure) and by non-magnified large area imaging using a CCD imager (as described in Step 6 of Detailed description section and shown in Figure 3A; using a filter set optimized for TRITC: (excitation 545/30, emission 610/75), 1 sec exposure).

Results: Figure 13 shows that fluorescence-based large-area imaging detects individual *Mycobacteria* cells stained with the Auramine-Rhodamine reagent. The fluorescent signals in the large area image were shown to correspond to individual *Mycobacterium* cells or small clusters of cells by using high magnification fluorescence microscopy (1000X; Figure 13). *E. coli* subject to the same procedure did not show significant fluorescence.

Variations. Differentiation of *M. tuberculosis* infection from other mycobacterial species (*e.g., M. avium*) is frequently important. Example 27 presents a variation of this example that identifies *M. tuberculosis* specifically using category-specific anti-*M. tuberculosis* antibodies.

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Example 10. Rapid antimicrobial susceptibility testing using large area imaging.

Overview. The goal of antimicrobial susceptibility testing is to determine which of various antimicrobial therapies is most effective at neutralizing a pathogen isolated from a patient. Rapid antimicrobial susceptibility testing is a critical – sometimes life-saving – application in infectious disease diagnostics. Timely and efficacious choice of antimicrobial therapy depends on the results of antimicrobial susceptibility testing.

After identification, infectious agents are generally tested for their ability to withstand various concentrations of several antimicrobial compounds. Drawbacks of entrenched methods are long turnaround time for results (most often 2 to 4 days from sample acquisition time). This is especially problematic for time-critical medical emergencies such as central nervous system and bloodstream infections.

In this example, large area imaging was used to rapidly detect the susceptibility of model *E. coli* cells to the antibiotic tetracycline. Cells of two strains, one resistant and one sensitive, were tested for their ability to grow in a period of several hours in the antibiotic. Growth was detected by large area imaging of individual cells stained with a fluorescent nucleic acid stain.

Experimental procedure. Tetracycline susceptibility testing was performed on two *E. coli* strains: MG1655 (sensitive strain) and MG1655/pLAFRI (resistant strain) following the broth dilution method outlined in the NCCLS guidelines for anti-microbial susceptibility (Methods for Dilution Antimicrobial Susceptibility Tests for bacteria That Grow Aerobically, Fifth edition, NCCLS, M7-A5 Vol. 20 No. 2, January 2000). At six time points following inoculation of the test media (0,1,2,4,6, and 18 hour), 1 ml aliquots were taken of both the resistant and sensitive strain cultures from each tetracycline dilution (0 μg/ml to 320 μg/m l and 0 μg/ml to 4 μg/ml for the resistant and sensitive respectfully). Also at these times points both a visible turbidity growth check and a plate count were performed. The turbidity check was performed according to NCCLS broth dilution guidelines. A "+" was recorded for a turbid culture and a "-" for non-turbid culture. The 1 ml aliquots were then stained with Syber Green I as in Example 2. Aliquots (10 μl) of the stained cells were placed in separate wells (with optically clear bottoms) of a 96 well plate (Greiner America, Inc.; cat. num. 655896), which contained 90 μl of water (Type I quality). The plates were spun in a centrifuge (Beckman Allegra) for 10 min at 600 g and then imaged. Fluorescence was detected by imaging using a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) with an FITC optical filter set (Chroma/excitation 470/40 nm, emission 522/40 nm). Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager. Positive signals detected by large area imaging were confirmed to be single *E. coli* cells by using an Axioplan II fluorescent microscope (Carl, Zeiss Inc., Thornwood, NY) equipped with the same filter set.

Results. Figure 14 shows the results of the antimicrobial susceptibility test using individual cell detection and quantification by non-magnified large area imaging. Comparing the bottom two panels of the figure shows that for determining cell growth, non-magnified large area imaging is comparable to culture. (Quantification by counting colonies on agar plates also gave comparable results). The minimum inhibitory concentration of antibiotic (MIC) as determined by CCD imaging and by culture were comparable, but somewhat lower than the MIC determined after overnight growth (second panel from the top of Figure 14).

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The figure demonstrates that this rapid imaging method easily discriminates an antibiotic resistant strain (right panel of Figure 14) from an antibiotic sensitive strain (left panel of Figure 14). After 4-hours of growth, dramatic growth of the resistant strain was evident in an antibiotic concentration (64 μg/ml) that was several hundred times than that that inhibited growth of the antibiotic sensitive strain (0.25 μg/ml). The results were comparable when assayed by CCD imaging (bottom panel), liquid culture (second panel from bottom), or colony counting (not shown).

Thus, after a 4-hour incubation, large area non-magnified imaging provides antimicrobial susceptibility testing results comparable to both the culture assays (scored as turbidity) and plate counts.

Advantages and applications. There are several clinically important advantages to the method described here over traditional antimicrobial susceptibility testing techniques. By labeling with category-specific binding molecules (*e.g.*, see Example 2 - Example 6), identification of the bacteria can be achieved at the same time as quantifying bacterial growth in the presence of an antibiotic. The ability to detect and quantify small numbers of identified cells would meet an important unmet diagnostic need: antimicrobial susceptibility testing in clinical samples without bacterial subculture. Achieving this goal would provide great patient benefit by significantly decreasing the time needed to determine the appropriate antimicrobial therapy.

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Example 11. Large area detection of individual fluorescently labeled *Candida albicans* cells that have been magnetically selected

Objective. This example demonstrates the utility of non-magnified large area imaging to detect individual cells that have been magnetically selected and deposited in a detection zone at the bottom of a microtiter plate. *Candida albicans* is a common human pathogen that causes diseases including bloodborne infections and vaginal yeast infections. In this example, *C. albicans* cells were stained with a fluorescent nucleic acid stain and labeled with a rabbit IgG anti-*Candida albicans* antibody, then mixed in the well of a microtiter plate with magnetic particles coated with anti-rabbit IgG antibodies. Application of a magnetic field causes the cell:particle complexes to become arranged on the bottom of the well. Cells were then detected using CCD-based non-magnified large area imaging.

Experimental procedure. *Candida albicans* strain 10453 (American type Culture Collection, Manassas VA) was grown in YM medium (BD, Sparks, MD) for 18 hours at 22°C. Cells were fixed in 2.5% solution of formaldehyde in PBS for five minutes, washed twice in PBS-B, and then stained and labeled with YOYO-1 (1 μM) nucleic acid stain (Y-3601 Molecular Probes, Eugene, OR) and a rabbit anti-*Candida albicans* polyclonal antibody (B65411R, Biodesign International, Saco, ME) for 30 minutes. Cells were then washed three times in PBS-TB to remove unbound stain and antibody. An aliquot of cells was mixed with a suspension of magnetic particles with surface-bound goat anti-rabbit IgG antibodies (8430050, Polysciences Inc., Warrington, PA) in PBS-TB buffer in the wells of an optically clear, flat, glass-bottomed microtiter tray (Uniview; Cat. #7706-2370, Whatman, Inc. Ann Arbor, MI). After 15 minutes, a magnetic field was applied using a magnetic ferrous block (Cortex Biochem, Inc., San Leandro, CA) such that the magnetic particles were caused to align substantially evenly over the surface of the bottom of the well. An image of the bottom of the well was captured using the CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) using the FITC filter set (480/40 nm excitation, 535/50 nm emission; Chroma Technology Corp., Brattleboro, VT) and MetaMorph image capture software (Universal Imaging Corp., West Chester, PA). Confirmation that discrete signals seen in large area imaging corresponded to single cells was achieved using high magnification microscopy at 1200 times magnification using a Zeiss Axioplan II fluorescent microscope, Carl Zeiss Inc., Thornwood, NY) using the same FITC filter set.

Results. Figure 15 shows that non-magnified large area imaging of microtiter tray reaction wells containing cells gave punctate fluorescencent signals. These fluorescent signals were shown to correspond to individual *Candida albicans* cells or small groups of cells by using high magnification (1200X) fluorescence microscopy of the same reaction wells. In contrast, wells containing no cells or wells containing unstained cells (negative control wells) presented no (or very few) positive signals. The small number of fluorescent objects in the negative control images was likely to be due to dust particles or other non-biological material.

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Example 12. Large area detection of individual *Candida albicans* cells specifically bound to fluorescent and paramagnetic polystyrene particles.

Objective. In this example, individual *C. albicans* cells were detected using non-magnified large area imaging following separation with cell-specific paramagnetic particles and labeling with *C. albicans*-specific fluorescent particles.

Experimental procedure. The following mixture was made in wells of an optically clear, flat, glass-bottomed microtiter tray (Uniview 7706-2370 , Whatman, Inc., Ann Arbor, MI): PBS-TB buffer containing YOYO-1 (50 nM), $5x10^7$ magnetic particles (MEO3N 1 μm diameter; Bangs Labs Inc., Fishers, IN) with surface-adsorbed rabbit anti-*Candida albicans* polyclonal antibody (B65411R, Biodesign International, Saco, ME), as previously described in example 6, and $5x10^7$ fluorescent particles (Fluospheres F-8821, 1 μm diameter, red fluorescent, Molecular Probes, Eugene, OR) with the same antibody covalently coupled to the surface using standard EDAC coupling chemistry. To covalently couple antibodies to particles, fluorescent carboxylated particles (1 μm,.25 ml, 2% solids, Red (580/605), polystyrene, Molecular Probes Cat No. F-8821) were washed in MESB (2 ml) by centrifugation (5 min; 11,000 x *g*; Eppendorf Centrifuge Model 5417C, Swinging Bucket Rotor A-12-11) at room temperature and resuspended by vortexing and pipetting. In coupling experiments using magnetic particles, washing was performed using SPHERO™ FlexiMag Separator Jr. device (Spherotech, Cat No. FMJ-1000) which enabled separation of magnetic particles from a suspension in 5 minutes. Particle washing was repeated twice followed by resuspension of the particles in MESB (500 μl; for a concentration of 10 mg/ml). Freshly prepared EDAC (Sigma Cat No. E-6383) was added to a final concentration of 0.2 mg/ml and mixed gently at room temperature for 5 minutes. To this suspension, rabbit anti-*C. albicans* antibody (5 nmol; type A polyclonal; Biodesign Cat No. B65411R, Lot No. 3B03200; purified as described in Example 8) was added dropwise with vortexing and incubated for 2 hours at room temperature with rotation (about 30 rpm). Particles were washed four times in MESB by centrifugation as described above. Particles were resuspended in MESB containing ethanolamine (0.03%; Sigma Cat No. E9508) and incubated for 30 minutes at room temperature with rotation. Particles were washed four times in MESB by centrifugation as described above, followed by three washes in 0.1 M sodium acetate pH 4.0. Lastly particles were washed two times in PBT and resuspended to 1% solids in PBT.

Candida albicans cells suspended in PBS-TB buffer were added such that the final reaction volume was 200 μl. The mixture was incubated on a shaker for 60 minutes. Any unbound fluorescent particles were separated from the magnetic particle complexes by three 200 μl PBS-TB buffer washes following application of a magnetic field using an array of neodinium-iron-boron magnets arranged to interface with 96-well microtiter trays (8MB4109S Polysciences Inc., Warrington, PA). Fluorescence was detected by non-magnified large area imaging using the CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) with optical filter sets appropriate for the red fluorescent particles (Texas Red filter set, 560/55 nm excitation, 645/75 nm emission; Chroma Technology Corp., Brattleboro, VT) and the nucleic acid-binding dye YOYO-1 (FITC filter set, 480/40 nm excitation, 535/50 nm emission). The presence of fluorescent particles co-purified with the magnetic particles indicates the presence of *Candida albicans* cells. Positive signals detected on the CCD imager were confirmed to be complexes of cells, fluorescent particles, and magnetic particles by using an Axioplan II fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with the same Texas Red filter set to view the fluorescent particles and a FITC filter set to view the nucleic acids of YOYO-1-stained cells. Images were captured using Image-Pro Plus

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image capture software (Media Cybernetics, Silver Spring, MD) using a 100 ms exposure time.

Results. Figure 16 shows that non-magnified large-area imaging of reactions containing cells gave punctate fluorescencent signals while reactions without cells gave no such signals. These fluorescent signals were shown to correspond to red fluorescent particles surrounding single YOYO-1 (green)-stained *Candida albicans* cells (or small groups of cells) by using high magnification fluorescence microscopy of the same reaction wells.

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Example 13. Large area detection of individual Candida albicans specifically bound to fluorescent antibodies and magnetic particles

Objective: This example demonstrates the utility of non-magnified large area imaging to detect and identify individual cells using fluorophore-labeled antibodies (signal moieties:category-binding molecules) and antibody-coated paramagnetic particles (selection moieties). In this example, non-magnified large area imaging in a microtiter dish format was used to separately detect *Candida albicans* and *Escherichia coli* cells following binding of paramagnetic particles and fluorophore-labeled antibody to the cells.

Experimental procedure: Wells of an optically clear, flat, glass-bottomed microtiter tray (Uniview 7706-2370, Whatman, Inc., Ann Arbor, MI) were blocked using 'Block Aid' (B-10710 Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Approximately $5x10^7$ magnetic particles (MEO3N 1 μm diameter Bangs Labs Inc., Fishers, IN) with surface-adsorbed rabbit anti-*C. albicans* (B65411R, Biodesign International, Saco, ME or anti-*E. coli* polyclonal antibody (B65003R, Biodesign International, Saco, ME) prepared as described in example 6 were added to a volume of PBS-TB buffer in the well to give a final reaction volume of 200 μl. *C. albicans* or *E. coli* cells were fixed in 2.5% solution of formaldehyde in PBS for five minutes and washed twice in PBS-B. Cells were added to the suspension of paramagnetic particles and the mixture was incubated at room temperature on a shaker for 30 minutes. FITC-labeled anti-*Candida albicans* polyclonal antibody (5 μg) (CR2155RF, Cortex Biochem, San Leandro, CA) or anti-*E. coli* antibodies (1 mg; Biodesign, Saco, ME. B65001R) labeled with with Alexa Flour ™ 488 (Molecular Probes, Eugene, OR., Cat# A10235) that were labeled according to the manufacturers instructions, were added and the mixture was incubated for a further 15 minutes. Unbound antibody was separated from the magnetic particle:cell complexes by repeated washing and application of a magnetic field using a magnetic ferrous block (CD2001, Cortex Biochem, Inc., San Leandro, CA) such that the magnetic particles were caused to align substantially evenly over the surface of the bottom of the well. Fluorescence was detected by imaging using the CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) using the FITC filter set (480/40 nm excitation, 535/50 nm emission filters for detection of the FITC and Alexa488 fluorophores. Fluorescent signals co-purified with the magnetic particles indicate the presence of *C. albicans* or *E. coli* cells. Positive signals detected on the imager were confirmed to be cell:fluorescent antibody complexes by high magnification fluorescence microscopy (1200X) using an Axioplan II fluorescent microscope (Carl Zeiss Inc., Thornwood, NY) after staining cellular nucleic acid with the red fluorescent nucleic acid-binding dye YOYO-3 (1 μM) (Y-3606, Molecular Probes, Eugene, OR).

Results. Figure 17 shows that wells containing *Candida albicans* or *E. coli* cells gave numerous punctate fluorescent spots. These fluorescent signals were shown to correspond to individual cells (or small clusters of cells) by using high magnification fluorescence microscopy (1200X) and staining cells for nucleic acid using the red fluorescent dye YOYO-3 (Molecular Probes, Eugene, OR). Wells containing no cells gave no (or very few) such signals. The small number of fluorescent objects in the negative control images was likely to be due to dust particles or other non-biological material.

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Example 14. Non-magnified large area detection of individual chemiluminescent yeast cells using a CCD camera

Objective: The goal of this example was to use non-magnified large area imaging to visualize individual cells labeled with chemiluminescent signaling moieties. In this embodiment, the surface of *C. albicans* cells were coated with fluorescein-conjugated antibodies, followed by alkaline phosphatase-conjugated anti-fluorescein antibodies and reacted with CDP Star substrate to generate light. The presence of individual cells in the chemiluminescence image was confirmed by fluorescence microscopy. An image intensifier was not required for detecting the individual cells.

Materials and Methods: *C. albicans* 90028 (American Type Culture Collection) were grown in YM medium (VWR cat # DF0711-17) for 48 hours at 25 C. The cells were fixed in 2.5% formaldehyde in PBS for 5 minutes followed by a wash in PBS, and stored at 4 C. Ten volumes of blocking buffer (0.5% NEN Blocking Reagent cat # FP329, 0.1 M Tris-HCl pH8, 0.15 M NaCl) were added to an aliquot of cells and the suspension was incubated for 30 minutes. Polyclonal rabbit anti-*C. albicans*, fluorescein isothiocyanate conjugated antibodies (BioDesign cat # B65411F) were added at a 1/100 dilution. The cell suspension was incubated with gentle mixing for 60 minutes. The cells were spun down and washed twice with blocking buffer, followed by resuspension in a 1/25 dilution of anti-fluorescein, alkaline phosphatase conjugated antibodies (NEN cat # NEF-709) in blocking buffer. Again the cells were incubated with gentle mixing for 60 minutes. The cells were spun down and washed 3 times in 0.1 M Tris-HCl pH 8, 0.15 M NaCl, followed by a wash in 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl. The final suspension of cells was in the pH 9.5 buffer.

For large area imaging, dilutions of the cells were spotted on CAST nylon coated glass slides (Schleicher and Schuell cat # 10484181) that were pre-moistened with the pH 9.5 buffer and overlayed with CDP Star reagent (NEN NEL-601). Chemiluminscence was visualized with 10 minute exposures using a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) with no emission filter. After the chemiluminescent image was captured, Slow Fade reagent (Molecular Probes cat # S-2828) was spotted onto the slide and a coverslip was added. The slide was viewed via fluorescence microscopy with an FITC filter cube (Chroma #SP101) at 400x magnification to identify individual cells.

Results: Individual *C. albicans* cells labeled with chemiluminescent signaling moieties could be detected without magnification as seen in Figure 18, The three different dilutions represent about 200, 60, and 20 cells respectively, from left to right. At the lowest dilution each spot on the chemiluminescent image could be correlated with a cell or a small group of cells when viewed by fluorescence microscopy. Spatial orientation was aided by the presence of penciled-in grid lines and the relative positions of cells. Larger, brighter spots, seen especially in the left panel, represent more than one cell.

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Example 15. Non-magnified large area detection of individual chemiluminescent yeast cells using direct exposure of instant film

Objective: The goal of this example was to use instant photographic film to achieve non-magnified large area detection of individual cells labeled with chemiluminescent signaling moieties. In this embodiment, the surface of *C. albicans* cells was coated with fluorescein conjugated antibody category-binding molecules, bound to alkaline phosphatase:antibody conjugates, and reacted with CDP Star substrate to generate light.

Materials and Methods: *C. albicans* 90028 (American Type Culture Collection) were grown and labeled as in Example 14. For imaging on Polaroid film, a piece of Hybond-N membrane (Amersham-Pharmacia RPN1782B) was placed on a piece of absorbent filter paper and pre-wet with 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl. Dilutions of cells were spotted on the membrane, followed by the addition of CDP Star reagent (NEN NEL-601). The membrane was mounted in a SpotLight camera (Boston Probes DT10000) according to the manufacturer's instructions and exposed to ASA 2000 film (Boston Probes DT20000) for 30 minutes.

Results: Individual *C. albicans* cells labeled with chemiluminescent signaling moieties could be detected directly on instant film as shown in Figure 19. The figure shows images of three different dilutions of labeled cells. The sample dilutions shown in Figure 19 are the same as those imaged in Example 14 (Figure 18) and represent about 200, 60, and 20 cells (from left to right, respectively in the figure). Large, bright spots, seen especially in the left panel, correspond to clusters containing numerous cells. The spots in the right panel of the figure represent either individual cells or clusters of several cells.

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Example 16. Detection of organisms involved in lower respiratory tract infections using non-magnified large area imaging

Objective: To identify organisms involved in lower respiratory tract infections: *Chlamydia pneumoniae, Mycoplasma pneumoniae* and *Legionella pneumophila* using non-magnified large area imaging.

Overview: Pneumonia is a major cause of death world-wide and is the sixth leading cause of death in the United States. Trends indicate that this figure is rising do an increase in the proportion of individuals >65 years of age as well as other factors. Currently diagnosis is often empirical, though a definitive diagnosis is thought to improve patient outcomes by prescribing the appropriate antibiotic. Detection of specific organisms (some of which are difficult to culture) by direct fluorescence staining of sputum combined with the power of non-magnified large area imaging would assist in diagnosis thereby decreasing the incidence of fatalities due to pneumonia as well as health care expenditures.

Experimental Methods: *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* control slides were obtained (Bion, Park Ridge, IL; Cat. CP-4212 and MP-1212 respectively). The slides were allowed to warm to room temperature and incubated in blocking solution (PBS/1% BSA, 15 minutes, 25°C). The blocking solution was removed via aspiration and a dilution of primary antibody (anti-*Chlamydia pneumoniae*; DAKO Corporation, Carpinteria, CA, Code NM660, Clone RR402, diluted 1/5, and anti-*Mycoplasma pneumoniae*; Fitzgerald International Industries, Inc., Concord, MA, Cat. No. 10-M40, diluted 1/1000) in blocking solution was added to individual wells of the *Chlamydia pneumoniae* control slide and the *Mycoplasma pneumoniae* control slide. The slides were incubated (30 minutes, 25°C) followed by a washing procedure (PBS-BT, 4 x 5 minutes each) and incubation with a secondary Cy3 conjugated antibody (15 minutes, 25°C,goat anti-mouse IgG-Cy3, Jackson ImmunoResearch Laboratories, West Grove, PA, Cat. No.111-165-144). Slides were washed with PBS-BT and sterile type 1 water by flooding the wells (4 x 5 minutes each and 2 x 5 minutes each respectively). For demonstration of large area imaging detection of *Legionella pneumophila* FITC-conjugated antibodies against *L. pneumophila* serogroup 1 (Cat. No. 92-103-FL) as well as heat fixed *Legionella pneumophila* serogroups 1 (Philadelphia 1, Cat. No. 92-103-H) and 6 (Chicago 2, Cat. No. 92-110-H) were obtained (mTECH™ Monoclonal Technologies, Alpharetta, GA). A smear of cells from the two heat killed *Legionella pneumophila* serogroups (1 and 6) was prepared on a poly-lysine coated slides (Sigma, Cat. No. P0425). Blocking solution was flooded onto the slide and allowed to incubate (15 minutes, 25°C) followed by aspiration. A FITC-conjugated primary antibody directed against *Legionella pneumophila* serogroup 1 was flooded onto the slide (30 minutes, 25°C) followed by aspiration and washing with PBS and type 1 water (2 x 5 minutes each and 2 x 5 minutes each respectively). All slides were air dried followed by image capture using non-magnified large area imaging (TRITC filter set, Chroma Id. No. 41002b, excitation 545/30 nm, emission 610/75 nm for Cy3 conjugates and FITC filter set, Chroma Id. No. SP101, 470/40 nm, emission 522/40 nm for FITC conjugates). Slides were mounted (Pro Long Antifade Reagent, Molecular Probes, Eugene, OR, Cat. No. P-4781) and fluorescence microscopy (Axioplan II fluorescent microscope, Carl, Zeiss Inc., Thornwood, NY; Cy3.5 filter set, Chroma Id. No. 41002b, excitation 545/30 nm, emission 610/75 nm for Cy3 conjugates or FITC filter set, Chroma Id. No. SP101 excitation 470/40 nm, emission 522/40 nm) was performed to observe detail (400x).

Results: This example demonstrates that organisms involved in lower respiratory tract infections can be detected using non-magnified large area imaging in conjunction with the direct fluorescent antibody staining technique. Large area imaging of positive and negative controls indicates that the detection is specific (Figure 20). That the signal

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seen using large area imaging is specific to that generated by labeled bacterial cells was confirmed by microscopic analysis.

Variations: The power of non-magnified large area imaging can be applied to the detection of other bacteria, yeasts and molds as well as other categories of disease including: urinary tract infection, sepsis, and sexually transmitted diseases.

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Example 17. A mulitiplexed direct fluorescence immunoassay that simultaneously scans a sample for 3 disparate microbes using non-magnified large area imaging

Overview. In this example, non-magnified large area imaging was used in a multiplex test that detects and identifies 3 types of microbes. The cells were incubated with a mixture of 3 category-specific (in this case, species-specific) antibodies, each of which was labeled with a distinct fluorescent dye. Individual cells were detected using a CCD imager.

Experimental procedure. *E. coli* and *Candida albicans* were grown and fixed according to Example 2 and Example 11 respectively. *Streptococcus pyogenes* was grow overnight at 37°C in Brain Heart Infusion (Difco Cat# 237500) and fixed and washed according to Example 2. All 3 types of cells were individually diluted in water until 20-50 cells per high power field (X40) could be seen by phase contrast microscopy. Aliquots (3 µl) of these dilutions were placed on poly-L-lysine coated coverslips and spread over an area approximately 5 mm in diameter and allowed to dry.

Anti-*E. coli* antibodies (1 mg; Biodesign, Saco, ME. B65001R), anti-*C. albicans* antibodies (1 mg; Biodesign, Saco, ME. B65411R), and anti-Steptococcus Grp A antibodies (1 mg; Biodesign, Saco, ME. B10601R) were labeled with Alexa Flour ™ 488 (Cat# A10235), Alexa Flour ™ 350 (Cat# A10170), and Alexa Flour ™ 546 (Cat# A10237), protein labeling kits respectively, according to manufacturers instructions (Molecular Probes, Eugene, OR.).

A mixture of the labeled antibodies was prepared in normal rabbit serum (Cat# 88-NR50; Fitzgerald, Concord, MA) each at 20 µg/ml. The cells, spotted on coverslips, were covered with the antibody mixture and incubated for one hour at room temperature in a humid plastic pipette box lid containing water-saturated paper towels and covered with Saran Wrap. Coverslips were then washed once in PBS containing 1% normal rabbit serum and then twice in PBS (10 minutes per wash). The coverslips were removed from the wash and any residual liquid was blown off using compressed air. The relevant area of each coverslip was imaged for 1 sec in a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) in the FITC (Ex 480nm/40nm and Em535nm/50nm) (Alexa488 specific), Texas Red (Ex 560 nm/55 nm and Em645 nm/75 nm) (Alexa 546 specific) and DAPI (Ex 360 nm/40 nm and Em460 nm/50 nm) (Alexa 350 specific) channels.

Results. The result of the multiplex bacterial assay is shown in Figure 21. For each distinct bacterial sample, the numbers and intensities of objects were greatest in the expected fluorescence channel. Thus, the numbers and intensities of the objects seen in the FITC (Alexa 488) channel were greatest for the *E. coli* samples. Similarly, *C. albicans* samples, and *S. pyogenes* samples had the strongest intensities and the most numerous objects in the DAPI and Texas Red channels, respectively. Thus, the assay can differentiate the 3 categories of cells with a mixture of antibody category-binding molecules using non-magnified large area imaging.

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Example 18. Solid phase capture assay for Adenovirus

Objective: This example shows that small numbers of viruses captured on a solid support can be labeled with fluorescent particles and visualized by non-magnified large area imaging. The wells of a 96 well plate were first coated with anti-adenovirus antibodies. Next adenovirus and particles coated with anti-adenovirus antibodies were added to the well, and the viruses and particles were captured by the antibody-coated surface. Unbound particles were removed and the captured particles, representing the captured virus, were detected by large area fluorescence image analysis.

Materials and Methods: The wells of a 96 well Greiner plate (Greiner Labortechnik cat# 655097) were coated with biotinylated-BSA (Sigma cat# A-8549) by adding 50 µl of a 0.2 mg/ml solution in 100 mM sodium bicarbonate pH10 and incubating overnight at room temperature. Wells were washed once with 100 µl PBS. The biotinylated-BSA layer was then coated with 50 µl 0.1 mg/ml streptavidin (Jackson Immunoresearch Laboratories cat# 016-000-113) in PBS for two hours at room temperature, followed by a wash of PBS, then coated with 50 µl biotinylated-anti-adenovirus antibodies (Chemicon cat# MAB8052 antibodies biotinylated with Molecular Probes kit cat# F-6347) at 0.1 mg/ml in PBS for two hours at room temperature. The wells were washed three times in 100 µl PBS-TB and stored at 4 C.

Adenovirus type 2 (ATCC VR-846) and Respiratory Syncitial Virus (RSV) (ATCC VR-1302) were fixed by reconstituting in 4% formaldehyde/PBS (final concentration) for 30 minutes at room temperature. Glycerol was added to a final concentration of 16% and aliquots were frozen at -80 C. Virus particle titer was estimated based on the titers provided by the manufacturer expressed as $TCID_{50}$ and converted to pfu (plaque forming units) by multiplying by a factor of 0.7 as recommended by the manufacturer (ATCC). The assumption was made that the pfu is approximately equal to the number of virus particles in the preparation. Before use, aliquots were thawed on ice. After thawing, unused portions were stored at 4 C for further experiments.

Particles were coated with anti-adenovirus antibodies (Chemicon cat# MAB8052) as described in Example 8 except that 0.2 µm diameter fluorescent particles at 1% solids (Molecular Probes cat# F-8848) were used.

To perform the assay, the storage buffer was removed from the well. About 10,000 virus particles and $3x10^8$ particles were added to a well at a final volume of 100 µl in Block Aid buffer (Molecular Probes cat# 10701). The microtiter tray was incubated for one hour at room temperature on a shaking platform. The wells were washed 3 times in PBS-TB, and then once in water. After the water was removed the wells were imaged using the CCD Imager (described in Step 6 of Detailed description section and shown in Figure 3) using the FITC excitation (470 nm/40 nm) and emission filter (522 nm/40 nm) set. Images were processed and analyzed using Image-Pro Plus software (Media Cybernetics). The object counting utility was used to estimate the number of particles in the wells.

Results: This example shows viral particles captured on a solid phase can be labeled with fluorescent particle signaling moieties and detected without magnification as seen in Figure 22. Analysis of the image (bottom panel) using Image-Pro Plus software indicates the number of objects (particles) is about 13,000, which roughly matches the approximate number of adenovirus particles added (~10,000). However, it should be noted that the number of virions in the preparation were not precisely determined. Also it was not known how many infected tissue culture cells or fragments of such cells were present in the preparation. The object count in the RSV negative control (top panel) was about 800, and represents the non-specific background.

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Example 19. Solid phase capture of a virus in blood

Objective: This example shows that small numbers of viruses in a blood sample can be captured on a solid support, labeled with fluorescent particles, and visualized by non-magnified large area imaging. Blood spiked with Adenovirus was added to anti-adenovirus antibody-coated wells in a 96 well plate. After capturing the virus, fluorescent particles coated with anti-adenovirus antibodies were added. Unbound particles were removed and the captured particles, representing captured virus, were detected by CCD-based fluorescence imaging.

Materials and Methods: The wells of a 96 well plate (Greiner Labortechnik cat# 655097) were coated with biotinylated-anti-adenovirus antibodies. Adenovirus type 2 (ATCC VR-846) and RSV (ATCC VR-1302) were reconstituted and fixed as in Example 18. Particles were coated with anti-adenovirus antibodies (Chemicon cat# MAB8052) as described in Example 8, except that 0.2 μm diameter fluorescent particles (Molecular Probes cat# F-8848) at 1% solids were used. To perform the assay, the storage buffer was removed from the wells. About 10,000 virus particles were added to a well in 50% Block Aid buffer (Molecular Probes cat# 10701), 50% mouse blood, in a final volume of 100 μl. The microtiter tray was incubated for one hour at room temperature on a shaking platform. The wells were washed two times in PBS-TB, followed by the addition of $3x10^8$ particles in 100 ul Block Aid buffer. After an hour of incubation with shaking, the wells were washed three times with PBS-TB, once with water, and visualized using a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) using the FITC excitation (470 nm/40 nm) and emission filter set (522 nm/40 nm).

Results: This example shows specific viral particles (in this case, adenovirus) can be captured on a solid phase in the presence of 50% blood, labeled with fluorescent particle signaling moieties and detected without magnification as seen in Figure 23. Analysis of the image (bottom panel) using Image-Pro Plus software indicates the number of objects (particles) is 13,489, which roughly matches the approximate number of adenovirus particles added (10,000). The object count in the RSV negative control (top panel) was 2,173, and represents the non-specific background. The results of this assay are similar to the results in Example 18 indicating that the presence of blood does not interfere with the capture of viral particles onto the solid phase, their labeling with fluorescent particles, or their detection without magnification.

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Example 20. Liquid phase assay for Adenovirus

Objective: This example shows that the invention can scan for small numbers of viruses using a liquid—phase sandwich-forming method. A sample containing adenovirus was incubated with a combination of magnetic and fluorescent particles that are coated with anti-viral antibodies. Complexes of magnetic and fluorescent particles were induced to form in the presence of adenovirus (but not a control virus). These fluorescent complexes were magnetically selected, deposited on an optically clear surface and imaged using non-magnified large area imaging.

Materials and Methods: Adenovirus and RSV were reconstituted and fixed as described in Example 18. Antibody coated fluorescent and magnetic particles were prepared as described in Example 8 with anti-adenovirus antibodies (Chemicon cat# MAB8052) used on 1 μm diameter red fluorescent particles (Molecular Probes cat# F-8851) and 2.8 μm diameter magnetic particles (Dynal; cat# 142.03). In this example, $5x10^5$ red fluorescent particles were mixed with $5x10^6$ magnetic particles in Block Aid buffer (Molecular Probes cat# B-10710) to a final volume of 250 μl. Adenovirus or RSV was added to the assay at a titer of about 1000 virus particles per sample. After four hours at room temperature with mixing, the magnetic particles and any bound particles were separated using a Polysciences BioMag magnetic separator for microcentrifuge tubes and washed three times in PBS-TB. The washed magnetic particles were transferred to microtiter wells (Greiner Labortechnik cat# 655097) and visualized in a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) with the Texas Red excitation (560 nm /55 nm)/emission (645 nm/75 nm) filter sets. Images were captured and analyzed with Image-Pro Plus software (Media Cybernetics) as in Example 18.

Results: This example shows specific viral particles can be captured using a liquid—phase sandwich-forming method with magnetic and fluorescent particles and detected without magnification as seen in Figure 24. Roughly 1000 virus particles were added to the assay. In the bottom panel of the figure, analysis with the Image-Pro Plus object counting utility detects 2324 adenovirus particles, while in the negative RSV control (top panel), only 46 particles which represent non-specific background binding, were detected.

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Example 21. Homogenous assay for Adenovirus in blood

Overview of the example. Efficient and cost-effective viral load testing – determining the concentration of viruses in human samples – represents a major unmet need in medical diagnostics. This example demonstrates how the invention can rapidly enumerate viruses in a sample. The example tests for Adenovirus, an important human pathogen. Technically, the example is similar to Example 8.

Coating particles with antibodies. Particles are coated with antibodies as in Example 8 and resuspended at a concentration of 2% solids. In this example, anti-Adenovirus monoclonal antibodies (anti-hexon; catalogue number MAB8052; Chemicon) is used to coat yellow-green fluorescent particles (TransFluoSpheres; catalogue number T-8871; Molecular Probes) and magnetic nanoparticles (carboxy polymer-coated ferrofluid; Immunicon; catalogue number F3000). Equal volumes of the fluorescent and magnetic particles are mixed so that the final concentration of each type of particle in the mixture is 1% solids. The particle mixture is dispersed using sonication as in Example 8.

Detecting and quantifying Adenovirus in blood. The test described in this example detects adenovirus virions in blood. Samples (10 µl in PBS-B) containing various amounts (0, 10^3, 10^4, 10^5 pfu) of purified adenovirus virions (CsCl gradient purifed; Frank Graham, McMaster University) are added to whole blood and to the particle mixture and the reaction is processed as in Example 8. The number of fluorescent particle clusters minus in the experimental sample minus the average number of clusters in the negative control samples indicates the number of Adenovirus particles in the sample.

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Example 22. Multiplex large area imaging immunoassay that simultaneously scans for a bacterium and a virus

Objective: This example demonstrates that the invention can be used to construct tests that simultaneously scan a sample for multiple diverse analytes – here, a bacterium and a virus. Paired antibody-coated microparticles – one fluorescent and one paramagnetic – were used to bind to targets, as in several previous examples (*e.g.*, Example 8). Here, however, two such pairs of particles were used, one set coated with anti-*E. coli* antibodies and one set coated with anti-adenovirus antibodies. To distinguish the two types of particle:analyte complexes from each other, distinctive fluorescent particles were used for each analyte. The *E. coli*-specific particles had green fluorescent character while the adenovirus-specific particles had red fluorescent character. After mixing the sample and the analyte-specific particles, the resulting particle:analyte complexes were magnetically selected to the detection zone and imaged using non-magnified large area imaging. The viral and bacterial targets were identified and quantified by analysis of two CCD images that were acquired while using optical filter sets tuned for either the red or green particles.

Materials and Methods: *E. coli* cells were grown and fixed as described in ,Example 8 and adenovirus was reconstituted and fixed as described in Example 18. Antibody coated fluorescent and magnetic particles were prepared as described in Example 8. Anti-adenovirus antibodies (Chemicon cat# MAB8052) were used to coat red fluorescent particles (Molecular Probes cat# F-8851) and anti-*E. coli* antibodies (KPL cat# 01-95-90) were used with green fluorescent particles (Molecular Probes cat# 8852) while batches of magnetic particles (Dynal.cat# 142.03) were made with each of the antibodies. For the assay, particles were mixed in a final volume of 250ul Block Aid buffer (Molecular Probes cat# B-10710) in the following amounts: $2.5x10^7$ particles for each fluorescent particle and $5x10^6$ particles for each magnetic particle. Adenovirus and/or *E. coli* were added to the assay at about 1000 virus particles/cells each. After one hour at room temperature with mixing, the magnetic particles and any bound particles were separated in a magnetic field and washed three times in PBS-TB. The washed magnetic particles were visualized in Greiner microtiter wells (Greiner Labortechnik cat# 655097) using a CCD imager (described in Step 6 of Detailed description section and shown in Figure 3) with both the FITC and Texas Red excitation/emission filter sets (see Example 18 and Example 20 for filter specifications).

Results: This example demonstrates that a bacterium and a virus can be scanned for in the same sample and detected using non-magnified large area imaging. As seen in Figure 25, when roughly 1000 adenovirus are added to the mixture, the majority of particles detected are the anti-adenovirus red fluorescent particles (Texas Red filter sets) (top row). When roughly 1000 *E. coli* are added, anti-*E. coli* green fluorescent particles are detected using FITC filter sets (second row), while when both adenovirus and *E. coli* were added, particles are detected in both channels (third row). When neither pathogen is added, an order of magnitude fewer particles were detected, representing the non-specific background (bottom row).

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Example 23. Filter flow-through assay for detecting individual bacteria using non-magnified large area imaging.

Overview. In this example, a rapid flow-through assay is combined with non-magnified large area imaging to detect small numbers of bacteria that are labeled with highly fluorescent particles. After incubating the bacteria and beads together, the resulting solution containing bacteria:bead complexes is passed through a filter, that had been coated with anti-bacterial antibody. The complexes that are captured by the filter are then detected using non-magnified large area imaging as in Example 1.

Experimental design. A nitrocellulose membrane (Pall Biodyne A; 5 μm pore; cat. num.BNCF810S) was cut into two 1 cm by 1 cm squares. The squares were soaked in a solution containing anti-*E. coli O157* (200 μg/ml in PBS). After drying at room temperature, the membranes were blocked (200 ml; 30 min; room temparature; 1% casein (Hammerston grade; EM sciences; cat. num.CX0525-1) and 0.1% Tween 20 (Sigma; cat. num. P1379) in PBS). The membranes were then blotted dry on absorbent paper. The membranes were transferred to parafilm and the membrane was saturated with PBS (300 μl). A solution of *E. coli O157* (10^5 cells) and red fluorescent beads (10^6 beads/ Molecular Probes; 1 μm; sulfate; 580/605 nm; cat. num.F-8851) that had been coated with anti-*E. coli O157* antibodies (Kirkegaurd and Perry Laboritories; cat. num. 01-95-90; antibodies passively adsorbed as described in Example 8) were mixed together in a tube, allowed to incubate for 15 minutes and then added to one of the nitrocellulose squares. On the other square, just the antibody coated fluorescent beads were added (this filter was the "no bacteria" negative control). After an additional 15 minute incubation, the membranes were put on a piece of filter paper (Whatman 50um pore) and the excess liquid was absorbed. The membranes were then washed (PBS-T; 50 ml; 20 min). Fluorescence was detected by imaging using a CCD imager with an FITC optical filter set (excitation 560/55 nm, emission 645/75 nm) for red fluorescent beads. Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD imager. Positive signals detected on the imager were confirmed to be beads bound to E. coli by using an Axioplan II fluorescent microscope (Carl, Zeiss Inc., Thornwood, NY) equipped with the same filter sets.

Results. Figure 26 shows the results of using a flow through assay to detect bacteria that were decorated with specifically bound highly fluorescent beads. The CCD image of the experimental filter through which the bacteria-containing sample was passed contained numerous white spots, while the control filter through which a sample lacking bacteria was passed contains few beads. High power microscopy confirmed that the experimental filter contained numerous complexes consisting of bacteria surrounded with beads, while the control filter contained none of the complexes but rather only dispersed single beads.

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Example 24. Quantification of bacteria using large area imaging of cells stained with a fluorogenic esterase substrate

Objective: In many applications it is useful to have a large dynamic range for quantifying live bacterial cells. An ideal system would be able to accurately count from zero or one bacterial cell up to millions or tens of millions, thus eliminating the serial dilutions and their inherent lack of precision that are necessary for traditional microbiological plating methods. In this example we show how staining live cells with fluorogenic substrates, coupled with CCD-based, non-magnified large area imaging can be used to quantify cells over at least 5 orders of magnitude.

Experimental Methods: *E. coli* ATCC 8739 cells were grown and processed as described in Example Y (Cell Direct example). Serial 10-fold dilutions of the cells were made in PBS and filtered in duplicate samples through black polyester membranes (Chemunex cat. # 200-C2010-01) mounted on absorbent pads (Chemunex cat. # 200-C3012-02) in a Millipore 1225 manifold and stained as described in Example Y (Cell Direct Example). In addition, 10 μl of the 10^{-5} dilution was plated in triplicate on TSA (BD catalogue no. 236950) and grown at 37°C overnight to get a cell titer. The fluorescent signals on the polyester membranes were captured using a CCD Imager (described in step 6 above; Figure 3) with an FITC optical filter set (Chroma/excitation 470/40 nm, emission 522/40 nm). Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager. The signal generated from each filter was defined as the sum of the pixel intensities of all objects (where objects are defined in this particular example as containing pixel intensities from 350-65301). This method of defining signal eliminates the background from regions of the filter that do not contain any stained cells, but does not mask or undercount intensities from overlapping objects. (Since the cells are small and fairly transparent, signals are additive as long as the layer of cells is thin.)

Results: As shown in Figure 28, the signal generated from this method is linear over at least 5 orders of magnitude, which is considered a large dynamic range for this test.

Variations. At low object numbers, accurate quantification can be achieved by counting individual objects rather than using the sum of their pixel intensities, since the objects will be unlikely to overlap. This can extend accurate counting down to one or zero cells, especially if multiple stains and multiple excitation and/or emission wavelengths are used to determine which objects represent viable cells. In addition, more sophisticated object finding algorithms can be employed to take into account local background intensities and variations in illumination.

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Example 25. Detecting individual stained bacteria on a filter using non-magnified large area imaging

Overview. In this example, large area imaging is used to detect individual *E. coli* bacterial cell targets that are stained with a fluorescent dye that binds to nucleic acids. The stained cells are filtered through a black polycarbonate filter, illuminated with white light, and imaged with a CCD camera.

Experimental design. A culture of *E. coli* MG1655 was stained with Syber Green I as in Example 2. The culture was diluted and approximately 10^5 cells were filtered through a black polycarbonate filter using a vacuum pump and a plastic funnel cup (Millipore Microfil V User Guide, PF07114, Rev A 3/00). The filters were then washed with water (50 ml; type 1 quality). Fluorescence was detected using a large area CCD-based imager (Figure 3) and a FITC optical filter set (excitation 470/40 nm, emission 522/40 nm) which is appropriate for detecting Syber green I. Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager. Positive signals detected on the Imager were confirmed to be cells by using an Axioplan II fluorescent microscope (Carl, Zeiss Inc., Thornwood, NY) equipped with the same filter sets.

Results.

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Figure 27 shows the capture and detection of bacteria on a filter. Syber green I stained cells on the surface of the filter are seen as white spots. When viewed under high power magnification the spots are seen as stained cells.

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Example 26. Non-instrumented detection of small numbers of bacterial cells without magnification

Overview. In this example, particles, which were coated with both alkaline phosphatase and anti-*E. coli* antibodies, were used to detect *E. coli* O157 bacteria in a liquid capture assay. Bacteria were bound to both the dually coated particles and antibody-coated magnetic particles in liquid and the particle: analyte complexes were separated and washed using magnetic force to separate the particle complexes from the unbound particles. The complexes were deposited on a 0.2 μm pore nitrocellulose membrane using filtration and then visualized using both chemiluminescent and colorimetric substrates.

Experimental procedure. The dually coated particles were made by adding both biotinylated alkaline phosphatase (5 μl of a 2.9 mg/ml stock; Pierce; cat. num. 29339) and biotinylated donkey anti-goat IgG antibody (5 μl of a 1.6 mg/ml stock; Jackson; cat. num. 705-065-147) to streptavidin-coated particles (10^8 particles; Bangs;0.95 um, non-fluorescent; cat. num. CP01N). The reaction level was brought up to 100 μl with PBS. After a 30-minute incubation, the particles were washed twice. A wash consisted of spinning the particles down in a microcentrifuge at 3000 g for 5 minutes, then discarding the supernatant and resuspended the particles in PBS (100 μl). After washing, goat anti-*E. coli O157* antibody (5ul of a 1mg/ml stock; Kirkegaurd and Perry Laboratories; cat. num. 01-95-90) was added to the particles. The particles were allowed to incubate for 30 minutes at room temperature and then washed twice as mentioned above. After making the dually coated particles, an *E. coli O157* culture was fixed in 2.5% formaldehyde (see example 1, Large area imaging of individual bacteria stained with a DNA-binding fluorescent dye) and serially diluted in ten fold increments to achieve 10^7 through 10^3 cells/ml. In separate 1.5 ml tubes, 10 μl of each dilution (done in duplicate) were combined with both goat anti-*E. coli O157* antibody coated magnetic particles (10^6 particles; Dynal; 2.8 um; tosylated; cat. num. M-280; prepared as in Example 8) and the dually coated particles (10^6 particles). The particle: bacteria suspension was brought up to 100 μl with PBS-TB and incubated with mixing for one hour at room temp. After incubation the tubes were washed four times in PBS-TB. A wash consisted of magnetic separation to draw the magnetic particle: bacteria:particle sandwich to one side of the tube followed by aspiration to remove the supernatant and resuspension in PBS-TB (100 μl). Both replicates of each dilution of the washed particle: bacteria sandwiches were filtered separately. Each replicate was added to PBS (50 ml) and filtered through a 0.2 μm pore nitrocellulose membrane using a vacuum pump and a plastic funnel cup (Millipore Microfil V User Guide, PF07114, Rev A 3/00). BM Purple AP substrate (500ul; Roche; cat. num. 1442074) was added to one set of filters. The other filter set had CDP-star (500ul; NEN; cat. num. NEL-601) added to them. After a 1-hour incubation the BM purple membranes were washed in water to remove left over BM Purple and the membranes were allowed to air dry. The CDP-star membranes were mounted in a SpotLight camera (Boston Probes; cat. num. DT10000) according to the manufacturer's instructions and exposed to ASA 2000 film (Boston Probes; cat. num. DT20000) for two seconds. The same filters were then exposed using non-magnified large area imaging. Image-Pro Plus software, version 4.1 (Media cybernetics) was used to capture and process images from the CCD Imager.

Results. Figure 29 shows that the bi-functional particle, that was conjugated to a category-specific binding molecule and an enzymatic signaling moiety, can be detected using either chromogenic or chemiluminescent signal elements. The assay described here using the dually labeled particles is sensitive, detecting low numbers of bacteria (approximately 100 cells). The ability to see with the naked eye, *E. coli*:bead complexes – which are normally only visible under a high power microscope – is due to the enormous signaling power of these particles that contain large numbers of enzyme mole-

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cules. In fact, single dispersed microscopic beads were detectable with the naked eye when prepared, deposited on filters, and treated with chromogenic substrate as in this example. The approach used in this example demonstrates the potential for the invention to provide simple, inexpensive, and non-instrumented point-of-care tests that are, in contrast to current rapid tests, extremely sensitive.

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Example 27. A rapid, homogenous, sensitive, and quantitative immunoassay for *Mycobacterium tuberculosis* in clinical samples

Overview. The medical significance of diagnostic testing for *M. tuberculosis* is discussed in the overview of Example 9. In this example, the invention is used to construct a homogenous immunoassay (see Example 8 and Figure 11) using category-specific anti-*M. tuberculosis* antibodies. This type of test is rapid, sensitive, inexpensive, and easy-to-use. The assay described here complements the acid-fast bacillus approach demonstrated in Example 9 as it differentiates *M. tuberculosis* from other mycobacterial species.

***M. tuberculosis*-specific antibodies.** The category-binding molecules in this example are antibodies that specifically bind to *M. tuberculosis*. This example makes use of a monoclonal antibody (MPB64-ICA) that is specific for species in the *M. tuberculosis* complex (Abe, *et al.*, J. Clin. Microbiol. 37:3693-2881, 1999). Alternatively, polyclonal antibodies can be used. For example, for the present application one could use a rabbit polyclonal antibody that reacts with *M. tuberculosis* (BioDesign catalogue number B65601R).

Category-specific polyclonal antibodies are preferably purified by immunoaffinity chromatography using methods that are familiar to those skilled in the art. In this example, *M. tuberculosis*-specific antibodies are purified using columns containing the target pathogens immobilized on activated particles (*e.g.*, Affigel 10; BioRad) (Harlow, *et al.*, *Using Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999)). After positive selection of the desired antibodies in this way, the antibodies are checked for cross-reaction with other lower respiratory tract pathogens and commensal flora typical of the respiratory tract (*e.g.*, see Chapter 3, Table 1 in Murray, *et al.*, eds. (1999). Manual of Clinical Microbiology. 7th ed. Washington, D.C.: American Society for Microbiology) including mycobacterial species that do not cause tuberculosis (*e.g.*, *M. avium*. Pathogens are fixed (Amann, *et al.*, Appl Environ Microbiol 56: 1919-25, 1990), spotted onto poly-lysine coated glass slides (Sigma; cat. num. P-0425) and treated with the anti-*M. tuberculosis* antibody (1:500 dilution in PBS-B; 20 min; RT), washed 4 times with PBS-TB (50 ml), and then similarly treated with fluorescein-labeled goat-anti-rabbit IgG. Organisms that cross-react with the antibodies are then used to remove the cross-reacting antibodies from the specifically reacting antibodies. The cross-reacting organisms are attached to particles and used to absorb the cross reacting antibodies by immunoaffinity chromatography as described above for positive selection of *M. tuberculosis*-specific antibodies. However, for removing cross-reactive antibodies, the unbound antibodies are collected and the bound antibodies discarded. The resulting antibodies are specific for *M. tuberculosis*.

Alternatively, non-commercial antibodies (polyclonal or monoclonal) or recombinant antibodies can be produced by using standard methods known to those familiar with the art that are described in various works and references therein (*e.g.*, Coligan, J, *et al.*, eds. (1994). *Current Protocols in Immunology*. New York: John Wiley & Sons; Knott, C, *et al.* (1997). Development of Antibodies for Diagnostic Assays. In Principles and Practice of Immunoassay, C. Price and D. Newman, eds.: Stockton Press; George, A. (1997). Antibody Engineering: Potential Applications for Immunoassays. In Principles and Practice of Immunoassay, C. Price and D. Newman, eds.: Stockton Press).

Coating magnetic and fluorescent microparticles with category-specific antibodies. The category-specific antibodies are used to coat magnetic particles and fluorescently-dyed polystyrene particles as described in Example 8.

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Binding *M. tuberculosis*-specific particles to a lower respiratory sample. In the next step, the fluorescent and magnetic *M. tuberculosis*-specific particles are allowed to bind to the *M. tuberculosis* cells in a lower respiratory sample. Liquefied lower respiratory samples (200 µl) are mixed with the *M. tuberculosis*-specific particle mixture (1 X 10^6 particles of each type) in a well of a 96-well microtiter dish with an optically clear bottom (Greiner Labs; catalogue number 665097). BAL lower respiratory samples are used without prior treatment. Sputum samples are prepared using the NALC-NaOH method (Isenberg, ed. (1992). Clinical microbiology procedures handbook. Washington, D.C.: American Society of Microbiology). Section 3.4).

Detecting and quantifying *M. tuberculosis* in the sample. Samples are processed, imaged and analyzed as described in Example 8.

Controls. Positive and negative control experiments are preferably processed in parallel to clinical samples. Positive control samples, which are treated identically to the clinical samples, preferably contain a known amount (about 1000 cells) *M. tuberculosis* in PBS. Detection of the correct amount of cells in the positive control indicates that the biochemical and image analysis procedures are working correctly. Negative control samples contain PBS, but no cells. No positive signals should be seen in the negative control experiment if all procedures are working properly.

Variations. Staining of the target can also be used for detection instead of or in addition to using fluorescent particles (see variations in Example 8. Specific staining procedures for *M. tuberculosis*, such as auramine O or auramine-rhodamine staining can also be used (see Example 9).

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Example 28. Rapid homogenous antimicrobial susceptibility testing of *Mycobacterium tuberculosis* in clinical samples using fluorescent staining

Overview. There is great medical importance attached to the rapid identification (see Example 9) and rapid susceptibility testing of *M. tuberculosis*. This example combines rapid growth of *M. tuberculosis* directly in a clinical sample with a simple but sensitive acid-fast smear technique (Example 9, Figure 13) a to cut weeks off of the time currently required to provide susceptibility data for patients with tuberculosis.

For *M. tuberculosis,* the turnaround time for susceptibility testing ranges from weeks to months, during which time a patient being treated with a sub-optimal antimicrobial therapy can be spreading the disease to many other people. Existing susceptibility tests start from a cultured sample. Thus, whereas for most organisms, the start of susceptibility testing is generally 24 to 48 hr after sample collection, for *M. tuberculosis*, the start of testing is generally delayed for several weeks. Current rapid testing regimes generally require 7-12 days of drug-free growth before antibiotic susceptibility testing. A more rapid susceptibility test for *Mycobacterium tuberculosis* is critically needed because of the growing worldwide epidemic of multi-drug resistant *Mycobacterium tuberculosis.*

The antimicrobial susceptibility assay described here offers clinically valuable features including: minimal growth requirements (several generations), simplicity, cost-effectiveness, parallel analysis of numerous antimicrobial regimes, the ability to directly test biologically complex clinical samples containing low titers of bacteria. The unique features of the test are consequences of the invention's ability to identify and enumerate small numbers of microbes in a sample without using microscopy. The invention cuts several weeks from the classical susceptibility testing turnaround time by bypassing the otherwise obligatory drug-free culture step, and by shortening the incubation with antibiotics to 4 days. The turnaround time for testing using the invention is less than half that of the fastest of the emerging rapid tests (*e.g.*, Norden *et al.*, J. Clin. Micro. 33:1231-1237,1995). The invention requires much less expensive instrumentation than that required by other rapid susceptibility testing methods (*e.g.*, BACTEC-460, and flow cytometry).

The approach used in this example can also be used for monitoring patients undergoing antimicrobial therapy – another important unmet clinical need. Monitoring viable cell counts in patients receiving antibiotics is important for determining therapeutic efficacy. Current rapid tests for *M. tuberculosis,* based on nucleic acid amplification, cannot be used for patient monitoring. This is because non-viable *Mycobacterium tuberculosis,* containing amplifiable nucleic acid, persist in patients for long periods of time even when therapy is effective. The number of viable organisms can be determined by assessing the number of cells in a sample before and after incubation for several generations of growth (in the absence of antibiotics in this application).

Identifying *M. tuberculosis*. Sputum samples are prepared and stained using the auramine-rhodamine acid-fast bacillus method (Isenberg, ed., 1992, *supra*) and tested for *M. tuberculosis* as described in Example 9. If *M. tuberculosis* is present, aliquots of sputum are used to analyze the antimicrobial susceptibility of the pathogens.

Direct growth of *M. tuberculosis* in the clinical sample with various antibiotics. Antibiotic susceptibility testing is performed on samples that contain *Mycobacterium tuberculosis* using the broth microdilution method. Aliquots of liquefied sputum (100 µl; prepared as on Example 27) are added to test tubes (2 ml polypropylene screw-cap tubes; Sarstedt) containing concentrated 7H9 broth (100 µl; prepared at three times the normal concentration; DIFCO #211417) and inoculated with various concentrations of

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ethambutol, isoniazid, and rifampin (100 µl antibiotic added to each tube; Sigma) as described previously (Moore, *et al.*, J Clin Microbiol 37: 479-83, 1999). Two tubes are inoculated with PBS (100 µl) rather than antibiotic. One of these tubes is incubated in parallel with the susceptibility test samples to measure drug free growth of the bacteria (positive control). The other tube is incubated at 4°C to prevent growth (negative control). All tubes are incubated for 4 days and then killed by treatment with 1% paraformaldehyde as described previously (Moore *et al.*, 1999, *supra*).

Ascertaining antimicrobial susceptibility using a fluorescent acid-fast bacillus test. The samples that have been grown in the presence or absence of antibiotics are next enumerated using the fluorescent acid-fast bacillus described in Example 9. Aliquots (*e.g.*, 100 µl) of the samples are smeared on slides, imaged using non-magnified large area imaging, and analyzed as before (Example 9). For each antibiotic tested, 2 curves are plotted using sample enumeration data. One curve is based on the total integrated intensity of the images and the other is based on the total number of objects found using the imaging software. Minimum inhibitory concentrations (MICs) of the various antibiotics are determined by examination of these curves (see Example 10).

Variation using a homogenous immunoassay format. Other embodiments of the invention can be applied to this application, such as the homogenous immunoassay format used in Example 27 (see also Figure 11). When using the liquid-phase format, aliquots (100 µl) of the samples grown in various antibiotic concentrations are added to a mixture of *M. tuberculosis*-specific fluorescent and magnetic particles and processed as described in Example 27.

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Example 29. Rapid, homogenous, quantitative testing for Human Immunodeficiency Virus in blood

Overview. Detecting and measuring HIV levels in blood is critically important at all stages of HIV infection. Early diagnosis of HIV infection depends on the ability to detect low levels of virus in plasma. Quantifying levels of virus in plasma is the key strategy for monitoring the health of AIDS patients and is essential for following the effectiveness of anti-viral treatments. The sensitivity required of HIV detection methods is less than 100 virions/ml. Currently ultra-sensitive methods are based on nucleic acid detection (*e.g.*, PCR, TMA, and bDNA). However, these methods are expensive and slow relative to immunoassays. Current immunoassays, however, are not sensitive enough to measure low levels of viruses in plasma.

This example demonstrates the power of the invention to offer the ultra-sensitivity of a nucleic acid amplification test in a simple, rapid, and economical homogenous immunoassay format. Key features of the invention, detecting and identifying individual targets in a non-microscopic sample, are essential for achieving the combination of sensitivity and simplicity.

Coating particles with antibodies. To make HIV-specific particles, yellow-green fluorescent particles (FluoSpheres; catalogue number T-8803; Molecular Probes) and magnetic particles (carboxy polymer-coated ferrofluid; Immunicon; catalogue number F3000) are coated with antibodies as in Example 8, except that anti-HIV gp160/120 (Chemicon; catalogue number MAB8836) is used.

Control particles. This example incorporates both positive and negative internal controls. If all procedures and reagents are working correctly the positive control particles will be evident, but an insignificant number of negative control particles will be detected. The positive control particles consist of orange fluorescent particles (TransFluoSpheres; 100 nm catalogue number T-8872) and magnetic nanoparticles (ferrofluid). The positive control particles are coated with anti-bacteriophage Fd antibody (Accurate; catalogue number BYA-3163-1). A known number (~1000) of bacteriophage Fd virions are added to each experimental sample. If the procedure is working correctly, all of the Fd virions will bind to both the Fd-specific orange fluorescent nanoparticles and to the Fd-specific magnetic nanoparticles.

The negative control particles are dark red fluorescent particles (TransFluoSpheres; 100 nm catalogue number T-8876) that are coated with anti-digoxygenin antibody (Roche; catalogue number 1 333 062). The dark red fluorescent particles that are detected in the assay indicate the background level (*i.e.,* the number of particles that are detected independent of association with a magnetically labeled target).

Making the particle ensemble. HIV-specific particles and control particles are mixed so that the total combined particle concentration is 2% solids. The particles are dispersed by sonication as in Example 8.

Preparing plasma. Blood is collected in sterile tubes (*e.g.*, Bection-Dickinson; catalogue number 6454) using EDTA as an anticoagulant. Plasma is separated from whole blood (1.5 ml) by centrifugation in a screw-cap microcentrifuge tube (*e.g.*, Sarstedt; catalogue number 782.694.006) at 900 RPM for 10 min at room temperature. Plasma can be stored frozen for several weeks at -20°C or used within a day if not refrigerated. Approximately 1000 Fd bacteriaphage (10 µl of100 pfu/µl stock in PBS; ATCC catalogue number 15669-B2) are added to each plasma sample (200 µl).

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Immunoassay and HIV detection. The particle mixture (100 µl) and samples are then mixed together in the well of a microtiter dish, incubated, magnetically selected, and imaged as in Example 22 except that 3 images are collected using filter sets appropriate for the 3 types of particles comprising a single excitation filter (Chroma HQ480/40x) and three different emission filters, Chroma HQ522/40m for yellow-green particles, Chroma HQ567/15m for orange particles, and Chroma HQ667/30m for dark red particles.). The number of yellow-green particles (HIV-specific) minus the number of dark red particles (negative control) indicates the number of viruses in the sample. The ratio of the number of dark red particles to the number of input Fd bacteriophage particles indicates the efficiency of the test.

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Example 30. Analysis of human cells by immunophenotyping: quantifying CD4+ cells in AIDS patients

Human disease diagnostics, including cancer and immunodeficiency diagnostics, depend on identification of human cells based on their distinct molecular constituencies. Immunophenotyping, an important tool identifying specific cell types, uses antibodies to tag and stain target cells. Common technical formats for immunophenotyping include flow cytometry and high magnification microscopy (immunohistochemistry). However, flow cytometry requires expensive instrumentation and high magnification microscopy can be insensitive. In contrast, immunophenotyping assays using the invention can be both sensitive and relatively inexpensive. This example uses the invention for a test that is key to determining the immune status, and thus the health, of patients with AIDS. The assay determines the concentration of $CD4^+$ T cells in blood. The example is technically similar to Example 8 except that in this example the target cells are human cells and that the sample is blood.

Preparing magnetic and fluorescent particles. CD4+-specific fluorescent and magnetic particles are prepared as in Example 8 except that CD4-specific antibody (Biodesign; cat # P54400M) is used here. The coated particles are mixed as in Example 8, and added (25 µl of the 1% suspension) to each of 6 wells of a 96-well microtiter dish with an optically clear bottom (*e.g.*, Greiner; catalogue # 655896) containing PBS-TB (175 µl).

Quantifying CD4+ cells in whole blood. Whole blood (5 µl) from a patient with AIDS is added to the particle mixture in each of 2 of the 6 microtiter dish wells (constituting duplicate experimental sample wells). Whole blood (5 µl) from a healthy donor (with known CD4+ cell concentration) is added to each of two wells (constituting duplicate positive control wells). Human serum (depleted of cells; Biochemed; cat. num. 758AB) is added to the third pair of wells containing the particle mixture (constituting duplicate negative control wells). Cells and particles are incubated at room temperature for 20 min. CD4+ cells that have associated with CD4+-specific magnetic particles are drawn to the bottom face of the wells using a magnet as in Example 8. The unbound material is removed by washing (3 washes; 200 µl PBS-TB each wash) while maintaining the magnetic field. The CD4+ cells are imaged and quantified as in Example 8. The efficiency of the test is determined by comparing the CD4+ cell count in the positive control to the known concentration. The background level of the assay is determined from the negative control wells.

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Example 31. A rapid homogenous immunoassay for Chlamydia trachomatis and Neisseria gonorrhoeae

Overview. The diagnostic assay developed in this example tests for two sexually transmitted pathogens, *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, which commonly cause urethritis. The infections have serious sequelae including pelvic inflammatory disease, infertility, and increased susceptibility to HIV infection. It has been estimated that about 150 million people were newly infected with one of these two pathogens in 1995 (almost 1 million people in the U.S. were infected in 1998). Almost 1 billion dollars per year is spent on testing for *C. trachomatis* and *N. gonorrhoeae*. Current testing uses methods range in complexity from Gram staining (for *N. gonorrhoeae*) to DNA amplification. Unfortunately, the tests tend to be either inexpensive and insensitive (*e.g.*, Gram staining, direct fluorescence assays) or sensitive and expensive (*e.g.*, nucleic acid amplification tests).

This example uses the invention to construct a test that inexpensively and sensitively detects, identifies, and enumerates *C. trachomatis* and *N. gonorrhoeae* in a urine sample.

Making pathogen-specific and control particles. The pathogen-specific particles are made using the procedures described in Example 8. Green fluorescent particles (FluoSpheres; catalogue number T-8803; Molecular Probes) and magnetic particles are coated with anti-*Chlamydia* antibodies (BioDesign; catalogue number C65641M). Red fluorescent particles (Molecular Probes TransFluoSpheres, cat# T-8861) and magnetic particles are coated with anti-*N. gonorrhoeae* antibodies (BioDesign; catalogue number C65618M). The positive and negative control particles are made as in Example 29.

Detecting and quantifying *N. gonorrhoeae* and *C. trachomatis* in urine. Urine samples (10 ml) collected using a commercial kit (B-D Urine Collection Kit (Becton-Dickinson, BD Vacutainer™ Brand urine collection cups). The urine is spun at 4000 x *g* for 10 minutes to and the supernatant removed. The pellet is resuspended in 400 µl PBS-B. Samples are mixed with particles, incubated, magnetically selected, imaged and analyzed as in Example 29. However, in this example 4 images are taken of the sample, three with the filter sets described in Example 29, and one with filters appropriate for imaging the red fluorescent particles (Chroma HQ617/40m). The number of yellow-green fluorescent particles indicates the number of *C. trachomatis* in the sample. The number of red fluorescent particles indicates the number of *N. gonorrhoeae* in the sample.

Variations. The test in this example can be extended to include scanning for other pathogens that can cause urethritis (*e.g., Trichomonas vaginalis, Ueraplasma urealyticum* and *Mycoplasma genitalium*). Additional targets can be detected and identified by using additional distinct signaling moieties (*i.e.,* particles with distinguishable fluorescence character) or a combinatorial labeling strategy. Quantitative testing for the presence of polymorphonuclear leukocytes, which is helpful in diagnosing urethritis, can be incorporated into the test by using particles coated with antibodies specific for these cells.

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Example 32. A rapid, multiplexed, homogenous, cytometry test for lower respiratory pathogens

Pneumonia. Pneumonia is the most common cause of death from infectious disease in the United States. The etiology of the disease is dependent on age and immune status. Viruses cause most childhood pneumonia, while bacterial pathogens are the most common pathogens causing adult pneumonia. The spectrum of pathogens that cause pneumonia in immunocompromised hosts varies greatly and differs for patients with cancers affecting the immune system or protective surfaces (mucosal or skin), transplant recipients, and HIV-infected patients.

For successful treatment of pneumonia, it is essential to rapidly identify the pathogen. However, current diagnostic practices are incapable of efficient pathogen identification. More than half of diagnostic efforts to determine the cause of pneumonia fail to identify the etiologic agent. (This does not include the large fraction of cases in which no attempt is made to identify the pathogen). Many bacterial and all viral and fungal pathogens that cause lower respiratory tract infections cannot be identified by routine microbiological culture methods. For example, special methods are required to identify the pathogens that cause tuberculosis, whooping cough, legionnaire's disease, and pneumonia caused by mycoplasma. Lower respiratory diagnostics are complicated by the difficulty in obtaining a lower respiratory sample that is uncontaminated with normal upper respiratory flora. Furthermore, normal flora that are harmless in the upper respiratory tract can cause pneumonia when aspirated by a patient. Discerning whether a strain identified by diagnostics is an upper respiratory contaminant or the etiologic agent requires careful sample quality control measures and microbiological quantification.

Pneumonia cases are generally considered in two classes: community acquired pneumonia and hospital acquired pneumonia. Community acquired pneumonia is a condition that causes about 20,000 deaths and that incurs more than $8 billion treatment costs annually in the United States. Hospital-acquired pneumonia is the most serious and the second most common type of hospital-acquired infection. More than 10% of patients in intensive care units get pneumonia while in the hospital. The fatality rate is high: about 1/3 of nosocomial pneumonia patients die of the disease. Rapid and accurate diagnostics is critical since the optimum life-saving antibiotic therapy depends on which of numerous potential pathogens are causing the disease. The broad range of potential pathogens, some of which are listed in Table 2 contributes to the challenge of lower respiratory diagnostics.

Patients with lower respiratory infections account for 75% of oral antibiotics prescribed in the United States. Nearly $1 billion a year is wasted on useless antibiotics, due to the failure of current diagnostics to identify the pathogen in most lower respiratory tract infections. Incorrect application of antibiotic therapy to viral infections is in large part responsible for the overuse of antibiotics and the current world-wide epidemic of antibiotic resistant pathogens. Thus, there is a great need for a single diagnostic assay that tests for a comprehensive set of lower respiratory pathogens.

Advantages of the test. In this example, the invention is used to make a homogenous immunoassay that is ultra-sensitive, quantitative, and highly multiplexed. By simultaneously testing for common bacterial, viral, and fungal pathogens, the method described here offers a substantial improvement over current practices. The sensitive immunoassay, with no culture, amplification, or enzymatic steps is user-friendly, cost effective, fast, and amenable to commercialization (see Example 8 for advantages of homogenous immunoassays). The test offers major improvements in diagnostic effectiveness leading to appropriate and timely antimicrobial therapy, ultimately saving many lives.

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Technical overview of the example. Figure 32 diagrams the scheme used in this example. A lower respiratory sample (*e.g.*, BAL or liquefied sputum) from a patient with lower respiratory infection symptoms is mixed with an ensemble of particles each of which is coated with antibodies that bind to a particular target pathogen. Each pathogen-specific particle is uniquely color coded by ratio labeling. An approach analogous to that used in Example 31 is used in this more highly multiplexed test.

Table 2. Common lower respiratory pathogens

Streptococcus pneumoniae	*Haemophilus influenzae*	*Mycoplasma pneumoniae*
Moraxella catarrhalis	*Chlamydia pneumoniae*	*Acinetobacter spp.*
Legionella spp.	*Staphylococcus aureus*	*E. coli*
Klebsiella spp.	*Serratia marcescens*	*Pseudomonas aeruginosa*
Proteus spp.	*Enterobacter spp.*	Respiratory Syncytial Virus
Adenovirus	Influenza A virus	Influenza B virus
Parainfluenza virus	Cytomegalovirus	*Candida albicans*
Aspergillus spp.	*Cryptococcus neofomans*	*Pneumocystis carinii*

Pathogen-specific antibodies. The category-binding molecules in this example are antibodies that specifically bind to the lower respiratory pathogens listed in Table 2. Antibodies for each pathogen are obtained, when possible, from commercial sources (*e.g.*, BioDesign, Biotrend Gmbh, Cologne, Germany, Fitzgerald Industries International, Inc., Concord, MA, and Accurate Chemical and Scientific Corporation, Westbury, NY)). Alternatively, antibodies can be obtained, affinity purified, and tested as described in Example 27.

Antibodies are tested for binding specificity and ability to label target pathogens. For obtaining optimal labeling, several antibodies specific for a particular pathogen are preferably compared to each other. The target pathogen (about 10^5 pathogens cells or virally infected cells in 10 µl PBS-T) are mixed with the category-specific antibody (about 10 µg in 10 µl PBS-T) and an excess (*e.g.*, 20 µg) of fluorescent labeled (*e.g.*, fluorescein) secondary antibody (*e.g.*, goat anti-mouse or goat anti-rabbit, depending on the source of the category-specific primary antibody) and incubated at room temperature for 20 minutes. Unbound antibodies are removed by washing 2 times in PBS-T (1 ml). Washing is accomplished by spinning the diluted organisms in a microcentrifuge (12,000 x *g*; 1 min). The fluorescently labeled target organisms are then imaged in a fluorescent microscope (Zeiss Axioplan 2) and recorded using imaging software (Image-Pro Plus, Media Cybernetics, Silver Spring, MD).

Using an analogous assay, antibodies that provide optimal labeling of target pathogens are tested for specificity by binding them to a panel of respiratory pathogens and commensal flora commonly found in the lower respiratory tract. Similarly, to insure that the antibodies do not react with endogenous components of the respiratory tract, each antibody is also tested for binding to panels of upper and lower respiratory samples from normal patients and patients with lower respiratory disease (that is known not to be caused by the target pathogen).

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Using these tests, antibodies are chosen that efficiently bind to the target pathogen, but not any other microorganism or component found in respiratory samples.

Constructing an ensemble of category-binding molecule-coated particles. The category-specific antibodies for each of the lower respiratory pathogens listed in Table 2 are used to coat two kinds of particles: polystyrene-coated magnetic particles and fluorescently-dyed polystyrene particles as in Example 8. In this example, however, each family of pathogen-specific antibodies is conjugated to fluorescent particles that are uniquely ratio-labeled. Thus, for each pathogen category, a paired set of category-specific particles is made that comprises magnetic particles and a coded set of fluorescent particles. The fluorescent particles used in this example are custom-made ratio-labeled sulfate-derivatized beads (Bangs Laboratories; combinations of 5 different concentrations of fluorescein (505/515) and Texas Red (595/615). The beads are labeled The particle ensemble is combined so that the final particle concentration is 2% solids (in PBS-TB). Positive and negative control particles are prepared and included in the particle ensemble as in Example 29, except that the control particles in this example are constructed using ratio-labeled particles, with color codings that are distinct from the pathogen-specific particles.

Detection, identification, and quantitation of the pathogens in the sample. Liquefied lower respiratory samples (200 µl BAL samples to which 1000 control phage have been added as in Example 29) are mixed with the particle ensemble (100 µl) in a microtiter dish. The samples are incubated, magnetically selected as in Example 8. Two images are collected using two filter sets appropriate for the two dyes used for ratio-labeling (for fluorescein: excitation Chroma HQ480/40x and emission Chroma HQ535/50m; for Texas Red: excitation Chroma HQ560/55x and emission (Chroma HQ645/75m). The images are aligned using Image-Pro Plus software. The fluorescent signature (*i.e.*, the ratio of the two dyes used for ratio-labeling) of each object found by the software is scored and compared to a look-up table (created empirically using the different classes of ratio-labeled fluorescent particles) that correlates fluorescent signature and pathogen-specificity. The number and classification of objects is tabulated using a customized software module created with the ImagePro Plus software package. The efficiency of the test is monitored by enumeration of the objects corresponding to the positive and negative control particles. The test background is estimated by analysis of a test run in parallel in which PBS (200 µl) is substituted for the BAL sample.

Chroma HQ560/55x) and emission (Chroma HQ645/75m) Chroma excitation 560/55, emission 645/75

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Example 33. An immunoassay that scans in parallel for numerous lower respiratory pathogens using non-magnified large area imaging

Overview. The medical goals and significance of this example are identical to those of Example 32. Here, as in that example, category-specific antibodies are used to simultaneously scan a lower respiratory sample for numerous diverse pathogens that cause pneumonia (including viruses, bacteria, and fungi). However, in this example, the sample is affixed to a glass slide. The identity of the lower respiratory pathogen is determined by parallel immunohistochemical analyses followed by imaging individual targets by non-magnified large area imaging. Figure 33 shows the scheme used in this example.

Indirect fluorescent assay of a lower respiratory sample. Smears of lower respiratory samples are prepared on glass slides and fixed as described previously (*e.g.*, Isenberg, ed., 1992, *supra*; section 9.5). To bind the different antibodies to different sections of the glass slides, adhesive wells (*e.g.*, adhesive silicone flexiPerm cell culture chambers, custom manufactured with 30 wells; IVSS; Sartorius) are affixed to the slides. The category-specific antibodies used in this example are described in Example 32. Each type of category-specific antibody (200 µl; 10 µg/ml in PBS) is added to a different well. As a negative controls, purified IgG from rabbit pre-immune serum (200 µl; 10 µg/ml in PBS; Rabbit IgG Reagent Grade, Sigma, Cat. No., I 5006) and purified IgG from mouse pre-immune serum (Mouse IgG Reagent Grade Sigma, Cat. No., 5381) are added to separate wells. Slides are incubated at room temperature for 30 min. Wells are washed with PBS (200 µl; 4 washes). The adhesive wells are then removed so that the entire slide can be efficiently processed as a single unit. After immersing the slide in PBS (in a Coplin jar) and then in distilled water, the slide is allowed to air dry. The sample is completely overlain with a mixture of goat-anit rabbit and goat anti-mouse biotinylated polyclonal secondary antibodies (5 µg/µl each polyclonal antibody in PBS) and allowed to incubate for 30 min at room temperature in a humidified slide chamber (Boekel). After rinsing (2 times in PBS; 1 time in distilled water; in a Coplin jar), the sample is completely overlain with streptavidin-coated fluorescent particles (Molecular Probes; cat. num. F-8780; 0.5% solids), incubated, and washed as in the previous step.

Imaging and analysis. The sample is imaged using a CCD-based imager as before (described in Step 6 of Detailed description section and shown in Figure 3). The imager acquires an image of each section of the slide corresponding to a well containing a pathogen-specific antibody or control antibody. Image analysis software (Image-Pro Plus) then calculates the total fluorescence and the number of fluorescently stained objects in each image. A well with significantly more fluorescent objects and fluorescent intensity than the negative control wells indicates the possibility of an infection caused by the corresponding pathogen.

Variations and related embodiments. It may be useful bundle tests in this compound assay (in additional wells) that test for commensal species normally found in the oral/pharangeal region that typically contaminate lower respiratory samples. It may also be useful to include antibodies specific for squamous epithelial cells (which can indicate the quality of the lower respiratory sample) and/or cells of the immune system (which can be informative about the likelihood of infection).

Other types of signaling moieties with the potential for attaining high signal complexity can be used in place of the ratio-labeled particles. For example, fluorophore labeled antibodies can be used analogously. Labeling two pools of each pathogen-specific antibody with two or more fluorophores can generate high signal complexities. The final antibody family used in the assay is created by mixing a unique ratio of the differentially

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labeled pathogen-specific antibodies. For example, the antibody family that is specific for pathogen X might have one part red fluorescent antibodies, one part yellow antibodies, and one part blue antibodies. The antibody family that is specific for pathogen Y might have two parts yellow antibodies, and one part blue antibodies, and so on. Note that the signal intensity of targets labeled with fluorescent antibodies will, in general be much reduced compared to the highly fluorescent particles used in the example. The signal intensity depends also on the number of antigens on the targets. Thus, for small targets, and other targets with relatively low numbers of category-specific binding sites, this method may require more sensitive signal detection instrumentation.

An approach analogous to the one in this example can also be used for an *in situ* nucleic acid hybridization-based test. In this case, cells would be prepared, fixed, and hybridized as described in Example 1 except that each family of category-specific probes would be labeled with biotin (using standard methods; *e.g.*, incorporation during PCR). After hybridization and washing the slides would be processed and imaged as in this example (*i.e.*, the steps that occur after removing unbound biotinylated secondary antibody).

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Example 34. Multiplexed identification of urinary tract infections without culture

Urinary tract infections (UTIs) are common – about 150 million cases per year – and represent healthcare costs in the billions of dollars. Confirmed diagnosis of UTI generally requires a urine culture which is time-consuming and, for some pathogens, not possible using standard microbiological methods and media (*e.g., Chlamydia trachomatis*). This example describes the use of the invention to construct a simple and sensitive UTI test that is highly multiplexed and does not require bacterial culture. The scheme of the test method is shown in Figure 34.

Antibodies that bind to common UTI pathogens. Table 3 indicates the cellular pathogens that commonly cause UTI. Antibodies that specifically bind to each pathogen are obtained and selected analogously to those in Example 27 and Example 32. In addition to the species-specific antibodies, antibodies that react to broad groups of bacteria are used in this example. Antibodies that bind to most gram-negative bacteria (*e.g.*, cat. #15306, QED Bioscience, Inc., San Diego, CA) and to most gram-positive bacteria (cat #15711, QED Bioscience, Inc., San Diego, CA) are included for detection of less common organisms for which species-specific antibodies are not included.

Table 3. Bacterial pathogens that cause urinary tract infections (UTI).

Escherichia coli	*Morganella* spp.
Staphylococcus saprophyticus	*Citrobacter* spp.
Klebsiella spp.	Group B streptococcus
Enterobacter spp.	Group D streptococcus
Proteus spp.	Enterococci
Chlamydia trachomatis	*Pseudomonas aeruginosa*
Neisseria gonorrhoeae	*Acinetobacter* spp.
Corynebacterium urealyticum	*Serratia* spp.

Attaching capture antibodies to a glass slide. Antibodies are covalently bound to aldehyde-containing glass slides (TeleChem International; SuperAldehyde Substrates). To bind the different antibodies to different sections of the glass slides, adhesive wells (*e.g.*, adhesive silicone flexiPerm cell culture chambers, custom manufactured with 24 wells; IVSS/Sartorius) are affixed to the slides. Each type of category-specific antibody (100 µg/ml in PBS; ~2.5 mm deep) is added to a different well and then processed as described previously (MacBeath, G., *et al.*, Science 289: 1760-3, 2000; including author supplied supplementary material on the world-wide web at http://www.sciencemag.org-/feature/data/1053284.shl). The silicone wells are removed after the free antibody is removed from the wells by washing and before the subsequent processing steps.

Direct binding of UTI pathogens in urine samples to immobilized capture antibodies. Urine samples are collected according to standard procedures (Isenberg, ed., 1992, *supra*). Samples (28 ml in 50 ml disposable polypropylene tube; Falcon) are neutralized by addition of 1M EPPS pH 8.0 (4 ml; sodium salt), 100 mM NaEDTA pH 8.0 (4 ml; sodium salt), and 10XPBS-TB (4 ml; see definitions). A microscope slide with bound capture antibodies is submerged in the urine sample and incubated at room temperature for 2 hr with gentle agitation (Bambino rotator; Boekel). During the incubation, bacteria contact and adhere to the section of the slide containing the corresponding capture antibodies. The feasibility of this assay is made possible by the fact that bacterial UTI infections generally occur at high pathogen titers (*e.g.*, $10^3 - 10^5$ cells/ml).

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Staining captured cells and imaging. The slide is removed from the urine sample, washed (3X; 50 ml PBS-TB ; 5 min; with gentle agitation; followed by a 50 ml EE wash; 15 seconds with gentle agitation), air dried, and heat fixed (5 min on the surface of a heat block heated to 100°C). The slide is then covered with a solution containing Syber Green I (1:1000 dilution of 10,000X stock solution; Molecular Probes; #S7563), incubated (10 minutes at room temperature), and washed (two 50 ml washes in EE;1 min each; with gentle agitation).

Each sector of the slide is analyzed by non-magnified large area fluorescent imaging using the imager shown in Figure 3A and described in Step 6 of the Detailed Description section above.

Alternative tests, methods, and formats. The test described in this example has numerous applications including, for example, identifying pathogens in respiratory, infected surgical wound, food, or environmental samples. Analogous assays could be constructed using alternative labeling techniques (*e.g.*, fluorescent particles, alkaline-phosphatase/antibody-coated particles, horseradish-peroxidase:antibody conjugates, fluorescent antibodies, RLS particles, magnetic particles), formats (*e.g.*, microfluidic or lateral flow and flow through formats, in which the capture antibodies are attached to bibulous membranes), and imaging methods (et *e.g.*, visual or reflectometer detection of chromogenic assays and film luminography of chemiluminescent and bioluminescent assays) many of which are described elsewhere in this application.

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Example 35. Rapid, multiplexed, identification of blood-borne viruses: HIV, HCV, HBV, and CMV

Detection of blood-borne viruses is an essential for testing individual patient samples and samples in blood banking applications. Serological tests (testing for antibodies that are specific for the target viruses) are commonly used. However, methods that directly test for the target virus have the advantage of detecting infected samples earlier in the infection process than serological methods. This is because antibodies specific for the pathogen generally do not appear in the blood of an infected individual (seroconversion) for three to four weeks. Unfortunately, direct tests for viruses are either insensitive (most antigen tests) or expensive (most nucleic acid tests).

This example describes use of the invention to construct a rapid, multiplexed test that can detect individual free virus particles in blood samples without using microscopy. The scheme of the test is displayed in Figure 35.

Coating fluorescent particles and wells with anti-viral antibodies. Antibodies specific for the target viruses can be purchased from numerous commercial sources. For example, the following antibodies are obtained from the indicated distributors: anti-HIV (anti-gp120; monoclonal; Biodesign; cat. # C65199M); anti-HCV (Biodesign; cat. # C8A024M); anti-HBV (anti-Hepatitis B Surface Antigen; monoclonal; Biodesign; cat. # C86132M); and anti-CMV ((anti-glycoprotein B; monoclonal; Biodesign; cat. # C8A024M).

Antibodies are bound to microtiter dish wells in four adjacent distinct spots per well (1 spot per antibody) by passive absorption. Each anti-viral antibody is spotted (1 µl; 1 µg/µl) in a well of a 96-well microtiter plate (Greiner America; cat. num. 55896) and incubated for 2 hrs at room temperature in a humidified chamber (Boekel Slide Moat; model 240000). Wells are then washed and blocked as described.

Color-coded virus specific fluorescent particles are made by coating fluorescently dyed polystyrene particles with distinct emission spectra with the anti-viral antibodies using methods described in Example 8. Fluorescent particles (Molecular Probes) are coded as follows: HIV-specific particles (Yellow-Green; cat #F8823); HCV-specific particles (Orange; cat #F8820); HBV-specific particles (Crimson; cat #F8816); and CMV-specific particles (Infrared; cat #F8818). The 4 types of antibody-coated particles are mixed at equal concentrations (10^7 particles/ml in PBS-T). Before use, the antibodies are sonicated and washed (Example 8). Note that, as the various antibodies are segregated in spots in the well, it is also possible to use the same type of fluorescent particle for each virus. However, use of distinct signaling moieties increases the robustness of the test.

Detecting viruses in blood samples. Blood samples are collected from various sources including, for example, from patients [using standard methods including treatment with the anti-coagulant EDTA as described in *e.g.*, (Isenberg, ed., 1992, *supra*)]; from units of donated blood; or from blood fractions (*e.g.*, plasma). Whole blood (200 µl) is added to a microtiter dish wells containing spots of each capture antibody. The sample is allowed to incubate at 37° for 2 hours to insure that viruses can adhere to capture antibodies on the well surface. The blood sample is removed from the well which are washed thoroughly (4X; 200 µl PBS-TB used for each wash). The mixture of virus-specific particles (200 µl) is added to the well and spun briefly (Beckman Allegra 6; GH-3.8 rotor; 1200g) to coat the bottom of the well with particles. After a brief incubation (10 min at room temperature) unbound particles are removed by washing with agitation (4 X 200 µl PBS-TB with vortexing; 1 min each wash). Finally, the wells are rinsed with EE (200 µl) and allowed to dry. The wells are then imaged and analyzed using a CCD imager as in Example 8 except that multiple images are acquired using the appropriate

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filter sets (yellow green: excitation Chroma HQ480/40x and emission Chroma HQ535/50m; orange: excitation Chroma HQ535/50X and emission Chroma HG610/75m; crimson: excitation Chroma HQ560/55x and emission Chroma HQ645/75m; and infared: excitation Chroma HQ710/75x and emission Chroma HQ810/90m). Viruses are identified by the spots to which bound particles adhere. Additional diagnostic robustness is provided by the fact that only particles of the expected color adhere to a particular spot if the assay is successful.

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Example 36. Ultra-sensitive lateral flow test for Influenza A virus using non-magnified large area imaging

Objective: This example demonstrates the use of the invention to rapidly detect low levels of virus with a user-friendly lateral flow assay format and non-magnified large area imaging. In the experiment described below, dilutions of Influenza A particles were applied to porous membrane strips, contacted with labeling particles in a conjugate pad, and moved by capillary action past capture antibodies for selecting virus:labeling particle complexes. These captured complexes were then visualized using large area non-magnified imaging.

Experimental Methods: The lateral flow test strips were assembled as described. Antibody lines were made by stripping both a Influenza A specific capture line (QED, cat. no. 1302) and control line (Jackson Immuno Research Laboratories, Inc.; biotin anti-mouse IgG, cat. no. 115-165-146) onto the membrane (5-10 mm from wicking pad). The lines were allowed to dry (at least 15 min) before use. Streptavidin labeled fluorescent beads (Bangs Laboratories Inc.; cat. no. CP01F-5121) were labeled with biotin anti-Influenza A antibody (Virostat; cat. no. 1307) by combining the beads (10 µl of 1.23 $X10^{11}$ stock), antibody (10 µl of 1.0 µg/ml stock) and PBS (80 µl) and mixing (1.5 hours/ room temp). The beads were then spun down (5000 g, 10 min) and resuspended in PBS-B (100 µl). The anti-Influenza A coated fluorescent beads (2 µl) were added to the conjugate pad of each strip. A stock of purified Influenza A (Advanced Biotechnologies Inc: Influenza A/ PR/8/34 (H1N1), cat. no. 10-210-000) was serially diluted using PBS-B. Test samples (100 µl of an IL-2 dilution) were combined with PBS-TB (50 µl) and added to the sample pad of the test strip. After running the assay (~15 minutes) the strips were imaging using non-magnified large area imaging.

Results: Figure 36 shows the results of lateral flow tests for Influenza A virus labeled with fluorescent labeling particles and analyzed using non-magnified large area imaging. The figure shows images of the capture and control lines from test strips onto which were applied samples containing (from left to right) 0, 10^5, 10^6,10^7 and 10^8 virion/ ml. The fluorescent signal increases with increasing concentration of Influenza A virions. The data shown shows the test can detect concentrations at least as low as 10^5 virions/ ml. This experiment therefore demonstrates the sensitivity of lateral flow tests based on the invention.

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Example 37. A rapid lateral flow test for M. tuberculosis using visual detection

Overview. Rapid lateral flow, or "strip", test formats are becoming increasingly important for offering point-of-care diagnostics (*e.g.*, in doctors' offices, emergency rooms, and home testing). However, lateral flow tests are often too insensitive to be used to detect infections caused by pathogens that may be present at low levels in clinical samples. For example, detecting *M. tuberculosis* infection in sputum requires detecting several thousand cells/ml of sputum – below the sensitivity threshold of current strip tests. There is a critical unmet public health need in the developing world for simple non-instrumented tests for *M. tuberculosis*. Although nucleic acid amplification tests can detect low titers of *M. tuberculosis*, these tests are too expensive and complex to be of use in most clinical settings.

This example describes an inexpensive, simple, fast, and highly sensitive lateral flow test for *M. tuberculosis*. The user interprets the test visually – without the use of an instrument.

Binding anti-*M. tuberculosis* antibodies and alkaline phosphatase to nanoparticles. Monoclonal anti-*M. tuberculosis* antibodies (MPB64-ICA; Abe, *et al.*, 1999, *supra*) and alkaline phosphatase (Pierce; 31391) in equimolar concentrations are bound to gold conjugate particles (30 nm; Nanoprobes; CG3054) by passive adsorption according to the manufacturer's recommendations and resuspended at 10^{11} particles/ml.

The lateral flow unit is designed and constructed using specifications, materials, and procedures that are understood by those skilled in the art of lateral flow testing. The design and construction issues are described in technical notes provided by manufacturer's of lateral flow tests (*e.g.*, *Millipore's Short Guide for Developing Immunochromatographic Test Strips*,2nd ed., Millipore, technical note # TB500, 1999; *Lateral Flow Tests*, 1st ed., Bangs Laboratories, technical note #303, 1999) and other literature (*e.g.*, Chandler, J, *et al.*, IVD Technology 6: 37-49, 2000; Weiss, Alan, IVD Technology 5: 48-57, 1999; Wild, D, ed. (2001). *The Immunoassay Handbood.* 2nd ed. New York, NY: Nature Publishing Groop, 2001)).

Lateral flow components are assembled from a kit (Millipore; catalog number HFMI-DAK015; Hi-Flow Plus Membrane Assortment Assembly Kit) according to the manufacturer's instructions. The antibodies in the test zone and the agents in the control zone are applied as stripes using a reagent dispensing apparatus (matrix 1600; Kinematics) according to the manufacturer's recommendations.

Liquefied sputum or BAL samples (200 µl; Example 27) are spotted onto the sample pad. The sample moves via capillary action through the membrane. After a brief period (3 min) PBS-T (200 µl) is applied to the sample pad to allow the sample to proceed through the membrane via capillary action. This PBS-T wash step is repeated after another brief period (3 min). After another 3 minutes, gold conjugates (10 µl; 10^9 particles coated with anti-*M. tuberculosis*/alkaline phosphatase) are then applied to the sample pad followed by two PBS-T washes (as before). Alkaline-phosphatase substrate (1 ml; BM purple; Boehringer Mannheim) is applied directly to the membrane and allowed to develop until spots are visible in the positive control zone (30 min - 1 hr) after which time the residual colorimetric detection reagent is poured off and water (1 ml) is applied directly to the membrane. After 3 min the water is poured off. If the test works correctly, there are about 1000 spots visible in the positive control zone. The number of spots in the test zone indicates the number of *M. tuberculosis* in the sample.

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Various alternative immunochromatographic configurations are also possible including "flow through" arrangements (see Example 23).

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Example 38. A rapid lateral flow test for detecting numerous disparate biowarfare agents using non-magnified large area imaging

Diagnosing infection due to agents of biowarfare presents several challenges beyond those encountered in routine infectious disease diagnostics. The agents that are likely to be employed in bioterrorism or biowarfare are expected to be different than those seen in normal medical practice and therefore may be difficult to recognize using standard diagnostic protocols. Diagnosing use of unconventional agents in the field requires scanning for disparate agents (including toxins, viruses, and bacteria) using portable user-friendly systems that can detect infection at early stages when titers are very low.

This example describes a lateral flow immunoassay that is rapid, portable, and user-friendly, and that can scan a sample for a broad range of agents present in low numbers in a clinical or environmental sample. Technically, the example resembles Example 36 except that numerous pathogens are detected in one assay and that chemiluminescent signals are detected electronically.

Table 4. Unconventional agents likely to be used in biowarfare or bioterrorism

Bacillus anthracis	*Shigella spp.*	Variola virus
Yersinia pestis	*Salmonella spp.*	Marburg virus
Francisella tularensis	*Vibrio cholerae*	Ebola virus
Brucella suis	Ricin toxin	
Coxiella burnetii	Botulinum toxin	

Nanogold-antibody conjugates specific for agents likely to be used in biowarfare or bioterrorism. The example scans a sample (*e.g.*, blood) for the agents listed in Table 4, some of which are bacteria, viruses, and protein toxins. Antibodies specific to each agent in Table 4 are obtained, affinity purified, and tested for specificity as described in Example 32 and Example 27. Note that for the protein toxins, monoclonal antibodies with distinct and non-overlapping epitopes are used for conjugation to nanogold particles and for the test zone (or capture zone) on the lateral flow membrane (see below).

Nanogold conjugates, specific for the agents in Table 4, are made by passive adsorption of each type of affinity purified antibody and equimolar alkaline phosphatase as described in Example 26. The resultant agent-specific conjugates in PBS-TB are mixed together in equimolar concentrations so that the final total conjugate concentration is solids in PBS-TB.

Lateral flow device. The lateral flow test in this example is constructed and implemented as is the assay in Example 36, except for the larger number of analytes tested, the different signal character (chemiluminescence in this example, colorimetric in Example 36), and the use of a conjugate pad in this example. Figure 38 shows the configuration of the lateral flow test. The figure has been simplified for clarity (*e.g.*, the test depicts an assay for only three of the agents in Table 4 and the three conjugates shown on the conjugate pad at the top of the figure represent a population of agent-specific conjugates). The disposable lateral flow device has a test zone and a control zone as in Example 36, but in this case the test zone contains parallel stripes of affixed agent-specific antibodies – one stripe for each agent-specific antibody. The control zone contains parallel stripes of affixed unconventional agents – one stripe for each target agent. The mixture of agent-specific conjugates are embedded in the conjugate pad as de-

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scribed in various references (*e.g.*, Millipore, technical note # TB500, 1999, *supra*; Bangs Laboratories, technical note #303, 1999, *supra*; Chandler, J, *et al.*, 2000, *supra*; Weiss, A., 1999, *supra*; Wild, 2001, *supra*).

Lateral flow assay. A liquefied sample (200 µl of *e.g.*, saliva, liquefied sputum, serum) is applied to the sample pad, followed by 3 applications of 200 µl PBS-TB to the sample pad (waiting 60 seconds between applications to the sample pad). The liquefied sample mobilizes the conjugates as it moves from the sample pad through the conjugate pad via capillary action (Figure 38). Any target agents that may be present in the sample bind to the corresponding nanogold:antibody:AP conjugates. The agent:nanogod complexes continue to move by capillary action until they encounter the corresponding agent-specific antibodies in the test zone stripe corresponding to the particular agent whereupon the agent:nanogold complexes become immobilized. Nanogold conjugates that have not bound to agents proceed to the control zone and bind to the corresponding stripe of control antigens.

The test strip is covered with CDP-Star (PE Biosystems) and allowed to incubate for 5 minutes. The strip is then imaged using a CCD imager (Orca II; Hamamatsu) as depicted in Figure 38. Individual agents (organisms, viruses, or proteins) are detected by virtue of the chemiluninescence emitted by the alkaline phosphatase coated particles to which the agent is bound. If the assay works correctly, all of the control stripes are coated with chemiluminescent particles. If one (or more) agents are present in the sample, the corresponding stripes will contain immobilized chemiluminescent particles. The identity of the agents are determined by the distance of the positive test stripe from the control stripes (the distances of each agent's test stripe from the control stripes is known). Imaging software (Image-Pro Plus) processes the image collected by the CCD camera using macros that quantify the number of spots in the test stripes and assign identity to the agent by measuring the distance from the control stripes.

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Example 39. A comprehensive test for respiratory pathogens using nucleic acid probes

Objectives and Advantages. In this example, a single assay comprehensively tests for the presence of a disparate array of common respiratory pathogens in a sample from a patient with symptoms of lower respiratory disease. The medical goals and significance of this example are identical to those of Example 32. This example, however, uses genomic DNA probes and an *in situ* hybridization format rather than antibody category-binding molecules and an immunoassay format.

Technical overview of the example. Category-specific sequences are isolated from various lower respiratory tract pathogens using genomic subtraction (bacteria and fungi), or computer analysis (viruses). A family of oligonucleotide probes is synthesized corresponding to the Category-specific sequences specific to each pathogenic group. Each family of category-specific probes is labeled with a distinct combination of fluorescent quantum dot labels. The entire probe ensemble (i.e., all of the probe families) is hybridized to the sample, which is fixed to a slide and then the unbound probes are washed away. Using a CCD camera, the identity of a pathogen in a clinical sample is determined by ascertaining which family of pathogen-specific probes – i.e. which combination of quantum dot labels – hybridizes to the microbes in the sample.

Isolating Category-specific sequences from pathogens that cause lower respiratory disease. Table 2 lists common pathogens that cause lower respiratory infections. The genomic subtraction method (Straus 1995, supra) is used to isolate category-specific sequences from the bacterial and fungal (i.e., non-viral) pathogens. The following section illustrates the subtraction strategy by describing the process for purifying and identifying diagnostic markers specific to *Streptococcus pneumoniae*. *S. pneumoniae* is by far the most common cause of pneumonia; it is responsible for about 30-50% of all cases of community acquired pneumonia (500,000 per year in the U.S.).

Using genomic subtraction to isolate a set of DNA fragments including *S. pneumoniae* category-specific sequences. Figure 39A shows the phylogenetic relationship between *S. pneumoniae* and its closest relatives (Kawamura, et al., International Journal Of Systematic Bacteriology 45: 406-8, 1995). The strategy for isolating category-specific sequences can be broken down into two steps: a genomic subtraction step (Figure 39B) and a screening step (Figure 39C). First, genomic subtraction (Straus, 1995, supra) is carried out using the DNA from the pathogenic strain (in this case *S. pneumoniae*) as the "+" genomic difference sample (Figure 39B). The "–" genomic difference sample is constructed by pooling several of the closest related strains – these strains do not commonly cause pneumonia. The resulting subtraction products are fragments that hybridize to the pathogen's genome but not to the genomes of the closely related species. These fragments are then cloned into the XhoI site of pBluescript I vector using the partial fill-in method (Corrette-Bennett, et al., Nucleic Acids Res 26: 1812-8, 1998).

Identifying subtraction products that are category-specific sequences. The next step is to isolate a subset of the *S. pneumoniae*–specific subtraction products that are category-specific, that is, that hybridize to all *S. pneumoniae* strains but that do not hybridize to any strains in other groups. Note that not all of the subtraction products are category-specific sequences. The set of subtraction products does contain all of the desired category-specific sequences fragments, but also contains some non-category-specific fragments. This is because the genomic subtraction experiment (see previous paragraph) selected for fragments that hybridize to a single *S. pneumoniae* strain (the "+" strain in the genomic subtraction) and that do not hybridize to single strains of several related species. Thus, for example, some of the subtraction products do not hybrid-

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ize to some other *S. pneumoniae* strains and some do hybridize to different strains of S. mitis than were used in the subtraction.

To identify the subset of subtraction products that are category-specific sequences, the cloned fragments are screened by hybridization to the genomic DNA of many different *Streptococcus* isolates. Because it is impossible to test a probe for hybridization to all *S. pneumoniae* strains, the best approximation is made by testing the probes for hybridization to hundreds of *S. pneumoniae* strains isolated from all over the world and that have been isolated over a period of several decades (for *S. pneumoniae* strains are obtained from collections archived at the American Type Culture Center, and the Center for Disease Control). Similarly, each probe is tested for hybridization to many non-*S. pneumoniae* strains of *Streptococcus*. Figure 39C shows a convenient method for testing whether a probe is a category-specific sequence. Genomic DNA is prepared from disparate isolates of *S. pneumoniae* and related *Streptococcus* species (using the method of Graves and Swaminathan Graves, et al. (1993). Universal Bacterial DNA isolation procedure. In Diagnostic molecular microbiology: principles and applications, D. Persing, T. Smith, F. Tenover and T. White, eds. (Washington, D.C.: American Society for Microbiology), pp. 617-621.), denatured, and spotted on a positively charged nylon filter (e.g., GeneScreen Plus, NEN) in the form of a dot blot array (Ausubel 1987, supra). (For species that are amenable to colony hybridization, rather than "dot blotting", strains can be grown and lysed in situ on nylon filters, thus avoiding purifying DNA from a large number of strains).

To test whether a cloned subtraction product is a category-specific sequence, it is hybridized to the arrayed genomic DNA. A clone is classified as a category-specific sequence if it hybridizes to genomic DNA from all of the *S. pneumoniae* strains but to none of the genomic DNA from related *Streptococcus* species (Figure 39C). The cloned subtraction products are labeled by incorporating digoxygenin during PCR amplification of the cloned recombinant plasmids. The standard PCR conditions are used (see definitions) with XhoL and XhoR primers (which directly flank the cloned subtraction products in the Bluescript vector) except that reactions are supplemented with digoxygenin-dUTP (100 μM; Boehringer Mannheim). Individual labeled probes are then hybridized to the dot blot arrays of genomic DNA using standard methods for high stringency hybridization (Ausubel 1987, supra) followed by detection using an alkaline-phosphatase:anti-digoxygenin antibody conjugate (Genius; Boehringer Mannheim) and CDP-Star (NEN) following the manufacturer's recommended protocol (Boehringer Mannheim). Chemiluminescent images are obtained using a CCD camera (ORCA II; Hamamatsu) housed in a light-tight enclosure (Alpha Innotech Corp., MultiImage II cabinet, Cat # DE-500-110) and analyzed using Image-Pro imaging software package (Media Cybernetics, Silverspring, MD).

Synthesizing a family of category-specific oligonucleotides. Next, oligonucleotide probes are synthesized that correspond to 30 of the category-specific clones identified by the dot blot analysis described above. First each of the 30 chosen *S. pneumoniae* category-specific sequences is sequenced. All DNA sequencing is carried out using an ABI 377 automatic sequencer from Perkin Elmer and using methods recommended by the manufacturer. For synthesizing oligonucleotide probes, 2 sub-sequences approximately 20 bases long from each of the 30 category-specific sequences are chosen using the program "Primer3" (S. Rozen, H. Skaletsky (1998) Code available at http://www-genome.wi.mit.edu/-genome_software/other/primer3.html) using the default parameter settings for internal hybridization oligonucleotide picking except that the minimum melting temperature is set to 59° and the maximum melting temperature is set to 61°.

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Each oligonucleotide is synthesized with an amino group modification at the 5' terminus (Midland Certified Scientific Reagent Co.). The amino-modified oligonucleotides is capable of being covalently attached to highly fluorescent quantum dots (see below).

Table 5. Combinatorial labeling using quantum dots. By combining 5 types of distinctly colored quantum dots, 31 ($2^5 - 1$) different combinations of colors are possible. For each of the 24 pathogens listed in top row of Table 2, a subset of the pathogen-specific oligonucleotides are bound to each type of quantum dot marked with a diamond in the column. Thus, a family of probes with a distinct signal signature is constructed for each pathogen. Note that 7 of the 31 possible combinations of the 5 label colors are not shown in the table (*i.e.*, the combinations corresponding to no colors, 1 color, and 5 colors).

	Streptococcus pneumoniae	*Haemophilus influenzae*	*Mycoplasma pneumoniae*	*Moraxella catarrhalis*	*Chlamydia pneumoniae*	*Acinetobacter spp.*	*Legionella spp.*	*Staphylococcus aureus*	*E. coli*	*Klebsiella spp.*	*Serratia marcescens*	*Pseudomonas aeruginosa*	*Proteus spp.*	*Enterobacter spp.*	Respiratory Syncytial Virus	Adenovirus	Influenza A virus	Influenza B virus	Parainfluenza virus	Cytomegalovirus	*Candida albicans*	*Aspergillus spp.*	*Cryptococcus neoformans*	*Pneumocystis carinii*
violet	♦	♦	♦	♦							♦	♦	♦	♦	♦	♦					♦		♦	♦
blue	♦				♦	♦	♦				♦	♦	♦				♦	♦	♦		♦	♦		♦
green		♦			♦			♦	♦		♦			♦	♦		♦	♦		♦	♦	♦	♦	
yellow			♦			♦		♦		♦		♦		♦		♦	♦		♦	♦	♦	♦	♦	♦
red				♦			♦		♦	♦			♦		♦	♦		♦	♦	♦		♦	♦	♦

Combinatorial labeling of category-specific oligonucleotide probe family with quantum dots. To achieve the goal of a comprehensive lower respiratory infection test, the *S. pneumoniae* probe family must be labeled in a way that distinguishes it from probe families corresponding to the other pathogens in Table 2. In this example, each probe family is labeled with a distinct combination of quantum dot labels. The principle of combinatorial labeling is shown in Figure 5. Quantum dots are nanometer-sized semi-conductor crystals that fluoresce brightly enough to be detected easily as single particles with simple and inexpensive instrumentation (Bruchez, *et al.*, Science 281: 2013-6, 1998; Chan, *et al.*, Science 281: 2016-8, 1998; Service, Science 281: 1930-1, 1998). In sum, the properties of quantum dots that are very useful for the invention include the fact that the emission spectrum is very narrow; the emission spectrum is determined by the size of the nanocrystal — by varying size, quantum dot labeling reagents with numerous distinct emission spectra are generated (important for this multicolor application); the luminescence is very high, so that single particles can be imaged; the size of the particles is small, comparable to that of a small protein (enhancing accessibility of probes to targets in fixed cells during *in situ* analysis); quantum dots can be covalently modified with biologically important molecules (*e.g.,* nucleic acids, proteins, biotin); and a single wavelength of light causes emission from quantum dots with different emission spectra.

To distinctly label each of the 24 category-specific oligonucleotide probe families (corresponding to the respiratory pathogen listed in Table 2) different combinations of quantum dots are used. By combining 5 types of distinctly colored quantum dots, 31 ($2^5 - 1$)

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different combinations of colors are possible (Table 2). From each set of pathogen-specific oligonucleoteides, 100 oligonucleotides are conjugated to each type of quantum dot marked with a diamond in the pathogen's column in Table 2. Thus, for *S. pneumoniae*, 100 different *S. pneumoniae* category-specific oligonucleotides are labeled with violet quantum dots. Similarly, 100 other *S. pneumoniae* category-specific oligonucleotides are labeled with blue quantum dots. The 200 quantum dot-labeled *S. pneumoniae* category-specific oligonucleotides constitute a *S. pneumoniae* category–specific probe family. *In situ* hybridization of the probe family to individual *S. pneumoniae* cells is detected as clusters of blue and violet quantum dots. Similarly, *Staphylococcus aureus* cells are detected as localized clusters of violet and green quantum dots (as indicated in column 2 of Table 2). Oligonucleotides are covalently bound to quantum dots (Quantum Dot Corp) using the manufacturer's recommended procedures.

An occasional oligonucleotide probe may not behave as a category-specific sequence, even though it is a sub-sequence of a category-specific sequence. This can occur due to patchy sequence divergence within the larger category-specific sequence. Confirmation that the oligonucleotides are category-specific sequences is achieved by hybridizing the quantum dot–labeled oligonucleotides to the dot blots as described above but using hybridization conditions appropriate for oligonucleotides (Ausubel 1987, *supra*). The dot blots are analyzed using an ORCAII CCD camera (Hamamatsu) with UV epi-illumination in a benchchtop enclosure (Alpha Innotech Corp., MultiImage II cabinet; Cat # DE-500-110) and imaging software (MetaMorph Universal Imaging Corporation, Downingtown, PA). Oligonucleotides in the probe set that do not behave as category-specific sequences (hybridizing to all *S. pneumoniae* strains but no other *Streptococcus* strains) are not included in the category-specific oligonucleotide probe family.

Note that other signal generating moieties can be substituted for quantum dots in this example. For example, carboxylate-modified fluorescently labeled particles (40 nanometer diameter TransFluoSpheres, Molecular Probes) can be attached to 5' terminal amino-modified oligonucleotides using EDAC chemistry (according to manufacturer's instructions). Five types of TransFluoSpheres with distinct emission spectra (560, 605, 645, 685, and 720 nm) but all with the same absorbance maximum (488 nm) are then used to label pathogen-specific oligonucleotides in the combinations analogous to the ones using 5 differently types of quantum dots in Table 5.

Alternatively, the larger cloned category-specific sequences can be labeled using a nucleic acid polymerase (*e.g.*, Klenow fragment of DNA polymerase I, reverse transcriptase, or Taq polymerase) in the presence of modified nucleotides. Examples of useful nucleotide modifications include nucleotides directly conjugated to fluorophores (*e.g.*, the Alexa series from Molecular Probes, or Fluorescein-12-dUTP (NEN Life Sciences; Cat. No., NEL413) nucleotides containing primary amines (which can be conjugated to fluorophores after incorporation into polynucleotides), and hapten modified nucleotides (*e.g.*, digoxygenin-dUTP; Boehringer Mannheim). For hapten-modified polynucleotides, the signaling moiety (*e.g.*, fluorescein-labeled anti-digoxygenin antibody) is often conjugated to the probe after the probe is hybridized to the sample. For combinatorial labeling using polymerase-labeled category-specific sequences, as for oligonucleotides, labeling schemes must use a set of distinguishable signaling moieties.

Isolating category-specific probes for other lower respiratory pathogens. Category-specific probe families for the other bacterial and fungal pathogens in Table 2 are made as described above for *S. pneumoniae*. However, as indicated above and in Table 5, each category-specific probe family is labeled with a different combination of quantum dots. Pathogens used for developing this test are obtained from the American Type Culture Collection (ATCC; Manassas, VA).

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Viral category-specific probe families are prepared similarly except that the viral category-specific sequences are isolated by comparison of publicly available genomic sequences. First the public databases (*e.g*, GenBank; http://www.ncbi.nlm.nih.gov) are scanned for sequences derived from a viral pathogen, *e.g.*, Influenza A Virus. These sequences are then imported and aligned in the program MegAlign (DNAStar). Category-specific sequences are chosen from regions that are invariant in the genomes and that have been sequenced from more than one isolate of the virus. Oligonucleotides are designed, synthesized, linked to quantum dots, and tested as described above for *S. pneumoniae*. Viral isolates are obtained for preparing genomic nucleic acid (for dot blots) from culture repositories such as the American Type Culture Collection (ATCC). For example, the ATCC maintains a large collection of Influenza A and Influenza B viruses. Nucleic acid from various isolates of a viral species is prepared for genomic dot blots by standard methods (see *e.g*,, Stephenson, *et al.*, eds. (1998). Diagnostic virology – Laboratory manuals. (Totaowa, NJ: Humana Press). 307 pp).

The pathogen-specific probe families are combined to create a lower respiratory pathogen probe ensemble. The probe ensemble is assembled in EE buffer (see definitions) so that the concentration of each probe is 10 nM.

Identifying pathogens present in a clinical sample. The pathogen-specific probe ensemble is used to scan a respiratory sample for 24 pathogens simultaneously (Figure 40). The preferred layout of the sample and controls is indicated in Figure 40. The microscope slide is etched with registration marks in the sample and control areas to facilitate alignment of images (see below).

A respiratory sample (*e.g.*, a sputum, bronchoalveolar lavage, or protected bronchial brush specimen) along with control targets are applied to a glass microscope slide and air dried. An internal control section on the slide is constructed on each slide, onto which are arrayed spots containing purified respiratory pathogens. The purpose of the control section of the slide is to confirm that hybridization proceeds correctly and to provide internal fluorescently labeled standards with which to compare the signal in the sample. Each of the 24 respiratory pathogens represented in the probe ensemble (listed in Table 2) is spotted (~250 organisms in 0.1 µl PBS) onto the slide and air dried. Samples and controls are then fixed using the method of Braun-Howland *et al* (Braun-Howland, *et al.*, Biotechniques 13: 928-34, 1992). Before hybridization, the nucleic acid in the fixed sample is denatured by covering the sample with 70% formamide at 75° for 10 min. The excess liquid is poured off, and the slide is rinsed in EE and allowed to air dry.

Next the probe ensemble is hybridized to the sample on the glass slide. Hybridization is carried out in HYB buffer (50 µl; see definitions) containing 5 µl of the probe ensemble (the final concentration of each probe is 1 nM). The hybridization solution is covered with a glass coverslip and incubated in a humidified slide moat (Boekel) at 50°C for 30 minutes. Slides are washed 5 times (30 seconds each wash) in HYB buffer at 50°. After cooling the slide to room temperature, they are rinsed with EE and air dried.

A CCD imaging apparatus similar to the one in Figure 3A (but without the automated stage) is used to acquire images of the sample and of the internal standards. The loading tray has 3 positions: open, first imaging position, and second imaging position. The slide, with bound quantum dot-labeled probes is inserted into the loading tray (in the open position) of the imaging apparatus with the internal standards section positioned towards the imager. The loading tray is moved to the first imaging position for viewing the internal standards section of the slide. After imaging the standards, the slide is moved into the second imaging position for analyzing the experimental sample.

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Image acquisition is preferably controlled by computer software (Image-Pro Plus). Five images are automatically acquired, one for each fluorophore signaling moiety. For imaging each fluorophore, the software automatically selects the correct excitation and emission filters, and collects the image according to user-defined parameters (*e.g.*, exposure time, intensity value of standards, *etc.*). The software constructs a composite image by combining information from each of the five images. The images are automatically aligned with the aid of registration marks (the marks are etched in the sample and control areas such that they are visible in each image). Alignment is necessary because successive images are generally offset slightly due to small mechanical perturbations during filter changes. The software then assigns a matrix of values corresponding to the intensity of each of the five images to each pixel in the composite image.

Software-based analysis of the standards has two main functions. First, it confirms that the experimental procedures are working correctly. Each of the 24 "spots" in the standards array contains individual cells or viruses of a target pathogen that have been treated identically to pathogens that might be in the experimental sample. The combination of signal moieties for each pathogen-specific family of category-binding molecules is known. Thus, the software checks whether the cells/viruses in each spot corresponds to the expected combination of signaling moieties. Furthermore, the number of pathogens observed in each spot is compared experimentally to the known number (about 250 organisms). If the correct number pathogens are labeled as expected by the bound probes, the experimental procedures are deemed have worked correctly. The software analysis includes comparing the results to rejection criteria, which can be set by the user. Experiments are rejected for reasons that preferably include failure of one or more pathogens to be labeled correctly or efficiently, excessive background (determined by analysis of sections of the image that have no pathogens), and poor signal to noise ratio. Values for background fluorescence assessed at this stage for each of the 5 images is preferably subtracted from the images of the standards and sample images.

The second function of analysis of the standards is to generate an accurate key for associating signal signatures with known pathogens. For each pathogen a matrix is constructed of expected values for each of the 5 images (*i.e.*, for each image collected with fluorophore-optimized exitation/emission filters). To establish the expected ranges for a pathogen in one of the images, intensity values for each of the ~250 organisms in a spot are tabulated. In preferred embodiments of the invention, threshold values are chosen based on the observed range of intensity values detected in the standards. Thus, for each pathogen, a set of expected ranges for each image is then constructed. A lookup table is also constructed that associates a pseudocolor with sets of values that fall within the ranges expected for a particular pathogen. The process of assigning pseudocolrs is similar to that used for karyotyping (Schröck, *et al.*, 1996, *supra*; Speicher, *et al.*, 1996, *supra*;).

The barcode for sample identification (constructed by applying black bars of various thicknesses) is preferably placed on the slide so that it is imaged simultaneously with the standards. Methods for barcode application and analysis are understood by average workers familiar with the art.

After analysis of the standards, the slide loader is moved to the second position, placing the sample section of the slide in the imaging area. The software drives the collection of 5 images of the sample, (automatically imaged as before for the standards). Using the lookup table constructed during analysis of the standards, pseudocolors are assigned to individual objects that correspond to the sets of color ratio values that fall within the expected ranges for a particular pathogen. The intensities for each type of pseudocolored objects are summed and divied by the average intensity value of a target in the

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control sample to obtain an estimate of the number of targets present in each sample. This first pass of the image analysis identifies and enumerates pathogens in objects that represent a single category of target. In a second pass, the software tests pixels registering ratios of values that are not consistent with multiples of any single target pathogen to determine if they have ratios that are consistent with registering more than one type of pathogen. Finally, the software displays for the user a diagnosis (*i.e.*, infection or no infection), the identities and numbers of pathogens in the sample, the sample ID number (extracted from the bar coded information on the slide), and composite images (with pseudocolored pixels) of both the standards and the sample.

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Example 40. Testing blood samples for numerous pathogens simultaneously using nucleic acid probes

Bloodstream infections. Pathogenic invasion of the cardiovascular system is one of the most serious infectious diseases. Of the approximately 200,000 bloodstream infections that occur every year in the United States, between 20 and 50 percent are fatal. Particularly at risk are immunocompromised patients, the very young and very old, those with skin or soft tissue infections and wounds, and the recipients of invasive medical procedures. All major types of pathogens can infect the bloodstream, including bacteria, viruses, fungi, and parasites. Rapid identification of a pathogen in a bloodstream infection is critical for instituting appropriate, potentially life-saving therapy.

The therapy of choice depends on the identity of the pathogen. Many tests and much expense can be required to determine the source of infection. However, promptly initiating the optimal therapy is often a life and death matter. Thus, there is a need for a single assay that rapidly determines the identity of a broad range of common bloodstream pathogens.

Overview of the example. The test described in this example scans for the presence of a broad range of bloodstream pathogens in a blood sample by using indirectly labeled pathogen-specific probes for *in situ* hybridization. By simultaneously testing for common bacterial, viral, and protozoan pathogens, the method offers a substantial improvement over current practices. The rapidity of the test (which does not require microbiological culture) makes it particularly useful for the critical task of quickly diagnosing bloodstream pathogens and for instituting appropriate and timely therapy.

Table 6. Pathogens that cause bloodstream infections.

Bacteria	Eukaryotes
Staphylococcus spp.	Plasmodium spp.
Staphylococcus aureus	*Leishmania donovani*
Viridans streptococci	Toxoplasma spp.
Enterococcus spp.	Microfilariae
Streptococcus spp.	*Histoplasma capsulatum*
Streptococcus pneumoniae	*Coccidoides immitis*
Escherichia spp.	*Cryptococcus neoformans*
Klebsiella spp.	Candida spp.
Pseudomonas spp.	
Enterbater spp.	**Viruses**
Proteus spp.	HIV
Bacteroides spp.	Herpes simplex virus
Clostridium spp.	Hepatitis C virus
Pseudomonas aueruginosa	Hepatitis B virus
Cornybacterium spp.	Cytomegalovirus
	Epstein-Barr virus

Assembling an ensemble of combinatorially labeled category-specific probes from pathogens that cause bloodstream infections. Table 6 lists some common pathogens that cause bloodstream infections. Category-specific sequences are isolated from the non-viral (*i.e.*, bacterial, fungal, and parasitic) pathogens using the representational difference analysis method, as modified by Tinsley *et al.* (1996, *supra*). This method is technically distinct from the one used in Example 39, yet it isolates subtrac-

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tion products with identical properties. The genomic difference samples are constructed as described in Example 39 and the products are cloned into the Bluescript II vector using standard methods (Ausubel 1987, *supra*) Pathogen-specific families of category-specific sequences are identified and sequenced as in Example 39.

Oligonucleotide probes are synthesized and tested as in Example 39. Combinatorial labeling using 5 types of spectrally distinct quantum dots is used, as in the previous example, using a scheme analogous to the one shown in Table 5. In this example, however, each oligonucleotide is designed with two moieties: a category-specific sequence and a tag sequence (Figure 6 and Figure 41). The tag moiety provides a means for indirect labeling of the oligonucleotide probes. To indirectly label a probe molecule, an anti-tag (or tag complement) sequence is labeled with a signaling moiety and is then hybridized to the bifunctional oligonucleotide probe (Figure 41). Five distinct tags are used, one for each color class. Suppose, for example, the "color code" for *S. aureus* is green, violet, and blue. About 100 *S. aureus*-specific probes are synthesized with the "green tag", 100 others with the "blue tag", and 100 others with the "violet tag". The tag sequences and category-specific sequences are designed so that the melting temperatures (T_m; calculated at 1M NaCl using the algorithm of Suggs (Suggs, S. V., *et al.*, Proc Natl Acad Sci U S A 78: 6613-7., 1981) are 60° C (± 1°) for each moiety and its complement. The tags are also designed so that they do not cross hybridize with each other, each other's complements, the category-specific oligonucleotides, or the complements of the category-specific oligonucleotides (*i.e.*, all such combinations of oligonucleotides preferably have melting temperatures ($T_{m(mismatch)}$) of 40°C (*i.e.*, $T_{m(perfect\ match)}$ – 20°C). Advantages of this strategy are that only 5 labeled anti-tag sequences need be synthesized (and they can be used for numerous applications) and that tags and anti-tags can hybridize to each other during hybridization to the sample. (Note that in alternative embodiments, a single universal tag sequence can be used on all oligonucleotides, or family-specific tags could be used).

Oligonucleotides are checked to make sure they behave as strict category-specific sequences by hybridization to dot blots of genomic DNA, as in Example 39. In this example, however, the probes are indirectly labeled with anti-tags that are coupled to the enzyme, alkaline phosphatase (Synthetic Genetics). Probes are hybridized to dot blots in HYB buffer at 52°C (a temperature equal to the melting temperature (T_m) of the oligonucleotide/genomic DNA (60°) hybrid minus 8°C). The blots are washed in the same buffer at the same temperature (3 10 min washes), rinsed briefly in a solution of 150 mM NaCl and 100 mM tris•HCl pH 9.5, and then overlain with CDPStar (NEN). Digital images of the blots are obtained as described in the previous example.

Thus, each set of oligonucleotides corresponding to a pathogen-specific probe family (each probe family hybridizes specifically to one of the 30 pathogens listed in Table 6) is labeled with a unique combination of fluorescent dyes using a combinatorial labeling scheme. The labeled probe families are combined to form a bloodstream pathogen-specific probe ensemble as in Example 39.

***In situ hybridization* of the ensemble of blood pathogen probes to blood samples.** Blood specimens are collected using standard methods (Isenberg, ed. (1992). Clinical microbiology procedures handbook. Washington, D.C.: American Society of Microbiology). Blood samples (0.5 ml) are prepared for *in situ* hybridization using the method of Lischewski *et al* (Lischewski, *et al.*, J Clin Microbiol 35: 2943-8, 1997). Adjacent to the blood sample are spotted an array of control spots as in the previous example, except that in this example the spots contain organisms from the bloodstream pathogens listed in Table 6. Slides are also barcoded as in the previous example. Hybridization and washing are carried out as in the previous example, except that in this example both the ensemble of probes (final concentration is 1 nM each probe) and anti-tags (equimolar to

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the corresponding concentration of the corresponding tag sequences in the ensemble of probes) are included.

The slides are imaged and analyzed as in Example 39.

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Example 41. A rapid concerted test for numerous central nervous system viruses using nucleic acid probes

Overview of the example. Infection of the central nervous system (CNS) is a medical emergency. Rapid diagnosis of the infectious agent is critical for optimum therapeutic outcome. Diagnosis of viral infection is particularly problematic and often expensive. The method described in this example can be used to test a cerebrospinal fluid (CSF) sample simultaneously for the presence of various types of viruses.

This example details a rapid highly multiplexed test that minimizes sample preparation, required instrumentation, and experimental complexity. CSF samples are spotted and fixed to a filter, denatured, and hybridized to an ensemble of viral probes (combinatorially labeled with quantum dots) using a lateral flow chromatographic method. The bound probes are imaged and identified by non-magnified large area CCD imaging.

Assembling ensembles of viral-specific sequences. Category-specific sequences are chosen that are specific for each of the groups of viruses in the panel of viruses listed in Table 7. In some cases, viral-specific category-specific sequences are already described in the literature. In other cases, sequences are chosen from viral genomic sequences in public databases after comparing the sequences to other viruses in the database. Sequence comparisons are made using standard methods (Ausubel *et al.*, 1987, *supra*). Category-specific oligonucleotides are chosen, synthesized, and labeled with quantum dots as in Example 39. The combinatorial labeling scheme is analogous to that shown in Table 5 except that the category-specific oligonucleotides from the viruses in Table 7 are conjugated to quantum dots. A central nervous system pathogen-specific probe ensemble is constructed by combining the various category-specific oligonucleotides in EE at a concentration of 10 nM (each oligonucleotide).

Table 7. Viruses that cause CNS infections.

coxsakievirus A	coxsakievirus B
herpes simplex virus	Togavirus
St. Louis encephalitis virus	measles virus
Epstein-Barr virus	Hepatitis
myxovirus	paramyxovirus
JC virus	mumps virus
Echovirus	equine encephalitis virus
Bunyavirus	Lymphocytic choriomeningitis virus
Cytomegalovirus	rabies virus
Varicella-zoster virus	BK virus
HIV	

Sample preparation. CSF is collected using standard methods (Isenberg, 1992, *supra*). Samples (1 ml) are spun briefly in a microcentrifuge (12,000X*g*; 1 min) to remove cells and particulate matter. Viruses in the supernatant (1 ml) are concentrated by spinning at 7,500g in a Biomax–5 grade Ultrafree-4 centrifugation unit (Cat # UFV4 510XB; Millipore, Bedford,MA). The retentate (10 µl) is heated at 95° C for 5 min to denature nucleic acid and spotted on a supported nitrocellulose strip (0.5 X 2 cm; 5 µm pore; Osmonics). In a region of the strip distinct from the sample, control samples of denatured genomic nucleic acid from each of the viruses in Table 7 ($1X10^5$ genomes each) laid down in adjacent stripes (as in a bar code) as described previously (Gravitt, *et al.*,

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Journal Of Clinical Microbiology 36: 3020-7, 1998). Reference stripes of ink are similarly applied

Capillary action-mediated hybridization. The quantum dot labeled probe ensemble is contacted with the denatured sample DNA of the sample and controls by using a paper chromatography hybridization assay described by Reinhartz *et al* (Reinhartz,1993, *supra*). Figure 42 diagrams the procedure used in this example. Probes in a hybridization solution are applied to one end of the strip allowed to flow past the immobilized denatured sample nucleic acid via capillary action. The hybridization solution is that used by Reinhartz *et al,* supplemented with 1% polyvinyl pyrrolidone, except that the DNA included in the solution is the bloodborne pathogen probe ensemble (the final concentration is 1 nM for each quantum dot oligonucleotide probe). The strips are dipped into the hybridization solution (100 µl) and hybridization chromatography is performed for 25 min at 50°. Samples are washed twice in 0.3% Tween/PBS for 5 min at 37°C and then allowed to air dry.

Identifying the pathogen by CCD imaging of filter strips. Pathogens are identified by imaging as in Example 39. Viral nucleic acid molecules in the sample hybridize to probes with a characteristic combination quantum dot labels. Each cluster of quantum dots is assigned a pseudocolor. Identification of the virus in the CSF sample is achieved by comparing the pseudocolor of the spots in the sample to the pseudocolors assigned to the control stripes (containing known viral genomes).

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Example 42. Rapid identification and antimicrobial susceptibility testing for *Mycobacterium tuberculosis* using genomic DNA probes

Overview. The medical significance of rapid susceptibility testing for *M. tuberculosis* is discussed in the overview of Example 28. This example differs from previous examples (Example 9, Example 27, and Example 28) in that *M. tuberculosis*-specific genomic DNA sequences are used as category-binding molecules rather than using *M. tuberculosis*-specific stains, non-specific stains, or antibodies.

The *M. tuberculosis*-specific genomic DNA sequences obtained by genomic subtraction and are modified by the hapten digoxygenin. Aliquots of a patient's sputum sample are incubated in growth media containing various antibiotics at various concentrations. After arraying samples on a filter, probing with hapten-labeled *M. tuberculosis*-specific sequences, the cells are enumerated using chemiluminescence and non-magnified large area CCD detection.

Preparing hapten-labeled *M. tuberculosis*-specific category-specific sequences. *M. tuberculosis*-specific sequences are isolated by genomic subtraction and dot blot screening as described in Example 39 and shown in Figure 40. For genomic subtraction, the plus genomic difference sample is *M. tuberculosis* and the minus genomic difference sample is *Mycobacterium avium*. To determine which of the cloned subtraction products are *M. tuberculosis*-specific sequences, the cloned products are used to probe 50 *M. tuberculosis* isolates (from disparate geographical locations) and 50 isolates of related *Mycobacterium* species in dot blots as in Example 39. The probes are labeled with digoxygenin and the dot blots are imaged as described in Example 39. Digoxygenin-labeled category-specific sequences (n=96) are pooled (final concentration of each probe is 250 pg/µl).

Testing a lower respiratory sample for *M. tuberculosis*. Sputum samples are prepared using the NALC-NaOH method (Isenberg, ed. (1992). The pretreated sputum is then applied (100 µl) to a nylon filter (GeneScreen; NEN) and prepared by placement on a series of filters as follows. The filter is successively laid (for 5 min each at room temperature, except where noted) on a stack several filters saturated with prelysis solution (see solution section) for 45 min at 37°C (wrapped in plastic wrap to prevent evaporation); a dry filter (Whatman 3MM); a filter saturated with a solution of 1% SDS/100 µg/ml proteinase K (Life Technologies) for 30 min at 37°C (wrapped in plastic wrap); a dry filter; a filter saturated with 0.5N NaOH; a dry filter; a filter saturated with 1M tris pH 7.5; and a dry filter. As a positive control, *M. tuberculosis* (~1000 organisms in 10 µl) is spotted adjacent to the sputum sample. As a negative control, *E. coli* (~1000 cells) is spotted adjacent to the positive control. *In situ* hybridization with hapten-labeled probes is carried out as described in Example 39. Anti-digoxygenin antibody conjugated to alkaline phosphatase (Genius kit; Boehringer Mannheim) is then bound to the hybridized probe, the filter is placed on a glass slide, and incubated with CDP-Star (NEN) according to the manufacturer's recommendations. The slide is imaged and analyzed using a non-microscopic CCD imager (Figure 3).

Incubating the clinical sample with various antibiotics. Antibiotic susceptibility testing is performed on samples that contain *M. tuberculosis* as in Example 28.

Arraying samples on a filter and preparing for hybridization. Aliquots of the cultured samples (100 µl) are arrayed on a gridded nylon filter using a disposable filtration device (Millipore; Microfil Funnel (100 ml), Cat # MIHAWG072) mounted on a vacuum flask. Samples (100 µl) are pipetted onto alternating grid squares while applying a vacuum (*e.g.*, using a Gast (Model; DOA-P104-AA) vacuum pump at 1/3 maximum vacuum

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level). The filter is removed and air-dried. The filter is then prepared for hybridization by laying on a series of filters as described earlier in this example

Hybridization, chemiluminescent imaging and analysis. *M. tuberculosis*-specific probes (digoxygenin-labeled above) are bound to the arrayed *M. tuberculosis* using high stringency hybridization and washing (Ausubel 1987, *supra*) followed by binding to an alkaline-phosphatase:anti-digoxygenin antibody conjugate (Genius; Boehringer Mannheim), and incubating with the chemiluminescent alkaline phosphatase substrate, CDP-Star (NEN) according to the manufacturer's recommended protocol (Boehringer Mannheim). Chemiluminescent images are obtained using non-magnified CCD camera-based imaging as above. The number of distinct objects and the integrated intensity in each sample spot are calculated as before in Description of invention, Step 6.

The numbers of targets in the experimental spots are compared to the negative and positive control spots to determine the amount of growth that occurred in the various antibiotic dilutions. A decrease in cell count of 45, 50, or 25% for samples containing ethambutol, isoniazid, and rifampin, respectively, indicates susceptibility to a given antibiotic regime (Moore *et al.*, 1999, *supra*).

The approach described above can be applied to other bacteria in the same fashion.

Rapid susceptibility testing by monitoring in-situ growth of microcolonies non-microscopically. Growth of microcolonies (*e.g.*, colonies consisting of between 2 and 1000 clonally related cells) on media containing various antibiotics can also be used to assess a strain's antibiotic susceptibility profile. In concept, this approach resembles the classical disk diffusion or agar dilution techniques or the newer fixed gradient method (*e.g.*, E-test). In all of these methods, cells are placed in solid growth media containing different concentrations of several antibiotics. To ascertain the antibiotic susceptibility profile of a strain, the bacterial isolate is scored for growth on the various antibiotics.

The invention can greatly accelerate acquisition of critical antibiotic susceptibility testing data using this approach For example, a disk diffusion susceptibility testing can be performed as recommended by the NCCLS (NCCLS Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard_Seventh Edition M2-A7 Vol. 20 No. 1 Jan. 2000). However, rather than plating the bacteria directly on agar plates, the bacteria are plated on nylon filters which are placed on agar media plates. Small filter disks (2.5 mm diameter) containing various antibiotics are placed on the nylon filters and the bacteria are allowed to grow for about 5 doubling times (usually several hours for fast growing bacteria). The filter is next placed successively (5 min at room temperature on each Whatman 3MM filter) on a dry filter; a filter saturated with 100% methanol; a dry filter; a filter saturated with propidium iodide (10 µg/µl in water); and a dry filter. A non-magnifying fluorescence imager, used in this example, is then used to directly visualize individual cells surrounding the disk. The diameter of the zone of growth inhibition is automatically measured by imaging software (Image-Pro Plus, version 4.1; Media cybernetics) analogously to the process of measuring the zone or halo around disks by eye in classical disk diffusion antibiotic susceptibility testing. Alternatively, cells can be imaged using methods such as pathogen-specific staining or category-binding molecules with light-scattering, or fluorescent, or chemiluminescent signaling moieties as described in this and other examples.

Rapid antiviral susceptibility testing. Many new antiviral compounds are currently being developed. The problem of antiviral resistance has come into prominence due to the rapid evolution of resistance in HIV. Susceptibility testing is thus becoming important. Although genotypic methods for determining resistance are becoming useful, assessing drug resistance by examining combinations of mutations in nucleic acid sequence is extremely complex and does not always correctly predict resistance. Unfortu-

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nately, phenotypic susceptibility testing on viral subculture is time consuming. However, using methods to monitor viral replication in cell cultures, analogous to those described for *M. tuberculosis* above, the invention could greatly improve the turnaround time for viral susceptibility testing.

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Example 43. A test for sexually transmitted disease pathogens using nucleic acid probes

Overview of example. This example exploits the invention to simultaneously test urine for two major sexually transmitted pathogens: *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. For more on the significance of the test refer to the overview of Example 31. Category-specific sequences from these strains are isolated using genomic subtraction (*Neisseria gonorrhoeae*) computer-based virtual subtraction strategy (*Chlamydia trachomatis*). Category-specific sequences are labeled with the haptens digoxygenin (*Chlamydia trachomatis*) or fluorescein (*Neisseria gonorrhoeae*) and hybridized to fixed cells that have been concentrated and immobilized on a filter. Intense signaling moieties, called resonance light scattering particles (RLS particles), are used to detect and identify the pathogens. The RLS particles, conjugated to anti-digoxygenin antibodies (red-scattering RLS particles) or anti-fluorescein antibodies (green-scattering RLS particles), are bound to pathogens in the sample. Because of the intensity of the signaling moieties, pathogens can be identified by simple visual inspection of illuminated samples. Red spots indicate a *Chlamydia trachomatis* infection and green spots signal a *Neisseria gonorrhoeae* infection.

Isolating *Chlamydia trachomatis* category-specific sequences using virtual subtraction. The existence of completed genome sequences for strains of *Chlamydia trachomatis* (Stephens, *et al.*, Science 282: 754-9, 1998), *Chlamydia pneumoniae* (Kalman, *et al.*, Nat Genet 21: 385-9, 1999) allow for computer-based determination of regions from one genome that differ from the other genome. This process is called virtual subtraction, by analogy to genomic subtraction experiments. The genome sequences, which are available in public databases (NCBI; http://www.ncbi.nlm.nih.gov/PMGifs/-Genomes/bacbt.html) are processed by software that aligns and compares whole genomes (MUMmer; Delcher, *et al.*, Nucleic Acids Res 27: 2369-2376, 1999). MUMer (using 20 bp for the minimum MUM length parameter) produces a detailed file depicting all of the gaps in the alignment. These gaps include insertion/deletions and highly polymorphic regions. Using the program Primer3 (S. Rozen, et al, 1998, *supra*) PCR primers were chosen for amplifying regions (~200– 500 bp) that occur in *Chlamydia trachomatis*, but not in *Chlamydia pneumoniae*. The PCR primers are designed with the bipartite structure shown in Figure 43. One primer has the XhoL primer sequence linked to a 20 bp primer corresponding to the "left" end of an insertion/deletion region in *Chlamydia trachomatis* (5'-XhoL-*Chlamydia trachomatis* left primer-3'). The other primer has the structure 5'XhoR-*Chlamydia trachomatis* right primer-3', where the *Chlamydia trachomatis* right primer is a 20 bp primer corresponding to the "right" end of the same insertion/deletion region. Using these primers, the virtual subtraction sequences are amplified from *Chlamydia trachomatis* cells (~10^5 cells in 10 µl EE; obtained from ATTC) after boiling the cells in EE buffer for 5 min. Amplification, products are labeled with digoxygenin-dUTP and screened for category-specificity using dot blot analysis as in Example 39.

Isolating *Neisseria gonorrhoeae* category-specific sequences using genomic subtraction. Potential *Neisseria gonorrhoeae* are isolated in this example using genomic subtraction as described in Example 39 (Straus, 1995, *supra*). The plus genomic difference sample is composed of a Sau3a digest of *Neisseria gonorrhoeae* genomic DNA and the minus genomic difference sequence is composed of sheared *Neisseria meningitidis* DNA. *Neisseria gonorrhoeae* and *Neisseria meningitidis* (obtained from ATTC) are grown in the appropriate medium (ATCC; 814 GC medium) and prepared using the CTAB method (Ausubel 1987, *supra*).The subtraction products are cloned, labeled, and screened against dot blots (containing DNA from various *Neisseria* species) as in Example 39. Fluorescein-labeled category-specific sequences are synthe-

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sized by amplifying recombinant plasmids using Fluorescein-12-dUTP (NEN Life Sciences; Cat. No., NEL413) and the primers XhoL and XhoR (see sequences in abbreviation section) according to manufacturer's recommended procedures.

Assembling a *Chlamydia trachomatis/Neisseria gonorrhoeae* probe mixture. Digoxygenin-labeled *Chlamydia trachomatis* category-specific sequences (n=96) are combined with fluorescein-labeled *Neisseria gonorrhoeae* category-specific sequences (n=96) (equal volumes of the amplification reactions are mixed together so that the final concentration of each probe is approximately 250 pg/µl).

Making probe-specific RLS signaling moieties. By binding RLS particles, which are easily visualized due to intense light scattering, to the bound probes individual pathogen cells are detected and identified. Two different types of RLS particles (prepared as described in US 6214560) are used to differentially label bound *Chlamydia trachomatis* probes and bound *Neisseria gonorrhoeae* probes. Red scattering RLS particles (160 nm diameter gold particles) are used to bind to the *Chlamydia trachomatis* probes, while green scattering RLS particles (60 nm diameter gold particles) are used to bind to the *Neisseria gonorrhoeae* probes. The red scattering particles are made specific for the *Chlamydia trachomatis* probes by conjugating anti-digoxygenin antibodies, (Roche, Cat. No. 1 333 062) to the particles as described previously (Hermanson, 1996. *supra*, and references therein). Similarly, the green scattering RLS particles are made specific for the *Neisseria gonorrhoeae* probes by conjugating anti-fluorescein antibodies (Roche, Cat. No. 1 426 320) to the particles. The red and green scattering RLS:antibody conjugates are mixed together at equal concentrations ($3.8X10^{-11}$M).

Preparing urine samples for *in situ* hybridization. Urine samples (10 ml) collected using a commercial kit (B-D Urine Collection Kit (Becton-Dickinson) and are filtered through a black polycarbonate membrane (0.22 µm; Osmonics) to concentrate bacteria that may be present. Bacterial DNA is denatured and made accessible by treating filters essentially as for colony lifts (Ausubel 1987, *supra*). The filter containing the sample is placed, in succession, on a filter (Whatman 3MM) saturated with 0.5M NaOH (5 min); a dry filter (5 min); a filter saturated with 1 M tris pH 7.5 (5 min); and a dry filter (5 min).

Control filters are prepared similarly. *Chlamydia trachomatis* (~1000 elemental bodies; ATTC) in 10 ml PBS are filtered through a filter and prepared as described for the experimental sample. *Neisseria gonorrhoeae* (~1000 cells) in GC media (10 ml) are similarly collected and prepared on a separate filter. Finally, a negative control filter containing *E. coli* (~1000 cells) in LB (10 ml) is prepared in the same fashion.

Note, that in alternative embodiments other substrates can be substituted for nylon filters (*e.g.*, glass slides, poly-lysine treated glass slides, silanized glass slides, nitrocellulose filters *etc.*). Also, urine samples can be concentrated by centrifugation (*e.g.*, at 12,000 X *g*, for 1 min) rather than by filtration. Alternatively, urine samples, in smaller volumes (*e.g.*, 100 µl), can, be applied to the substrates directly followed by immobilization and treatment to render the genomic DNA accessible.

Binding the hapten-labeled *Chlamydia trachomatis/Neisseria gonorrhoeae* probe ensemble to filtered urine samples and controls. Filters are hybridized in 1 ml HYB solution (see definitions) containing a 1:2 dilution of the probe ensemble mixture described above (final concentration of each probe is approximately 125 ng/µl). The reactions are carried out for 4 hours at 65°C in a in an incubator (Hybaid). Unbound probe is removed by washing twice (20 min each wash) in 0.2XSSC (100 ml; see definitions) in glass hybridization bottles (Hybaid).

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Binding probe-specific RLS signaling moieties to probe-labeled pathogen cells. Filters are "blocked" by incubation in BB (blocking buffer; 100 ml; see definitions) in a plastic tray (RubberMaid) on a rotating platform with gentle shaking (~60 rpm) at room temperature (~22°C). RLS particles (200 µl of the mixture from above) in 1XBB (1 ml) are added to the filters in a sealed pouch (Seal-A-Meal) and incubated at room temperature with gentle shaking for 1 hr. Unbound RLS:antibody conjugates are removed from the filters by washing twice in BB (100 ml) at room temperature with gentle shaking. Filters are air dried on glass slides prior to visualization.

Visual identification of pathogens in the samples. Light from the Xenon-arc lamp in the CCD imager (described in Step 6 of the Detailed Description) is trained on filters at an oblique angle to the plane of the filters. Two images are processed using excitation and emission filters that correspond to the two classes of RLS particles. The experimental procedures are evaluated by assessing the control filters. The *Chlamydia trachomatis* control filter should have ~1000 red spots, the *Neisseria gonorrhoeae* filter a similar number of green spots, and the negative control filter (containing *E. coli*) should not have green or red spots. Finally, the filter containing cells from the clinical urine sample is evaluated for the presence of *Chlamydia trachomatis* (red spots), *Neisseria gonorrhoeae* (green spots), a mixed infection (red and green spots), or no *Chlamydia trachomatis*/*Neisseria gonorrhoeae* infection (lack of a statistically significant number of spots over negative controls).

Other related applications. Many related clinically important diagnostic tests are easily developed along the same lines as the one described in this example. Tests for other urinary tract pathogens (*e.g.*, *E. coli*) can be constructed. Such tests can include a large number of pathogens, if combinatorial labeling is used. Tests for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (and other infectious agents of the uro-genital tract) that use samples collected by swabs represent important related applications (Isenberg, 1992, *supra*). For example, samples collected using urethral (male or female), cervical, and vaginal swabs are important for uro-genital pathogen diagnostics. Samples on swabs can be directly smeared on filters or slides in these examples. Such tests are also valuable for diagnosing important viral pathogens of the uro-genital tract (*e.g.*, Human Papilloma Virus, Herpes Simplex 1, and Herpes Simplex 2), fungi (*e.g.*, *Candida*), and parasites (*e.g.*, *Trichomomas vaginalis*). The methods used in this example (in conjunction with the appropriately modified sample collection techniques) are applicable to many different types of clinical samples (*e.g.*, surgical wound, blood, and respiratory samples). Furthermore, in conjunction with various types of category-binding molecules (*e.g.*, antibodies, probes for mRNA), similar methods can be applied to non-pathogen analytes including diseased tissue, serum proteins, and toxins.

Technical variations. Besides the variations in sample collection and substrate mentioned above, the invention can incorporate other useful technical variations. For example, an operationally simpler test can be constructed using fluorescent category-specific antibodies labeled with fluorophores or fluorescent particles. Such a test allows several steps (*e.g.*, hybridization) to be eliminated. Many types of signaling moieties can be incorporated (*e.g.*, fluorescent nanoparticles, quantum dots, and traditional fluorophores) and automated imaging using a CCD camera. Rather than restriction-fragment sized category-specific sequences, labeled oligonucleotides can also be used, as described in some of the other examples. High throughput applications of this example can incorporate microtiter filter plates (for example 384-well plates) in which each well contains a different sample. After the binding step, the labeled cells on each well's filter can be imaged using a microtiter plate reader or CCD imager capable of detecting light scattering.

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Other Embodiments

All patents, patent applications, and publications referenced in this application are hereby incorporated by reference. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims. Examples of other embodiments that may be adapted to the methods described herein are found in U.S. Application No. _____, entitled "RAPID AND SENSITIVE DETECTION OF REPLICATING CELLS", filed September 6, 2002 and U.S. Application No. _____, entitled "RAPID AND SENSITIVE DETECTION OF MOLECULES", filed September 6, 2002, each of which is hereby incorporated by reference.

Other embodiments are in the claims.

What is claimed is:

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CLAIMS

1. A method for detecting target cells or viruses in a sample, wherein said targets
   a. measure less than 50 microns in at least two orthogonal dimensions, and
   b. are randomly dispersed in a detection zone comprising a detection area at a density of less than 100 target cells per mm2,

wherein said method comprises the steps of:

   a. illuminating said target cells to generate a detectable signal, and
   b. simultaneously detecting said signal in a section of said detection area, wherein the longest linear dimension of said section is greater than 1 mm, using magnification of less than or equal to 20 times.

2. The method of claim 1, wherein prior to step (a) said targets are contacted with category-binding molecules under conditions that allow for specific binding of said category-binding molecules to category-specific binding sites on said targets to form complexes.

3. The method of claim 2, wherein category-binding molecules are labeled, either directly or indirectly, with one or more signaling moieties.

4. The method of claim 3, wherein said detecting detects said complexes.

5. The method of claim 1, wherein said sample is contacted with signaling moieties that associate either directly or indirectly with said target cells.

6. The method of claim 2, wherein said complexes are randomly dispersed in a detection zone at a density of less than 10 complexes per mm^2 of the detection area.

7. The method of claim 1, wherein said targets are randomly dispersed in a detection zone at a density of less than 1 complex per mm^2 of the detection area.

8. The method of claim 1 wherein said detection does not entail magnification of more than 5X.

9. The method of claim 8, wherein said detection does not entail magnification of more than 2X.

10. The method of claim 9, wherein said detection does not entail magnification of more than 1X.

11. The method of claim 10, wherein said detection does not entail magnification of more than 0.2X.

12. The method of claim 1, wherein said targets are bacterial or eukaryotic cells.

13. The method of claim 1, wherein said targets are viruses.

14. The method of claim 1, wherein the category complexity is .

15. The method of claim 14, wherein said targets.

16. The method of claim 1, wherein said target cells include cells from two or more of the taxonomic groups: viruses, bacteria, fungi, plants, multi-cellular animals, and protists..

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17. The method of claim 1, wherein said detecting detects and identifies more than one non-overlapping category of targets.

18. The method of claim 2, wherein said category-binding molecules bind specifically to natural or recombinant antibodies or aptamers.

19. The method of claim 2, wherein said category-binding molecules bind specifically to DNA, RNA, or PNA probes.

20. The method of claim 2, wherein said category-binding molecules bind specifically to sites immediately adjacent to nucleic acid polymorphisms including single nucleotide polymorphisms.

21. The method of claim 1, wherein said sample comprises a fluid or tissue obtained from a multicellular organism.

22. The method of claim 21, wherein said samples comprise the bodily fluids or tissues of an animal.

23. The method of claim 22, wherein said samples is derived from a human.

24. The method of claim 22, wherein said samples is derived from a non-human vertebrate.

25. The method of claim 22, wherein said sample is a member of, or derived from a member of the group consisting of: respiratory, urogenital, reproductive tract, central nervous system, urine, blood, dermal, plasma, serum, saliva, wound tissue, wound exudate, biopsy, feces, and solid tissue samples.

26. The method of claim 1, wherein said samples is derived from a plant.

27. The method of claim 1, wherein said samples is obtained by sampling environmental air or water, or surfaces, objects, or organisms exposed to the environment.

28. The method of claim 1, wherein said sample is chosen from, derived from, or obtained from the group consisting of: raw, finished or in-process material in the manufacture pharmacological, cosmetic, blood, or other products for topical or internal use in humans or animals; raw, in-process or finished material in the manufacture of foods or beverages; raw, in-process or finished material in the manufacture of medical or *in vitro* diagnostic devices; chemical products; industrial surfaces; instrumentation; and machinery.

29. The method of claim 1, wherein said method detects the effect of one or more substances or treatments on said target cells.

30. The method of claim 1, wherein a selection method is used to deposit said targets on said detection surface, and wherein said selection method is from the group consisting of magnetic selection, centrifugation, settling, and filtration.

31. The method of claim 30, wherein said method comprises contacting said sample with magnetic particles that are conjugated to category-binding molecules.

32. The method of claim 30, wherein targets are deposited in said detection zone using one of said selection methods without using a selection moiety.

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33. The method of claim 30, wherein said targets are contacted in a liquid with target-specific selection moieties that have an average density greater than the average density of said liquid and are subsequently deposited on said detection surface using gravitational, centrifugal, or centripetal force.

34. The method of claim 1, wherein said sample is treated to liquefy and/or homogenize said sample.

35. The method of claim 1, wherein said contacting occurs in the liquid phase.

36. The method of claim 1, wherein said contacting occurs at the interface between a liquid and solid phase.

37. The method of claim 1, wherein said sample is treated to remove substances or objects other than said targets.

38. The method of claim 2, wherein said targets are immobilized on said detection surface prior to said contacting.

39. The method of claim 1, wherein said targets are specifically bound in the detection zone by category-binding molecules that are bound to the matrix or substrate of the detection zone.

40. The method of claim 1, wherein said targets are specifically bound in the detection zone by forming chemical bonds to matrix or substrate of the detection zone.

41. The method of claim 1, wherein said targets are immobilized in said detection zone by a process selected from the group consisting of air drying, heat fixation, and chemical fixation.

42. The method of claim 2, wherein said sample is treated so that the category-specific binding sites on said targets become accessible to contact by said category-binding molecules.

43. The method of claim 3, wherein unbound signaling moieties are removed from said complexes before said detecting.

44. The method of claim 3, wherein said signaling moieties have photonic signaling character, and wherein a colloidal or soluble substance is added to absorb the signal emitted by signaling moieties that are not in said detection zone.

45. The method of claim 1, wherein said sample is subdivided into individual aliquots that are tested, in parallel, for the presence of different non-overlapping categories of targets.

46. The method of claim 45, wherein each of said aliquots is contacted with a population of labeling particles that is conjugated to a different non-overlapping family of category-binding molecules.

47. The method of claim 45, wherein said sample is contacted successively with distinct families of category-binding molecules that specifically bind to non-overlapping categories of target-entities.

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48. The method of claim 1, wherein said detection zone comprises material selected from the group consisting of solid glass, solid plastic, the surface of the wells of microtiter plates, bibulous membranes, plastic strips, the surfaces of capillary tubes, the surfaces of microfluidic chambers, and the surfaces of microfluidic channels.

49. The method of claim 1, wherein said method includes lateral flow chromatography.

50. The method of claim 1, wherein said method is automatically repeated on a series of samples.

51. The method of claim 50, wherein said samples are automatically loaded into an instrument that contains the means for said detecting.

52. The method of claim 50, wherein said samples are automatically deposited in a series of detection zones that are physically associated and that are automatically and successively loaded into an instrument that contains the means for said detecting.

53. The method of claim 3, wherein said method detects light emitted, scattered, reflected, or absorbed as a result of said illumination of said complexes.

54. The method of claim 1, wherein said detecting detects fluorescence.

55. The method of claim 1, wherein said detecting detects chemiluminescence. non-illuminated!

56. The method of claim 1, wherein the means for illuminating comprises one or more lasers.

57. The method of claim 1, wherein the means for illuminating comprises one or more light-emitting diodes.

58. The method of claim 1, wherein the means for illuminating comprises a source of white-light.

59. The method of claim 1, wherein the means for illuminating comprises one or more optical filters adapted for illuminating said sample with light of a wavelength appropriate for detecting said complexes.

60. The method of claim 3, wherein the means for illuminating comprises one or more optical filters adapted for illuminating said sample with light of a wavelength appropriate for detecting said signaling moieties.

61. The method of claim 3, wherein the means for detecting said emitted, scattered, transmitted, or absorbed light comprises optical filters adapted to detect the signals derived from the illumination of said signaling moieties.

62. The method of claim 1, wherein said detecting detects thermal radiation.

63. The method of claim 1, wherein said detecting detects optical absorbance.

64. The method of claim 63, wherein said optical absorbance is in the infrared region.

65. The method of claim 1, wherein said detecting detects fluorescence.

66. The method of claim 1, wherein said detecting detects cellular autofluorescence

67. The method of claim 1, wherein said detecting detects fluorescence polarization.

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68. The method of claim 1, wherein said detecting detects optical reflectance.

69. The method of claim 1, wherein said detecting detects light scattering.

70. The method of claim 1, wherein said detecting detects Raman scattering.

71. The method of claim 2, wherein said category-binding molecules are conjugated directly or indirectly to labeling particles.

72. The method of claim 71, wherein said labeling particles are less than 20 microns in size.

73. The method of claim 71, wherein said labeling particles are less than 10 microns in size.

74. The method of claim 73, wherein said labeling particles are less than 5 microns in size.

75. The method of claim 74, wherein said labeling particles are less than 1 micron in size.

76. The method of claim 75, wherein said labeling particles are less than 100 nm in size.

77. The method of claim 76, wherein said labeling particles are less than 10 nm in size.

78. The method of claim 71, wherein said labeling particles comprise enzymatic signaling moieties at an average density of greater than or equal to 2 enzymatic signaling moieties per cubic micron of particle volume.

79. The method of claim 71, wherein said labeling particles comprise signaling moieties are particles dyed with or conjugated to signaling moieties that have fluorescent signal character and that are selected from the group consisting of: organic fluorophores, up-regulated phosphors, lanthanides, quantum dots, and enzymes that generate fluorescent product form non-fluorescent substrates.

80. The method of claim 71, wherein said labeling particles are latex particles, silica particles, quantum dots, resonance light scattering particles, up-converting phosphors, or particles composed chiefly of gold or silver.

81. The method of claim 3, wherein said signaling moieties are enzymatic signaling moieties.

82. The method of claim 81, wherein said signaling moieties are alkaline phosphatase or horseradish peroxidase enzymes.

83. The method of claim 3, wherein said signaling moieties are selected from the group consisting of organic fluorophores, up-regulated phosphors, lanthanides, quantum dots, and enzymes that generate fluorescent product from non-fluorescent substrates.

84. The method of claim 3, wherein said signaling moieties have fluorescent signaling character.

85. The method of claim 3, wherein said signaling moieties have chemiluminescent signaling character.

86. The method of claim 85, wherein said molecules are acridinium esters.

87. The method of claim 3, wherein said signaling moieties have chromogenic signaling character.

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88. The method of claim 3, wherein said signaling moieties have light-scattering signaling character.

89. The method of claim 2, wherein said category-binding molecules comprise antibodies.

90. The method of claim 2, wherein, wherein said category-binding molecules comprise aptamers.

91. The method of claim 2, wherein, wherein said category-binding molecules comprise nucleic acids or peptide nucleic acids.

92. The method of claim 2, wherein, wherein said category-binding molecules comprise ligands.

93. The method of claim 2, wherein, wherein said category-binding molecules comprise molecules with molecular weights less than 100 kD.

94. The method of claim 93, wherein said category-binding molecules comprise molecules with molecular weights less than 10 kD.

95. The method of claim 94, wherein said category-binding molecules comprise molecules with molecular weights less than 1 kD.

96. The method of claim 2, wherein said sample is contacted with an ensemble of said category-binding molecules, wherein said ensemble comprises one family of category-binding molecules specific for each non-overlapping category of target entities to be detected.

97. The method of claim 96, wherein all of said families of category-binding molecules are labeled with signaling moieties that emit signals of the same signal character and signal class.

98. The method of claim 96, wherein each of said families of category-binding molecules is labeled with signaling moieties that emit signals of a distinct signal class.

99. The method of claim 96, wherein said ensemble of category-binding molecules has a family complexity of 1.

100. The method of claim 96, wherein said ensemble of category-binding molecules has a family complexity that is greater than 1.

101. The method of claim 100, wherein said ensemble has a family complexity ≥ 5.

102. The method of each of claims 101, wherein said ensemble has a family complexity $\geq$ 10.

103. The method of each of claims 102, wherein said ensemble has a family complexity $\geq$ 20.

104. The method of claim 96, wherein said ensemble contains at least ten distinct types of category-binding molecules.

105. The method of claim 104, wherein said ensemble contains at least fifty distinct types of category-binding molecule.

106. The method of claim 96, wherein each category-specific family of category-binding molecules contains at least two distinct category-binding molecules.

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107. The method of claim 106, wherein each category-specific family contains at least 10 distinct category-binding molecules.

108. The method of claim 96, wherein said non-overlapping categories of target cells are identified by their differential localization in said detection area.

109. The method of claim 96, wherein each family of category-binding molecules has the same signal signature.

110. The method of claim 96, wherein said non-overlapping categories of target cells are identified by detection of and discrimination between the distinct signal signatures conferred on said category-specific families of category-binding molecules by said signaling moieties.

111. The method of claim 1, wherein said **labeling particles** comprise different populations, wherein each population is conjugated to a different non-overlapping family of category-binding molecules.

112. The method of claim 96, wherein each of said families binds specifically to a category of targets that also binds specifically to a family of category-binding molecules that is stably bound to a section of the detection zone, wherein each of said families that are bound to the detection zone are bound distinct sites, wherein said sites can be distinguished by said detecting.

113. The method of claim 96, wherein each of said families has the same signaling class and signature.

114. The method of claim 96, wherein each of said populations has a distinct signaling signature or signaling class.

115. The method of claim 96, wherein said method comprises optical filters adapted to discriminate between the signal signatures of said families of category-binding molecules.

116. The method of claim 96, wherein said families of category-binding molecules have a family complexity that is greater than 1.

117. The method of claim 96, wherein said families of category-binding molecules have a family complexity ≥ 5.

118. The method of claim 96, wherein said families of category-binding molecules have a family complexity ≥ 10.

119. The method of claim 96, wherein said families of category-binding molecules have a family complexity ≥ 20.

120. The method of claim 1, wherein said detecting occurs in a vessel that is a bar code or equivalent label for tracking the sample automatically.

121. The method of claim 1, wherein said detecting occurs on a surface with registration marks to facilitate alignment of multiple images of the same surface.

122. The method of claim 1, wherein said method detects control marks or control cells in a region of the in a specified region of the detection zone.

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123. The method of claim 53, wherein the means for detecting said emitted, scattered, transmitted, or absorbed light comprises optical filters adapted to detect the signals derived from the illumination of said **complexes**.

124. The method of claim 1, wherein said detecting comprises use of a photoelectric detector.

125. The method of claim 1, wherein said detecting comprises use of a photoelectric array detector.

126. The method of claim 125, wherein said photoelectric detector comprises a CCD or CMOS detector.

127. The method of claim 53, wherein said means for detecting said emitted, scattered, or absorbed light does not include an image intensifier.

128. The method of claim 1, wherein said detecting comprises use of a photomultiplier tube detector.

129. The method of claim 1, wherein said detecting comprises use of a photodiode detector.

130. The method of claim 1, wherein the means for detecting and identifying said targets comprises photosensitive film.

131. The method of claim 1, wherein the said detecting comprises direct visual detection.

132. The method of claim 1, wherein the number of targets is inferred from said detecting by analyzing images acquired by said detecting.

133. The method of claim 1, wherein the category of targets is inferred from said detecting using image analysis software.

134. The method of claim 133, wherein said image analysis software further comprises functions for discerning the signals generated by said complexes from other signals.

135. A method for detecting target cells in a sample, wherein said target cells

a. measure less than 50 microns in at least two orthogonal dimensions, and

b. are randomly dispersed in a substantially planar detection area at a density of less than 100 target cells per mm^2,

c. wherein said method comprises using a photoelectric detector that does not include an image intensifier to simultaneously detect said target entities, without illumination, in a section of said detection area, wherein the longest linear dimension of said section is greater than 1 mm.

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136. A method for detecting target cells in a sample, wherein said target cells

a. measure less than 50 microns in at least two orthogonal dimensions, and

b. are randomly dispersed in a substantially planar detection surface comprising a detection area at a density of less than 100 target cells per mm^2,

c. wherein said method simultaneously detects said target cells in a section of said detection area, wherein the longest linear dimension of said section is greater than 1 mm, and wherein said detection does not comprise:

i. using a photodetector, or

ii. illuminating said detection area

iii. cell or viral culture.

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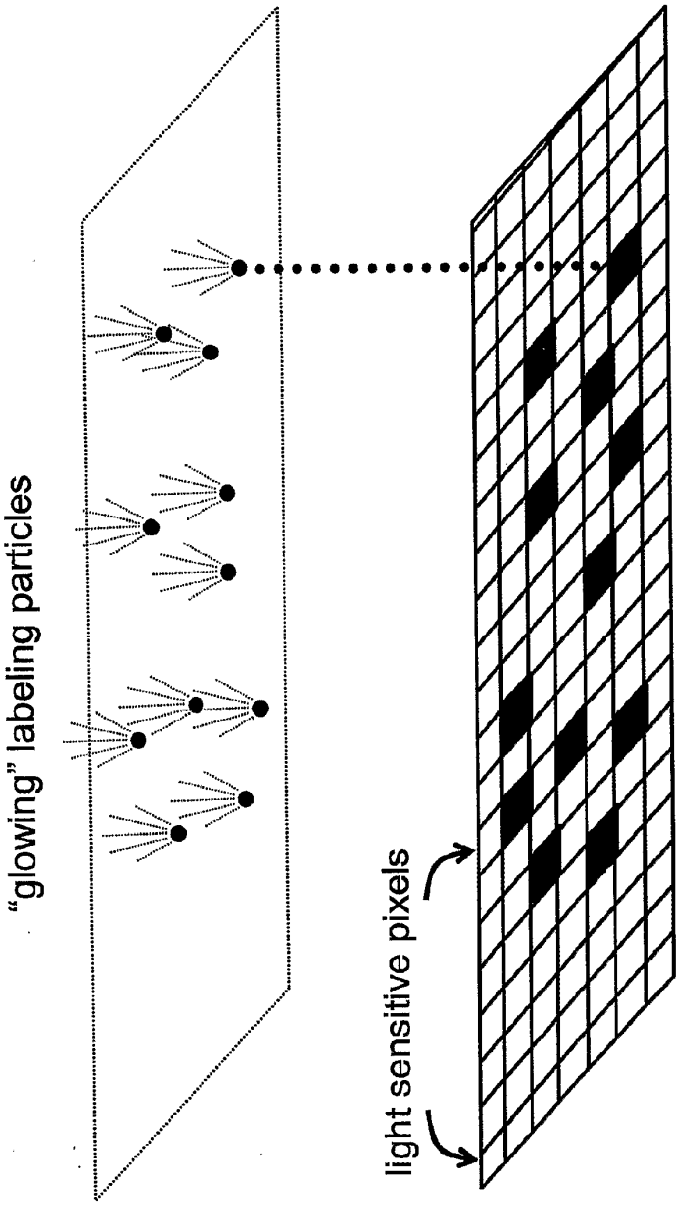


Figure 1. Detecting individual labeling particles using a CCD array.

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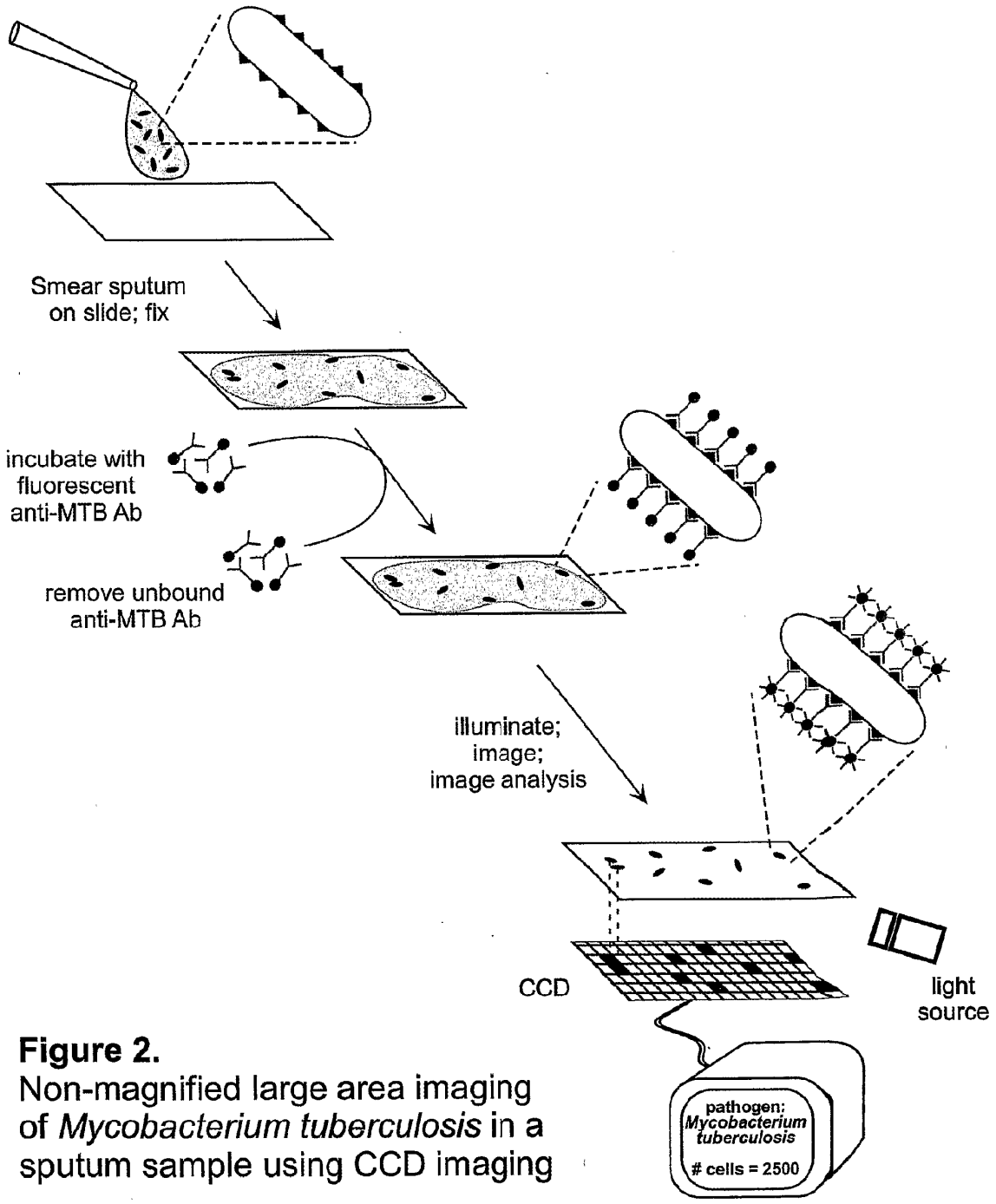


Figure 2.
Non-magnified large area imaging of *Mycobacterium tuberculosis* in a sputum sample using CCD imaging

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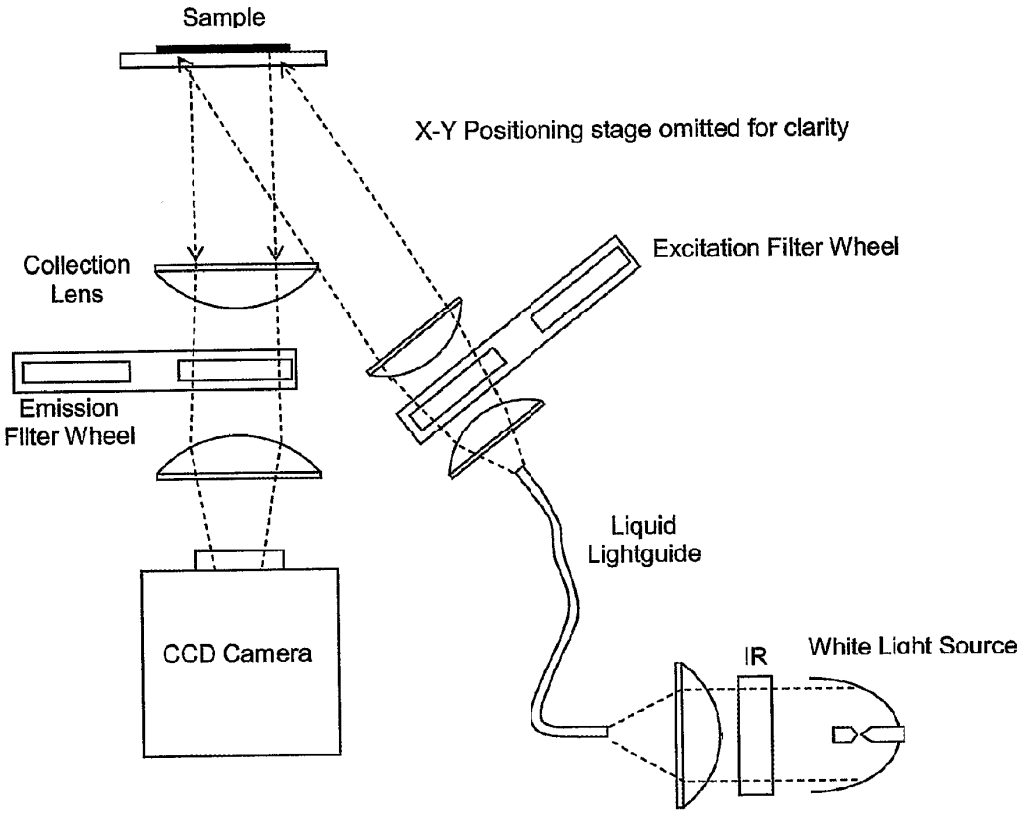


Figure 3. A CCD imaging device for large area imaging.

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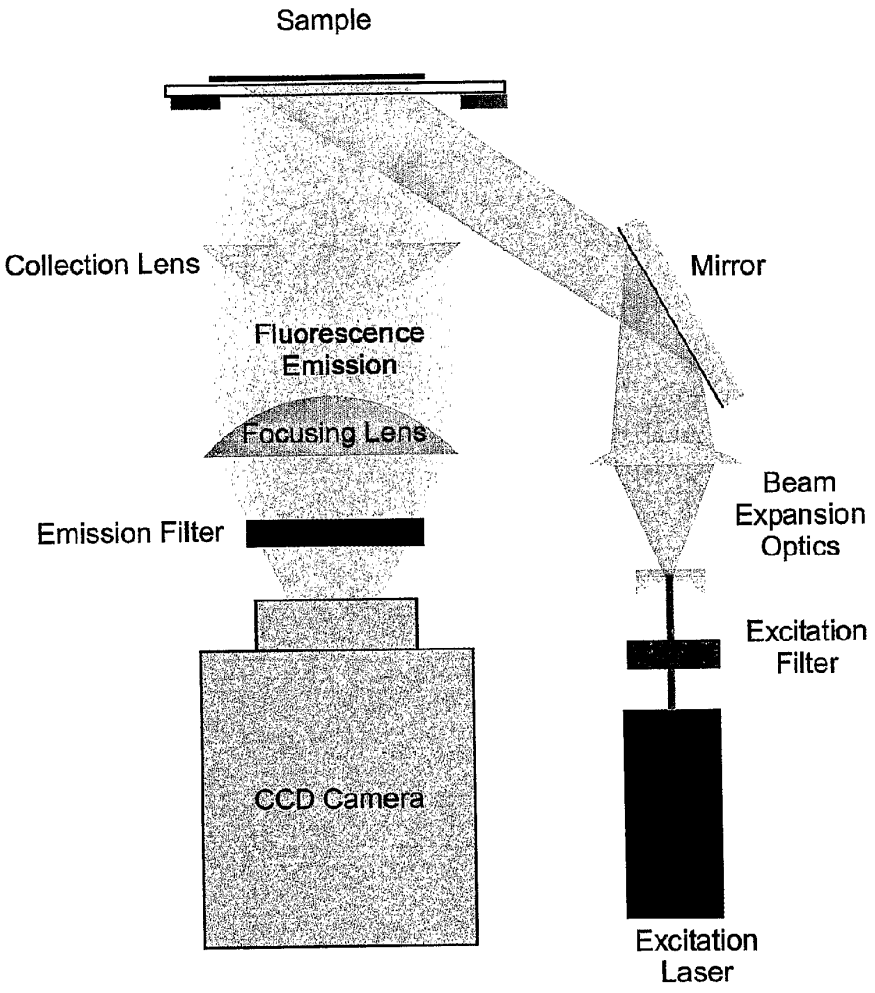


Figure 4. A CCD imaging system for non-magnified large area imaging.

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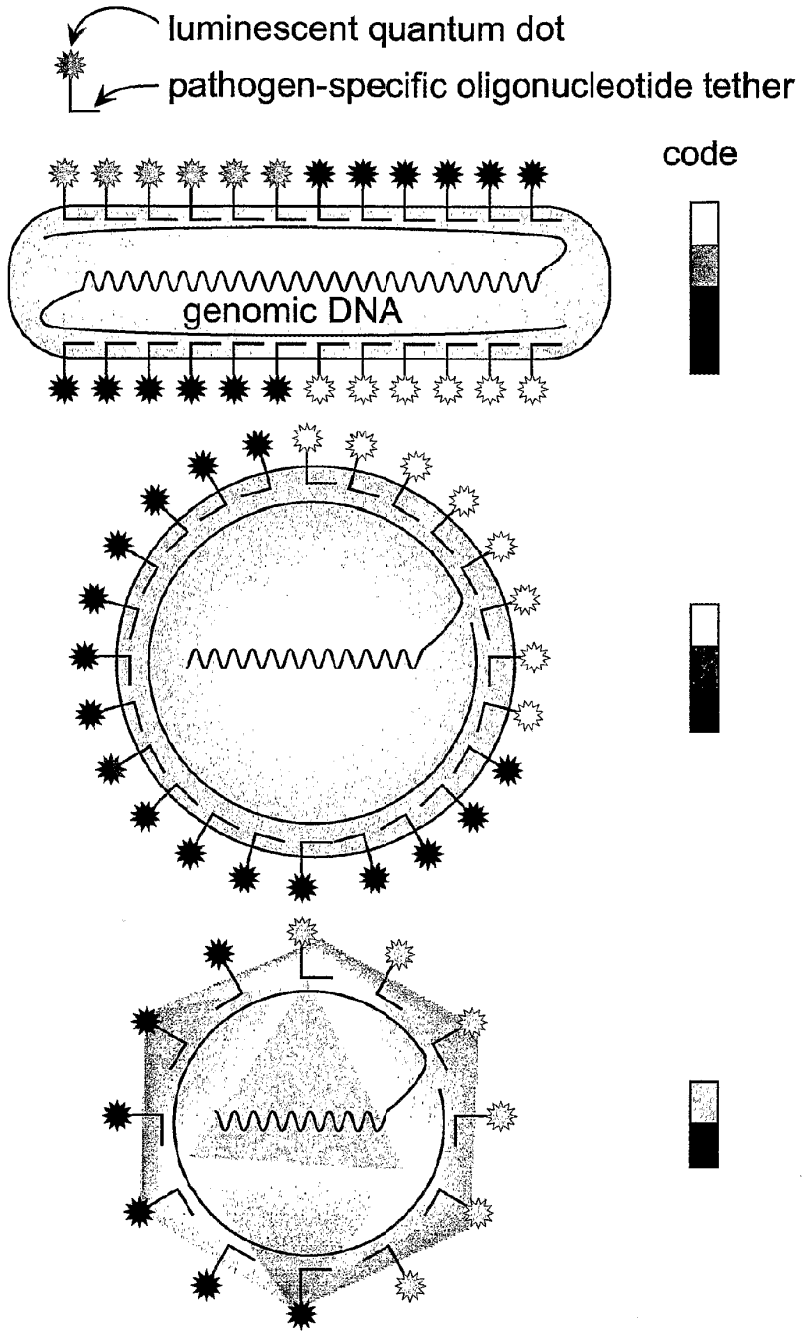


Figure 5.
Distinct labeling of different pathogens using combinatorial labeling

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Figure 6. Indirect labeling of an oligonucleotide target-binding molecule using a tag sequence and labeled tag complement

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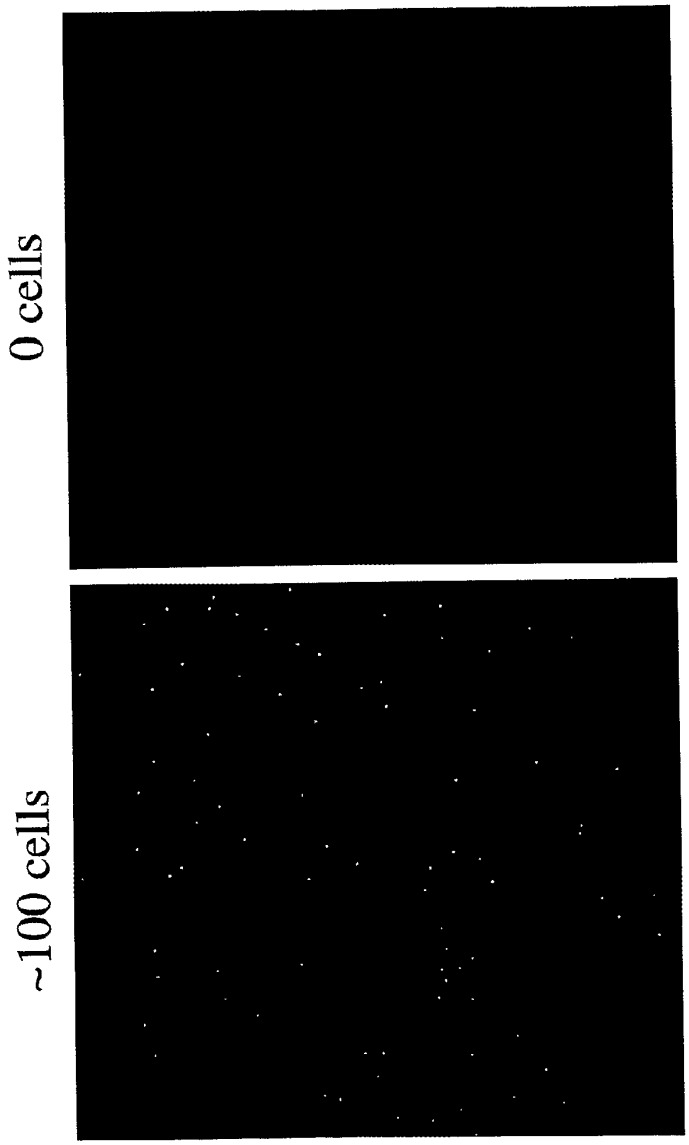


Figure 7. Large area imaging of individual bacteria labeled with a fluorescent DNA-binding stain (Example 1).

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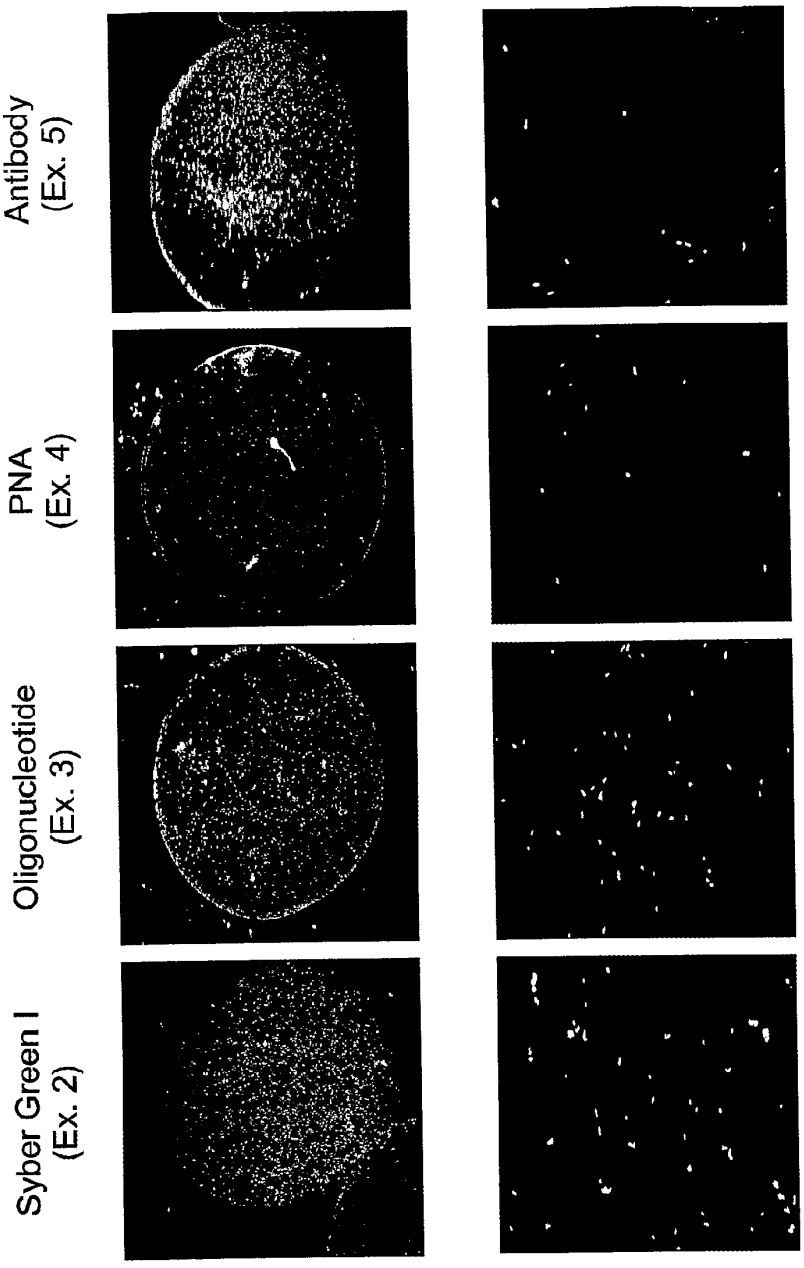


Figure 8. Large area non-magnified imaging of single *E.coli* cells using various signaling moieties (Examples 2-5).

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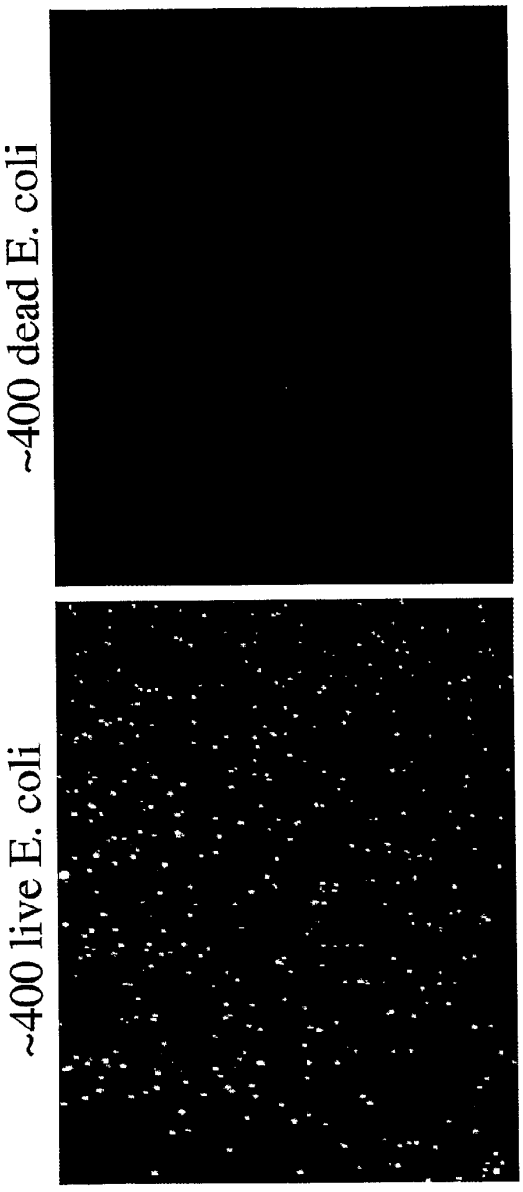


Figure 9. Large area imaging of individual live bacteria stained with a fluoro-genic esterase substrate (Example 6).

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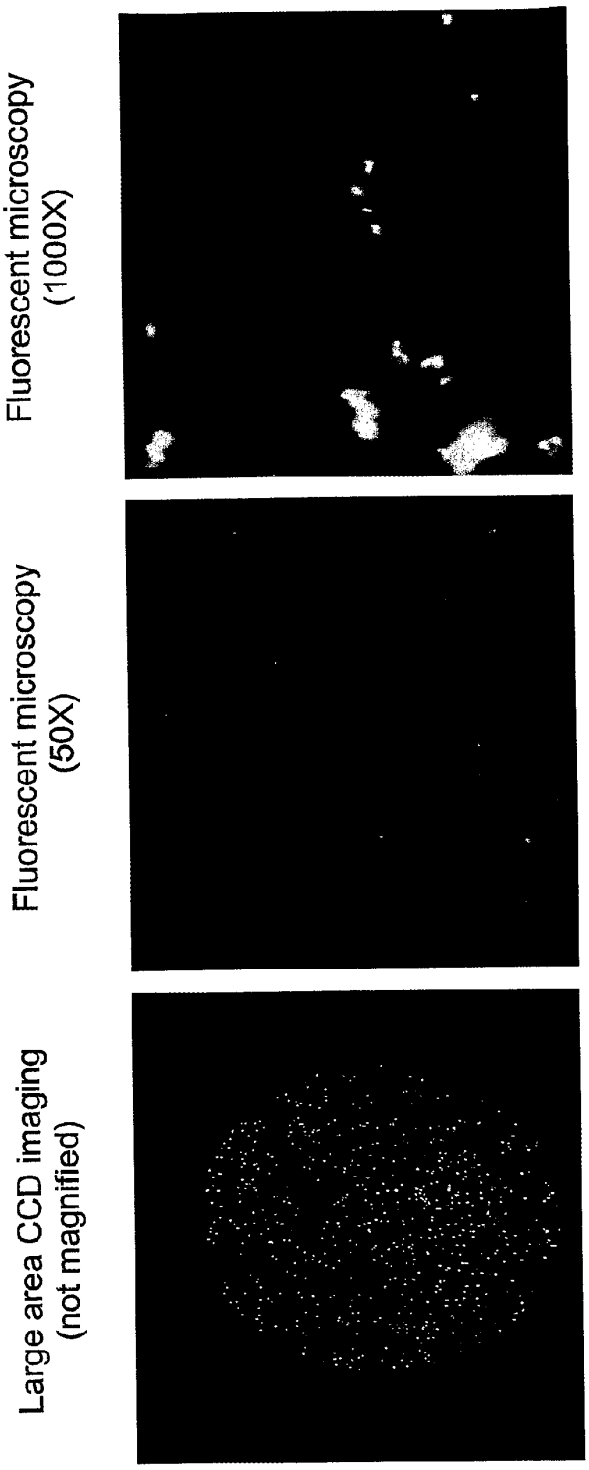


Figure 10. Large area non-magnified imaging of single bacteria labeled with highly fluorescent beads (Example 7).

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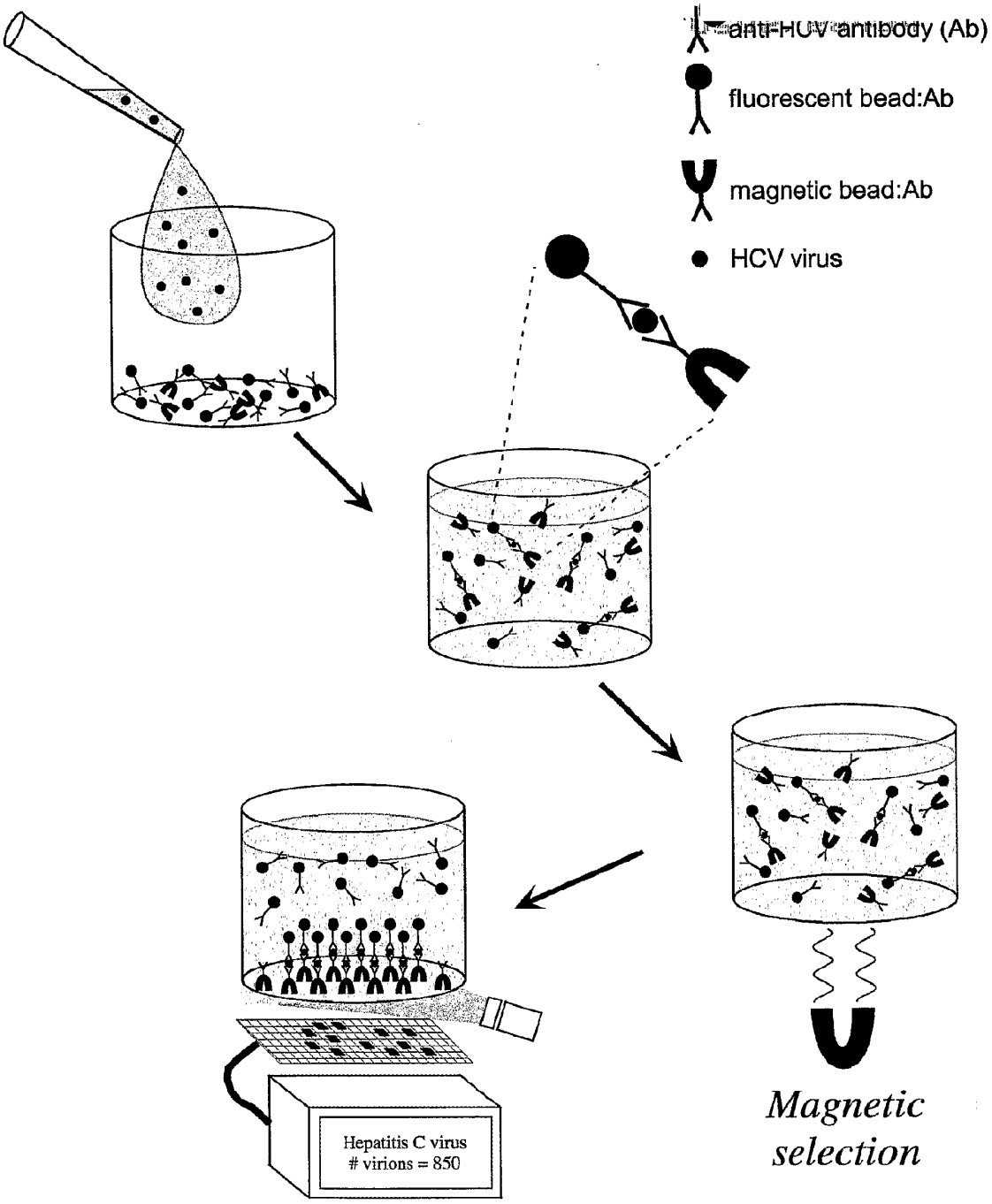


Figure 11.
Homogenous immunoassay for a virus (HCV) using liquid-phase magnetic selection of fluorescent bead:virus:magnetic bead complexes followed by non-magnified large area imaging.

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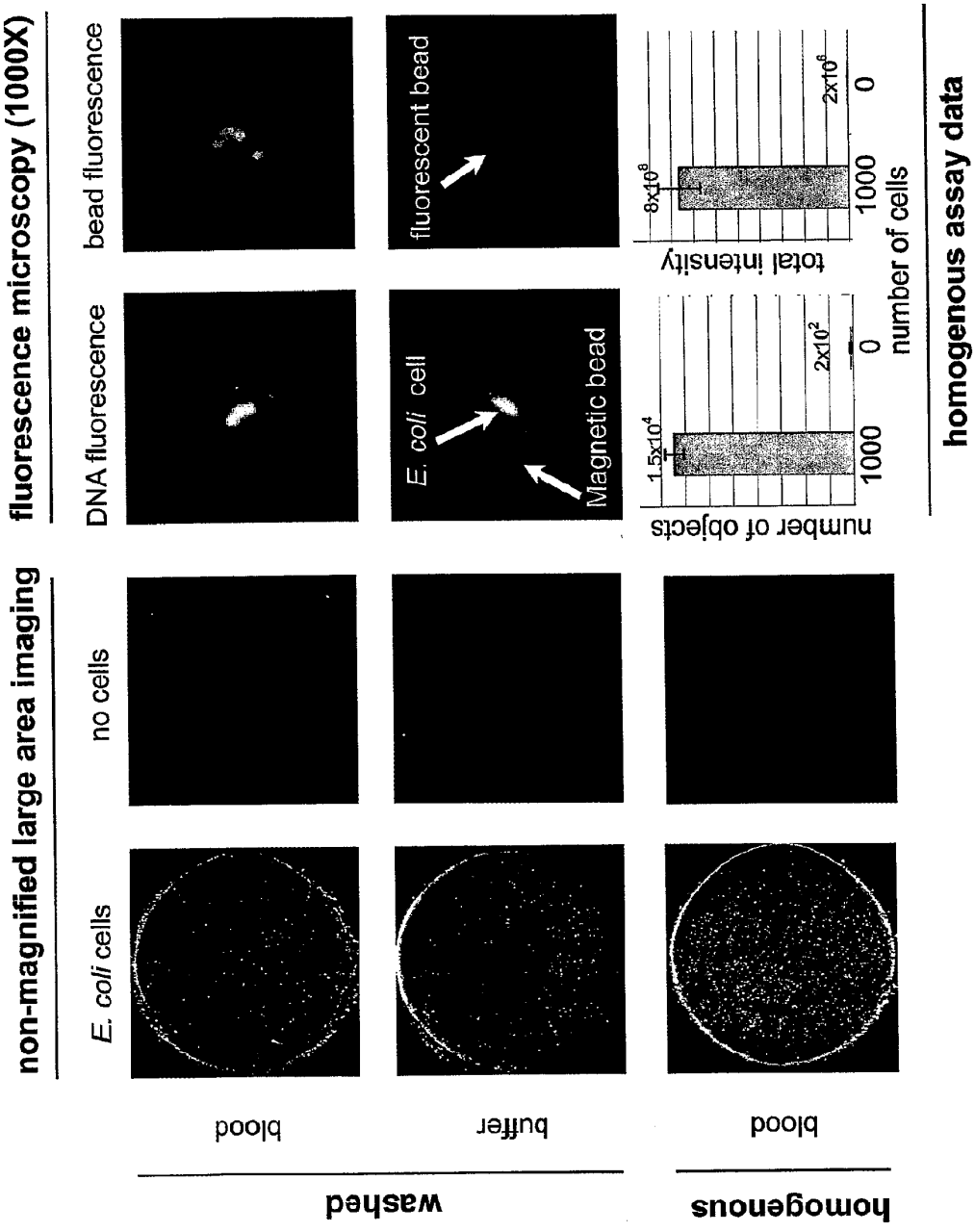


Figure 12. An homogenous immunoassay that detects individual bacteria using non-magnified large area imaging (Example 8).

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Figure 13. Large Area Imaging of Acid Fast Stained *Mycobacteria* in "simulated sputum" (Example 9).

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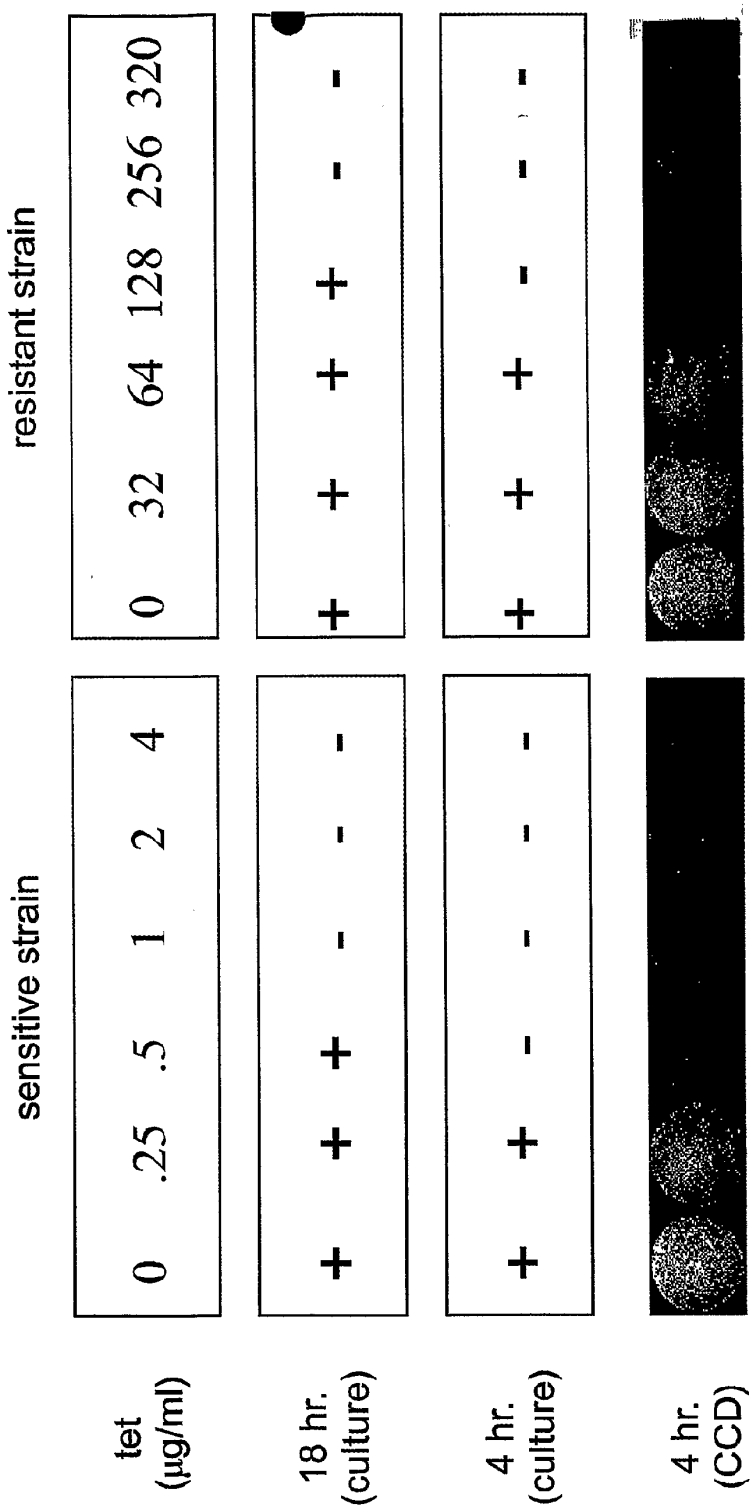


Figure 14. Rapid antimicrobial susceptibility testing using large area imaging. (Example 10).

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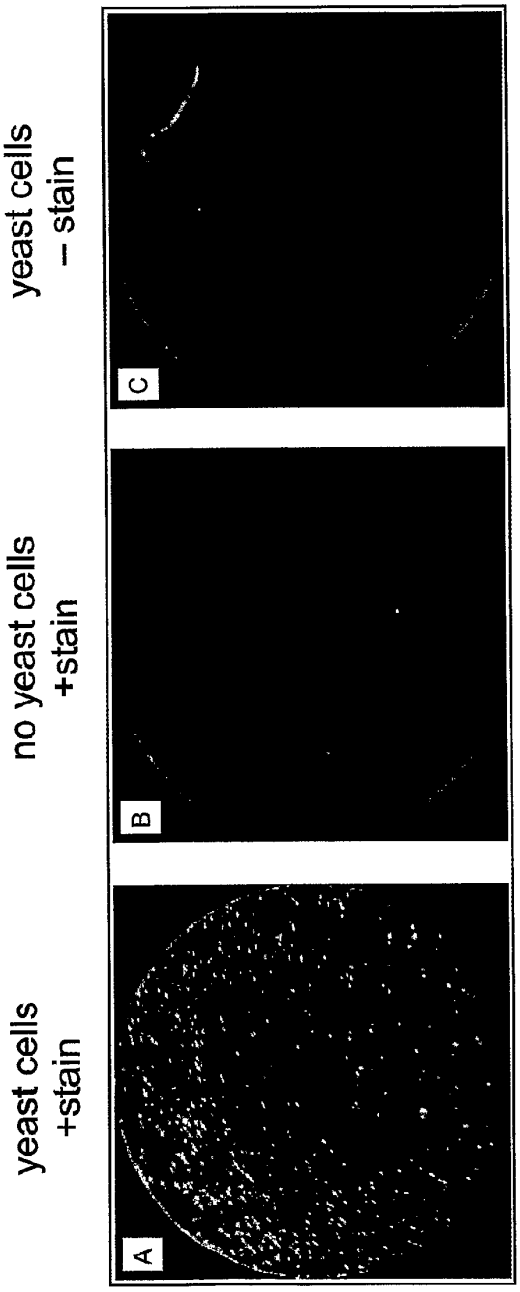


Figure 15. Large area detection of individual fluorescently labeled *Candida albicans* cells that have been magnetically selected (Example 11).

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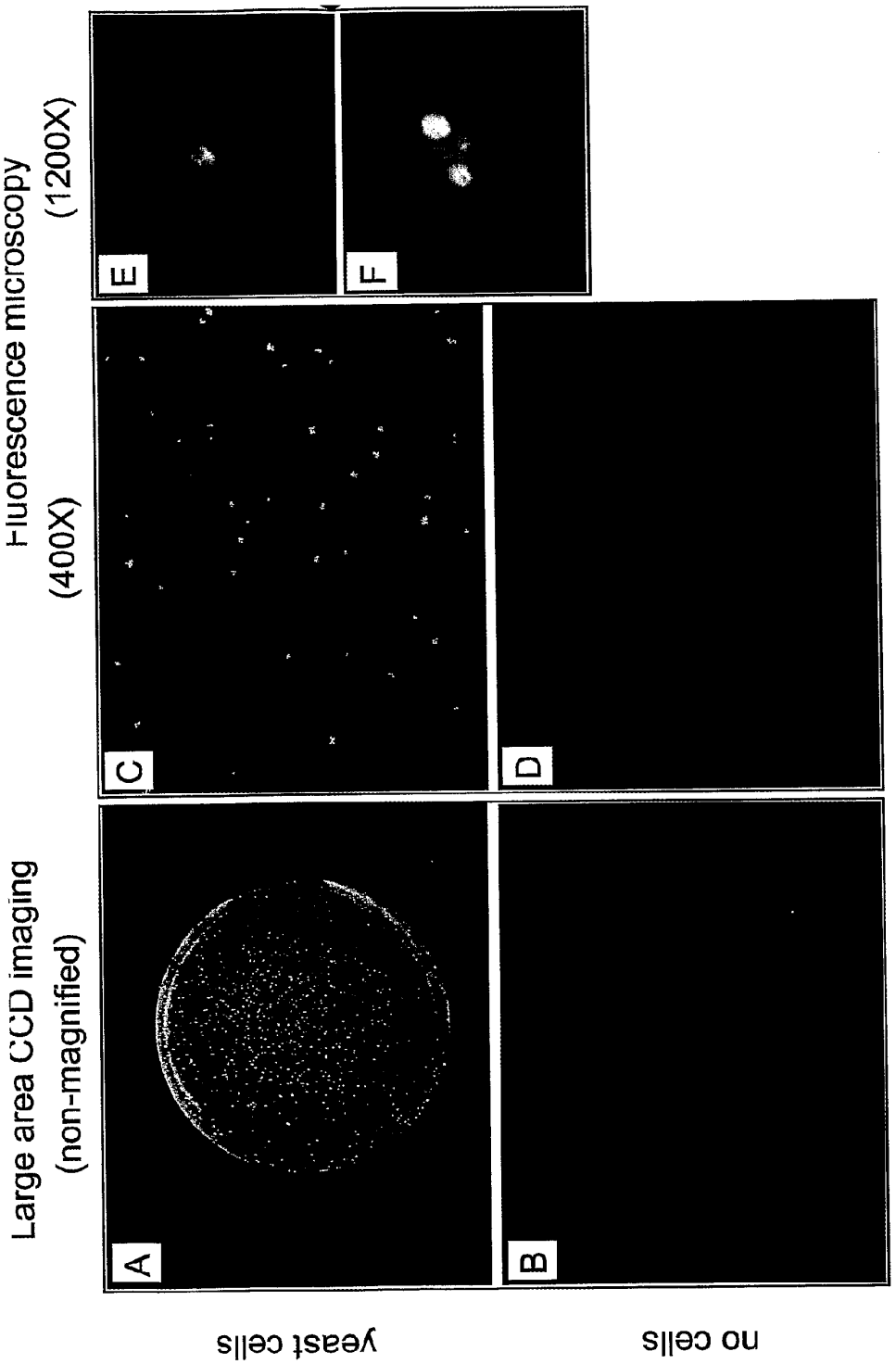


Figure 16. Large area detection of individual *Candida albicans* cells specifically bound to fluorescent and paramagnetic polystyrene particles (Example 12).

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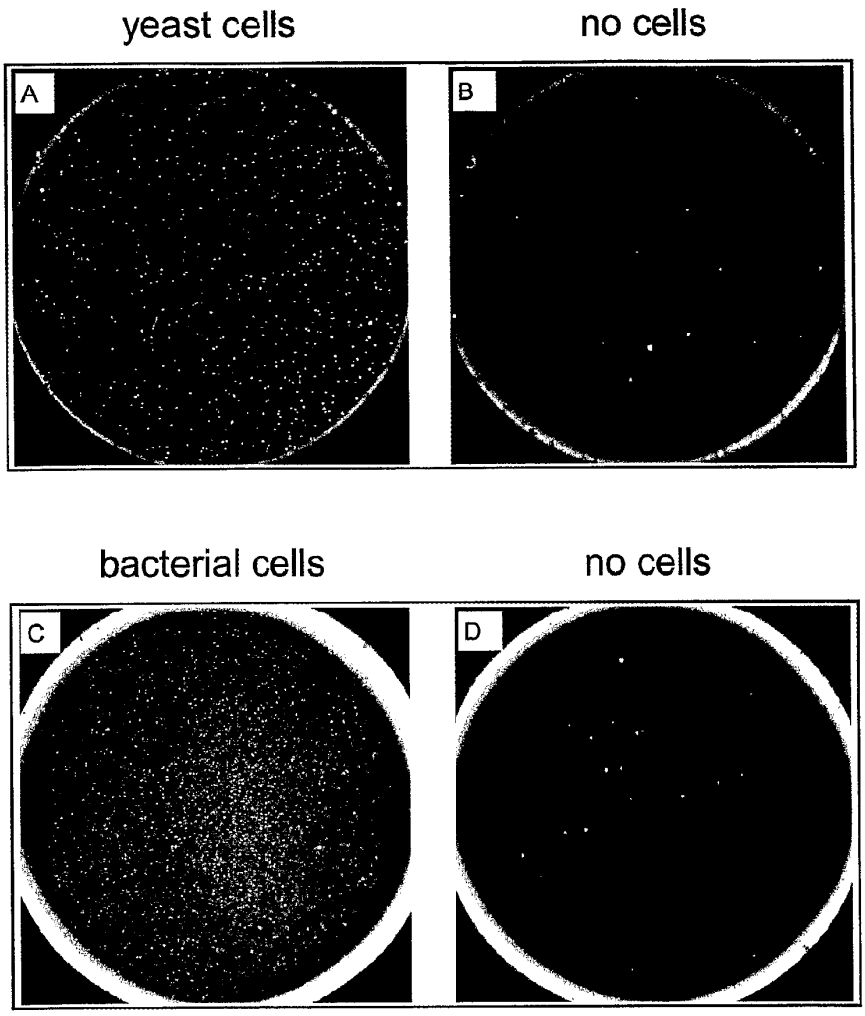


Figure 17. Large area detection of individual Candida albicans specifically bound to fluorescent antibodies and magnetic particles (Example 13).

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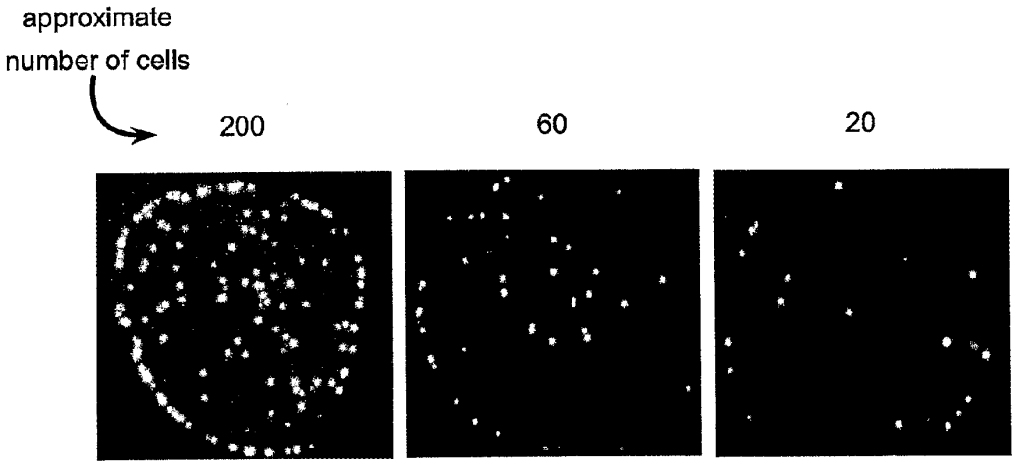


Figure 18. Non-magnified large area detection of single chemiluminescent yeast cells (*C. albicans*) using a CCD camera (Example 14).

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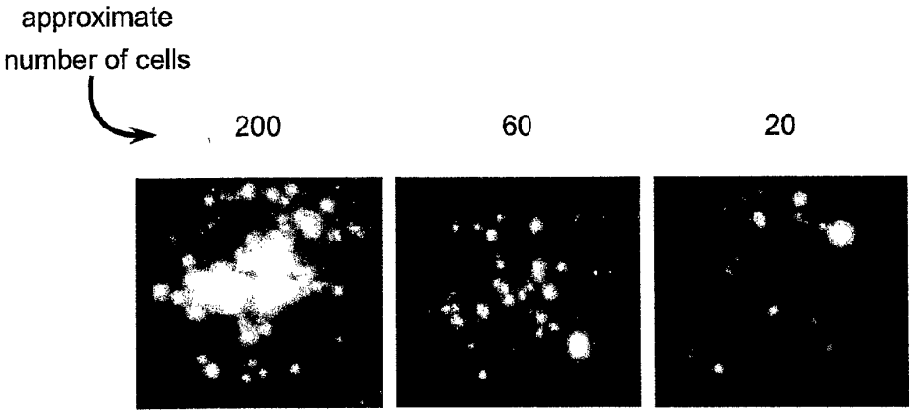


Figure 19. Non-magnified large area detection of single chemiluminescent yeast cells (*C. albicans*) using direct exposure of instant film (Example 15).

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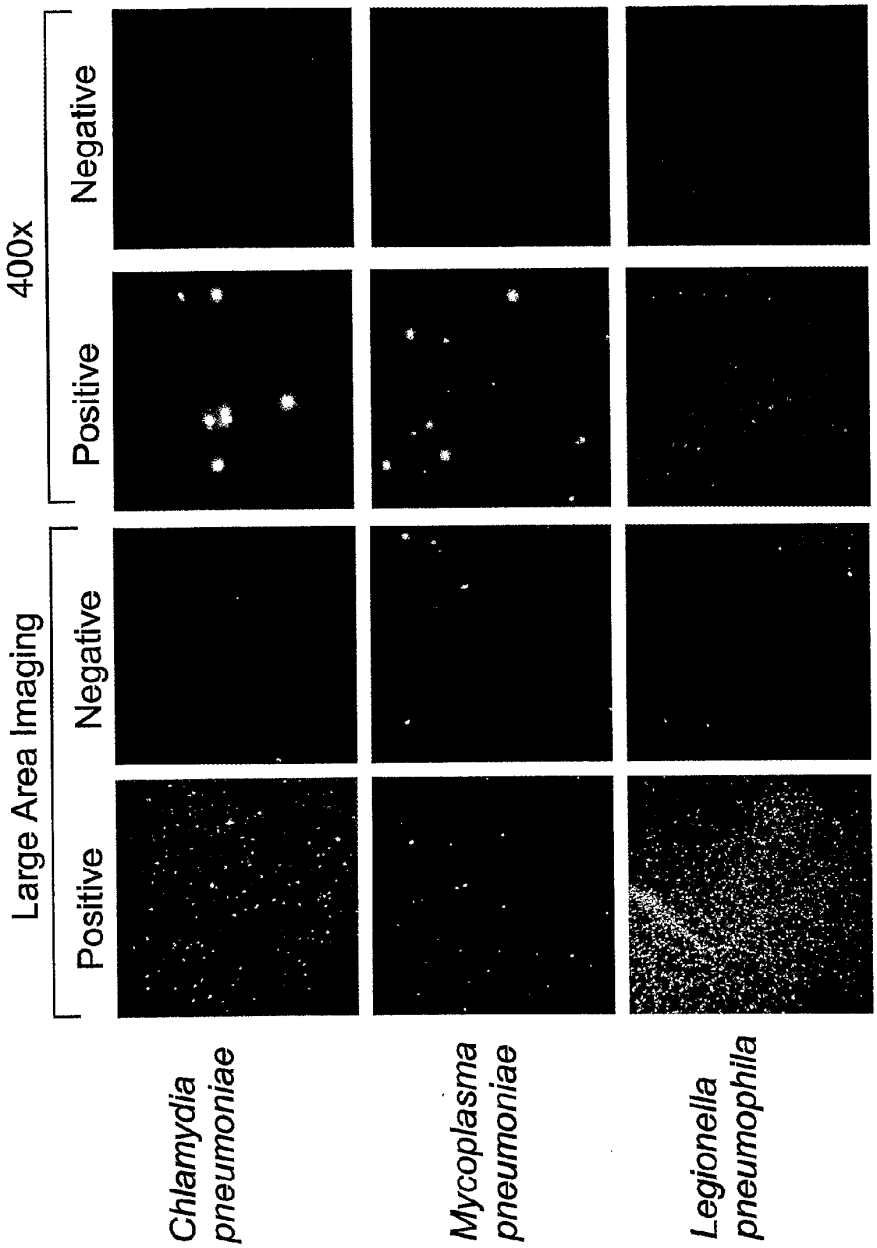


Figure 20. Detection of organisms involved in lower respiratory tract infections using non-magnified large area imaging (Example 16).

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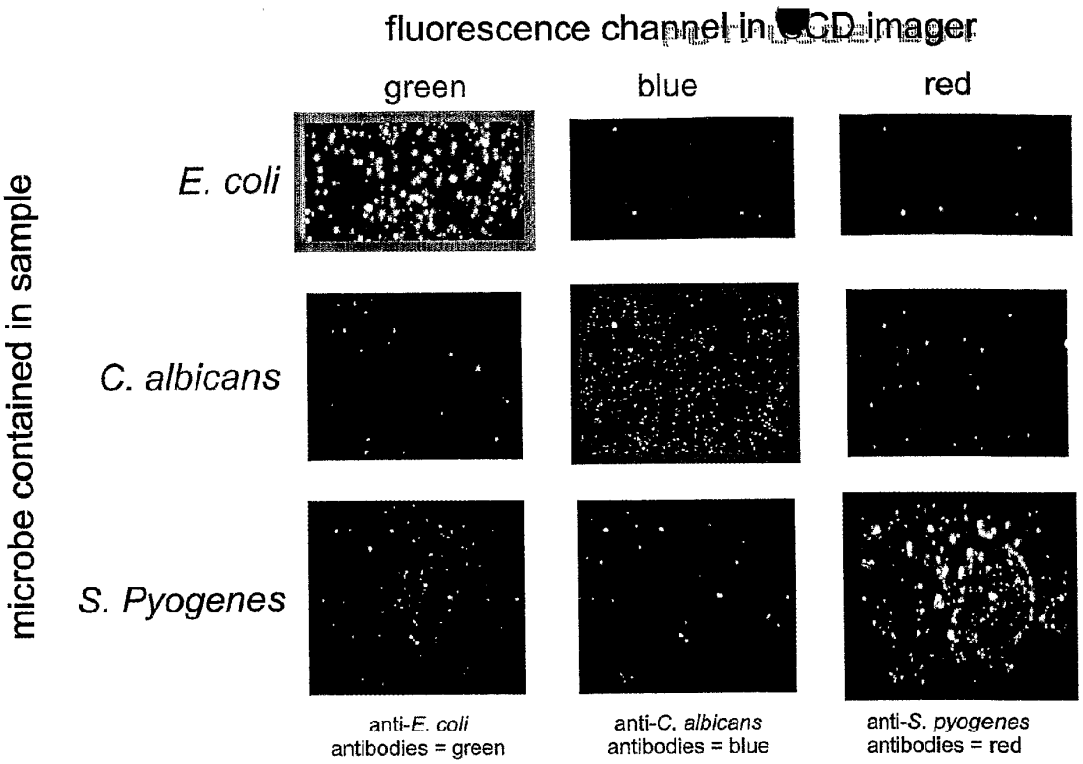


Figure 21. A mulitiplexed direct fluorescence immunoassay that simultaneously scans a sample for 3 disparate microbes using non-magnified large area imaging (Example 17).

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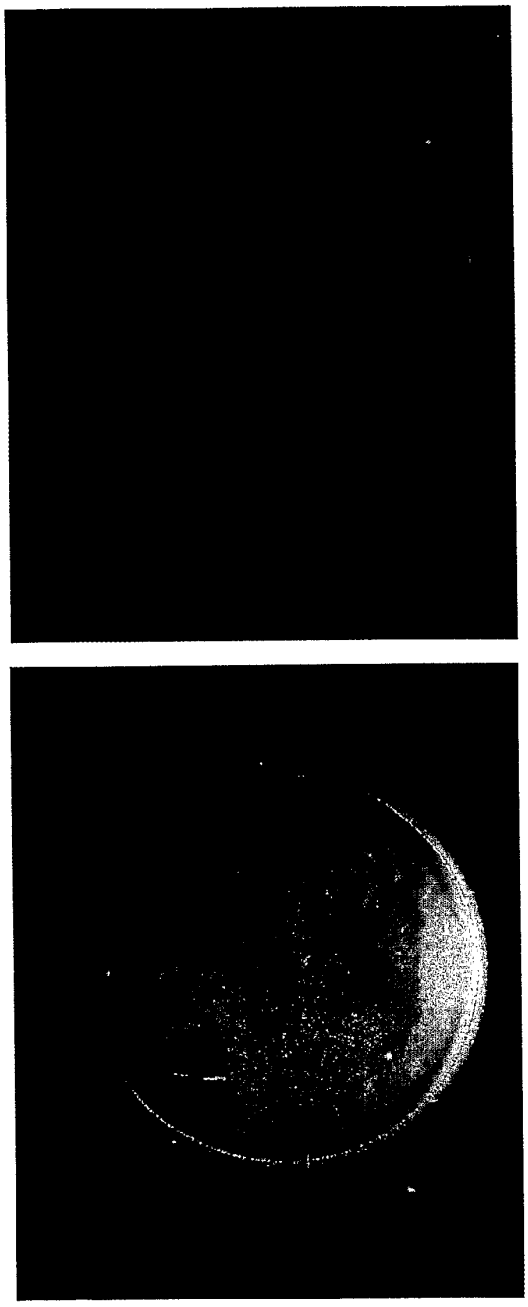
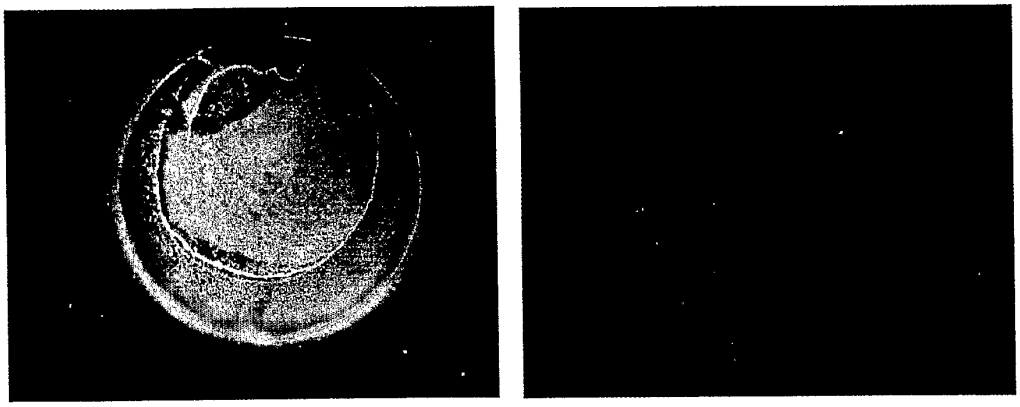


Figure 22. A viral immunoassay using solid-phase capture and non-magnified large area imaging (Example 18).

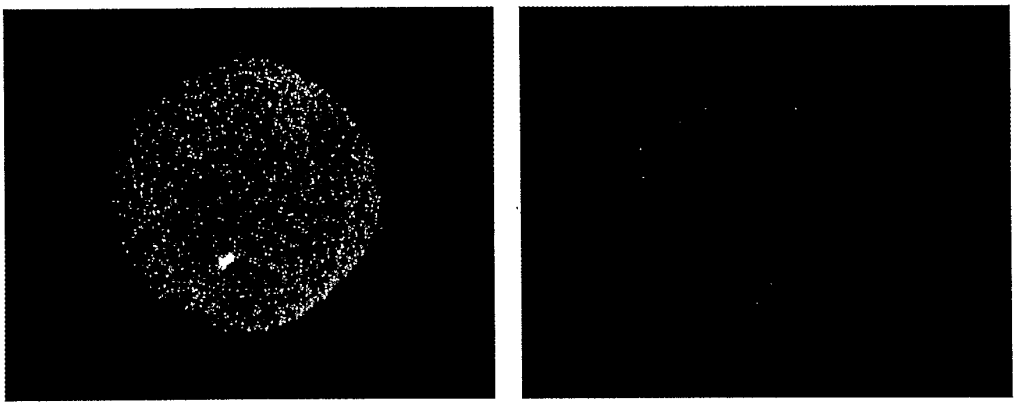
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adenovirus RSV (negative control)

Figure 23. A viral immunoassay in blood using solid-phase capture and non-magnified large area imaging (Example 19).

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adenovirus RSV (negative control)

Figure 24. A viral immunoassay using magnetic and fluorescent beads to form a liquid phase sandwich followed by non-magnified large area imaging (Example 20).

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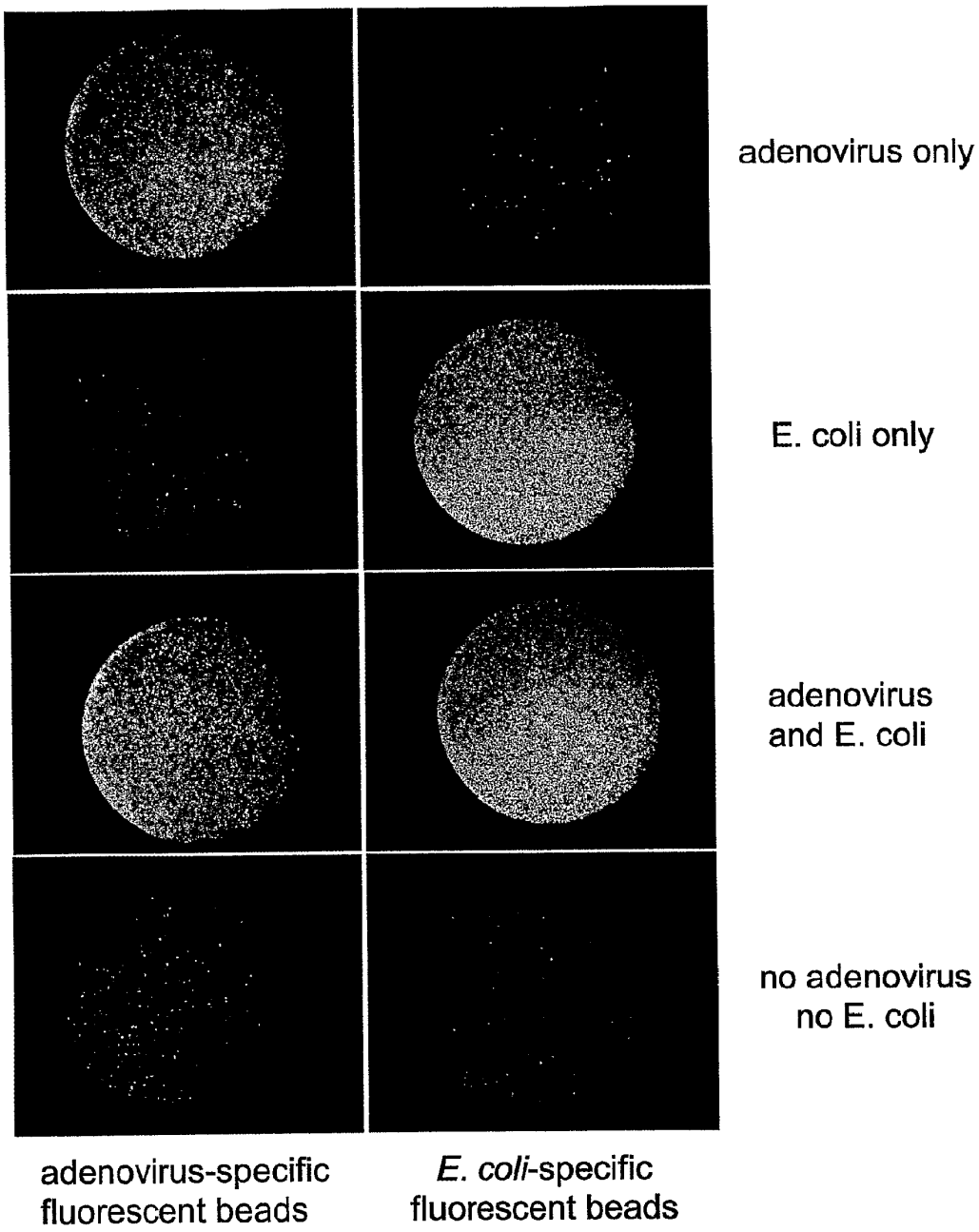


Figure 25. Multiplex large area imaging immunoassay that simultaneously scans for a bacterium and a virus (Example 22).

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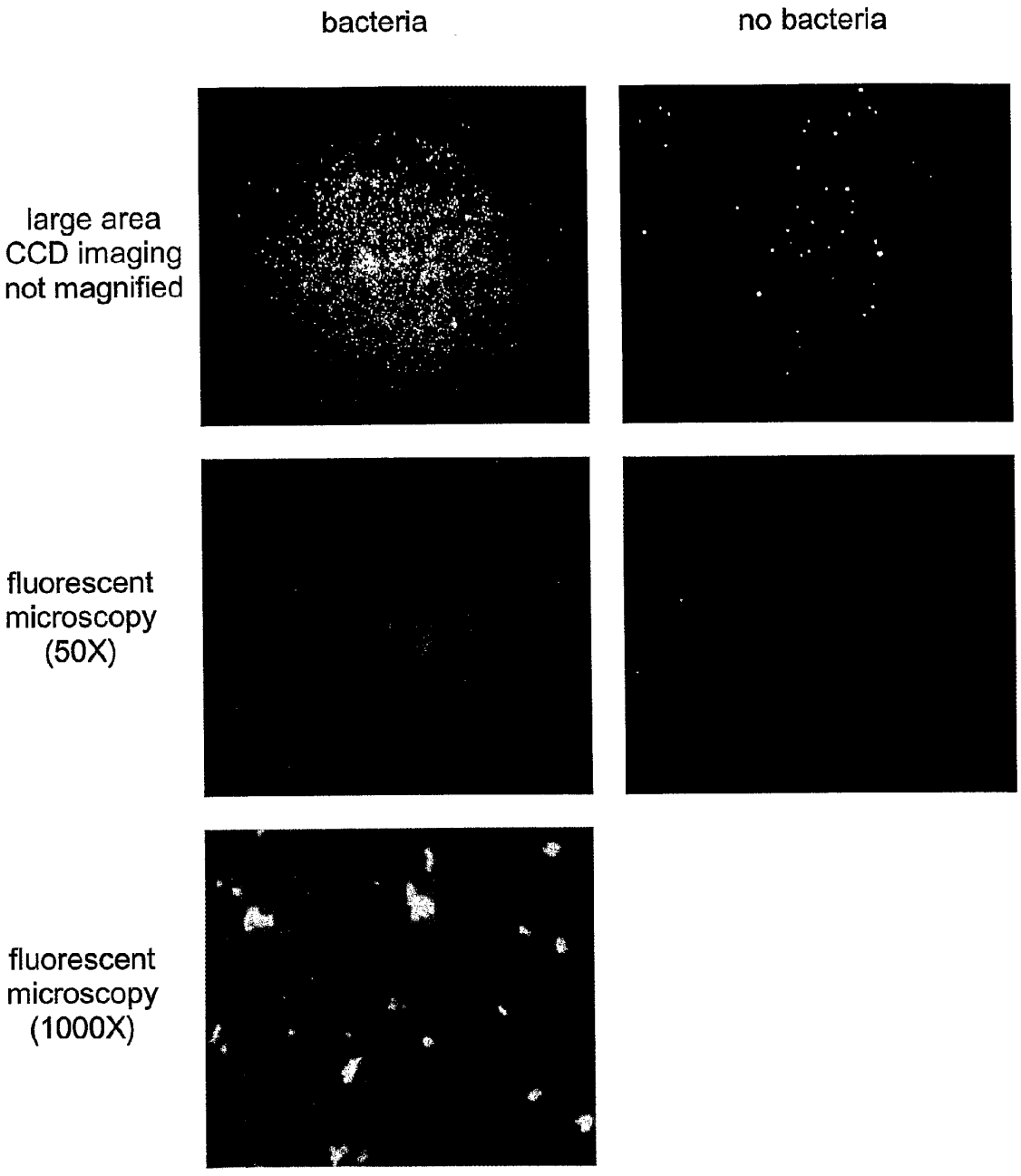


Figure 26 . Filter flow-through assay for detecting single bacteria using non-magnified large area imaging (Example 23).

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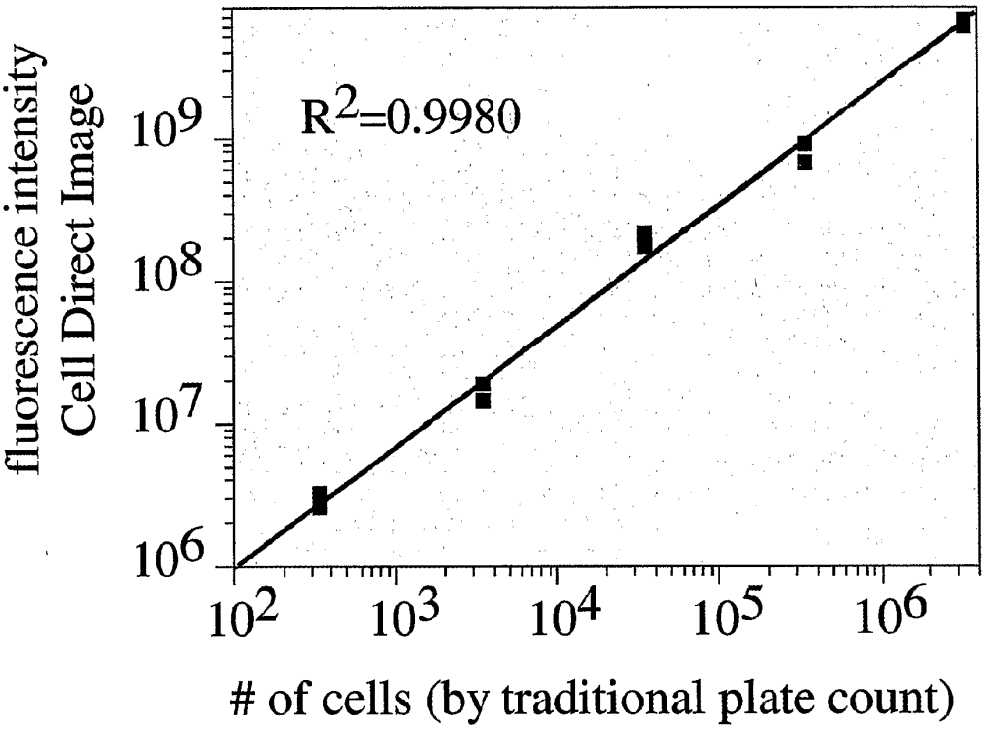


Figure 27. Quantification of bacteria using large area imaging of cells stained with a fluorogenic esterase substrate (Example 24).

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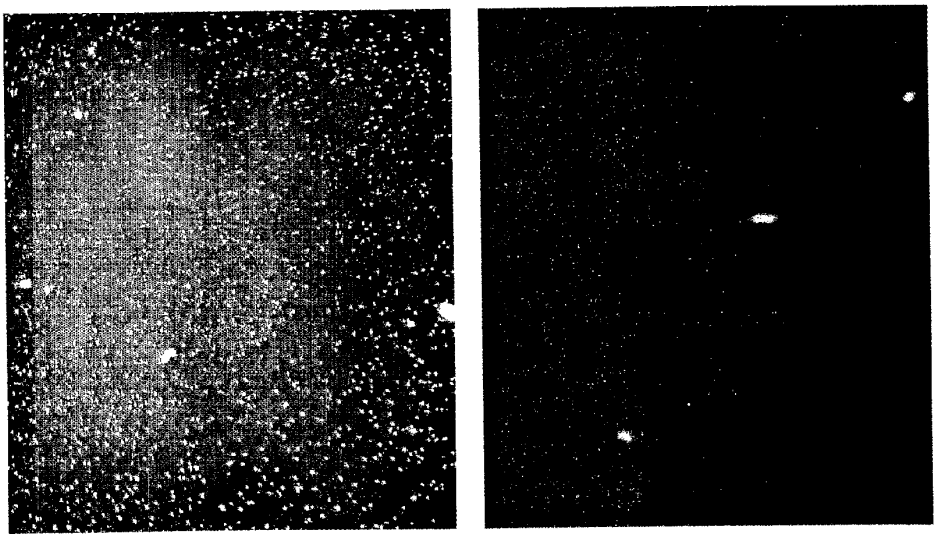


Figure 28. Detecting individual stained bacteria on a filter using non-magnified large area imaging (Example 25).

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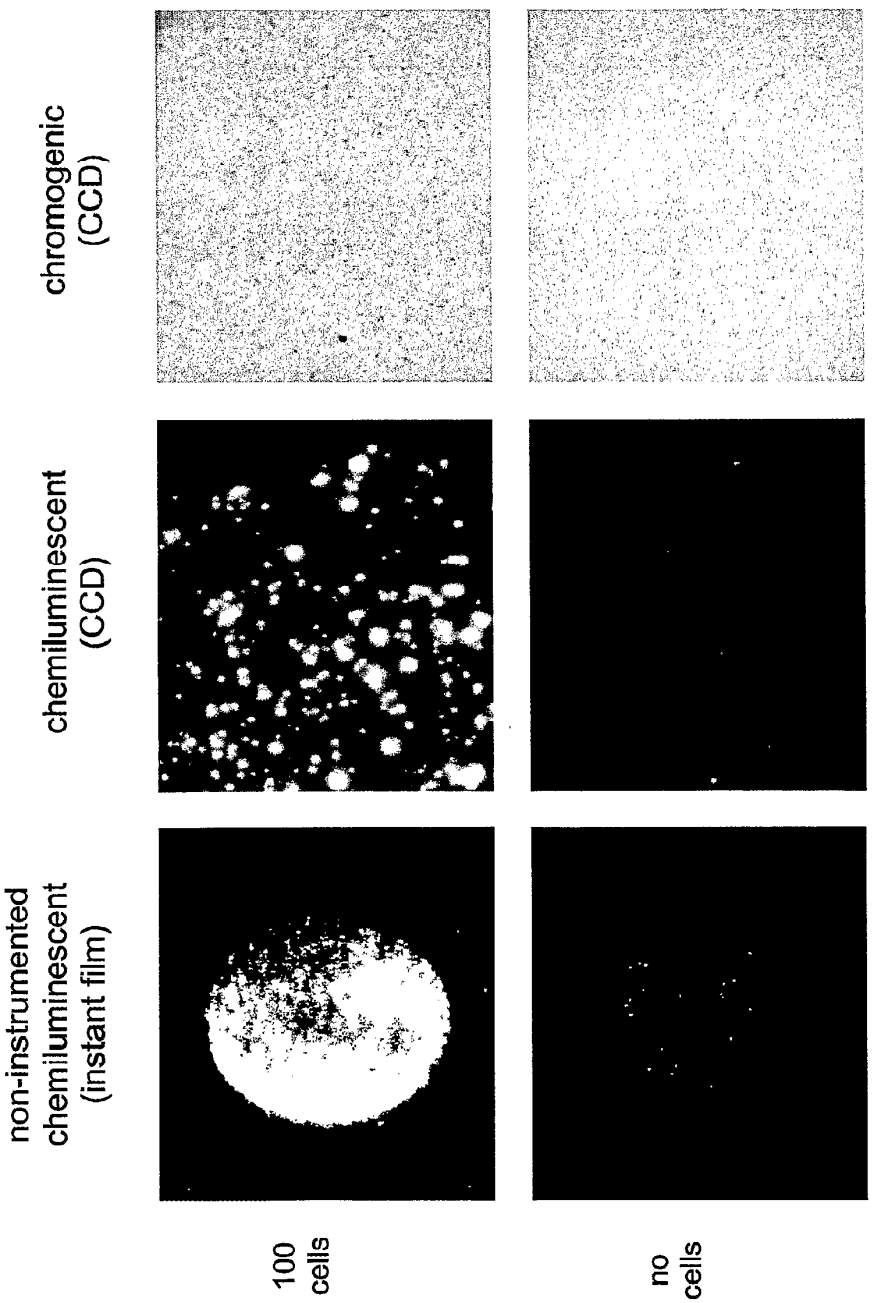


Figure 29. Non-instrumented detection of small numbers of bacterial cells without magnification using enzyme-coated beads (Example 26).

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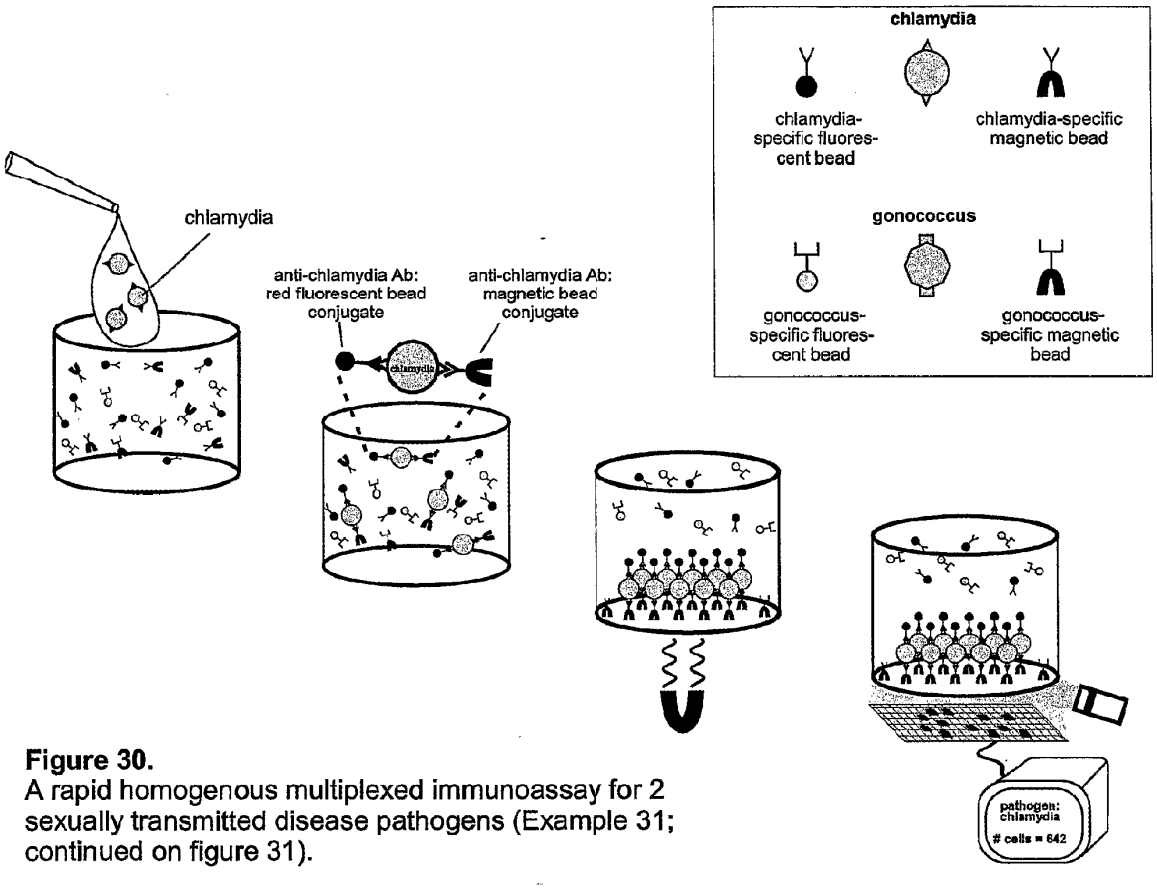


Figure 30.
A rapid homogenous multiplexed immunoassay for 2 sexually transmitted disease pathogens (Example 31; continued on figure 31).

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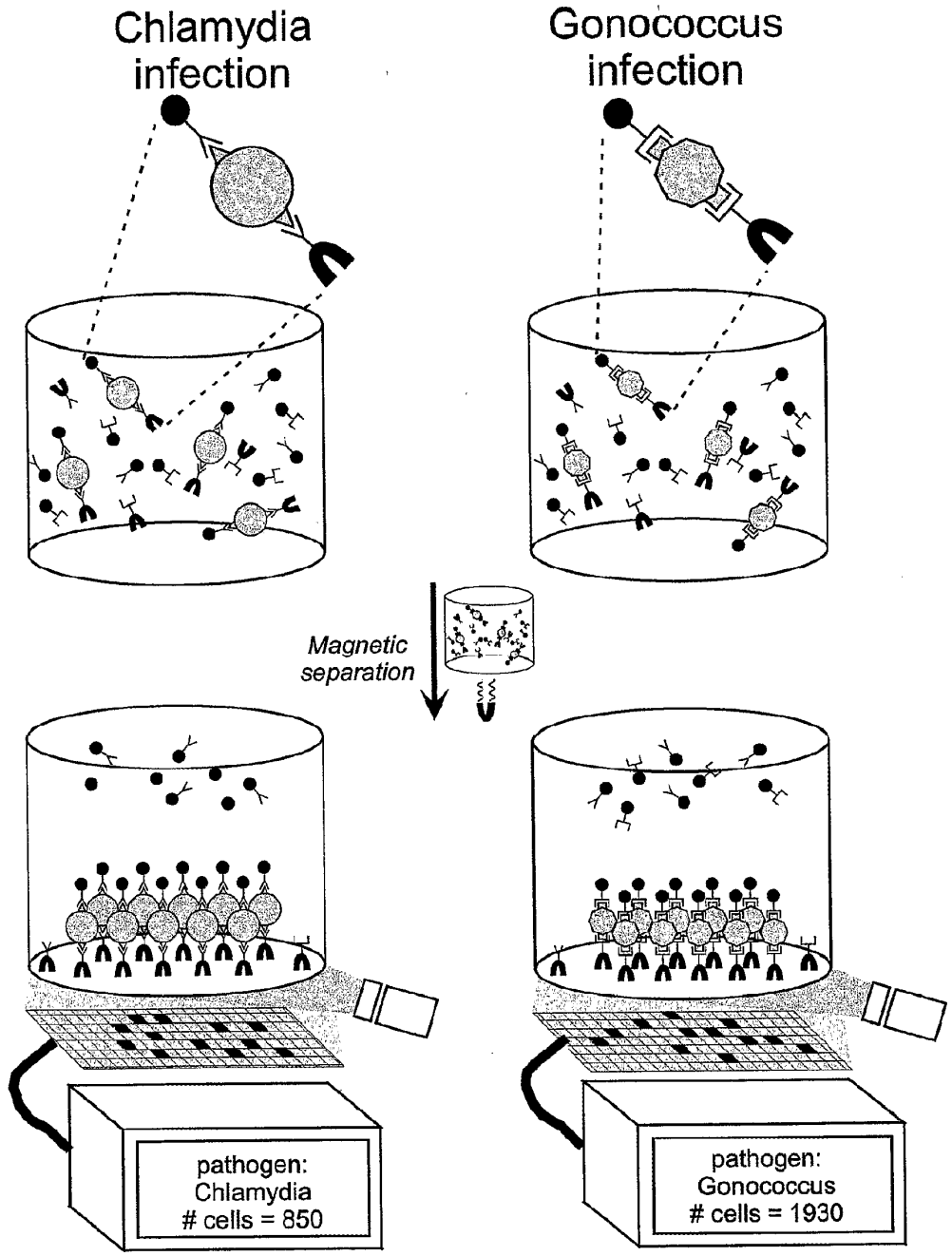


Figure 31. A rapid homogenous multiplexed immunoassay for 2 sexually transmitted disease pathogens (Example 31; continued from figure 30).

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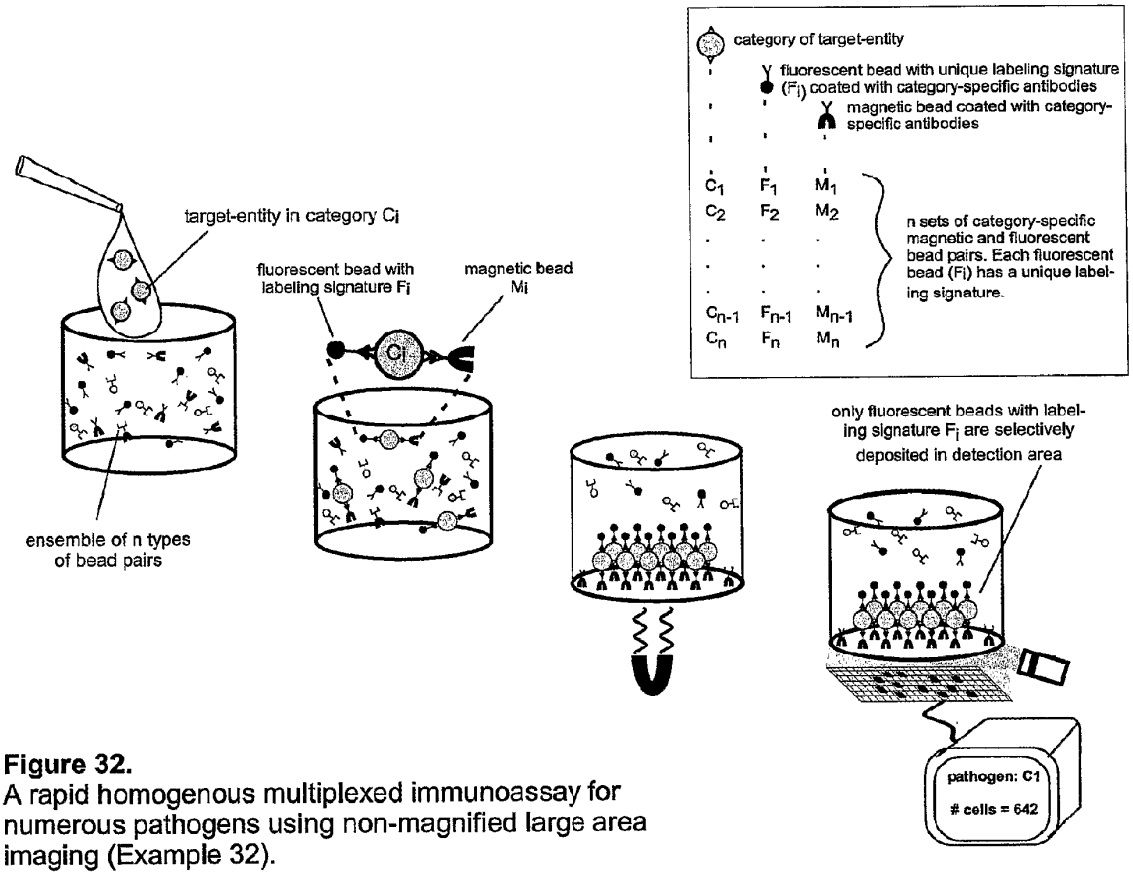


Figure 32.
A rapid homogenous multiplexed immunoassay for numerous pathogens using non-magnified large area imaging (Example 32).

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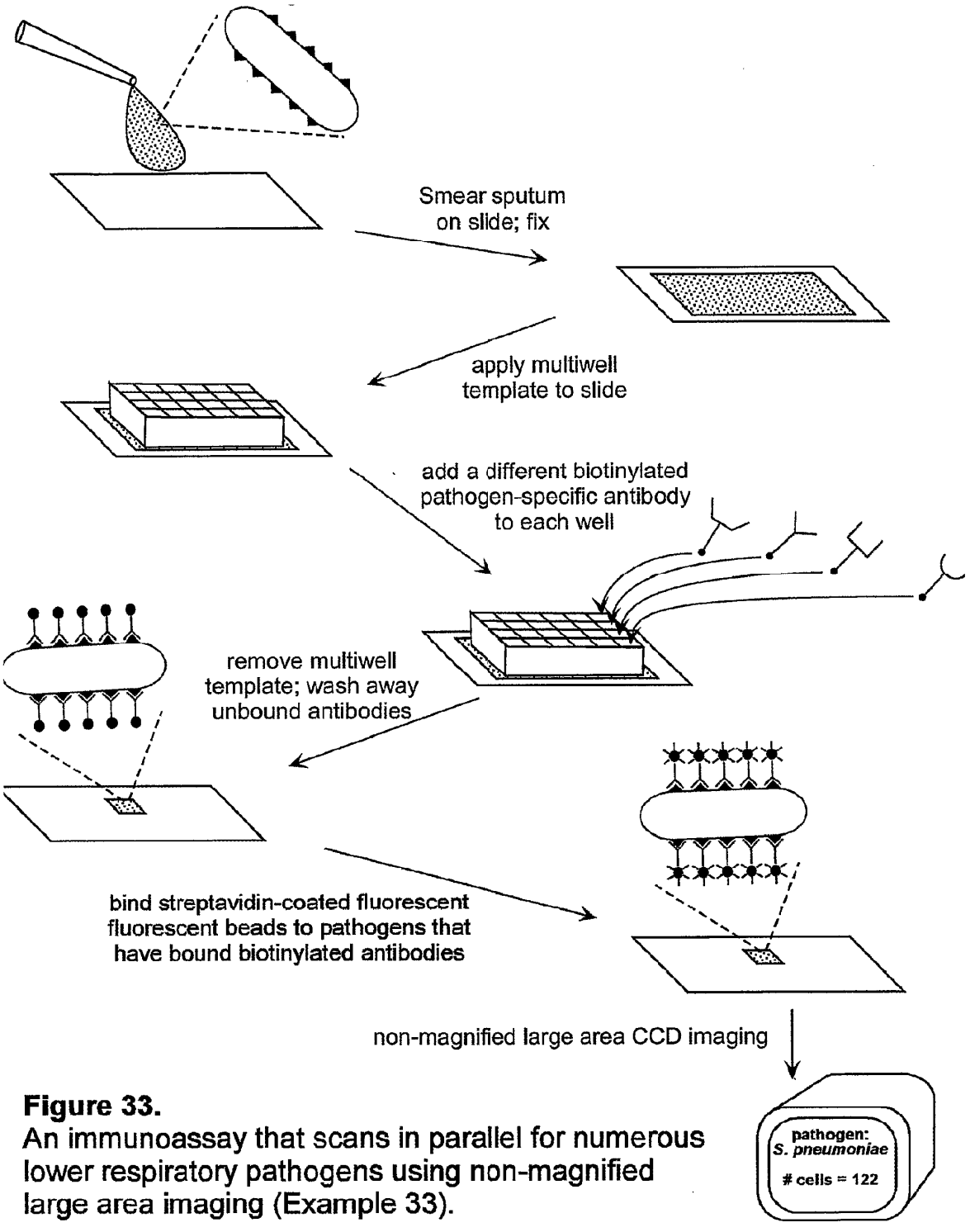


Figure 33.
An immunoassay that scans in parallel for numerous lower respiratory pathogens using non-magnified large area imaging (Example 33).

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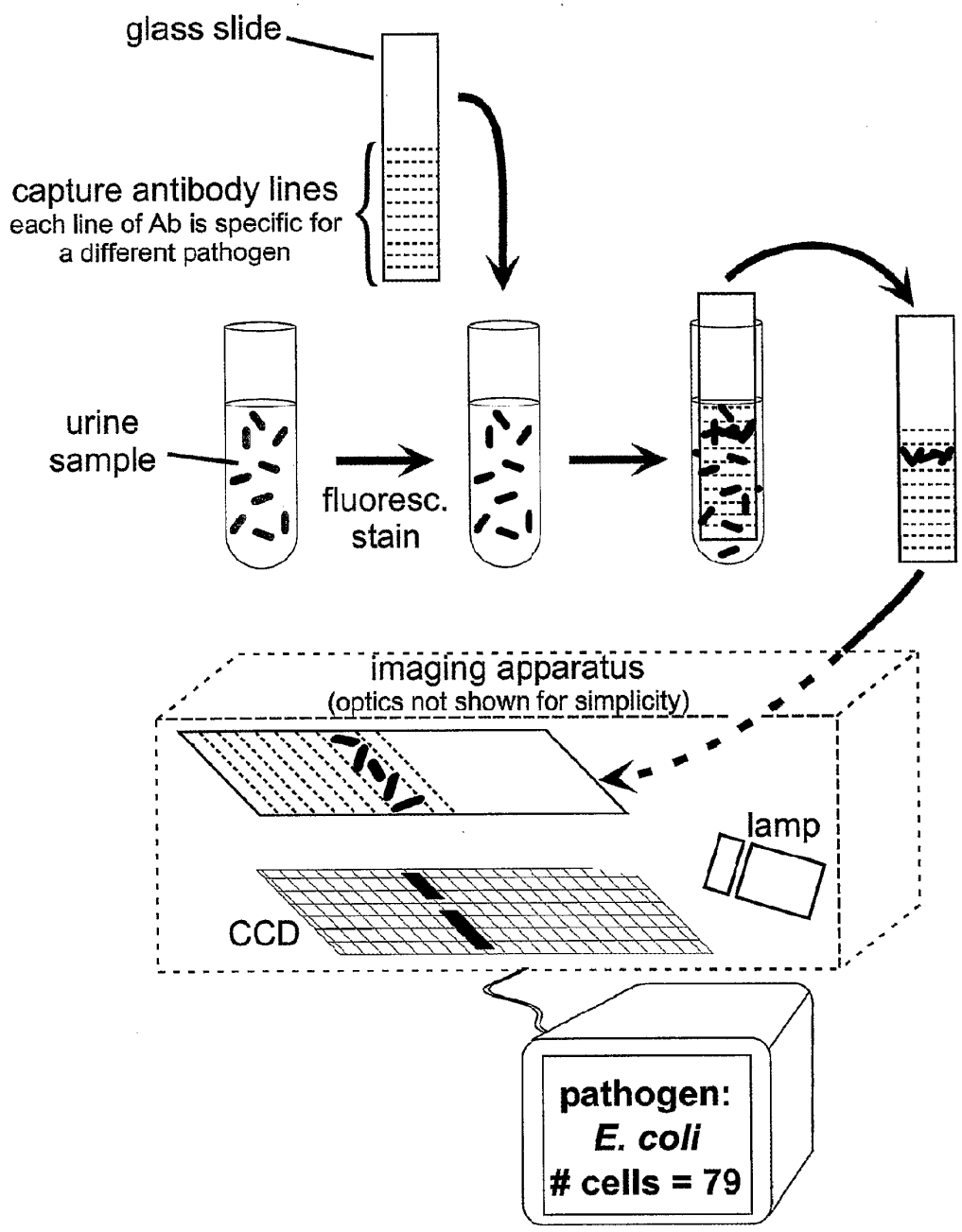


Figure 34.
Multiplexed immunoassay for urinary tract infection that detects single cells using non-magnified large area imaging (Example 34).

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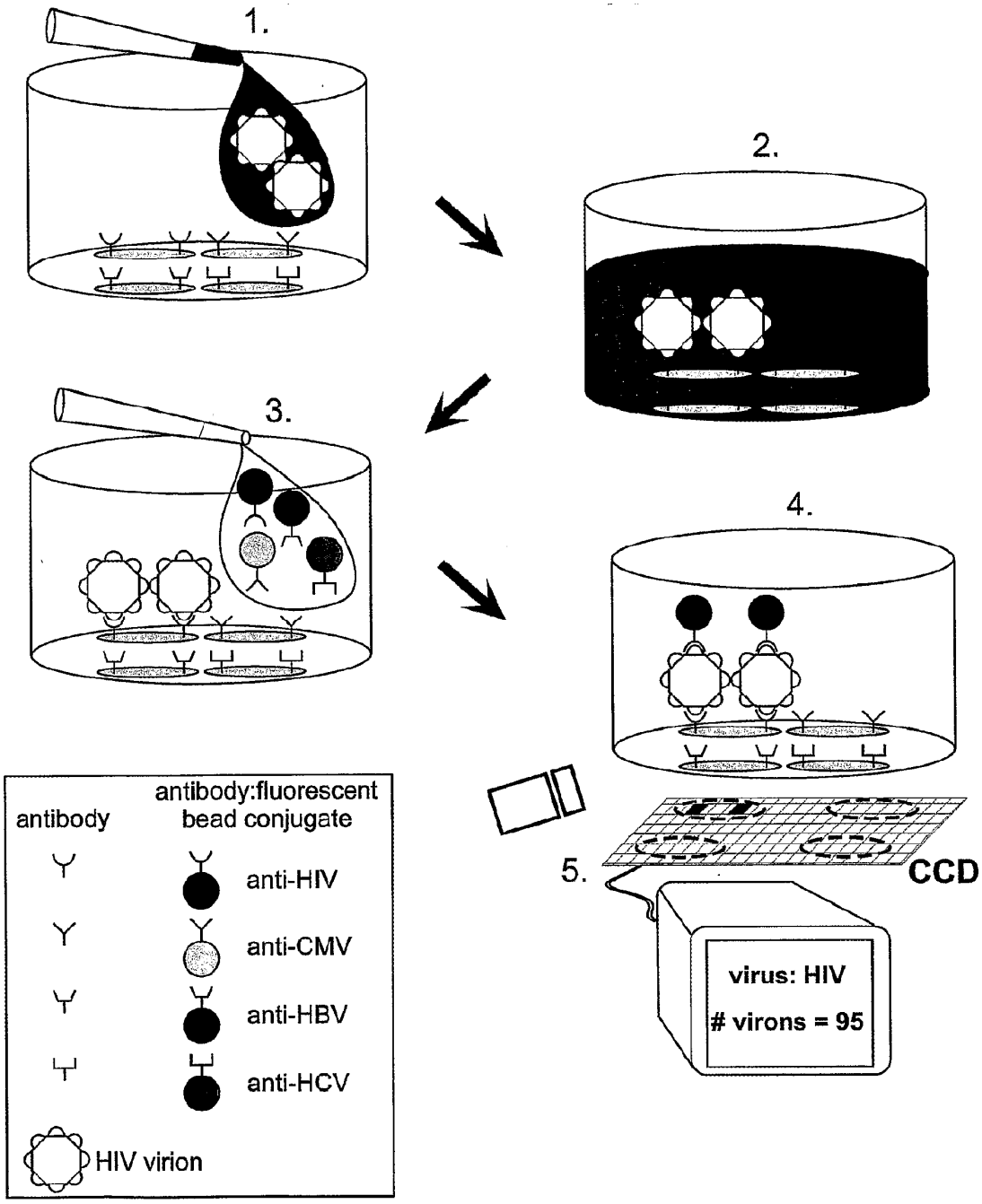


Figure 35.
Multiplexed immunoassay for bloodborne viruses using non-magnified large area imaging (Example 35).

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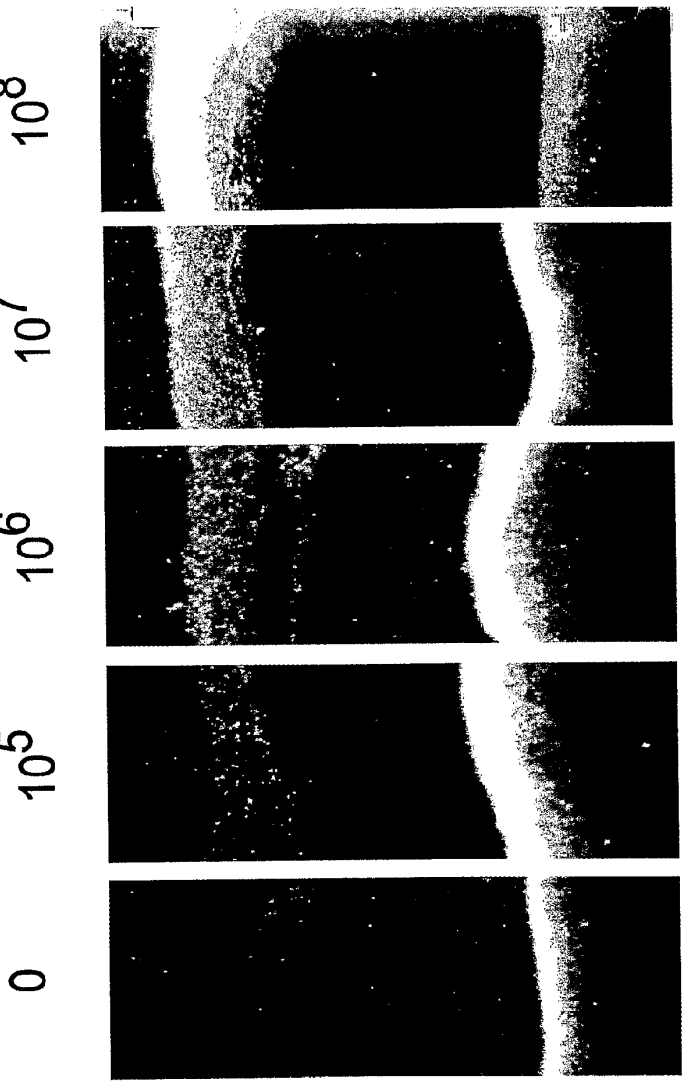


Figure 36. Ultra sensitive lateral flow assay for detecting low levels of virus using non-magnified large area imaging (Example 36).

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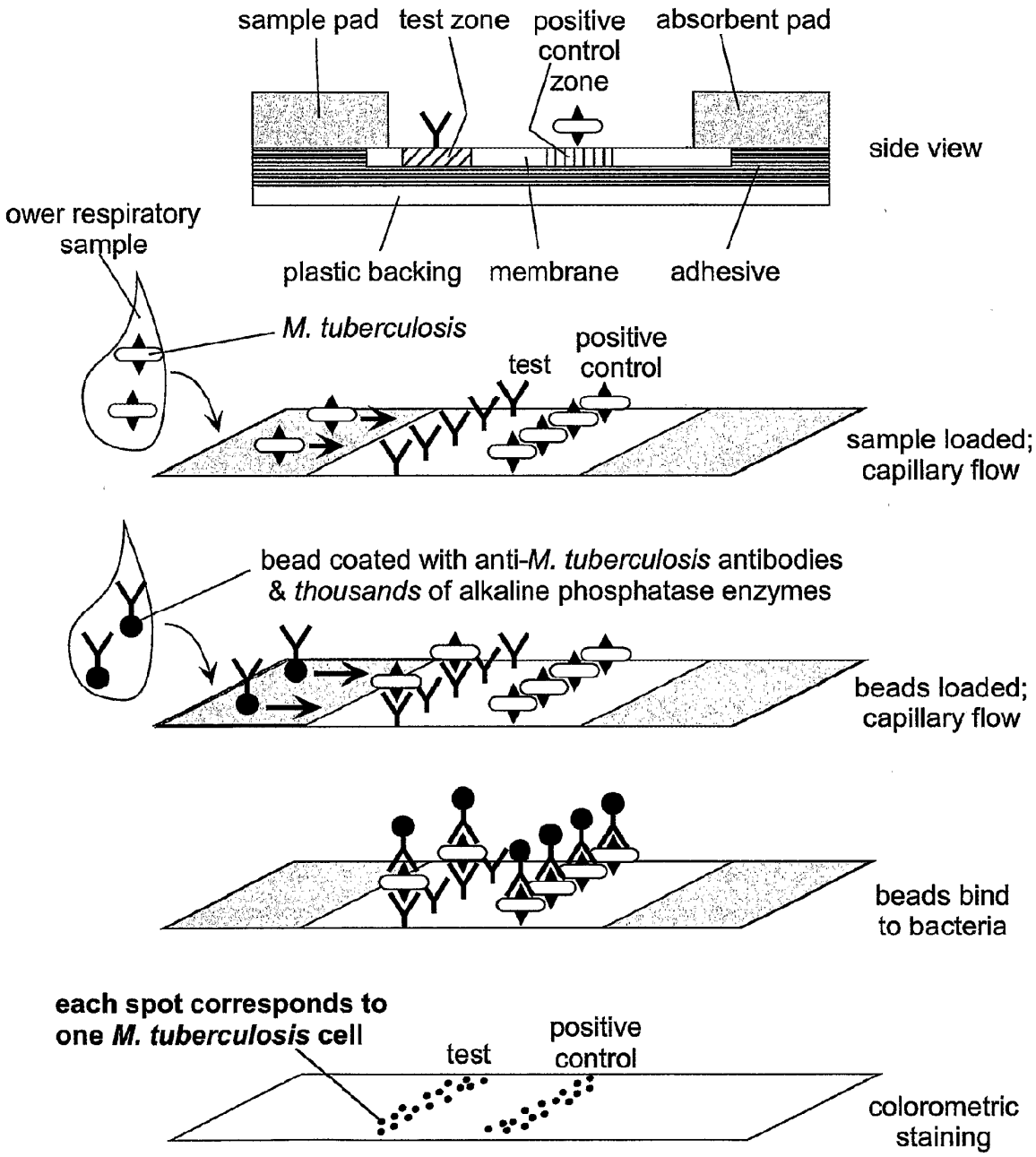


Figure 37. Rapid lateral flow test for *M. tuberculosis* using visual detection (Example 37).

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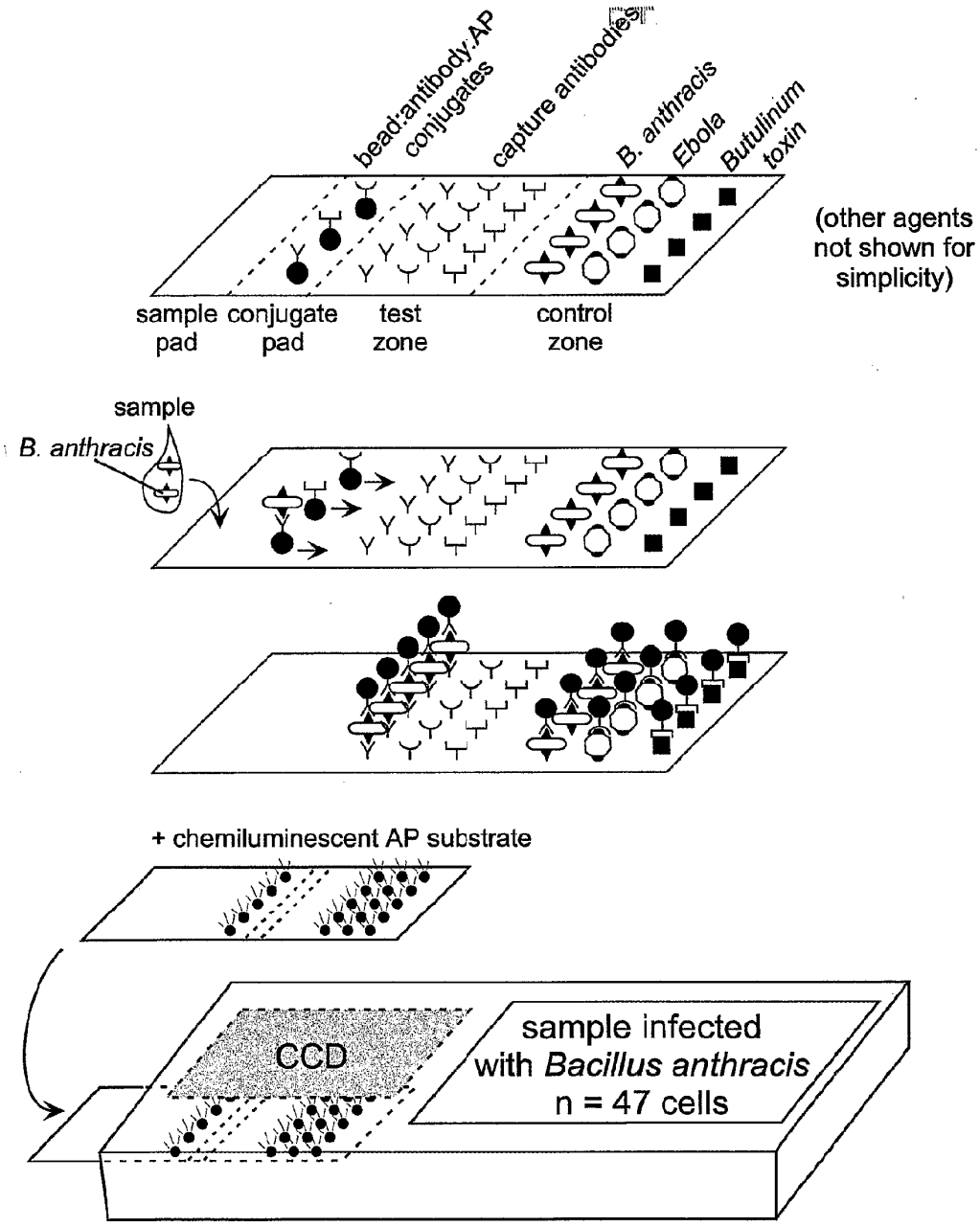
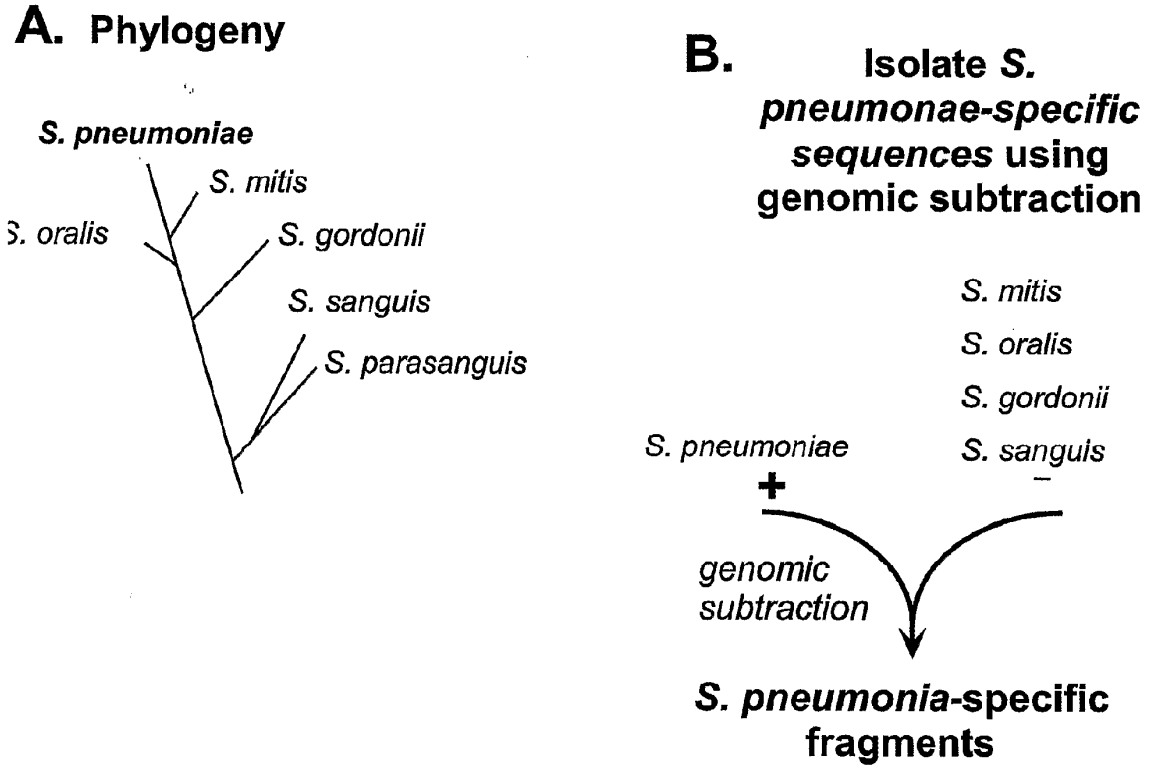
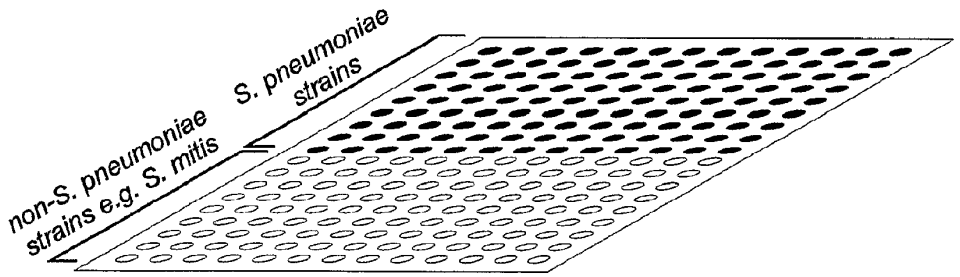


Figure 38.
Rapid lateral flow test for detecting numerous disparate biowarfare agents using non-magnified large area imaging (Example 38).

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a. probe dot blots containing *Streptococcus* genomic DNA with oligonucleotides corresponding to genomic subtraction products

b. choose oligonucleotide probes that are group-specific, *i.e.* that hybridize to ***all*** *S. pneumoniae* strains, but to ***no*** related species

Figure 39. Isolating a family of *S. pneumonia*-specific sequences using genomic subtraction (Example 39).

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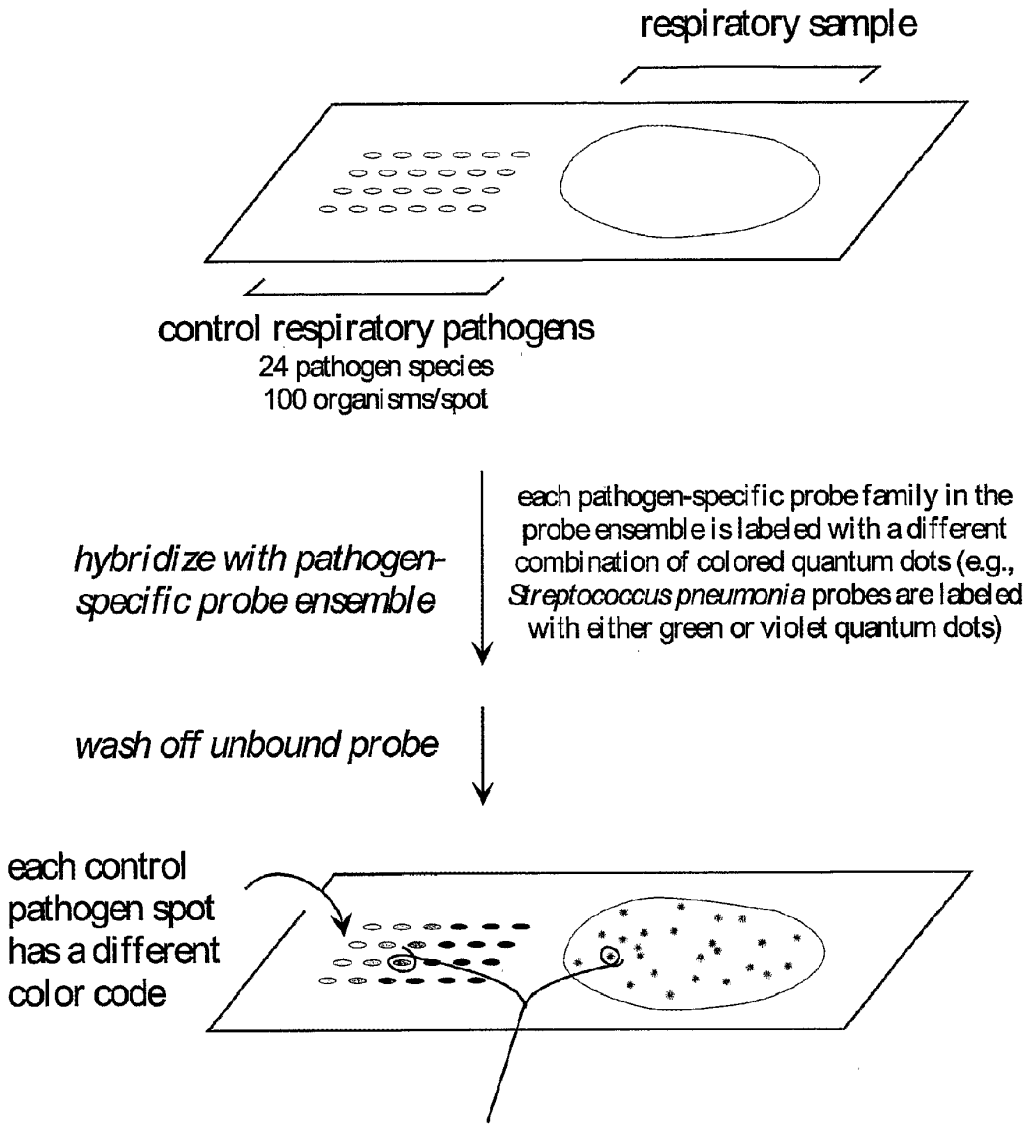


Figure 40 Scanning a respiratory sample for 24 different pathogens simultaneously using large area imaging (Example 39).

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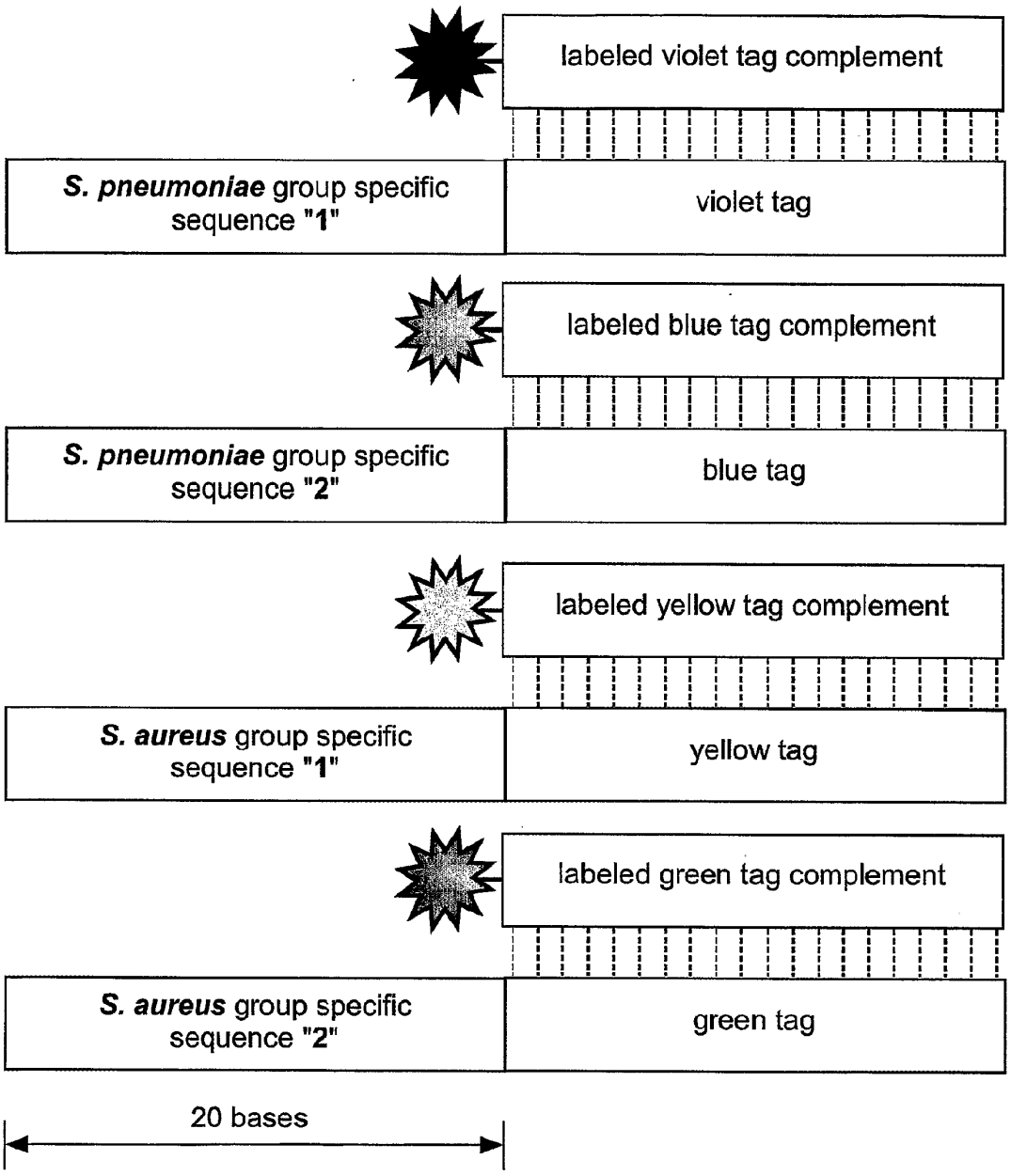


Figure 41. Indirect combinatorial labeling of group-specific sequences (Example 40).

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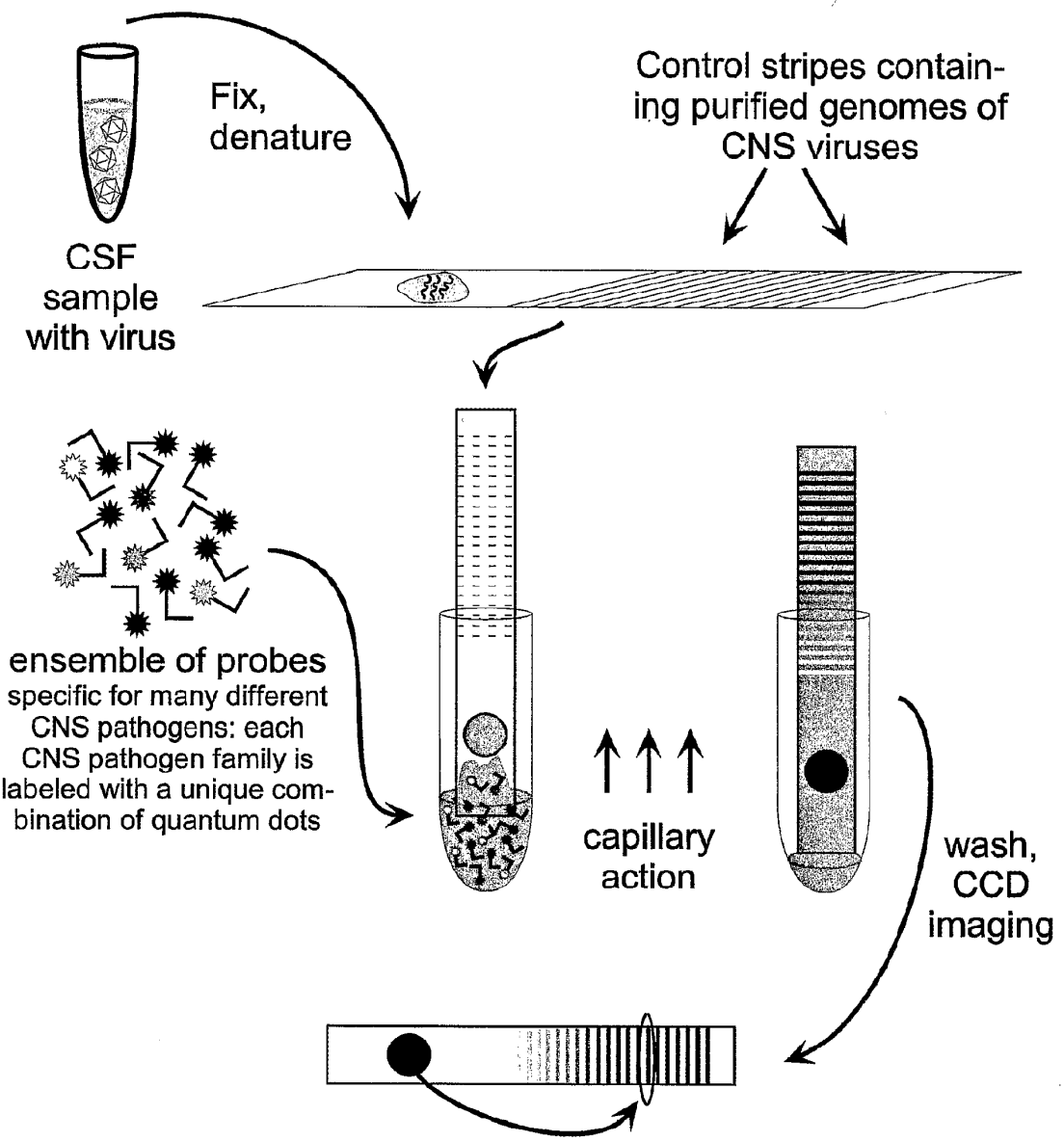


Fig 42. Scanning for numerous CNS viral pathogens simultaneously using a rapid hybridization chromatography assay (Example 41).

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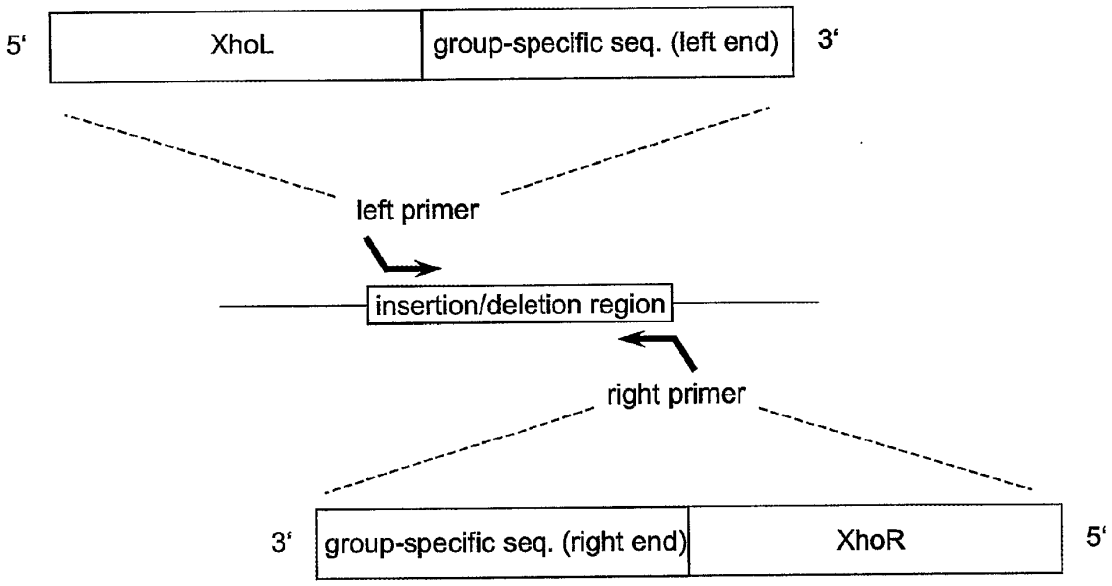


Figure 43. Bipartite primers for PCR amplification of the products of "virtual subtraction" (Example 43).

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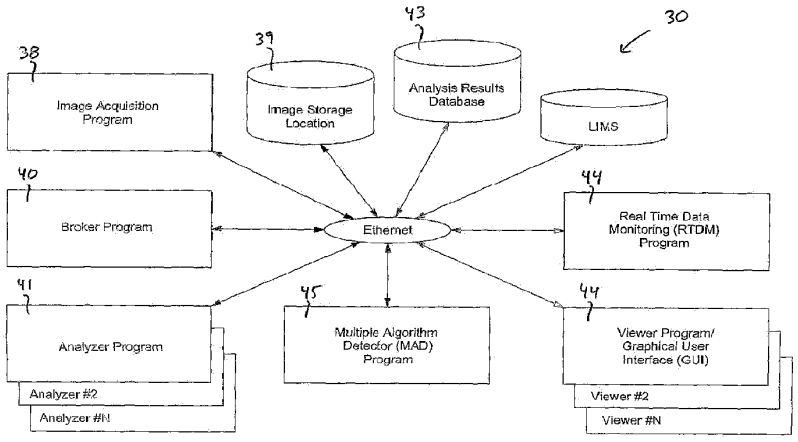
(74) Agents: SILVA, Robin, M. et al.; 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111 (US).

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[Continued on next page]

(54) Title: CHEMICAL REACTION MONITOR



(57) Abstract: An apparatus for parallel monitoring of a plurality of chemical reactions, each chemical reaction occurring within a respective one of a plurality of wells. The apparatus includes a lighting device for illuminating the plurality of wells, the lighting device having an light emitting diode (LED) array, a camera device configured to obtain images of the plurality of wells and saving the images to an image storage location, the camera device having a charge couple device (CCD) capable of imaging the plurality of wells simultaneously, a viewing program for viewing one or more of the saved images, and an analyzing program for opening each saved image and geometrically registering each saved image in order to determine a specific value corresponding to each one of the chemical reactions at the time the image was obtained, the analyzing program saving the specific values to an analysis results database. A method of utilizing the apparatus is also disclosed.

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

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CHEMICAL REACTION MONITOR

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 60/441,752 filed January 21, 2003, entitled CHEMICAL REACTION MONITOR, the entire contents of which is incorporated herein by this reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates to the field of monitoring chemical reactions. More particularly, the present invention relates to a new and improved chemical reaction monitor for use in monitoring parallel chemical reactions.

[0003] The chemical reaction monitor of the present invention is particularly suited for real-time monitoring of oligonucleotide synthesis. The chemical reaction monitor of the present invention is also particularly suited for providing a quality control (QC) measure for oligonucleotide production.

Description of Related Art

[0004] Oligonucleotide synthesis is a cyclical process that assembles a chain of nucleotides. Nucleotides are added one by one through a cycle of chemical reactions, in which a particular molecule (*e.g.*, a nucleotide) is added to a

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growing DNA molecule (e.g., a growing DNA chain), sometimes via catalysis, until the desired chain is complete. Generally, each cycle of chemical reactions includes the steps of detritylation, coupling, capping and oxidation.

[0005] During the detritylation or "deprotection" step, a dimethoxytrityl (DMT) group is removed from the last nucleotide of the growing DNA chain to allow the addition of the next nucleotide. The amount of DMT released from each cycle is monitored to insure a high coupling efficiency. The release of DMT is apparent because a bright orange color is emitted as DMT is released.

[0006] Monitoring of the detritylation or deprotection step in known commercial synthesizers is done in the form of a spectrophotometer monitoring a cuvette through which wash waste is passed. Such monitoring is done on a discrete per sample basis in systems that process as many as 32 oligonucleotide samples simultaneously. During the detritylation step the waste is monitored for the presence and magnitude of the orange color indicating the release of DMT. As the number of simultaneous reactions increases, the ability to use known methods to monitor reaction progress becomes prohibitively inefficient.

[0007] What is needed is a chemical reaction monitor that allows parallel monitoring of chemical reactions in which each sample may be observed and monitored both discretely and as a collection. The present invention satisfies this need and provides other advantages as well.

BRIEF SUMMARY OF THE INVENTION

[0008] One aspect of the present invention is directed to a chemical reaction monitoring system for parallel monitoring of a plurality of chemical reactions wherein each chemical reaction occurs within a respective one of a plurality of wells. The system includes a lighting device for illuminating the plurality of wells, a camera device configured to obtain a fresh image of the plurality of wells and saving the fresh image to an image storage location, a viewer program for

viewing the saved fresh image, and an analyzer program for accessing the saved fresh image, geometrically registering the saved fresh image in order to determine a specific value corresponding to each one of the chemical reactions within each well at the time the image was obtained, and saving the specific values to an analysis results storage location.

[0009] A system of the invention can further include optics for directing light from a lighting device to the sample and for directing the light from the sample to the camera.

[0010] In one embodiment, the chemical reaction is oligonucleotide synthesis. Thus, the device can be integrated into an oligonucleotide synthesis instrument. The plurality of wells may be located in a multi-well plate.

[0011] The lighting device may include a light emitting diode (LED) array. The LED array may have a first array and a second array positioned on either side of a multi-well plate-viewing window. Alternatively, the LED array can include a single array positioned pivotally mounted on one side of a multi-well plate-viewing window.

[0012] In one embodiment, the camera device is a charge couple device (CCD) capable of imaging the plurality of wells simultaneously. The camera device can be coupled with imaging optics for simultaneous imaging of a plurality of wells. The analyzer program may process the fresh image and save a corresponding processed image to the analysis results storage location.

[0013] The invention further provides a chemical synthesis system. The system may include one or more of the following components: (a) a sample holder placed to support a plurality of wells; (b) a liquid dispenser placed to dispense a liquid sample to the plurality of wells; (c) a liquid removal device placed to remove the liquid sample from the plurality of wells; (d) an automated device for

controlling said liquid dispenser or said liquid removal device; (e) a lighting device for illuminating the plurality of wells; (f) a camera device configured to obtain a fresh image of the plurality of wells and save the fresh image to an image storage location; (g) an computer system configured to: (i) access the saved fresh image; (ii) determine a specific value corresponding to each one of the chemical reactions within each well at the time the image was obtained; and (iii) communicate an instruction to the automated device.

[0014] Also provided is a chemical synthesis system that may include one or more of the following components: (a) a sample holder placed to support a plurality of wells; (b) a liquid dispenser placed to dispense a liquid sample to the plurality of wells; (c) a liquid removal device placed to remove the liquid sample from the plurality of wells; (d) a lighting device for illuminating the plurality of wells; (e) a camera device configured to obtain a fresh image of the plurality of wells and save the fresh image to an image storage location; and (f) a computer system configured to: (i) access the saved fresh image; (ii) determine a specific value corresponding to each one of the chemical reactions within each well at the time the image was obtained; and (iii) communicate a representation of the specific values to a graphical user interface.

[0015] In particular embodiments a liquid dispenser can be placed to independently dispense various liquid samples to the plurality of wells. Thus, different samples can be added to each well. A liquid dispenser can be placed under computer control. Accordingly, commands can be sent from the computer directing the liquid dispenser to dispense liquid, stop dispensing liquid, dispense a particular volume of liquid, or to dispense to a particular well in a plurality of wells being monitored.

[0016] Another aspect of the present invention is directed to a method for parallel monitoring of a plurality of chemical reactions, wherein each chemical reaction occurs within a respective one of a plurality of wells. The method includes the

steps of illuminating the plurality of wells, and obtaining images of the plurality of wells with a camera device and saving the images to an image storage location, the camera device being capable of imaging the plurality of wells simultaneously.

[0017] The method may further include the step of viewing one or more of the saved images. The method may further include the step of analyzing the saved images by opening each saved image and geometrically registering each saved image in order to determine a specific value corresponding to each one of the chemical reactions at the time the image was obtained, and saving the specific values to an analysis results storage location.

[0018] The invention further provides a method for synthesizing a plurality of different polymers. The method may include one or more of the following steps: (a) providing a plurality of wells containing support-bound monomeric or oligomeric precursors of the polymers; (b) dispensing second monomeric precursors of the polymers to the plurality of wells under conditions for forming an intermediate in which the support-bound monomeric or oligomeric precursors are bound to the second monomeric precursors; (c) obtaining a fresh image of the plurality of wells and saving the fresh image to an image storage location; (d) executing commands in a computer system to access the saved fresh image, geometrically register the saved fresh image in order to determine a specific value corresponding to each one of the chemical reactions within each well at the time the image was obtained, and save the specific values to an analysis results storage location; and (e) if the specific values are within a pre-defined passing range then repeating steps (a) through (d) and if the specific values are within a pre-defined failing range then preventing repetition of steps (a) through (d) for at least one of the wells in the plurality of wells.

[0019] An object of the present invention is to provide a method and apparatus for monitoring parallel chemical reactions in which each one of a plurality of samples may be observed and monitored both discretely and as a collection.

[0020] Another object of the present invention is to provide a method of using a camera to allow each one of a plurality of samples to be observed and monitored both discretely and as a collection.

[0021] Another object of the present invention is to provide a method of using a single or collection of optical filtering, remote triggering of the camera, remote triggering of an array of photodiodes, other suitable processes, or a combination thereof to allow a measurement of photons of a specific spectral quality using the monitoring apparatus.

[0022] The chemical reaction monitor of the present invention has other features and advantages which will be apparent from or are set forth in more detail in the accompanying drawings, which are incorporated in and form a part of this specification, and the following Detailed Description of the Invention, which together serve to explain the principles of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] FIG. 1 is a perspective view of a chemical reaction monitor of the present invention including a camera device and a lighting device mounted on an oligonucleotide synthesizer for capturing images of a multi-well plate.

[0024] FIG. 2 is an enlarged perspective view of the lighting device of FIG. 1.

[0025] FIG. 3 is a schematic view of a chemical reaction monitor in accordance with the present invention utilizing the chemical reaction monitor of FIG. 1.

[0026] FIG. 4 is a graphical representation of DMT measurements of a well of the multi-well plate taken during an exemplary successful oligonucleotide synthesis.

[0027] FIG. 5 is a graphical representation of DMT measurements of a well of the multi-well plate taken during an exemplary unsuccessful oligonucleotide synthesis.

[0028] FIG. 6 is a schematic representation of a graphical user interface of the chemical monitor of FIG. 3 illustrating stored data regarding the status of a specified multi-well plate during a specified process.

[0029] FIG. 7 is a schematic representation of a graphical user interface of the chemical monitor of FIG. 3 providing an overview of a plurality of networked oligonucleotide synthesizers.

[0030] FIG. 8 is a graph of an exemplary exponential decay showing yields as a function of "cycle" based on a fictitious substance and coupling efficiency.

[0031] FIG. 9 is a graphical representation of DMT measurements of a well of the multi-well plate taken during an exemplary successful oligonucleotide synthesis as compared to an expected DMT value.

[0032] FIG. 10 is a perspective view of another chemical reaction monitor of the present invention including a camera device and a lighting device mounted on an oligonucleotide synthesizer for capturing images of a multi-well plate.

[0033] FIG. 11 is an enlarged perspective view of the lighting device of FIG. 10.

[0034] FIG. 12 is a perspective view of a chemical reaction monitor of the present invention including a camera device and a lighting device mounted on an oligonucleotide synthesizer for capturing images of a multi-well plate.

DETAILED DESCRIPTION OF THE INVENTION

[0035] Reference will now be made in detail to the preferred embodiments of the present invention, examples of which are illustrated in the accompanying drawings. While the invention will be described in conjunction with the preferred embodiments, it will be understood that they are not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover alternatives, modifications and equivalents, which may be included within the spirit and scope of the invention as defined by the appended claims.

[0036] The chemical reaction monitor of the present invention is configured for use in monitoring parallel chemical reactions and is particularly suited for real-time monitoring of polymer synthesis such as oligonucleotide synthesis or peptide synthesis or both. The chemical reaction monitor of the present invention is also particularly suited for providing a quality control (QC) measure for oligonucleotide or peptide production. In one embodiment, the chemical reaction monitor is an automated DMT monitoring system used to track the yield, quality and general state of oligonucleotides (DNA) being synthesized at any one time. Although the present invention is exemplified herein in the context of polynucleotide synthesis one should appreciate that the chemical reaction monitor of the present invention is not limited to oligonucleotide synthesis and may be used in the monitoring of other types of chemical reactions.

[0037] A chemical reaction monitor of the present invention can be used to monitor any reaction that produces an optical signal indicative of reaction progress. Accordingly, an optical signal that results directly from a reaction can be monitored. For example as set forth below, the release of optically inactive DMT during deprotection steps of a polynucleotide synthesis can be monitored. In particular embodiments, a reagent can be added to a reaction to react with a product of the reaction thereby changing an optical property of the reaction mixture. For example, a reagent that becomes optically active in the presence of

a desired product, modifies a particular product to make the product optically active, reduces optical activity of a desired product, or becomes less optically active in the presence of a desired product can be used. In particular embodiments, polypeptide synthesis can be monitored based on the addition of a monitoring reagent such as bromophenol blue. Methods for peptide synthesis utilizing bromophenol blue to monitor the status of peptide coupling are known as described, for example, in U.S. patent 5,342,585, entitled APPARATUS FOR MAKING MULTIPLE SYNTHESIS OF PEPTIDES ON SOLID SUPPORT and issued to Lebl et al. on August 30, 1994. Other reagents that can be used in a polypeptide synthesis and used in an apparatus of the invention for simultaneously monitoring a plurality of reactions include, for example, trinitrobenzene sulfonic acid which can be added to a polypeptide reaction or ninhydrin which can be added to an aliquot removed from a peptide reaction in accordance with the Kaiser test kit (Fluka Chemicals, Cat No. 60017),.

[0038] Any of a variety of known optical properties can be monitored in accordance with the invention including, without limitation, absorbance, fluorescence, chemiluminescence, polarization, circular dichroism, fluorescence resonance energy transfer (FRET), light scattering or the like. Furthermore, those skilled in the art will readily recognize that an optical detection device, such as those set forth herein can be modified or replaced with any of a variety of known detection devices appropriate for monitoring such optical properties. Further still, the invention can be used to detect changes in optical properties that occur in one or more range of the electromagnetic spectrum including, without limitation, infrared, visible, ultraviolet, x-ray, microwave, sub-regions thereof such as red, blue or yellow sub-regions of the visible region, combinations of these regions or subregions, or other regions of the spectrum.

[0039] In accordance with the present invention, an oligonucleotide synthesizer is configured to acquire an image of a plurality of wells, such as the wells of a multi-well plate. As used herein the term "multi-well plate" is intended to mean a

substrate having a plurality of discrete chambers suitable for holding a liquid. A substrate included in the term can be, for example, molded plastic such as polystyrene or polypropylene. Exemplary multi-well plates include, for example, microplates, microtiter plates or n-well plates where "n" is the number of wells including, for example, 8-, 16-, 96-, 384-, or 1536-wells. As used herein, the term "microplate" is intended to mean a multi-well plate that has dimensions and properties consistent with the definition provided by the Society for Biomolecular Screening (Danbury, CT, USA). A multi-well plate can have wells with any of a variety of cross-sectional shapes including, for example, cylindrical, square, rectangular, multisided, interlocking shapes wherein the bottom of wells are flat, conical, pointed, or round.

[0040] A plurality of wells imaged or processed in accordance with this disclosure can be located in a single substrate or in multiple substrates. In one embodiment each well can be a separate tube occurring in a plurality of tubes. Such tubes can occur at fixed locations in a holder or substrate or can be moveable such that one or more of the tubes changes relative location with respect to another tube in the plurality.

[0041] The apparatus and methods set forth herein can be used to monitor large pluralities of reactions simultaneously including, for example, at least 96, 100, 384, 500, 1000, 1536, 2000, 3000, 4000, 5000, 10,000, 100,000 or more reactions. The reactions can occur simultaneously such that all are in the same stage of a reaction cycle or, alternatively, reactions proceeding through different stages of a reaction cycle can be monitored. Furthermore, different types of reactions can be simultaneously monitored. For example, an apparatus or method of the invention can be used to simultaneously monitor synthesis of a plurality of peptides and a plurality of polynucleotides.

[0042] An apparatus of the invention can be configured to acquire an image during any step of synthesis including, but not limited to, wash cycles, coupling

cycles, capping cycles and oxidation cycles. One will appreciate that image acquisition may be useful in debugging machine or software issues and for other technical means. Image acquisition is particularly suited for obtaining images of the wells of a multi-well plate during the deprotection step in order to determine DMT intensity.

[0043] Generally, an image is acquired, uniquely named and sent as a "fresh" or unprocessed image file to an image storage location. Upon arrival at the storage location, an analyzer program can process the fresh image file. For example, upon detecting a fresh image, the analyzer program can open the image, register the relative position of the multi-well plate to mitigate positional errors, and extract an optical intensity value from each well of the multi-well plate. Upon completion, the analyzer program can write the optical intensity data to an analysis results storage location. The process image file may be renamed to indicate that the image has been processed. In accordance with the present invention, this process may take place in as little as approximately 8 seconds thus allowing the analyzer program to populate the analysis results database with near real-time data. An apparatus or method of the invention generally includes a computer system such as those based on INTEL® microprocessors and running MICROSOFT WINDOWS® operating systems. Other systems such as those using the UNIX® or LINUX® operating system and based on IBM®, DEC® or MOTOROLA® microprocessors are also contemplated. The systems and methods described herein can also be implemented to run on client-server systems and wide-area networks such as the Internet.

[0044] As used herein the term "storage location" is intended to mean a computer readable memory. Exemplary forms of computer readable memory include, but are not limited to, a database, hard disk, floppy disc, compact disc, magneto-optical disc, Random Access Memory, Read Only Memory or Flash Memory. The memory or computer readable medium used in the invention can be contained within a single computer or distributed in a network. A network can be any of a

number of conventional network systems known in the art such as a local area network (LAN) or a wide area network (WAN). Client-server environments, database servers and networks that can be used in the invention are well known in the art. For example, the database server can run on an operating system such as UNIX®, running a relational database management system, a World Wide Web application or a World Wide Web server. Other types of memories and computer readable media are also contemplated to function within the scope of the invention.

[0045] Data stored at a storage location can be in any of a variety of forms known in the art including, for example, a text file, .xml file, .jpg file, TIFF file, or BMP file. Software to implement a method of the invention can be written in any well-known computer language, such as Java, C. C++, Visual Basic, FORTRAN or COBOL and compiled using any well-known compatible compiler. The software of the invention normally runs from instructions stored in a memory on a host computer system. A database or data structure of the invention can be represented in a markup language format including, for example, standard generalized markup language (SGML), hypertext markup language (HTML) or extensible markup language (XML).

[0046] A computer system useful in the invention can further include a laboratory management system (LIMS). A LIMS system can contain information relating to the sequence of a polymer to be synthesized as information about manipulations that have been and are to be carried out on the polymer. The entire sequence information regarding the synthesis of a polymer can be copied from LIMS into the analysis results storage location for quick, native retrieval by a technician or other authorized personnel. This can provide the advantage of minimizing the calls to LIMS to just one per synthesis.

[0047] The chemical reaction monitor of the present invention generally includes a hardware component and a software component. The hardware component

captures images of a plurality of wells of a multi-well plate containing samples in which chemical reactions occur. The software component can be subdivided into a storage subcomponent, an analyzer subcomponent, an analysis results and a viewing subcomponent.

[0048] In one embodiment shown in FIG. 1, a chemical reaction monitor 30 includes a hardware component mounted on an oligonucleotide synthesizer system 31 of the type including, but not limited to the OLIGATOR® DNA synthesis platform, developed by Illumina, Inc. of San Diego, California. Similarly, the hardware component may be mounted on oligonucleotide synthesizers such as, but not limited to, those disclosed by U.S. Patent Application No. 09/881,052, filed June 13, 2001, entitled OLIGONUCLEOTIDE SYNTHESIZER, and now U.S. Patent No. 6,663,832, the entire contents of which is incorporated herein by this reference. Other polymer synthesizers useful in the invention are described in U. S. Patent Nos. 5,338,831 and 6,121,054 and WO 00/44491, the entire contents of which patents are incorporated herein by this reference. Reagents can be dispensed to wells and removed by centrifugation as described in the above patent and patent application. Polymer precursors including, but not limited to, monomeric and oligomeric precursors can be attached to wells or other solid phase supports using methods known in the art as described for example in the above patent and patent application.

[0049] One should appreciate that the hardware component or the software components of the present invention or both may be used in combination with other systems in which multiple chemical reactions occur in parallel. Thus, reagents can be dispensed, manipulated, and removed from wells using methods utilized in synthesizers from Applied Biosystems, Perkin Elmer and other commercial vendors. Furthermore, several chemical synthesizers, each having appropriate hardware components such as those set forth herein, can be networked for communication with a centralized software component such that multiple instruments can be monitored.

[0050] Chemical reaction monitor 30 can include a hardware component having a computer accessible camera device 32, a lighting device 33 and a client server based software component for controlling the camera device and/or the lighting device. The camera device and the lighting device facilitate capturing an image of a multi-well plate 34, including a plurality of wells 35 located therein, during specific points of a chemical synthesis process.

[0051] The lighting device is positioned in a manner to illuminate a multi-well plate of a synthesizer apparatus and the wells thereof, for example, as shown in FIG. 2 for an oligonucleotide synthesizer. Any lighting source that allows wells to be photographed, captured as an image, or otherwise evaluated to determine optical properties can be used in the invention. Typically, a lighting device provides homogeneous illumination to all wells being monitored. . A lighting device can also be selected to transmit light of a particular range of wavelengths including, for example, visible, ultraviolet, infrared, sub-ranges thereof such as red, blue or yellow sub-ranges within the visible range, or combinations of these ranges or sub-ranges.

[0052] In particular embodiments, the lighting device includes a light emitting diode (LED) array 36 arranged adjacent the multi-well plate. A cold DC power source may be utilized to power the LED array. Advantageously, the DC power to each LED or the mechanical position of the various LEDs can be independently controlled to optimize the imaging of the camera. The DC/LED configuration of the present invention can be used to minimize and/or prevents dark or dim images. In particular embodiments, other forms of lighting such as incandescent or fluorescent lighting can be used. Those skilled in the art will know or be able to determine appropriate illuminating, optical manipulation and imaging configurations to provide a sufficiently homogeneous illumination for monitoring a chemical reaction occurring in a plurality of wells.

[0053] As shown in FIG. 2, two opposing LED arrays can be provided on either side of an aperture or rectangular plate glass window 37, which window provides a view of the multi-well plate beneath the glass window. In one embodiment, one LED array may be positioned slightly higher than the other. Typically, the LED arrays are positioned out of field of view of the camera.

[0054] One should appreciate that the LED array may vary in accordance with the present invention. For example, one, two, three or more LED arrays may be used. Furthermore, the LED arrays may be configured to utilize different wavelengths, diffusion patterns, voltages, etc. in order to influence the imaging of the camera, for example, improving the usefulness of data captured for a particular reaction being monitored.

[0055] Other lighting devices that can be used include, for example, one or more lamps, lasers or bulbs. Such devices are typically selected to provide homogeneous illumination to a plurality of wells being monitored. However, in embodiments in which the light source itself is not homogeneous a diffuser module can be used to provide homogenous lighting conditions to wells being monitored. One or more lenses can also be used in combination to provide homogeneous illumination.

[0056] If desired the wavelength of light that contacts wells to be monitored can be provided by a particular combination of light source and optical filters. An optical filter useful in the invention can be any device for selectively passing or rejecting passage of radiation in a wavelength, polarization or frequency dependent manner. Exemplary filters include an interference filter in which multiple layers of dielectric materials pass or reflect radiation according to constructive or destructive interference between reflections from the various layers. Interference filters are also referred to in the art as dichroic filters, or dielectric filters. The term can include an absorptive filter which prevents passage of radiation having a selective wavelength or wavelength range by absorption.

Absorptive filters include, for example, colored glass or liquid. Those skilled in the art will know or be able to determine an appropriate combination of light source and optical filters to produce illumination in a desired wavelength range.

[0057] Beam splitters, lenses, or other devices for changing the path of illumination can be used in an apparatus of the invention. Beam splitters, lenses and their properties are known in the art and can be obtained from commercial sources including, for example, Melles Griot (Irvine, Calif.), or Oriel Corp. (Stratford, Conn.). In particular embodiments, a beam splitter can be used for perpendicular illumination and viewing a multi-well plate or other plurality of wells. Thus, optical components can be arranged differently from the arrangements exemplified herein while providing illumination properties similar to those described herein. Accordingly, an apparatus of the invention can be configured for compact or modular placement suitable for a variety of laboratory environments.

[0058] If desired, a mask can be used in an apparatus of the invention to preferentially illuminate one or more wells in a multi-well plate or other plurality of vessels. For example, a mask can be placed to selectively illuminate wells of a multi-well plate compared to regions between the wells to prevent unwanted light scatter or to enhance contrast of images and ease registration of images obtained from the multi-well plate.

[0059] In particular embodiments, the intensity of each LED in an LED array can be independently controlled. For example, each LED intensity to be controlled by a manual adjustment such as adjustment with a potentiometer. Such control over the LED intensity can also be achieved programmatically, for example, via a networked GUI or computer system. An advantage of computer controlled illuminator electronics is that lighting intensity for all or part of the LED array can be changed remotely via software. Thus, an individual user can interact with a GUI to send commands that alter intensity of one or more LED in an array. A

further advantage is that illumination intensity can be controlled by a feedback loop in which one or more properties of the illumination are monitored, automatically compared by a computer algorithm to expected ranges for the one or more properties and intensity of one or more LEDs automatically adjusted in accordance with commands sent by the computer algorithm. Thus, a user need not intervene and properties such as LED intensity, homogeneity of the field of light or color of illumination can be adjusted in an automated fashion. Such properties can be determined using an image analyzer such as those described below in further detail.

[0060] In one embodiment, camera device 32 is a charge couple device (CCD) camera of the type including, but not limited to, the AXIS 2100 Network Camera provided by AXIS Communications, Inc. of Lund Sweden. One should appreciate that other types of cameras may be used in accordance with the present invention. A camera used in the invention can be any device that converts a detectable optical property into a signal in a location dependent manner. Thus, exemplary cameras useful in the invention include, for example, complementary metal oxide semiconductor (CMOS) camera, video camera, internet camera, and other imaging devices capable of converting a picture into a digital image. A camera device of the invention can be functionally connected to a computer such that a digital image or other data indicative of a signal detected by the camera can be communicated to the computer for further storage or processing or both. The camera device can include a built-in microprocessor for example, in one embodiment, the camera includes a built-in LINUX device and web server. Such configuration allows the camera device to be connected directly to the central network and be configured to store images in a designated image location on the central network. Camera modules discussed here incorporate imaging optics, photon detection devices, digitizing electronics, and electronic communication capability. These tasks can also be accomplished with separate components as opposed to an integrated camera module.

[0061] A camera device can be positioned on a chemical synthesizer over a multi-well plate or other plurality of wells such that it is capable of obtaining an image which includes all of the wells, as is shown in FIG. 1 for an oligonucleotide synthesizer. The multi-well plate generally includes 96 or 384 wells, however, one should appreciate that the multi-well plate may include more than 384 wells. Advantageously, the camera device not only allows observation of the multi-well plate as a whole, but also allows observation of each well individually. A camera device can incorporate an optical filtering stage rendering the detector capable of spectral measurements and tunable to specific chemicals that exhibit specific absorptivities in spectrum. Exemplary optical filters that can be used to filter light entering the camera include, without limitation, those set forth above with respect to lighting devices. An optical filter used for a camera device can match the emission of a particular lighting device in order to create a powerful absorption measurement in reflection mode and for selective detection of desired signals in a well to be detected or selective rejection of unwanted background signals. An RGB (Red, Green, Blue) camera device can also be used instead of a single color device. As with other cameras described herein, an RGB camera can be used without an optical filter if desired.

[0062] A camera device useful in the invention can include an internal camera microprocessor having an image acquisition program 38 that can be signaled to take an image and transfer the image electronically to a storage location such as a central network file repository or image storage location 39 on the central network shown in Figure 3. The image storage location can be a path location on the network. The image storage location is not machine specific and may be provided on different platforms including, but not limited to LINUX®, WINDOWS®, and other suitable operating systems such as those set forth herein previously. The storage location can be dynamically configurable by each of the chemical reaction monitor's components, so if a problem occurs all subcomponents of the software component can be pointed to a different location with minimal effort.

[0063] In the exemplary embodiment shown in Figure 3, the oligonucleotide synthesizer system is configured to control the camera device. For example, OLIGATOR® control software may be configured to signal the camera device, via the central network, when to take an image of a multi-well plate of the oligonucleotide synthesizer. The image may be transferred to the image storage location (see FIG. 3) in the form of a JPEG, TIFF, BMP file or other suitable file format.

[0064] In particular embodiments, the image files are named according to batch number, plate number, cycle number and loop number within the synthesis protocol that the synthesis process is at when the image is taken. In this respect, the term "cycle" refers to the complete cycle of steps for the addition of each base to the growing DNA chain, including the deprotection, coupling, capping, and oxidation steps. The term "loop" refers to an operation that is performed several times within the same cycle. For example, the deprotection step may repeat two or three times within the same cycle whereby a "loop" designation may be used to differentiate the particular deprotection operation within a cycle.

[0065] Turning now to FIG. 3, which figure illustrates an exemplary software component of chemical reaction monitor 30 in accordance with the present invention, separate software subcomponents or programs can be provided to independently or autonomously store, analyze, and view the images. For example, the camera device may include means such as image acquisition program 38 to autonomously send images to image storage location 39 on the central network. Similarly, the various subcomponents including, but not limited to, the broker, analyzer, viewer, multiple analogue and/or real-time data management programs described below may be separate and discrete from one another in order to remove dependencies. The network architecture of Figure 3 is exemplary and those skilled in the art will recognize that a variety of other architectures and modifications of the exemplified architecture can be used to

produce a chemical reaction monitoring system having the properties set forth herein.

[0066] An autonomous computer system configuration provides independence that allows images to be obtained and/or viewed without overburdening resources necessary for image analysis. This also allows individual development of the different subcomponents in different languages and/or platforms, by different engineers. Such autonomy also allows any one of the many subcomponents to be taken offline, causing at most, the other subcomponents to patiently wait for the off-line subcomponent to return to service, as opposed to a major system shut down. Thus, if one of the imaging, analyzing, or viewing subcomponents malfunction, the malfunction will not affect the remaining programs. For example, if the viewer program crashes, the discrete camera imaging software still allows the camera to continue capturing and storing images, which images may continue to be analyzed and, once the viewer program is again running, allow one to again view the stored images.

[0067] In one embodiment, when a synthesis starts, and in particular, when a de-protection step is encountered, an image is taken of the multi-well plate (see FIG. 6) and sent to the image storage location. The image storage location may be local or it can reside at a particular centralized network address. A broker program 40 can be configured to constantly observe the image storage location for new images. For example, the broker program can constantly observe the image storage location and define images as being "new" merely by their presence in this location, and by having a specific file extension, for example ".jpg" in the case of JPEG images, and/or other suitable image format extensions. The broker program can build a list of new image file names every time the queue is empty.

[0068] A broker program, replete with an internal queue of unique image filenames, can reside at an IP address on a network and can assign a unique file

name to each new image. A filename can encode information that is specific to a particular synthesis and step of the synthesis, such as the plate, the batch, the cycle, loop, machine name, etc.

[0069] A separate analyzer program 41 can be responsible for processing stored images. The analyzer program can run in the background autonomously and independent of the image storage program and other programs of the software component.

[0070] The analyzer program can scan for fresh images, that is, unprocessed or to-be-analyzed images, through a broker program. Once the analyzer program gets the filename of a fresh image, it can retrieve the file from the network location and perform image analysis. The analyzer program can individually open each image stored in the image storage location (see FIG. 3), register the image geometrically, then sample specific regions of the image for specific values that relate to the magnitude of the hue, saturation and luminance of the bright orange color which is emitted as DMT is released during the deprotection step of oligonucleotide synthesis. The specific regions of the image sampled can correspond to each well of the multi-well plate that has been imaged or photographed. Typically, the specific regions correspond to the center of each well. However, if desired any part of each well can be correlated with specific regions of the image.

[0071] The analyzer program can analyzes one or more optical property, such as DMT intensity, for every well in a multi-well plate, extract the intensity value and store the intensity value in memory. For example, the specific values which relate to the magnitude of the hue, saturation and luminance of each well of the image can determined and stored. Once in memory, the values for a particular optical property can be formed into a structured query language (SQL) statement and appended to an SQL database or analysis results database. After the database update is complete, the analyzer program can move the file to a new "processed"

location and changes the extension of the filename, for example, from ".jpg" to ".jpr" to indicate in a human-readable form that the image has indeed been processed.

[0072] At this point, the analyzer program may request another image from the queue. The analysis or processing cycle for each image typically takes less than approximately 10 to 20 seconds and can be about 8 seconds or less. The chemical reaction monitor may be provided with multiple copies of the analyzer program, running from different personal computers throughout a company's network. For example, 1, 2, 3 or more analyzer programs may simultaneously connect to the network and simultaneously analyze different images. Thus, as workload (e.g., the number of images to be analyzed) increases, additional analyzer programs can be started to ensure that the rate of analysis meets or exceeds a desired rate of image generation to ensure that throughput demands are met.

[0073] In some instances, the synthesizer requires the use of multi-well plates having filters or frits 42 (see FIG. 6). It has been determined that frits have a time varying intensity that is not linearly proportional to DMT intensity or concentration. It is hypothesized that this is due to the individual absorption rates of each frit, and drain rate of each well. In the event that frits are utilized, the frit is generally clearly visible in the bottom of each well. The analyzer program can be configured to programmatically exclude the image of the frit from data collection. Similarly other features present in an image of a plurality of wells can be programmatically excluded from data collection.

[0074] Continuing with the exemplary oligonucleotide synthesizer shown in the figures, the analyzed images can be stored elsewhere on the central network (e.g., with the ".jpr" extension) and specific values which relate to the magnitude of the hue, saturation, luminance or the grayscale intensity of each well of the image can then be logged into an analysis results database 43 on the central

network (see FIG. 3). This database can then be queried for either "real-time" monitoring and/or "historical" monitoring. Such configuration allows a technician to query the analysis results database while a process is going and visually identify problems in a near real-time fashion. Such configuration also allows future review of the storage location in a quality control (QC) fashion to look at images of completed batches for confirmation of problems that have happened in the past (i.e., historical monitoring) during the completed batches. The data analysis storage location can contain two table structures, one for laboratory information management system (LIMS) sequence data and one for real-time DMT values. The storage location may be comprised of any suitable database software including, but not limited to MICROSOFT® SQL SERVER® and MICROSOFT ACCESS®.

[0075] Certain determinations about the synthesis may be made from the observed images and/or the stored data. For example, the degree of color degradation may be tied to coupling efficiency in which a lower degree of color degradation indicates a higher coupling efficiency.

[0076] Observing a significant decline in the magnitude of the orange color emitted by the release of DMT can provide an early indication that a synthesis is suffering a general or catastrophic failure. In this regard, relatively quick color degradation within a couple of cycles is indicative of failure. Observation of such color degradation allows the synthesis to be stopped, automatically or by a technician, and restarted with fresh materials, thus saving time and money. Alternatively the system can be configured to automatically stop synthesis when changes in optical characteristics deviate substantially from a predefined threshold or range of acceptable values.

[0077] Individual wells or groups of wells that lose color can indicate a clogged nozzle in either a base or bulk delivery nozzle array of a synthesizer. For example, if a loss of color is observed in the same well of all plates, the color loss

may indicate that a bulk reagent nozzle used in delivering reagents to multiple plates is clogged. Similarly, if a loss of color is observed in an entire row of a single plate, the color loss may indicate that a dispenser which should be delivering regents to the row is clogged.

[0078] The analyzer program can have access to the source sequence or protocol file for polymers to be synthesized in order to avoid confusing a failed individual polymer with one that is completed. The architecture of this software can be such that an independent viewer program 44 is provided to allow a technician to monitor the synthesis in near real-time that, in turn, allows for subjective evaluation apart from the analyzer program.

[0079] In one embodiment, a chemical reaction monitoring device of the invention is provided with a multiple algorithm detector (MAD) program 45. The MAD program obtains a list of current batches that are being synthesized and uses these for analysis as follows. Once the list is established, the MAD program retrieves all of the DMT data for each well and each plate for an entire synthesis batch while the synthesis is occurring. The MAD program then works its way through all of the wells and all of the plates for that batch, examining the data for each well and rendering a pass/fail decision of the chemical reaction within that well. The pass/fail decision is written back to the results analysis storage location. The list of batches is re-evaluated every few minutes to update this list and the pass/fail analysis repeated for the new list.

[0080] In some processes, oligonucleotide synthesis may have a protocol that contains N-number of deprotection loops (n being an integer value of one or more), in which case, such information is stored in a protocol file. The MAD program of the present invention may be configured to autonomously determine the number of deprotection loops. In this regard, the MAD program determines the number of deprotection loops based on the image file names. As noted above, the cycle, loop, and other information can be encoded in to the image

filename. Thusly, the MAD program may calculate the summation of the data for all of the loops (1, 2, 3, ... N number of loops per cycle). Similar to the analyzer program described above, the MAD program can take about 10 to 20 seconds, and, in particular embodiments can take about 8 seconds or less, to analyze the data for an entire synthesis. The MAD program can continuously perform analyses as new data is constantly being added to the analysis results storage location.

[0081] Though the structure of the MAD program can support N-number of failure detection algorithms, a primary algorithm in the MAD program is an exponential fit algorithm. For example in the case of oligonucleotide synthesis using DMT deprotection steps, it is known and has been observed that DMT intensity decreases at a predictable rate. In general, this rate is clearly observable given typical molar concentrations of DMT and the range of cycles. DMT intensity "slightly" or "gently" decreases as synthesis progresses and has been found to decay in a negative exponential decay according to the following equation:

Eq. 1: $$\text{DMT Value} = e^{-kx}$$

where the value of "k" is the experimentally determined extinction coefficient which is a function of coupling efficiency and extinction coefficient that dictates the rate or degree in which the DMT diminishes, and "x" represents the cycle of the particular data point.

[0082] The MAD program may be configured to detect synthetic failures by calculating an "expected" optical signal for each well at any given cycle and comparing a detected value to the expected value. Detected values that differ substantially from the expected value are indicative of failure, whereas detected values that are sufficiently similar to the expected value are indicative of a passing score. For example, in the case of oligonucleotide synthesis, an expected DMT intensity value can be calculated based on the initial deprotection

values and the particular cycle. The expected value is determined by a function derived where the expected DMT value (EV) is a function of initial reading, coupling efficiency, and cycle as follows:

Eq. 2: $$EV = I_o * e^{-(100 - eff)/100 * C}$$

where "I_o" is the average initial intensity of the first three values, "eff" is the desired or assumed coupling efficiency in percent and "C" is the particular cycle expressed in an integer value from 0 to 100. During operation, the MAD program continuously compares the current DMT value against the expected value. Once the difference between the expected and real DMT value exceeds a pre-determined threshold, the well or oligo is flagged as "failed". For example, FIG. 9 illustrates DMT values for 1st, 2nd and 3rd deprotection loops of a particular well within a oligonucleotide synthesis cycle. In particular, there are three DMT loop values (y-axis) for every cycle (x-axis) and trityl value for the z-axis. The rear row represents an expected DMT value. In this case, it is apparent that the oligonucleotide suffered a catastrophic event around cycle no. 3 as the DMT values of the third cycle deviate substantially with respect to the expected value. In turn, the well can be flagged as "failed".

[0083] Acceptable values for a passing or failing synthetic step can be selected based on a tolerance level chosen by a particular user. For example, in the case of a relatively short polymer a relatively high loss in yield can be tolerated including, for example, a loss resulting in at least 85%, 90%, 91%, 92%, 93% or 94% yield per step. However, for synthesis of longer polymers lower loss of yield per step is typically tolerated since total yield loss is additive. In such cases a tolerable step yield can be, for example, at least about 95%, 96%, 97%, 98%, 99% or 99.5% yield per step. The pre-determined threshold may be set very conservatively such that exceeding its limits all but ensures the oligonucleotide within the particular well contains too many failure products for use. One should appreciate, however, that other algorithms may be provided to perform more

detailed analysis, corroborate with known standards, and/or define new standards.

[0084] As illustrated in FIG. 1, a graphical user interface (GUI) such as a flat-panel monitor or touch screen 46, a personal computer (PC) or other suitable means, may be provided on the oligonucleotide synthesizer to facilitate near real-time monitoring in which the GUI provides a user-friendly software interface on the oligonucleotide synthesizer. This configuration allows a technician to access the viewer program and thus view an image of the chemical reaction within the wells of the multi-well plate while the oligonucleotide synthesizer is running. Such configuration also allows a technician to browse the latest images (see, e.g., Photograph - Plate 4 in FIG. 6) and thus provides a near real-time monitoring configuration. Accordingly, the technician can be notified of a detected failure in a substantially real-time manner such that the user can stop the synthesis if desired.

[0085] In one embodiment, the viewer program is a graphical user interface (GUI) program, which is provided to readily provide user-friendly data to a technician or other authorized personnel. The GUI program provides a component of the chemical reaction monitoring system that is directly used by humans allowing human interaction with the system. Accordingly, the system can communicate information to a user through the GUI. Conversely, a user can provide information or a command to the system through the GUI program. The GUI program may connect to the analysis results storage location and may continuously retrieve the latest set of analyzed data. The GUI program can be configured to output a visual representation of the stored data. In one embodiment, the GUI program visually provides the user information regarding synthesis by way of touch screen 46 or other suitable means. The GUI program also serves as centralized real-time nerve center that allows anyone on a PC connected to the network to use, review or manipulate data in connection with ongoing or previously performed chemical reactions.

[0086] In one embodiment, the GUI program provides a windows-type graphical interface that may include a digital photograph of a specified plate or other representation of a plate. The GUI may also provide a visual representation as to the status of the chemical reactions taking place within each well. For example, wells that are marked as "failed" may be shown in red (e.g., vertical cross-hatching of wells A2 through A8 and A10), while wells that marked as "pass" may be shown in green (e.g., angled cross-hatching of wells A1, A9, A11 and A12). The GUI program may graphically provide the DMT intensity values of each well of a multi-well plate as shown in the lower left-hand corner of FIG. 6, and/or the cumulative DMT intensity values within a specified well, as shown in the upper right-hand corner of FIG. 6. One will appreciate, however, that the GUI program may be configured to visually represent the data of the analysis results storage location in other suitable means including, but not limited to, numeric representations, graphs, charts and/or tables.

[0087] In one embodiment, the chemical reaction monitor of the present invention is configured for real-time data monitoring (RTDM). A RTDM program 47 may be provided independently of, or integral to, the graphical user interface program or other program of the invention. The RTDM program mines data, such as that described above, for patterns of failure and renders a "pass/fail" decision on whole or partial processes.

[0088] The typical cycle time of an oligo coupling, one step of an oligonucleotide synthesis, takes approximately 10 to 15 minutes. As noted above, the time of a complete analysis or processing cycle of the analyzer program and the MAD program may be approximately 8 seconds or less. As the processing cycle represents a small percentage time as compared to the oligo coupling (e.g., 0.8% to 1.3%), the chemical reaction monitor of the present invention may be considered "real-time". Furthermore, the rate of analysis or processing by the analyzer and MAD programs may be substantially equal to or greater than the rate of image generation or acquisition by the camera. In particular, the analyzer

program may process an image and export the data extracted from the image to the analysis results storage location substantially as fast as an image is created. In other words, as soon as a picture is taken, the analyzer program analyzes the image and the data is logged.

[0089] Given that the data is available from the analysis results storage location in "real-time", the RTDM program can be configured to occur continuously. In particular, the RTDM program may serve as a quality-control program that continuously scans the analysis results storage location for new records and performs data mining from the stored data to ascertain whether or not a particular chemical reaction has passed or failed quality control.

[0090] In the case of oligonucleotide synthesis, there are many different failure patterns that one may observe in DMT intensity. For example, the intensity, or "concentration" of DMT directly correlates to the amount of and the quality of the oligonucleotide. In general, for a given oligonucleotide within a well, if the same DMT level is maintained throughout the entire synthesis, it is generally accepted that the quality of the oligonucleotide will be acceptable (see e.g., FIG. 4). On the other hand, should any significant changes be observed in the DMT level, such as an increasingly negative slope, or an exponential decay, etc, the oligonucleotide quality would likely be unacceptable (see e.g., FIG. 5).

[0091] FIG. 4 shows a graphical representation of DMT intensity measurements of a well of a multi-well plate taken during each cycle, and loop thereof, during an exemplary successful oligonucleotide synthesis. The rear row represents the final or summed measurement of all of the discrete DMT measurements that have occurred throughout the synthesis. In this example, the rear row "approximates" a straight line indicating that the DMT level is substantially maintained throughout the entire synthesis. The rear row does not significantly slope up or down, nor does the rear row have any aberrations. Instead, the substantially flat graph indicates that the oligonucleotide in question is "good".

[0092] In contrast, FIG. 5 shows a graphical representation of DMT measurements of a well of a multi-well plate taken during an exemplary unsuccessful oligonucleotide synthesis. The DMT intensity drops off sharply around cycle 9. In addition, the rear row, which again represents the final or summed measurement, resembles the unit step function indicating that the level of DMT intensity as significantly decreased. As opposed to the "flat" response of the "good" oligonucleotide in FIG. 4, the step response of FIG. 5 indicates that the oligonucleotide is "bad". Accordingly, a failed polymer synthesis can be identified due to a departure from an expected trend in a plot of optical signal change vs. cycle number.

[0093] As the DMT intensity can be monitored to determine whether an oligonucleotide is "good" or "bad", an RTDM program may be configured to mine the data of an analysis results storage location for patterns similar to those described above, alerting the appropriate personnel when failures are detected.

[0094] Advantageously, the chemical reaction monitor of the present invention allows parallel observation of a plurality of wells and allows observation of each well individually. Furthermore, the monitor of the present invention allows monitoring a plurality of wells with one imaging device.

[0095] Advantageously, the chemical reaction monitor of the present invention allows capturing data, analyzing the data, and using the data in a near real-time manner or in a historical manner to measure performance of the oligonucleotide synthesizer. The monitor is configured to detect and announce suspected failures of oligonucleotides, and may be configured to render a quantifiable number relating to oligonucleotide quality, akin to coupling efficiency and so on.

[0096] Advantageously, the imaging, analyzing, and viewing processes can be discrete and autonomous. If any one or more of the processes crashes or otherwise fails, the chemical reaction monitor of the present invention can render

a diagnosis based on the nature of the failure and communicate one or more potential solutions to a graphical user interface. The monitor can further interrupt one or more steps of a synthetic reaction. In one embodiment synthesis in all wells can be paused until a further command is provided by an external user. In other embodiments, delivery of reagent to a subset of wells that have experienced a failure can be discontinued. An advantage of the monitor is that interruption of only a subset of synthetic steps, such as suspending reagent delivery to a subset of failed wells, allows the remaining processes to continue operation while avoiding wasteful delivery of reagents to previously failed wells. Furthermore, the system can be configured such that if the analyzer program malfunctioned, the chemical reaction monitor of the present invention would allow the imaging and viewing processes to continue.

[0097] A chemical reaction monitor of the present invention can be configured to make decisions regarding whether or not to continue synthesis on a multi-well plate based on one or more criteria including, without limitation, the cost of proceeding with the synthesis vs. the value of the synthesis, time constraints on instrument scheduling based on the number of plates to be processed after the particular plate being monitored, the state of repair of the machine based on identification of global failures as well as other quality metrics determined in a manufacturing environment. Information regarding the optical properties of a reaction can be collected, displayed or manipulated in the time domain (i.e. intensity vs. time). In particular embodiments, time domain data can be converted by Fourier transform to provide data in the frequency domain. Thus, periodicity in optical property data will result in an observable frequency component. The Fourier transform can be coupled with wavelet analysis such that the frequencies can be correlated with different variables, such as the particular monomer being reacted in a given cycle and well (for example, an A, C, G, or T phosphoramidite monomer), the particular instrument being used (in cases where multiple instruments are being monitored) or a particular subcomponent of an instrument (for example, a valve or delivery nozzle). Wavelet analysis incorporates a third

dimension of the transform so that such variables can be represented in the third dimension and evaluated to determine if criteria important to a manufacturing environment are being properly met. If criteria are not being met the information from wavelet analysis can be used to direct adjustment of instrument activity to achieve a desired manufacturing goal. Such adjustments can be communicated to a GUI in the form of suggested solutions to a perceived malfunction. Alternatively, adjustments can be made in an automated fashion using a feedback loop in which the function of a component of the instrument is automatically altered in response to the perceived malfunction.

[0098] In one embodiment, the chemical reaction monitor includes a data registration and extraction algorithm for determining the DMT intensity within each well, which algorithm may include one or more of the following steps:

1) Read image;
   a) Determine plate configuration (e.g., 96 or 384 well plate);
2) Register;
   a) Use fiducial mark on plate holder (384 wells);
      i) Move to middle top well and pattern recognize;
      ii) Move to left and right of top row and recognize wells;
      iii) Create fit of line of centers of top row wells;
      iv) Use to calculate angle of rotation;
   b) OR use 2 corners of plate well detection (96 and/or 384 well plates);
      i) Use to calculate angle of rotation;
   c) Rotate image;
3) Find frit location;
   a) Find all well centers using plate measurements;
   b) Apply algorithm of optical parallax to identify frit position with respect to well center as viewed by camera;
      i) OR use 2x2 algorithm and thresholding to pattern recognize frit features independent of well positions on plate;
   c) Create circle around frit with known diameter;

d) Create circle around center of well with known diameter;

e) Average intensity of pixels inside outer circle but w/out frit area;

4) Store values in analysis results database per plate per cycle per well per run;

5) Repeat above for new image automatically.

[0099] In one embodiment, the chemical reaction monitor includes a data analysis and Pass/Fail algorithm for identifying specific failed oligonucleotides, which algorithm may include one or more of the following steps:

1) Read image data per loop per plate per synthesis cycle per well from analysis results database;

2) Read info for subject oligonucleotide from LIMS database (e.g., sequence length);

3) Sum all 3 loops per synthesis cycle (see, e.g., FIG. 4);

4) Determine average Intensity of first 3 cycles for each well;

a) If no value, assume no data and then set initial Intensity value accordingly for monitor cameras from configuration file;

b) If low value, FAIL oligonucleotide;

5) Check last synthesis step for modifier from LIMS;

a) If modifier does not have DMT then exclude last cycle from trending;

6) Determine Intensity exponential decay vs. cycle index;

a) Assume coupling efficiency at 98.5%;

b) Calculate expected value of DMT for each cycle based on assumed coupling efficiency;

c) Assume allowed variation of calculated vs. measured DMT value at set % value form configuration file;

d) Compare summed loop value with calculated value for every cycle;

e) If difference > +- se value then FAIL oligonucleotide;

7) Determine scaling of DMT intensity between loops;

a) Loop1/loop2/loop3 Intensity ratio for all cycles;

b) Compare with set criteria;

c) If outside set criteria then FAIL oligonucleotide;

8) Step detection in synthesis;
   a) Check all loops and sum of loops for appearance of step (decrease or increase) in DMT intensity;
   b) Compare step with set value from configuration file;
   c) If step > set value FAIL oligonucleotide;
9) Store PASS/FAIL values in database per plate per cycle per well per run;
10) Repeat above for new image automatically.

[00100] In one embodiment, the chemical reaction monitor includes a further data analysis and Pass/Fail algorithm for identifying trends for improving quality control and quality assurance, which algorithm may include one or more of the following steps:

1) Read Pass/Fail result per loop per plate per synthesis cycle per well from database;
2) Read info for this oligonucleotide from LIMS database, e.g. sequence length;
3) Pattern recognize Row-wise or Column-wise global failures;
4) Pattern recognize local area failures;
   a) If concentration of FAILED oligonucleotides within specific area of plate;
5) Compare with failure mode from configuration file;
6) Alert Real-Time data monitoring for failure mode;
7) Pause oligonucleotide synthesis;
8) Send Failure mode result to LIMS database;
9) Repeat above for all runs.

[00101] In one embodiment, the chemical reaction monitor includes a further algorithm for monitor and control synthesis, which algorithm may include one or more of the following steps:

1) Get Pass/Fail per well per oligonucleotide;

a) Calculate from previous algorithm submitted;
b) Receive data form database real time using RTDM GUI monitor for specific synthesis batch;

2) According to pass/fail criterion for whole plate and/or whole synthesis batch and using the above P/F numbers for individual wells, decide on plate/batch quality;
3) Alarm operators on quality;
   a) Use % number of failed/good wells in plate/batch;
   b) Use a "score" of goodness for plate/batch calculated using above criterion;
4) Proceed to pause only synthesis;
5) Suggest different modes/action plans of hardware configuration for rest of synthesis cycles;
   a) E.g. not firing all banks;
   b) E.g. resynthesizing only one of plates in-situ in synthesizer system;
   c) Etc.;
6) Operator confirms actions suggested form menu;
   a) Possibly, for specific, already confirmed or emergency modes, operator input is bypassed and action is taken by computer on stopping/continuing synthesis in specific mode;
7) Action taken and control of synthesizer system is performed by software component of monitor or via native software of synthesizer system;
8) Synthesizer system returns to normal operation with monitoring only;

Parallel, Return log and values of actions taken above in analysis results database.

[00102] In another embodiment of the present invention, chemical reaction monitor 30a is similar to chemical reaction monitor 30 above but includes a modified lighting device 33a and a modified software component. Like reference numerals have been used to describe like components of 30 and 30a. In this embodiment, the lighting device includes a single LED array 36a pivotally mounted and

positioned to one side of glass window 37a, as shown in FIG. 10. In this embodiment, a transparent reflector 48 is provided which directs the light rays of the LED array downward to illuminate multi-well plate 34a. The transparent reflector allows camera device 32a to take a picture of the multi-well plate when the LED array is pivoted downward and in place about the glass window illuminating the multi-well plate. A diffuser or optical filter or both can be applied prior to the light hitting the transparent reflector (beam splitter) if desired, as set forth previously herein.

[00103] In this embodiment, the software component includes an integrated viewer program and RTDM program, generally referenced by the numeral 49 in FIG. 12. One will appreciate that any two or more of the software subcomponents may be integrated into a single program. Thus, the programs and network locations set forth above are exemplary and not intended to limit the number or type of functions that can be performed by particular components or programs.

[00104] For convenience in explanation and accurate definition in the appended claims, the terms "up" or "upper", "down" or "lower", "left" and "right" are used to describe features of the present invention with reference to the positions of such features as displayed in the figures.

[00105] In many respects the modifications of the various figures resemble those of preceding modifications and the same reference numerals followed by subscript "a" designate corresponding parts.

[00106] The foregoing descriptions of specific embodiments of the present invention have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application, to thereby

enable others skilled in the art to best utilize the invention and various embodiments with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the Claims appended hereto and their equivalents.

WHAT IS CLAIMED IS:

1. A chemical reaction monitoring system for parallel monitoring of a plurality of chemical reactions, each chemical reaction occurring within a respective one of a plurality of wells, said system comprising:

a lighting device for illuminating the plurality of wells;

a camera device configured to obtain a fresh image of the plurality of wells and saving said fresh image to an image storage location;

a graphical user interface program for viewing said saved fresh image; and

an analyzer program for accessing said saved fresh image, geometrically registering said saved fresh image in order to determine a specific value corresponding to each one of the chemical reactions within each well at the time the image was obtained, and saving said specific values to an analysis results storage location.

2. The chemical reaction monitoring system of claim 1, wherein the chemical reaction is oligonucleotide synthesis.

3. The chemical reaction monitoring system of claim 1, wherein said plurality of wells comprises a multi-well plate.

4. The chemical reaction monitoring system of claim 1, wherein said lighting device comprises a light emitting diode (LED) array.

5. The chemical reaction monitoring system of claim 4, wherein said LED array includes a first array and a second array positioned on either side of a multi-well plate-viewing window.

6. The chemical reaction monitoring system of claim 4, wherein said LED array includes a single array positioned pivotally mounted on one side of a multi-well plate-viewing window.

7. The chemical reaction monitoring system of claim 1, wherein said camera device comprises a charge couple device (CCD) capable of imaging the plurality of wells simultaneously.

8. The chemical reaction monitoring system of claim 1, wherein said analyzer program processes said fresh image and said specific values comprise a processed image.

9. A chemical synthesis system comprising:

(a) a sample holder placed to support a plurality of wells;

(b) a liquid dispenser placed to dispense a liquid sample to said plurality of wells;

(c) a liquid removal device placed to remove said liquid sample from said plurality of wells;

(d) a lighting device for illuminating said plurality of wells;

(e) a camera device configured to obtain a fresh image of said plurality of wells and save said fresh image to an image storage location; and

(f) a computer system configured to:

(i) access said saved fresh image;

(ii) determine a specific value corresponding to each one of the chemical reactions within each well at the time the image was obtained; and

(iii) communicate a representation of said specific values to a graphical user interface.

10. The system of claim 9, wherein said computer system is further configured to write said specific values to a data storage location.

11. The system of claim 9, wherein said representation comprises a warning message if said specific values are within a pre-defined range of failure.

12. The system of claim 9, wherein said liquid removal device comprises a centrifuge rotor for orbiting said plurality of wells about an axis of rotation.

13. The system of claim 9, wherein said liquid removal device comprises a liquid aspirating tube.

14. The system of claim 9, wherein the chemical reaction is oligonucleotide synthesis.

15. The system of claim 9, wherein said plurality of wells comprises a multi-well plate.

16. The system of claim 9, wherein said lighting device comprises a light emitting diode (LED) array.

17. The system of claim 14, wherein said LED array includes a first array and a second array positioned on either side of a multi-well plate-viewing window.

18. The system of claim 14, wherein said LED array includes a single array positioned pivotally mounted on one side of a multi-well plate-viewing window.

19. The system of claim 9, wherein said camera device comprises a charge couple device (CCD) capable of imaging the plurality of wells simultaneously.

20. A method for synthesizing a plurality of different polymers comprising:

(a) providing a plurality of wells containing support-bound monomeric or oligomeric precursors of said polymers;

(b) dispensing a liquid comprising second monomeric precursors of said polymers to said plurality of wells under conditions for forming an intermediate in which the support-bound monomeric or oligomeric precursors are bound to the second monomeric precursors;

(c) obtaining a fresh image of said plurality of wells and saving said fresh image to an image storage location;

(d) executing commands in a computer system to access said saved fresh image, geometrically register said saved fresh image in order to determine a specific value corresponding to each one of the chemical reactions within each well at the time the image was obtained, and save said specific values to an analysis results storage location; and

(e) if said specific values are within a pre-defined passing range then repeating steps (a) through (d) and if said specific values are within a pre-defined failing range then at least temporarily preventing repetition of steps (a) through (d) for at least one of the wells in said plurality of wells.

21. The method of claim 19, wherein step (e) further comprises communicating a representation of said specific values to a graphical user interface.

22. The method of claim 20, further comprising:

(f) if said specific values are within a pre-defined failing range and repetition of steps (a) through (d) is temporarily prevented for at least one of the wells in said plurality of wells then receiving a command from said graphical user interface to repeat steps (a) through (d).

23. The method of claim 19, wherein step (c) further comprises removing said liquid from said plurality of wells after obtaining said fresh image.

24. The method of claim 19, wherein removing said liquid comprises centrifugation or aspiration.

25. The method of claim 19, wherein said polymer comprises a polynucleotide and said monomeric precursor comprises a nucleoside.

26. The method of claim 19, wherein said plurality of wells comprises a multi-well plate.

27. The method of claim 19, wherein step (c) further comprises illuminating said plurality of wells with a light emitting diode (LED) array.

28. The method of claim 19, wherein said fresh image is obtained with a charge couple device (CCD) capable of imaging the plurality of wells simultaneously.

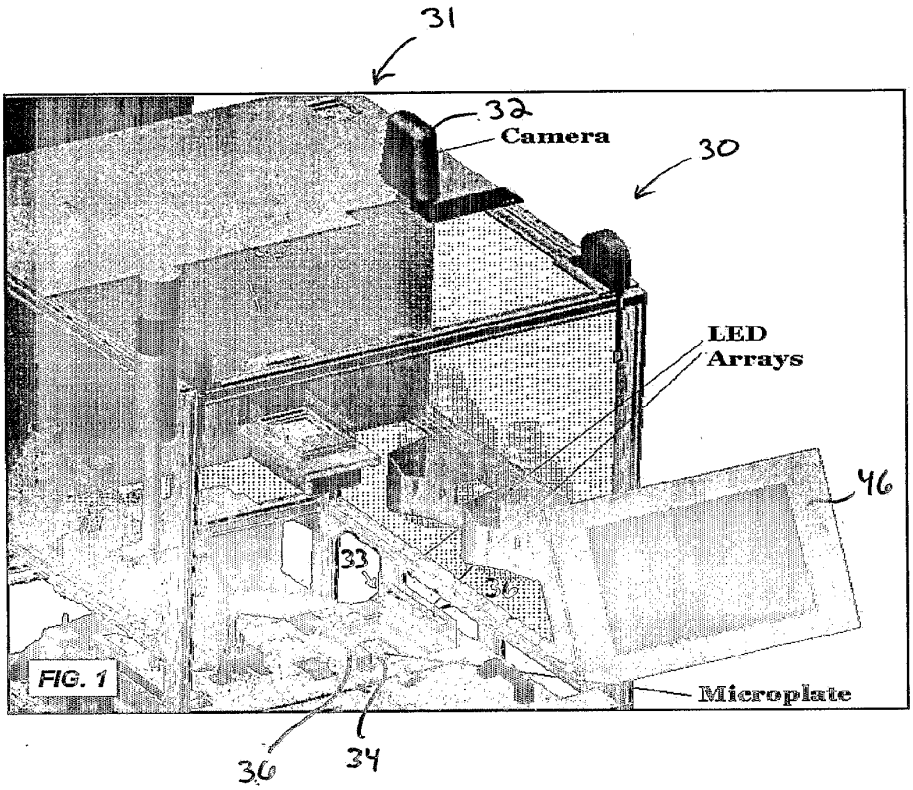


FIG. 1

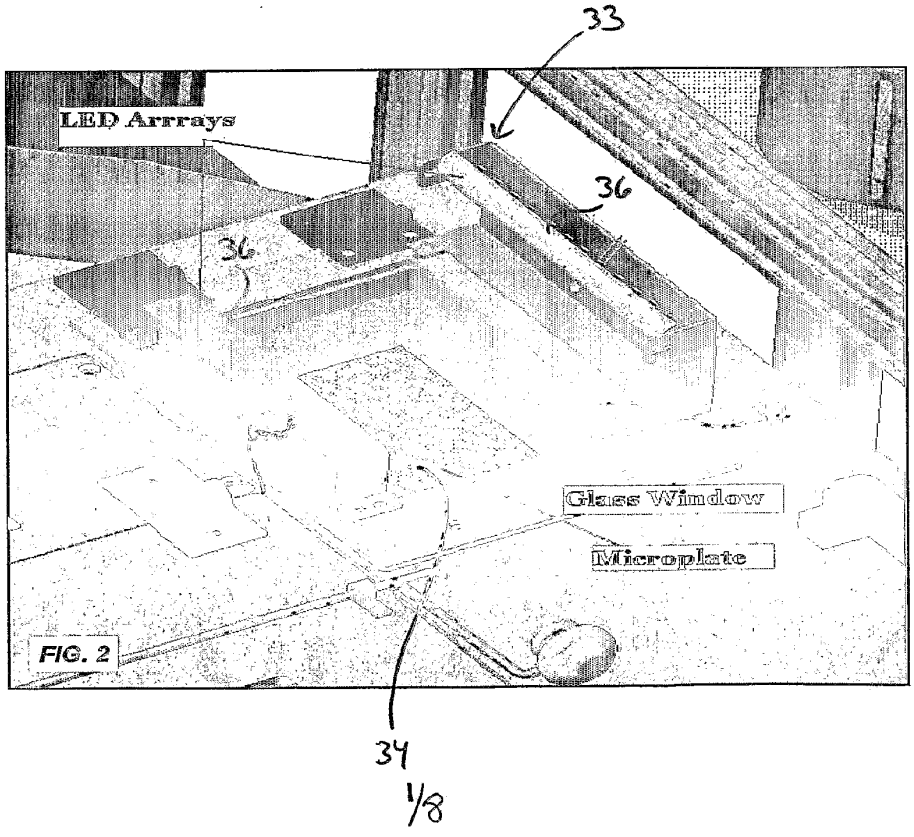


FIG. 2

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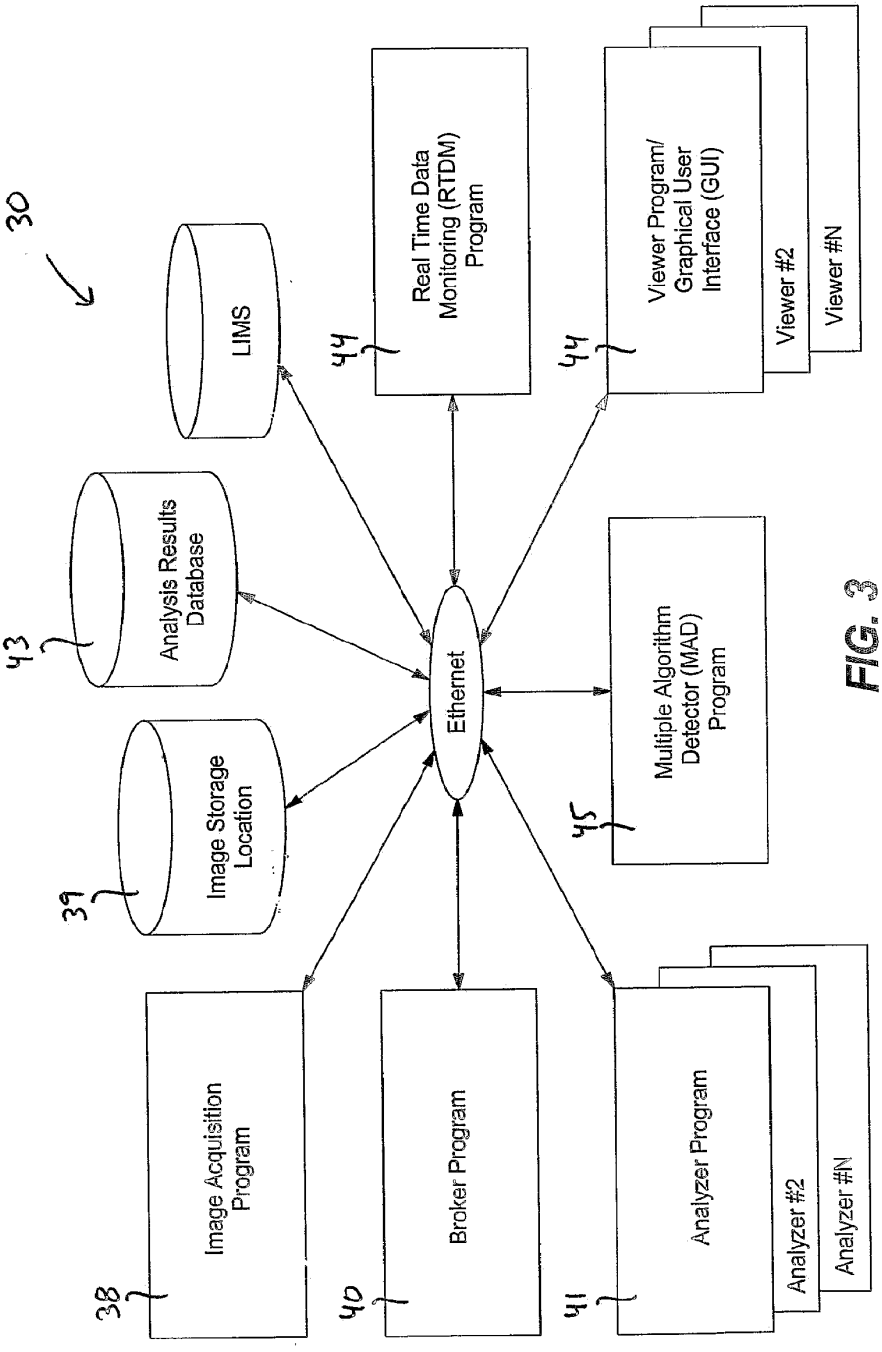


FIG. 3

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FIG. 4

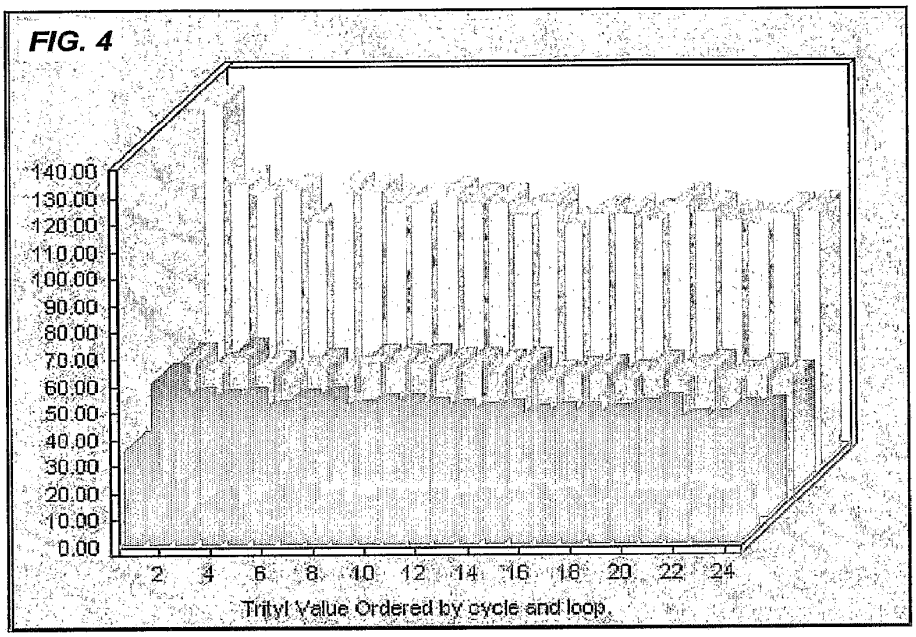
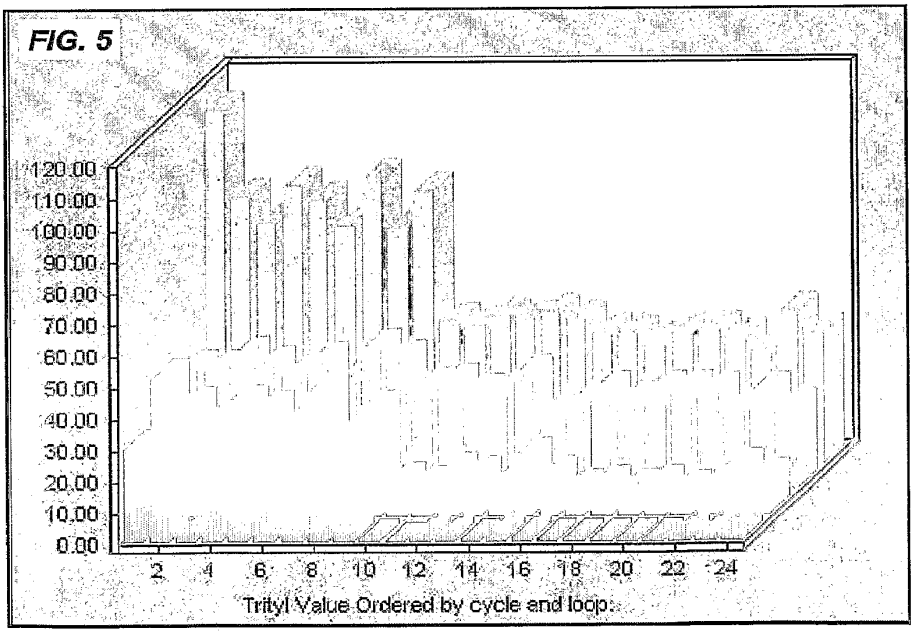


FIG. 5



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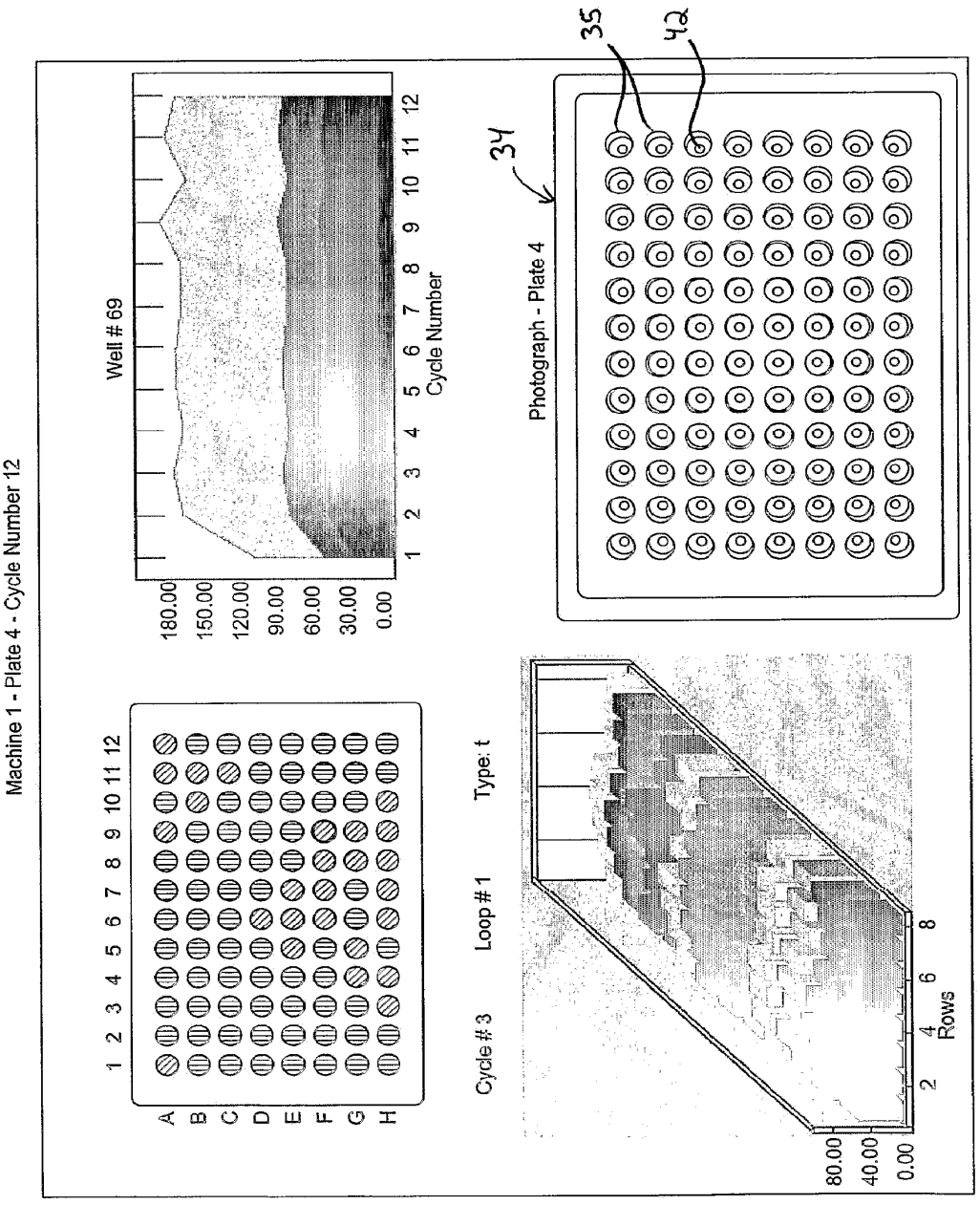

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Well # 69
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150.00
120.00
90.00
60.00
30.00
0.00
1 2 3 4 5 6 7 8 9 10 11 12
Cycle Number
Photograph - Plate 4
34
35
42
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
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80.00
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2 4 6 8
Rows

FIG. 6

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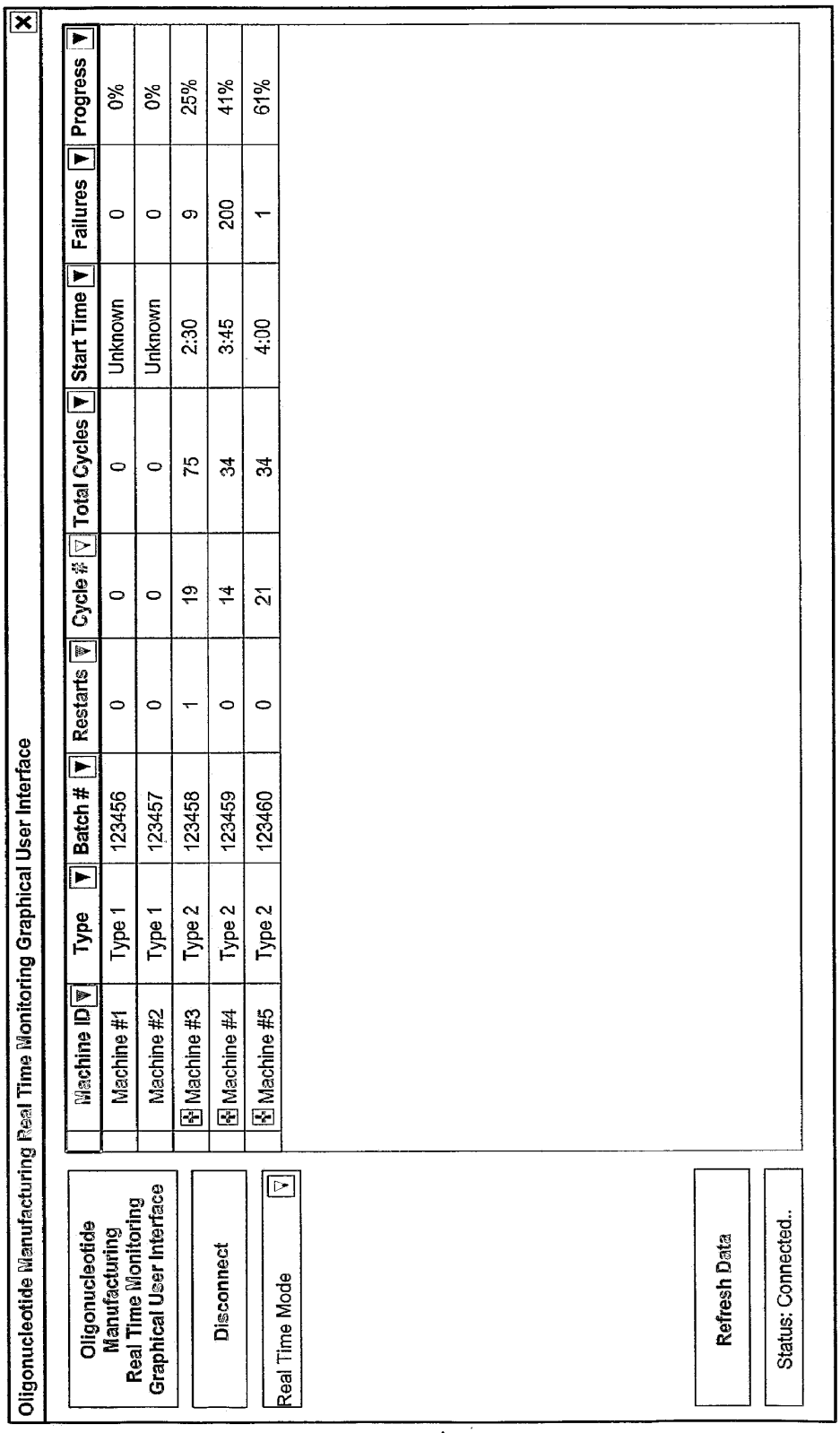

Oligonucleotide Manufacturing Real Time Monitoring Graphical User Interface
Oligonucleotide Manufacturing Real Time Monitoring Graphical User Interface
Disconnect
Real Time Mode
Refresh Data
Status: Connected..
Machine ID
Type
Batch #
Restarts
Cycle #
Total Cycles
Start Time
Failures
Progress
Machine #1 Type 1 123456 0 0 0 Unknown 0 0%
Machine #2 Type 1 123457 0 0 0 Unknown 0 0%
Machine #3 Type 2 123458 1 19 75 2:30 9 25%
Machine #4 Type 2 123459 0 14 34 3:45 200 41%
Machine #5 Type 2 123460 0 21 34 4:00 1 61%

FIG. 7

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FIG. 8

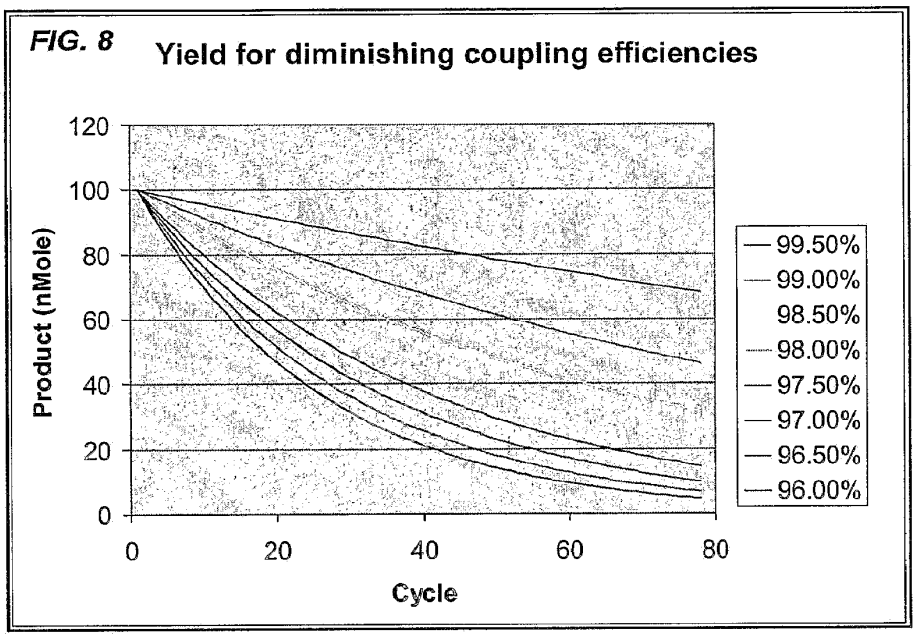
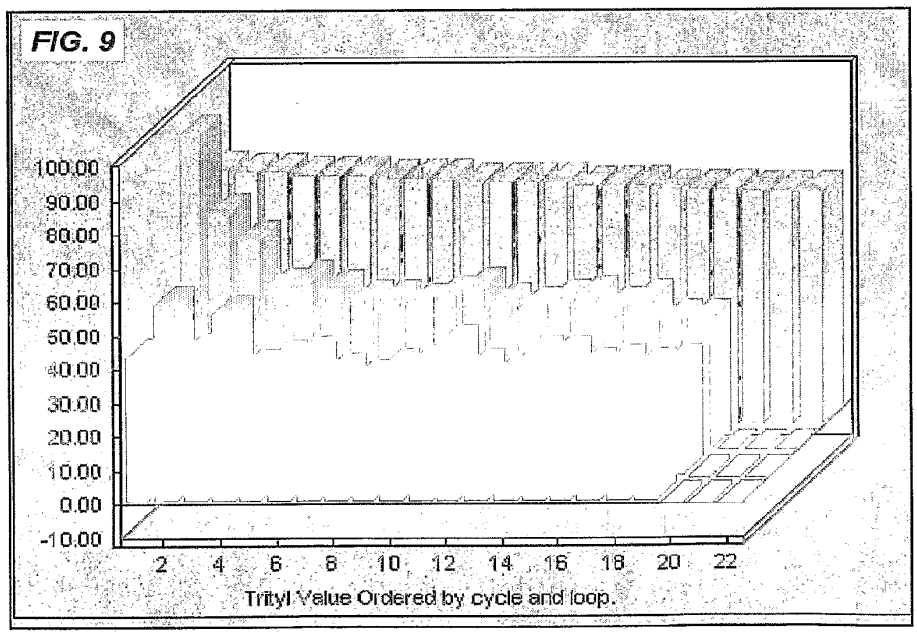


FIG. 9



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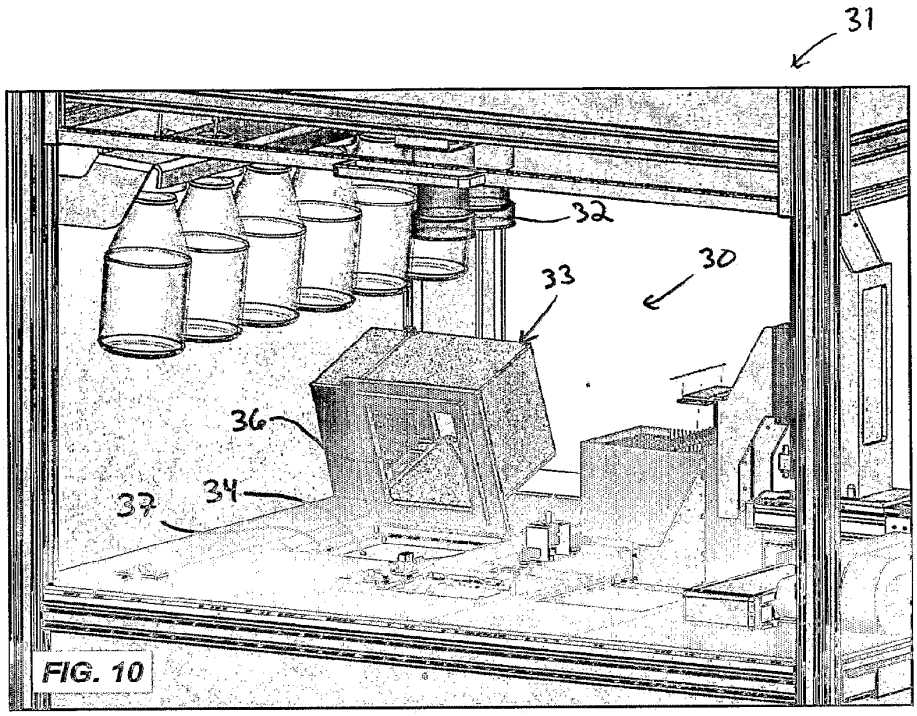


FIG. 10

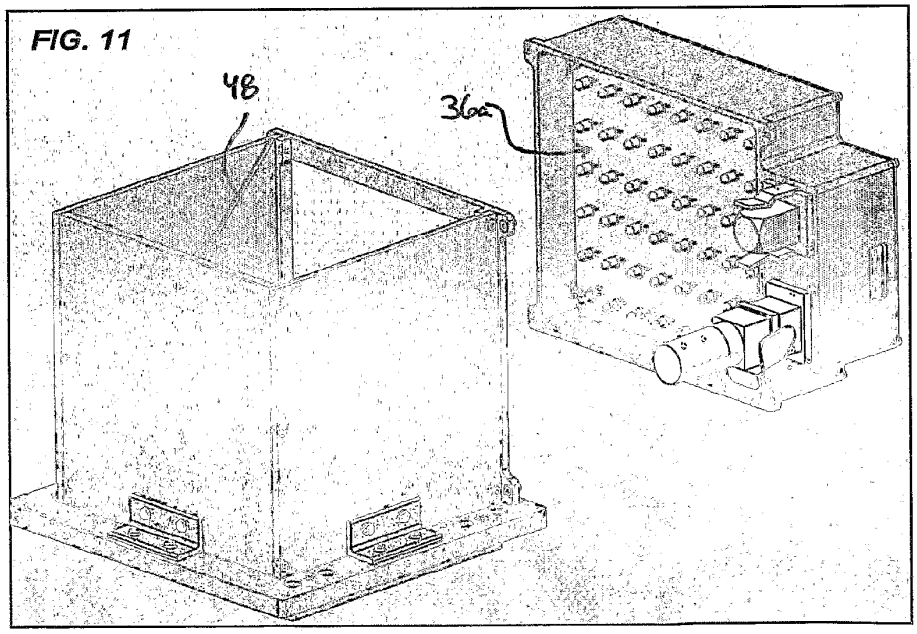


FIG. 11

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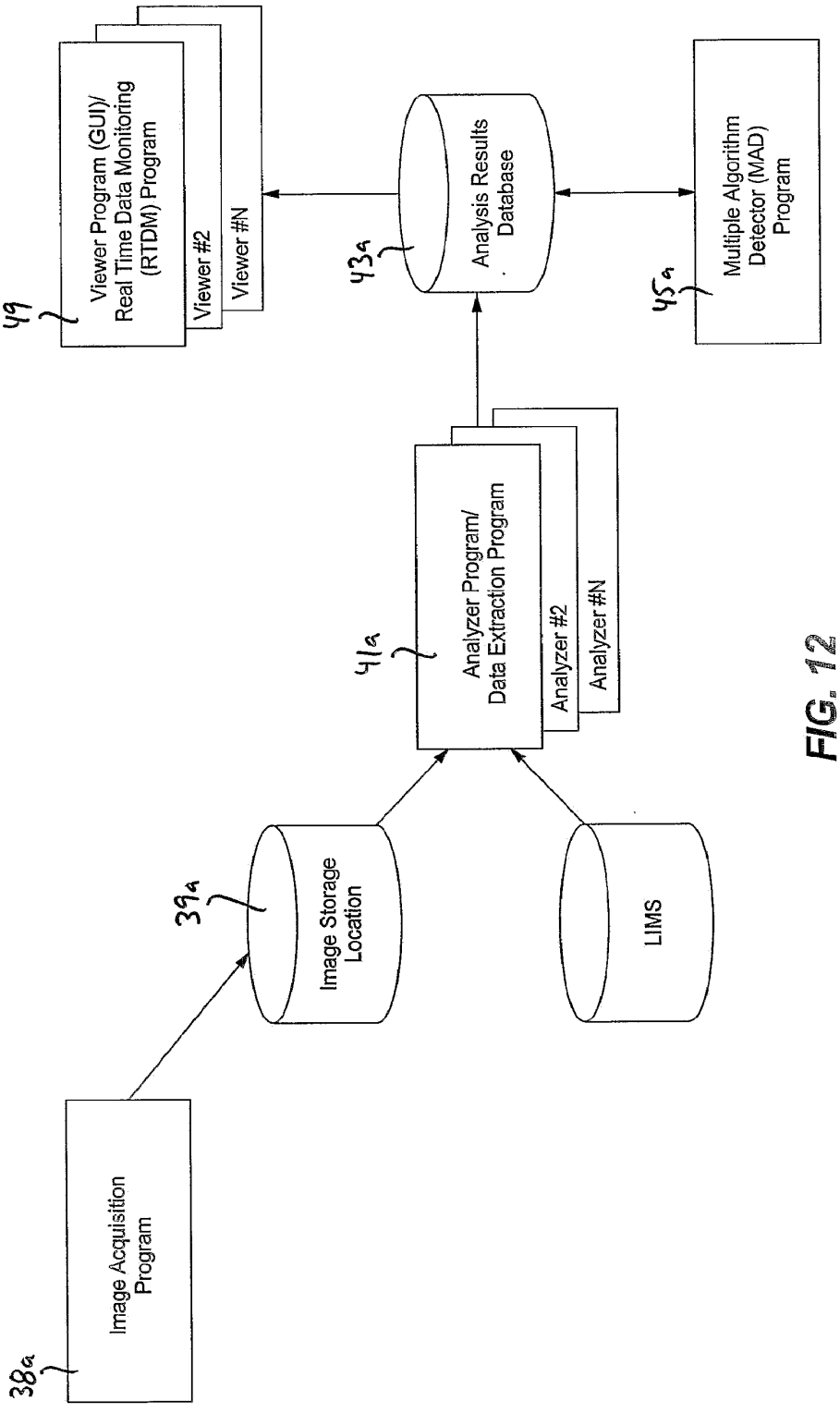


FIG. 12

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/001646

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01J19/00 G06F19/00

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)
IPC 7 B01J G06F

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 practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 128 310 A (AGILENT TECHNOLOGIES INC) 29 August 2001 (2001-08-29) abstract paragraph '0003! paragraph '0007! - paragraph '0011! paragraph '0020! - paragraph '0030! paragraph '0034! - paragraph '0035!; figure 6	1-28
X	WO 02/04123 A (BROVOLD CHRISTOPHER T ; GANZ BRIAN L (US); MICKLEY MANDEL W (US); MOUL) 17 January 2002 (2002-01-17) page 2, paragraph 6 - page 3, paragraph 1 page 9, paragraph 3 - page 10, paragraph 4 page 13, paragraph 3 page 14, paragraph 3 - page 16, paragraph 2 page 17, paragraph 2	1-28

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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"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 July 2004	16/07/2004

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nazario, L

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INTERNATIONAL SEARCH REPORT

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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 99/04228 A (CRAWFORD KIMBERLY L ; MARQUISS SAMUEL A (US); FRENCH TODD E (US); MINE) 28 January 1999 (1999-01-28) abstract page 6, paragraph 1 - paragraph 20 page 12, line 5 - page 19, line 4 page 55, line 15 - page 63, line 25 page 67, line 7 - page 69, line 21	1-28
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X	US 6 130 046 A (SMITH DAVID P ET AL) 10 October 2000 (2000-10-10) abstract column 2, line 9 - line 63 column 3, line 41 - column 5, line 46; figures 3,11,12	1-28
X	WO 02/16040 A (UNIV CHICAGO) 28 February 2002 (2002-02-28) page 4, line 12 - line 19 page 7, line 2 - line 7 page 16, line 28 - page 19, line 9	1-28

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US2004/001646

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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60/462,384 11 April 2003 (11.04.2003) US

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(74) Agents: **QUINE, Jonathan, Alan** et al.; Quine Intellectual Property Law Group, P.C., P.O. Box 458, Alameda, CA 94501 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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: SINGLE MOLECULE AMPLIFICATION AND DETECTION OF DNA

(57) Abstract: Methods and systems for performing single molecule amplification for detection, quantification and statistical analysis of rare nucleic acids are provided.

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SINGLE MOLECULE AMPLIFICATION AND DETECTION OF DNA

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This Application is a regular Utility Application corresponding to Provisional Patent Application USSN 60/462,384 "SINGLE MOLECULE AMPLIFICATION AND DETECTION OF DNA IN A MICROFLUIDIC FORMAT" by Knapp et al. filed April 11, 2003, and Provisional Patent Application USSN 60/436,098 "SINGLE MOLECULE AMPLIFICATION AND DETECTION OF DNA IN A MICROFLUIDIC FORMAT" by Knapp et al. filed December 20, 2002. The subject application claims priority to and benefit of each of these prior applications, which are incorporated herein by reference in their entirety.

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

[0002] Part of the technology for this application was developed under NIST-ATP grant 70NANB8H4000. The Government may have certain rights in this invention.

FIELD OF THE INVENTION

[0003] The invention is in the field of single molecule detection, e.g., by amplification of single molecules from complex mixtures, e.g., for disease diagnosis, detection of pathogens, environmental contaminants, or the like. Amplifications are conducted in high throughput systems, e.g., microfluidic systems, to provide an ability to detect rare molecules in complex samples that are aliquotted into low copy number reaction mixtures, whereby a rare copy nucleic acid of interest is detected, e.g., by amplifying large numbers of aliquots of the complex samples.

BACKGROUND OF THE INVENTION

[0004] The detection of nucleic acids is central to medicine, forensic science, industrial processing, crop and animal breeding and many other fields. The ability to detect disease conditions (e.g., cancer), infectious organisms (e.g., HIV), genetic lineage, genetic markers, and the like, is ubiquitous technology for disease diagnosis and prognosis, marker assisted selection, correct identification of crime scene features, the ability to propagate industrial organisms and many other techniques.

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[0005] One of the most powerful and basic technologies for nucleic acid detection is nucleic acid amplification. That is, in many typical formats, such as the polymerase chain reaction (PCR), reverse-transcriptase PCR (RT-PCR), ligase chain reaction (LCR), and Q-β replicase and other RNA/transcription mediated techniques (e.g., NASBA), amplification of a nucleic acid of interest precedes detection of the nucleic acid of interest, because it is easier to detect many copies of a nucleic acid than it is to detect a single copy.

[0006] PCR, RT-PCR and LCR are in particularly broad use, in many different fields. Details regarding the use of these and other amplification methods can be found in any of a variety of standard texts, including, e.g.,: Sambrook et al., Molecular Cloning - A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2000 ("Sambrook"); Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2002) ("Ausubel")) and PCR Protocols A Guide to Methods and Applications (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis). Many available biology texts have extended discussions regarding PCR and related amplification methods.

[0007] More recently, a number of high throughput approaches to performing PCR and other amplification reactions have been developed, e.g., involving amplification reactions in microfluidic devices, as well as methods for detecting and analyzing amplified nucleic acids in or on the devices. Details regarding such technology is found in the technical and patent literature, e.g., Kopp *et al.* (1998) "Chemical Amplification: Continuous Flow PCR on a Chip" Science, 280 (5366):1046; USP 6,444,461 to Knapp, et al. (September 3, 2002) MICROFLUIDIC DEVICES AND METHODS FOR SEPARATION; USP 6,406,893 to Knapp, et al. (June 18, 2002) MICROFLUIDIC METHODS FOR NON-THERMAL NUCLEIC ACID MANIPULATIONS; USP 6,391,622 to Knapp, et al. (May 21, 2002) CLOSED-LOOP BIOCHEMICAL ANALYZERS; USP 6,303,343 to Kopf-Sill (October 16, 2001) INEFFICIENT FAST PCR; USP 6,171,850 to Nagle, et al. (January 9, 2001) INTEGRATED DEVICES AND SYSTEMS FOR PERFORMING TEMPERATURE CONTROLLED REACTIONS AND ANALYSES; USP 5,939,291 to Loewy, et al. (August 17, 1999) MICROFLUIDIC METHOD FOR NUCLEIC ACID AMPLIFICATION; USP 5,955,029 to Wilding, et al. (September 21, 1999) MESOSCALE POLYNUCLEOTIDE AMPLIFICATION DEVICE AND METHOD; USP

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5,965,410 to Chow, et al. (October 12, 1999) ELECTRICAL CURRENT FOR CONTROLLING FLUID PARAMETERS IN MICROCHANNELS, and many others.

[0008] Despite the wide-spread use of amplification technologies and the adaptation of these technologies to truly high throughput systems, certain technical difficulties persist in amplifying and detecting nucleic acids, particularly rare copy nucleic acids. This is particularly true where the amplification reagents amplify a high copy nucleic acid in a given sample in addition to the rare nucleic acid and the two nucleic acids differ by only one or a few nucleotides in the same sample. For example, if a set of primers hybridizes to a high copy nucleic acid, as well as to a low copy nucleic acid in a given sample, the geometric amplification of the high copy nucleic acid proportionately dominates the amplification reaction and it is difficult or impossible to identify the low copy nucleic acid in any resulting population of amplified nucleic acids. Thus, low copy number alleles of a gene can be very difficult to detect, e.g., where a primer set cannot easily be identified that only amplifies the rare nucleic acid (and the practitioner will realize that perfect reagent specificity is rare or non-existent in practice). Amplification of the higher copy number nucleic acids in the sample swamps out any signal from the low copy nucleic acid. In spite of such difficulties, identification of rare copy nucleic acids can be critical to identifying disease or infection in the early stages, as well as in many other applications.

[0009] It is worth noting that these problems simply have not been addressed by the prior art. While a few authors have described single copy amplification as a theoretical exercise (e.g., Mullis et al (1986) Cold Spring Harbor Symp. Quant. Biol. 51:263-273; Li et al. (1988) Nature 335:414-417; Saiki et al (1988) Science 239:487-491, and Zhang et al (1992) Proc. Natl. Acad. Sci. USA 89:5847-5851), and others have described stochastic PCR amplification of single DNA template molecules followed by CE analysis of products in a microscale device (Lagally et al. (2001) Anal. Chem. 73:565-570), none of these prior approaches are suitable for detection of rare copy nucleic acids in samples. That is, none of these approaches are suitable to high throughput automation and the devices in the prior art cannot be adapted to practicably detect rare copy nucleic acids. For example, the device of Lagally et al., *id.*, flowed sample to be amplified into chambers, stopped flow of the system, ran the amplification reaction, manually reconfigured the device to flow amplification products out of the chambers, ran the amplification products out of the chambers for one reaction at a time, and detected the product. This cumbersome process results in few

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amplification reactions being made and analyzed in any useful time period and required almost continuous user intervention to make the system operate.

[0010] Another difficulty with amplification methods that is completely unaddressed in the prior art is that it can be quite difficult to perform quantitative analysis on rare nucleic acids. The problems noted above for detection apply to quantitative analysis as well, with the additional problem that quantification is impacted by the presence of high copy number nucleic acids in the sample, even if the rare nucleic acid can be amplified. This is because, even if the amplification is sufficiently specific that detection of the rare nucleic acid can occur, the high copy number nucleic acids still have competitive effects on the amplification reaction, in that they compete with the rare nucleic acid for reaction components during the amplification reaction. Thus, it is not generally possible to assess accurately the concentration of rare nucleic acids in a sample, particularly where the components of the system have not previously been characterized (it is, of course, somewhat simpler to assess amplification products quantitatively if the materials selected for amplification are already characterized). While amplification of materials that have already been fully characterized is of academic interest, this approach is of little practical value if it cannot be adapted to characterization of unknown materials. For example, the inability to quantify rare nucleic acids limits, e.g., the ability to diagnose disease, to establish disease prognosis and to perform accurate statistical assessments of the nucleic acid of interest.

[0011] The subject invention overcomes these difficulties by providing robust high throughput methods of identifying and quantifying rare nucleic acids of interest in a sample. A number of related methods and systems for identifying and quantifying rare nucleic acids of interest in the sample are provided herein.

SUMMARY OF THE INVENTION

[0012] The present invention relates to the surprising discovery that single molecule amplification can be used for the detection and statistical characterization of rare nucleic acids of interest in a sample, e.g., for disease diagnosis (e.g., cancer diagnosis), detection of pathogens, detection of rare environmental nucleic acids, and the like. For example, many individual amplification reactions can be performed on reaction mixtures derived from a sample comprising a rare nucleic acid of interest, where each reaction mixture has few (e.g., 1) or no copies of the rare a nucleic acid of interest, e.g., until the nucleic acid of interest is

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identified in a reaction mixture. Additional nucleic acids in the sample can also be amplified in low copy number reactions and statistical methods can be used to determine the relative ratio of the nucleic acid of interest to the additional nucleic acid, e.g., to determine relative or absolute concentration of the nucleic acid of interest. Desirably, most or all of the steps in the methods herein can be performed in a continuous flow format to greatly speed the rate of the overall method. Alternately, one or more of the steps can be performed in a stopped flow mode, e.g., where the detector is configured to simultaneously scan multiple amplification regions at once (simultaneous detection provides for increased throughput in these embodiments).

[0013] High throughput amplification systems such as those embodied in high throughput microfluidic systems are particularly well adapted to performing these methods, which can be used to detect nucleic acids of interest that are present at exceedingly low concentrations in a sample to be analyzed, e.g., by performing many low copy number amplification reactions until the nucleic acid of interest is detected, and/or until enough copies of the nucleic acid of interest are detected that reliable statistical evaluations can be performed. In a related aspect, the invention also provides new ways of determining whether and how many copies of an initial nucleic acid are present in a reaction mixture (or whether the initial nucleic acid is present in a reaction mixture) by considering how much a signal from the initial nucleic acid disperses during amplification and comparing the dispersion to expected dispersion arising from thermal diffusivity and/or Taylor Aris dispersion, or related phenomena (or even simply by comparison of the observed dispersion to empirically observed control reactions). This can include monitoring the shape (amplitude, signal width, and/or other signal shape features) of a signal generated from an aliquot comprising the nucleic acid of interest to a predicted or empirically observed signal shape. These shape features of the signal are extremely reproducible, which provides an ability to distinguish signals of interest from background random signal fluctuations. Both the number of molecules in an aliquot and the ability to distinguish signals of interest from background signal fluctuations can be performed by this class of methods. Systems and kits adapted to performing the various methods herein are also a feature of the invention. The nucleic acids that are quantified can be known (e.g., controls) or unknown in composition. They can include experimental nucleic acids (the nucleic acids of primary interest in the experiment at issue) or can be other unknown nucleic acids (e.g., uncharacterized genomic and/or cDNA from a biological sample of interest).

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[0014] Accordingly, in a first aspect, methods of detecting a nucleic acid of interest are provided. In the methods, a sample comprising the nucleic acid of interest and one or more additional nucleic acid is aliquotted into a plurality of reaction mixtures. At least two of the reaction mixtures are single copy reaction mixtures, each comprising a single copy of the nucleic acid of interest. The plurality of reaction mixtures additionally comprise at least one additional reaction mixture comprising at least one copy of the additional nucleic acid. The plurality of reaction mixtures are subjected to one or more amplification reaction (in this context, the amplification reaction may or may not amplify the nucleic acid of interest, i.e., if the reaction has zero copies of the nucleic acid of interest, it will not be amplified; if it has one or more copy it will). The nucleic acid of interest is detected in one or more of the single copy reaction mixtures.

[0015] In a closely related aspect, the invention includes methods of detecting a low copy nucleic acid of interest in a sample that has one or more higher copy additional nucleic acid that is different from the low copy nucleic acid. The method includes aliquotting the sample into a plurality of reaction mixtures. The mixtures include a plurality (e.g., about 5, or more, about 10 or more, about 50 or more, about 100 or more, about 150 or more, or about 500 or more) of zero copy reaction mixtures that include zero copies of the nucleic acid of interest and at least one single copy reaction mixture comprising a single copy of the nucleic acid of interest. The zero and single copy reaction mixtures are subjected to an amplification reaction (whether an amplification actually occurs or not). The nucleic acid of interest is then detected in the single copy reaction mixture (this includes the possibility that the nucleic acid of interest is detected in one or in multiple individual single copy reactions).

[0016] In an additional related class of embodiments, related methods of quantifying a nucleic acid of interest in a sample are provided. In the methods, the sample is aliquotted into at least 25 reaction mixtures comprising 2 or fewer copies of the nucleic acid of interest each (and generally 1 or fewer). The reaction mixtures are subjected to one or more amplification reactions. The nucleic acid of interest is then detected in a plurality of the reaction mixtures. In a number of embodiments, statistical evaluations of the nucleic acid of interest are performed based upon the detection of the nucleic acid of interest in the plurality of reaction mixtures. In one class of embodiments, at least 50 or more, at least 75 or more, or at least 100 or more reaction mixtures, comprising the 2 or fewer copies, are subjected to the one or more amplification reactions.

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[0017] In an additional class of related embodiments, methods of detecting a low copy nucleic acid of interest are provided. In the methods, a sample comprising the low copy nucleic acid of interest is aliquotted into a plurality of reaction mixtures. A plurality of the reaction mixtures contain zero copies of the nucleic acid of interest and at least one of the reaction mixtures comprises at least one copy of the nucleic acid of interest. A plurality of the plurality of zero copy reaction mixtures is subjected to one or more amplification reaction in a microfluidic device comprising at least one microchamber or microchannel. The nucleic acid of interest is determined not to be present in the zero copy reaction mixtures. At least one additional zero copy reaction mixture and the reaction mixture comprising the nucleic acid of interest are subjected to one or more amplification reaction. The nucleic acid of interest is detected in the reaction mixture comprising the nucleic acid of interest. Put another way, the reaction mixtures are amplified and checked for the presence of the nucleic acid of interest in the microfluidic device, at least until the nucleic acid is detected. For a low copy number nucleic acid, this can require a large number of amplification reactions be performed on the zero copy reaction mixtures until the nucleic acid of interest is found.

[0018] The invention also provides methods for quantifying a nucleic acid of interest in a sample, e.g., by taking diffusion/dispersion into consideration. In the methods, a sample comprising a copy of the nucleic acid of interest, or a complement thereof, is aliquotted into at least one reaction mixture. The reaction mixture is subjected to at least one amplification reaction, thereby amplifying the copy of the nucleic acid of interest. A shape, volume, width, length, height, area, or the like, in which the nucleic acid of interest, or a signal corresponding thereto, is present is detected. The shape, volume, width, height, length, or area is correlated to a number of copies of the nucleic acid of interest in the reaction mixture or sample, thereby quantifying the nucleic acid of interest in the sample. Because these shape features of the signal are extremely reproducible, it is straightforward to distinguish signals of interest from background random signal fluctuations. In a related aspect, knowledge of diffusion/dispersion and the reproducibility of these phenomena can be used to reliably distinguish the signal of a one or more target molecule(s) from random baseline system fluctuations. In any case, this correlation can be performed in any of a variety of ways, e.g., by comparing the shape, volume, width, height, length and/or other signal shape features to predicted values taking thermal diffusivity and/ or Taylor-Aris dispersion into account and/ or by back calculation from empirically observed values for

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known reactions performed in the system. It is worth noting that this method is particularly relevant to continuous flow systems, where materials disperse during flow.

[0019] In yet another class of embodiments, high throughput stopped flow methods of detecting rare nucleic acids are provided. For example, methods of detecting a nucleic acid of interest are provided, in which a sample comprising the nucleic acid of interest is aliquotted into a plurality of reaction mixtures. At least two of the reaction mixtures are single copy reaction mixtures, each comprising a single copy of the nucleic acid of interest. The reaction mixtures are flowed throughout a network of microchannels and subjected to one or more amplification reaction under stopped flow conditions in the network of microchannels. The nucleic acid of interest is detected in the single copy reaction mixtures under the stopped flow conditions. Desirably, the detection step can include detection of multiple reaction products simultaneously. For example, a CCD array or appropriate image processor can be used to scan an entire chip (or sub-regions thereof) for "clouds" of signal from amplified products. That is, an entire channel or network of channels can be scanned simultaneously after amplification and any or all regions where signal arising from amplification can be detected simultaneously (or in more than one pass of the scanner/ detector, if desired).

[0020] It will be appreciated that the above methods overlap with one another and that any of the above methods can be performed in combination with one another. Similarly, any or all of the above methods can be practiced in a continuous flow format to improve throughput of the relevant method, and/ or can use stopped flow in combination with image analysis of multiple regions of (or an entire) microchannel network.

[0021] For any or all of the methods herein, the reaction mixture can comprise the nucleic acid of interest and one or a plurality of additional nucleic acids, with the relevant method including detecting the nucleic acid of interest and/or the plurality of additional nucleic acids in the reaction mixture. The methods optionally include adding up the number of nucleic acids of interest, or the plurality of additional nucleic acids, or both, in the reaction mixture or the sample, or both. A ratio of the nucleic acid of interest or the plurality of additional nucleic acids in the reaction mixture to the sum of the nucleic acid of interest and/or the plurality of additional nucleic acids in the reaction mixture or sample can be determined. From this, a concentration of the nucleic acid of interest in the reaction mixture or sample can be determined. Similarly, the sum of the number of nucleic acids of

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interest and the plurality of additional nucleic acids can provide an indication of the total number of nucleic acids in the reaction mixture.

[0022] For any or all of the methods herein, aliquotting the sample or reaction mixture can comprise diluting the sample into a plurality of reaction containers (e.g., wells in a microtiter plate), and/or flowing the sample into a microfluidic dilution channel or chamber. In microfluidic embodiments, the sample is optionally diluted in the microfluidic dilution channel or chamber, whereby the sample is aliquotted into multiple diluted aliquots in the microfluidic dilution channel or chamber. Optionally, the part or all of the aliquotting/ dilution process can be multiplexed, e.g., by flowing a plurality of samples into the device or reaction containers simultaneously. Samples, aliquots, reaction mixtures, etc., can be flowed under pressure (e.g., into the microfluidic device) or via electroosmosis, or by any other available method. For convenience in microfluidic embodiments, the sample can be diluted from a common reaction component reservoir, e.g., comprising some or all of the reaction and/or buffer components for the amplification reactions (e.g., polymerase, primers, locus specific reagents, labels, salts, magnesium, water and/or the like). Alternately, one or more component can be located in one or more additional reservoir and the components can be mixed prior to amplification. Desirably, any or all of these steps can be practiced in a continuous flow format, or utilizing the stopped flow/ simultaneous image analysis methods noted herein.

[0023] The concentration of the nucleic acids of interest and/or any additional nucleic acid is optionally low in the methods of the invention, e.g., about 1 molecule per aliquot. For example, the sample can be diluted to a concentration of about 1 molecule of interest per nanoliter or less. Optionally, diluted aliquots are each diluted to the same degree; however, diluted aliquots can also be differentially diluted (e.g., to form a dilution series). The shape, volume of the aliquots can be quite low to keep reagent costs low, e.g., in microfluidic applications. For example, the aliquots can be less than about 100 nl in volume, e.g., less than about 10 nl in volume, or, e.g., about 1 nl in volume or less.

[0024] In a number of embodiments, at least one of the reaction mixtures is in an aqueous solution (the enzymes used in typical amplification reactions typically function well in an aqueous environment). This can take the form of sample plugs in a microfluidic device, fluid in reservoirs of a microtiter plate, or other forms such as where at least one of the reaction mixtures is formulated in an aqueous phase of an emulsion comprising aqueous droplets suspended in an immiscible liquid (in this embodiment, amplification can be

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performed on the reaction mixture when it is formulated in the emulsion). In the emulsion embodiment, the nucleic acid of interest is optionally present as a single copy in at least one aqueous droplet of the aqueous phase prior to performing the amplification reaction. The nucleic acid of interest is detected in the emulsion after the amplification reaction is performed. Optionally, a plurality of additional nucleic acids are also formulated in the aqueous phase of the emulsion and the method comprises detecting the plurality of additional nucleic acids. As with other embodiments herein, statistical analysis can be performed on, e.g., the ratio of the additional nucleic acids in the emulsion to the nucleic acid of interest, e.g., to determine the concentration of the nucleic acids of interest in the emulsion.

[0025] In any of the methods herein, at least 10 of the reaction mixtures are optionally low copy reaction mixtures (e.g., comprising 100 or fewer, usually 50 or fewer, typically 10 or fewer, generally 2 or fewer and often 1 or fewer copies of the nucleic acid of interest or the additional nucleic acid). Optionally, at least 25, at least 50, at least 100, at least 150, at least 500 or more of the reaction mixtures are low copy reaction mixtures. The low copy reaction mixtures can comprise at least 10, at least 25, at least 50, at least 100, at least 150 at least 500 or more single or zero copy reaction mixtures comprising 1 or fewer copies of the nucleic acid of interest. The reaction mixtures can, and often do, comprise no copies of the nucleic acid of interest. Thus, a plurality of the reaction mixtures can comprise a plurality of zero copy reaction mixtures that comprise no copies of the nucleic acid of interest. That is, at least about 10, 25, 50, 100, 150, 500, 1,000 or even 10,000 or more of the reaction mixtures can be zero copy reaction mixtures that have no copies of the nucleic acid of interest. In one aspect, the invention provides the ability to rapidly search through many such zero copy reaction mixtures to identify a nucleic acid of interest.

[0026] In several embodiments of the invention, the sample comprises at least one additional nucleic acid that is different than the nucleic acid of interest. The additional nucleic acid can, and often does, exist at a higher copy number in the sample than the nucleic acid of interest. The additional nucleic acid can be a known nucleic acid (e.g., a control or hybridization blocking nucleic acid) or can itself be unknown with respect to part or all of the composition (a common occurrence where the nucleic acid of interest is to be detected in a biological sample, e.g., a cell or tissue sample from a patient. For example, the additional nucleic acid can be present at a concentration at least about 100x, at least about 1,000x, at least about 10,000x, at least about 100,000x, at least about 1,000,000x or greater

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as high as the nucleic acid of interest in the sample (that is, can have at least about 100x, at least about 1,000x, at least about 10,000x, at least about 100,000x, at least about 1,000,000x or greater as many copies as the nucleic acid of interest in the sample). By screening sufficient numbers of sample aliquots, the nucleic acid of interest can be detected regardless of its relative concentration.

[0027] Optionally, the additional nucleic acid can be detected independent of the nucleic acid of interest. A ratio of the nucleic acid of interest to the additional nucleic acid can be determined, e.g., for statistical analysis of the nucleic acid of interest and/ or the additional nucleic acid. The number of nucleic acids in the reaction mixture (whether the nucleic acid(s) of interest, the additional nucleic acids or other nucleic acids) can be added up and the concentration of the nucleic acids (or the relative concentrations) can be determined in the sample, or in any of the various aliquots and reaction mixtures herein.

[0028] The nucleic acid of interest can be essentially any detectable nucleic acid. Examples include SNPs, low copy nucleic acids, cancer associated nucleic acids, infective or pathogen associated nucleic acids, forensic nucleic acids, and the like. Because of the ability of the methods of the invention to identify extremely low copy number nucleic acids, the invention is suitably applied to early stage disease diagnosis where cancer cells or pathogens are present at low concentrations. For example, colon cancer cells can be present in stool samples, but, at least in the early stages of colon cancer, the concentration of cancer cell DNA is small compared to the overall DNA in such a sample (typically much less than 1% of the cells from which the DNA sample was derived). The present invention can be used to identify and quantify cancer DNA in such a sample, providing a new method for disease diagnosis and prognostication. Similar approaches can be used to identify cancerous DNAs or pathogen nucleic acids from any fluid or tissue from which such samples are normally taken or derived, e.g., blood, urine, saliva, tears, sputum, stool, ejaculatory fluid, vaginal secretions, or the like. From these samples, infective/pathogenic agents such as viruses (e.g., HIV, herpes virus, pox virus, etc.), parasites (e.g., malarial parasites (*Plasmodium*), nematodes, etc.), bacteria (e.g., pathogenic *E. coli*, *salmonella*, etc.) can be identified. Where the pathogen is present at a relatively low concentration relative to related non-pathogenic organisms (e.g., pathogenic *E. coli* are present at an initially low concentration in the gut, as compared to non-pathogenic E. *coli*), the methods are particularly suitable.

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[0029] Most typically, the methods of the invention utilize thermocyclic amplification reactions, although non-thermocyclic reactions (e.g., using denaturants in place of heat, a procedure that is relatively practical in microscale applications) can also be used. In one typical class of embodiments, the reaction mixtures are subjected to one or more amplification reaction(s) by thermocycling the reaction mixtures in one or more microscale amplification chamber or channel. A variety of thermocycling methods can be used in a microscale device (or in reaction containers), e.g., heating by applying electrical current to fluid of the reaction mixture (e.g., in the microscale amplification chambers or channels), resistively heating a heating element that contacts or is in proximity to the reaction mixture (e.g., in the microscale amplification chambers or channels), heating with a Joule-Thompson or Peltier device, or any other available heating or heating and cooling method(s).

[0030] Optionally, the components of the system can be treated with one or more reagent between operational runs to reduce cross contamination between operations. For example, the amplification channel can have acid or base flowed into the channel between amplification reactions to reduce unwanted contamination from one or more previous amplification products.

[0031] In a convenient class of embodiments, detecting can include real time PCR detection, e.g., via use of TaqMan™ probes (operating by detecting a double-labeled probe before, during, or after polymerase-mediated digestion of the double labeled probe), use of molecular beacons, or the like. Real time detection can be omitted, e.g., simply by detecting amplicons via labeled probes, e.g., after separation of the amplicon from unlabeled probe.

[0032] Optionally, the detecting step(s) can include quantifying the nucleic acid of interest in the reaction mixtures, or the sample, or both. Alternately, the nucleic acid can be quantified separate from the detection step. In either case, quantifying the nucleic acid of interest optionally comprises detecting the nucleic acid in a plurality of single-copy reaction mixtures and performing statistical or probabilistic analysis to determine a percentage or distribution of reaction mixtures comprising a single copy of the nucleic acid of interest. The statistical or probabilistic analysis can comprise any available technique or combination thereof, e.g., Poisson analysis, Monte Carlo analysis, application of a genetic algorithm, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, partial least squares (PLS) analysis, or principle component analysis (PCA).

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[0033] In any of the methods, the initial starting concentration of a nucleic acid of interest can be determined, e.g., by detecting a reproducible shape, length, width, height, volume or area of signal for the nucleic acid of interest in a given reaction mixture. For example, the signal can be detected from a label bound to the nucleic acid of interest. The shape, length, width, height, volume or area is optionally correlated to a number of nucleic acids interest present in one of the reaction mixtures, and/or present in the sample based upon a Taylor-Aris dispersion calculation, or a thermal diffusivity calculation, or both, or by comparison to an empirically observed set of reaction mixtures having a known number of starting nucleic acids for amplification. Thus, in one aspect, the invention comprises calculating diffusion, or dispersion, or both, of one or more amplified nucleic acids in the given reaction mixture, and correlating the diffusion, or the dispersion, or both, to a number of copies of the nucleic acid of interest in one of the given reaction mixtures prior to amplification.

[0034] Systems and/or kits adapted for practicing the methods herein are a feature of the invention. The systems and/or kits can include system instructions (e.g., embodied in a computer or in a computer readable medium, e.g., as system software) for practicing any of the method steps herein. Fluid handling elements for aliquotting/ diluting samples, e.g., microfluidic handling elements, and detector elements can also be components of the systems and kits herein. In addition, packaging materials, integration elements (e.g., instrument cases, power supplies, etc.), instructions for using the systems and kits and the like can be features of the invention.

[0035] In one embodiment, the invention provides a system for detecting low copy nucleic acids of interest in a sample. The system includes a dilution module that dilutes the sample into multiple aliquots and a microfluidic device comprising an amplification channel or chamber configured to thermocycle one or more of the multiple aliquots. A detector integral with or proximal to the microfluidic device is also included, where the detector is configured to detect one or more amplified copies of the nucleic acid of interest in or on the microfluidic device. System instructions that direct the dilution module to aliquot the sample into a plurality of aliquots, including a plurality of zero copy aliquots comprising no copies of the nucleic acids of interest and one or more single copy aliquot comprising a single copy of the nucleic acid of interest are also included. Typically, the system also includes system software that correlates a reproducible signal shape, length, width, volume or area occupied by amplified copies of the nucleic acid of interest, as detected by the

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detector, to the number of copies of the nucleic acid of interest present in one of the aliquots, or to the number of copies of the nucleic acid of interest present in the sample, or both. Any or all of the system components can be selected to operate such that a sample of interest is continuously flowed during operation of the system. Alternately, the stopped flow/ simultaneous image analysis methods noted herein can be applied.

[0036] In a related embodiment, systems for quantifying one or more low copy nucleic acid of interest in a sample are provided. In the systems, a dilution module dilutes the sample into multiple aliquots. A microfluidic device comprising an amplification channel or chamber is configured to thermocycle one or more of the multiple aliquots. A detector integral with or proximal to the microfluidic device is configured to detect a reproducible shape, length, width, volume or area occupied by amplified copies of the nucleic acid of interest present in one of the aliquots following thermocycling of the aliquots. The system also includes system software that correlates the shape, length, width, volume or area occupied by amplified copies of the nucleic acid of interest to the number of copies of the nucleic acid of interest present in one of the aliquots, or to the number of copies of the nucleic acid of interest present in the sample, or both. Optionally, the system includes system instructions that direct the dilution module to aliquot the sample into a plurality of aliquots, including a plurality of zero copy aliquots comprising no copies of the nucleic acids of interest and one or more single copy aliquot comprising a single copy of the nucleic acid of interest.

[0037] For either of the above system embodiments, the dilution module is optionally integral with the microfluidic device. The microfluidic device also can include one or more electrodes positioned to flow electrical current into the microchamber or channel. Flow of current into the microchamber or channel can be used to heat fluid in the microchamber or channel. The microfluidic device optionally includes or is coupled to one or more heating element (e.g., a resistive heating element, a Peltier device or a Joule Thompson device) positioned within or proximal to the microchamber or channel, which heats fluid in the microchamber or channel.

[0038] The detector is typically configured to detect one or more electromagnetic energy signal in or on the microfluidic device, although other in device sensors (e.g., pH, conductivity, etc.) can also be used. For example, the detector can detect fluorescence, luminescence and/or fluorescence polarization of the sample.

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[0039] The system optionally comprises software with instructions for performing any of the method steps herein. For example, the system can include statistical or probabilistic system software that performs one or more statistical or probabilistic analysis of signals received from one or more of the aliquots subjected to thermocycling. For example, the statistical or probabilistic analysis can include Poisson analysis, Monte Carlo analysis, application of a genetic algorithm, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, PLS analysis, and/or PCA analysis. The statistical or probabilistic analysis optionally comprises quantitatively determining a concentration, proportion, or number of the nucleic acids of interest in the sample.

[0040] The systems above also optionally includes fluid handling or storage features such as sample storage modules that stores the sample until it is diluted by the dilution module, a sample retrieval module that retrieves the sample from the sample storage module and delivers it to the dilution module, or the like. These features are optionally designed to provide for continuous flow of fluid (e.g., comprising the sample) through the system (thereby providing for higher sample throughput). Alternately, or in combination, stopped flow/ simultaneous image analysis can be used in the systems herein.

[0041] Any of the above methods or systems can be used in combination. Additional features of the invention will become apparent upon review of the following.

BRIEF DESCRIPTION OF THE FIGURES

[0042] Figure 1 schematically illustrates a chip design for an 8 channel PCR sipper chip used in the Examples herein.

[0043] Figure 2 is a graph of percent amplification versus input copy number for 2 experimental runs, with a comparison to a predicted (Poisson) value.

[0044] Figure 3, Panels A and B provide peak area and peak width bar graphs.

[0045] Figure 4, Panels A-D are graphs illustrating peak width for amplification reactions.

[0046] Figure 5 is a graphical analysis of single molecule amplification peak widths.

[0047] Figure 6 is a schematic representation of a system of the invention.

[0048] Figure 7 is a schematic representation of a system of the invention.

[0049] Figure 8 is a schematic representation of a stopped flow system that uses simultaneous image processing of a network of channels to scan for nucleic acids of interest.

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[0050] Figure 9 is a schematic of a fluidic network after thermocycling. Spots represent the fluorescence "clouds" from single copy amplification reactions. The spots are counted for quantitative PCR analysis.

[0051] Figure 10 is a data graph showing single molecule DNA amplification.

[0052] Figure 11 is a data graph showing single molecule DNA amplification (6 passes).

[0053] Figure 12 is a data graph showing single molecule DNA amplification (3 panels).

[0054] Figure 13 provides a graph of detection of 2 mutation sites relevant to cancer detection developed on-chip using TaqMan probes.

DETAILED DESCRIPTION

[0055] The present invention derives, in part, from a surprising conceptual shift in considering how rare nucleic acids can be amplified and detected in or from a sample. In the past, detection of rare nucleic acids was performed by trying to find ways of improving the specificity and sensitivity of amplification and detection reactions. This is because the better the reaction can specifically amplify and identify a nucleic acid of interest, the better the reliability and throughput of the system. Considering a simple analogy, when trying to find a needle in a haystack, prior art thinking focuses on more efficient ways of extracting the needle from the haystack.

[0056] The present invention takes an entirely different approach to identifying nucleic acids of interest. Instead of trying to fish the nucleic acid of interest out of a complex sample directly, the entire sample is simply deconstructed into low copy number aliquots and the low copy number aliquots are subjected to amplification reactions until the nucleic acid of interest is found. Continuing with the simple analogy, the entire haystack is broken apart into individual pieces of hay and each is examined to see if it is hay or needle.

[0057] Modern high-throughput systems make this new conceptual approach possible, i.e., the ability to run massively high numbers of amplification reactions at low cost, e.g., using microfluidic amplification technologies, makes it possible to much more exhaustively sample for any particular nucleic acid of interest in a sample. The continuous flow or high throughput stopped flow nature of these systems further facilitates the approach. Furthermore, examination of a sample by such exhaustive sampling methods provides a great deal of quantitative information (and the concomitant possibility of

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statistical analysis) with respect to the composition of the sample and the abundance of the nucleic acid of interest. This, in turn, provides diagnostic and prognostic information relevant to the abundance (or relative abundance) of the nucleic acid of interest.

DEFINITIONS

[0058] It is to be understood that this invention is not limited to particular devices or biological systems, or amplification methods, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a", "an" and "the" optionally include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a microfluidic device" optionally includes a combination of one, two or more devices.

[0059] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

[0060] An "aliquot" is a portion of a component of interest (e.g., a sample). The aliquot can be diluted, concentrated or undiluted as compared to the component of interest.

[0061] A "nucleic acid of interest" is any nucleic acid to be amplified, detected and/or quantified in a sample.

[0062] An "amplification reaction" is a reaction that 1) results in amplification of a template, or 2) would result in amplification of a template if the template were present. Thus, an "amplification reaction" can be performed on a sample aliquot that comprises a nucleic acid to be amplified, or on a sample aliquot that does not comprise the nucleic acid. Actual amplification of a template is not a requirement for performing an amplification reaction.

[0063] A "zero copy" reaction mixture or aliquot is a reaction mixture or aliquot that has no copies of the relevant nucleic acid (e.g., a nucleic acid of interest, or an additional nucleic acid). It can comprise nucleic acids from a sample other than the relevant nucleic acid(s), or it can be completely devoid of any template nucleic acids from the sample.

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[0064] A "single copy" reaction mixture or aliquot is a reaction mixture or aliquot that has 1 copy of the relevant nucleic acid.

[0065] A "low copy" reaction mixture or aliquot is a reaction mixture or aliquot that has only a few copies of the relevant nucleic acid(s). Typically, such a reaction will have 50 or fewer, generally 25 or fewer, usually 10 or fewer and often 5 or fewer, 2 or fewer or 1 or fewer copies of the relevant nucleic acid(s).

[0066] A "high copy" nucleic acid reaction mixture or aliquot has at least 1 order of magnitude more copies than the low copy number reaction mixture or aliquot, and generally 2, 3, 4, or even 5 or more orders of magnitude more than the low copy number reaction mixture.

[0067] A nucleic acid is "quantified" or "quantitated" in a sample when an absolute or relative number of the nucleic acid in a sample is determined. This may be expressed as a number of copies, a concentration of the nucleic acid, a ratio of the nucleic acid to some other constituent of the sample (e.g., another nucleic acid), or any other appropriate expression.

NUCLEIC ACIDS AND SAMPLES OF INTEREST

[0068] The nucleic acid of interest to be detected in the methods of the invention can be essentially any nucleic acid. The sequences for many nucleic acids and amino acids (from which nucleic acid sequences can be derived via reverse translation) are available. No attempt is made to identify the hundreds of thousands of known nucleic acids, any of which can be detected in the methods of the invention. Common sequence repositories for known nucleic acids include GenBank EMBL, DDBJ and the NCBI. Other repositories can easily be identified by searching the internet. The nucleic acid can be an RNA (e.g., where amplification includes RT-PCR or LCR) or DNA (e.g., where amplification includes PCR or LCR), or an analogue thereof (e.g., for detection of synthetic nucleic acids or analogues thereof). Any variation in a nucleic acid can be detected, e.g., a mutation, a single nucleotide polymorphism (SNP), an allele, an isotype, etc. Further, because the present invention is quantitative, one can detect variation in expression levels or gene copy numbers by the methods.

[0069] In general, the methods of the invention are particularly useful in screening samples derived from patients for the nucleic acid of interest, e.g., from bodily fluids and/or waste from the patient. This is because samples derived from relatively large volumes of

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such materials can be screened in the methods of the invention (removal of such materials is also relatively non-invasive). The nucleic acids of interest (e.g., present in cancer cells) can easily comprise 1% or less of the related nucleic acid population of the sample (e.g., about 1%, .1%, .001%, .0001% or less of the alleles for the gene of interest). Thus, stool, sputum, saliva, blood, lymph, tears, sweat, urine, vaginal secretions, ejaculatory fluid, or the like, can easily be screened for rare nucleic acids by the methods of the invention, as can essentially any tissue of interest. These samples are typically taken, following informed consent, from a patient by standard medical laboratory methods.

[0070] Prior to aliquotting and amplification, nucleic acids are optionally purified from the samples by any available method, e.g., those taught in Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook et al., Molecular Cloning - A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2000 ("Sambrook"); and/or Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2002) ("Ausubel")). A plethora of kits are also commercially available for the purification of nucleic acids from cells or other samples (*see*, e.g., EasyPrep™, FlexiPrep™, both from Pharmacia Biotech; StrataClean™, from Stratagene; and, QIAprep™ from Qiagen). Alternately, samples can simply be directly subjected to amplification, e.g., following aliquotting and dilution. One advantage of single molecule detection is that the low concentration of sample components in the reaction can reduce the need for nucleic acid purification. That is, dilution of the sample reduces the abundance of unwanted components at the same time it distributes the nucleic acid of interest into reaction mixtures.

[0071] One preferred class of nucleic acids of interest to be detected in the methods herein are those involved in cancer. Any nucleic acid that is associated with cancer can be detected in the methods of the invention, e.g., those that encode over expressed or mutated polypeptide growth factors (e.g., sis), overexpressed or mutated growth factor receptors (e.g., erb-B1), over expressed or mutated signal transduction proteins such as G-proteins (e.g., Ras), or non-receptor tyrosine kinases (e.g., abl), or over expressed or mutated regulatory proteins (e.g., myc, myb, jun, fos, etc.) and/or the like. In general, cancer can often be linked to signal transduction molecules and corresponding oncogene products, e.g., nucleic acids encoding Mos, Ras, Raf, and Met; and transcriptional activators and

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suppressors, e.g., p53, Tat, Fos, Myc, Jun, Myb, Rel, and/or nuclear receptors. p53, colloquially referred to as the "molecular policeman" of the cell, is of particular relevance, as about 50% of all known cancers can be traced to one or more genetic lesion in p53.

[0072] Taking one class of genes that are relevant to cancer as an example for discussion, many nuclear hormone receptors have been described in detail and the mechanisms by which these receptors can be modified to confer oncogenic activity have been worked out. For example, the physiological and molecular basis of thyroid hormone action is reviewed in Yen (2001) "*Physiological and Molecular Basis of Thyroid Hormone Action*" Physiological Reviews 81(3):1097-1142, and the references cited therein. Known and well characterized nuclear receptors include those for glucocorticoids (GRs), androgens (ARs), mineralocorticoids (MRs), progestins (PRs), estrogens (ERs), thyroid hormones (TRs), vitamin D (VDRs), retinoids (RARs and RXRs), and the peroxisome proliferator activated receptors (PPARs) that bind eicosanoids. The so called "orphan nuclear receptors" are also part of the nuclear receptor superfamily, and are structurally homologous to classic nuclear receptors, such as steroid and thyroid receptors. Nucleic acids that encode any of these receptors, or oncogenic forms thereof, can be detected in the methods of the invention. About 40% of all pharmaceutical treatments currently available are agonists or antagonists of nuclear receptors and/or oncogneic forms thereof, underscoring the relative importance of these receptors (and their coding nucleic acids) as targets for analysis by the methods of the invention.

[0073] As already mentioned, one preferred class of nucleic acids of interest are those that are diagnostic of colon cancer, e.g., in samples derived from stool. Colon cancer is a common disease that can be sporadic or inherited. The molecular basis of various patterns of colon cancer is known in some detail. In general, germline mutations are the basis of inherited colon cancer syndromes, while an accumulation of somatic mutations is the basis of sporadic colon cancer. In Ashkenazi Jews, a mutation that was previously thought to be a polymorphism may cause familial colon cancer. Mutations of at least three different classes of genes have been described in colon cancer etiology: oncogenes, suppressor genes, and mismatch repair genes. One example nucleic acid encodes DCC (deleted in colon cancer), a cell adhesion molecule with homology to fibronectin. An additional form of colon cancer is an autosomal dominant gene, hMSH2, that comprises a lesion. Familial adenomatous polyposis is another form of colon cancer with a lesion in the MCC locus on chromosome #5. For additional details on Colon Cancer, *see*, Calvert et al.

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(2002) "The Genetics of Colorectal Cancer" Annals of Internal Medicine 137 (7): 603-612 and the references cited therein. For a variety of colon cancers and colon cancer markers that can be detected in stool, *see*, e.g., Boland (2002) "Advances in Colorectal Cancer Screening: Molecular Basis for Stool-Based DNA Tests for Colorectal Cancer: A Primer for Clinicans" Reviews In Gastroenterological Disorders Volume 2, Supp. 1 and the references cited therein. As with other cancers, mutations in a variety of other genes that correlate with cancer, such as Ras and p53, are useful diagnostic indicators for cancer.

[0074] Cervical cancer is another preferred target for detection, e.g., in samples obtained from vaginal secretions. Cervical cancer can be caused by the papova virus and has two oncogenes, E6 and E7. E6 binds to and removes p53 and E7 binds to and removes PRB. The loss of p53 and uncontrolled action of E2F/DP growth factors without the regulation of pRB is one mechanism that leads to cervical cancer.

[0075] Another preferred target for detection by the methods of the invention is retinoblastoma, e.g., in samples derived from tears. Retinoblastoma is a tumor of the eyes which results from inactivation of the pRB gene. It has been found to transmit heritably when a parent has a mutated pRB gene (and, of course, somatic mutation can cause non-heritable forms of the cancer).

[0076] Neurofibromatosis Type 1 can be detected in the methods of the invention. The NF1 gene is inactivated, which activates the GTPase activity of the ras oncogene. If NF1 is missing, ras is overactive and causes neural tumors. The methods of the invention can be used to detect Neurofibromatosis Type 1 in CSF or via tissue sampling.

[0077] Many other forms of cancer are known and can be found by detecting associated genetic lesions using the methods of the invention. Cancers that can be detected by detecting appropriate lesions include cancers of the lymph, blood, stomach, gut, colon, testicles, pancreas, bladder, cervix, uterus, skin, and essentially all others for which a known genetic lesion exists. For a review of the topic, *see*, The Molecular Basis of Human Cancer Coleman and Tsongalis (Eds) Humana Press; ISBN: 0896036340; 1st edition (August 2001).

[0078] Similarly, nucleic acids from pathogenic or infectious organisms can be detected by the methods of the invention, e.g., for infectious fungi, e.g., *Aspergillus*, or *Candida* species; bacteria, particularly *E. coli*, which serves a model for pathogenic bacteria (and, of course certain strains of which are pathogenic), as well as medically important bacteria such as *Staphylococci* (e.g., *aureus*), or *Streptococci* (e.g., *pneumoniae*); protozoa

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such as sporozoa (e.g., *Plasmodia*), *rhizopods* (e.g., *Entamoeba*) and flagellates (*Trypanosoma*, *Leishmania*, *Trichomonas, Giardia*, etc.); viruses such as (+) RNA viruses (examples include Poxviruses e.g., *vaccinia*; Picornaviruses, e.g. *polio*; Togaviruses, e.g., *rubella*; Flaviviruses, e.g., HCV; and Coronaviruses), (-) RNA viruses (e.g., Rhabdoviruses, e.g., VSV; Paramyxovimses, e.g., RSV; Orthomyxovimses, e.g., influenza; Bunyaviruses; and Arenaviruses), dsDNA viruses (Reoviruses, for example), RNA to DNA viruses, i.e., Retroviruses, e.g., HIV and HTLV, and certain DNA to RNA viruses such as Hepatitis B.

[0079] A variety of nucleic acid encoding enzymes (e.g., industrial enzymes) can also be detected according to the methods herein, such as amidases, amino acid racemases, acylases, dehalogenases, dioxygenases, diarylpropane peroxidases, epimerases, epoxide hydrolases, esterases, isomerases, kinases, glucose isomerases, glycosidases, glycosyl transferases, haloperoxidases, monooxygenases (e.g., p450s), lipases, lignin peroxidases, nitrile hydratases, nitrilases, proteases, phosphatases, subtilisins, transaminase, and nucleases. Similarly, agriculturally related proteins such as insect resistance proteins (e.g., the Cry proteins), starch and lipid production enzymes, plant and insect toxins, toxin-resistance proteins, Mycotoxin detoxification proteins, plant growth enzymes (e.g., Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase, "RUBISCO"), lipoxygenase (LOX), and Phosphoenolpyruvate (PEP) carboxylase can also be detected.

ALIQUOTTING THE SAMPLE

[0080] The sample can be aliquotted and/or diluted using standard or microfluidic fluid handling approaches (or combinations thereof). Standard fluid handling approaches for dilution/ aliquotting include, e.g., pipetting appropriate volumes of the sample into microtiter trays and adding an appropriate diluent. These operations can be performed manually or using available high throughput fluid handlers that are designed to use microtiter trays. High throughput equipment (e.g., incorporating automated pipettors and robotic microtiter tray handling) is preferred, as the present invention contemplates making and using high numbers of aliquots of a sample of interest.

[0081] Many automated systems for fluid handling are commercially available and can be used for aliquotting and/or diluting a sample in the context of the present invention. For example, a variety of automated systems are available from the Zymark Corporation (Zymark Center, Hopkinton, MA), which utilize various Zymate systems (*see also*,

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http://www.zymark.com/), which typically include, e.g., robotics and fluid handling modules. Similarly, the common ORCA® robot, which is used in a variety of laboratory systems, e.g., for microtiter tray manipulation, is also commercially available, e.g., from Beckman Coulter, Inc. (Fullerton, CA). In any case, a conventional high throughput systems can be used in place of, or in conjunction with microfluidic systems (for example, conventional systems can be used to aliquot samples into microtiter trays, from which microfluidic systems can draw materials) in practicing the methods of the invention.

[0082] In one aspect, emulsions are created, where sample aliquots comprise or consist of droplets within the emulsions. The emulsions can be amplified by standard thermocyclic reactions and amplified nucleic acids detected within droplets of the emulsions using standard equipment (e.g., microscope stations or CCD arrays).

[0083] Microfluidic systems provide a preferred fluid handling and amplification technology that can conveniently be applied to the present invention. In typical embodiments, samples are drawn into microfluidic devices that comprise networks of microscale cavities (channels, chambers, etc., having at least one dimension less than about 500 μM in size and often less than about 100μM) and the samples are mixed, diluted, aliquotted or otherwise manipulated in the network of cavities. For example, the microscale device can comprise one or more capillary, in fluid communication with the network, extending outward from a body structure of the microscale device. Negative pressure (vacuum) is applied to the capillary and fluids are drawn into the network from a container (e.g., a well on a microtiter tray). This process can be multiplexed by using a device that comprises multiple capillary channels, permitting many samples to be drawn into the network and processed simultaneously. Sample interfaces with dried samples can also be performed using this basic system, e.g., by partly or completely expelling fluid from the capillary to hydrate samples prior to drawing them into the microfluidic device (the fluid is typically contacted to the samples as a hanging drop on the tip of the capillary and then drawn back into the capillary). For either approach, *see also*, USP 6,482,364 to Parce, et al. (November 19, 2002) MICROFLUIDIC SYSTEMS INCLUDING PIPETTOR ELEMENTS; USP 6,042,709 to Parce, et al. (March 28, 2000) MICROFLUIDIC SAMPLING SYSTEM AND METHODS; USP 6,287,520 to Parce, et al. (September 11, 2001) ELECTROPIPETTOR AND COMPENSATION MEANS FOR ELECTROPHORETIC BIAS and USP 6,235,471 to Knapp, et al. (May 22, 2001) CLOSED-LOOP BIOCHEMICAL ANALYZERS. Essentially any fluid manipulation

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(aliquotting, diluting, heating and cooling) can be performed in the network using available methods. Details regarding dilution and aliquotting operations in microscale devices can be found in the patent literature, e.g., USP 6,149,870 to Parce, et al. (November 21, 2000) APPARATUS FOR IN SITU CONCENTRATION AND/OR DILUTION OF MATERIALS IN MICROFLUIDIC SYSTEMS; USP 5,869,004 to Parce, et al. (February 9, 1999) METHODS AND APPARATUS FOR IN SITU CONCENTRATION AND/OR DILUTION OF MATERIALS IN MICROFLUIDIC SYSTEMS; and USP 6,440,722 to Knapp, et al. (August 27, 2002) MICROFLUIDIC DEVICES AND METHODS FOR OPTIMIZING REACTIONS. Samples and components to be mixed/ diluted or aliquotted can be brought into the microscale device through pipettor elements or from reaction component reservoirs on the device itself, or, commonly, both. For example, the sample can be brought into the microfluidic device through a pipettor channel and diluted and supplied with common reagents from an on device dilution and/ or reagent reservoir(s). Locus specific reagents (e.g., amplification primers) can be on the device in wells, or stored off the device, e.g., in microtiter plates (in which case they can be accessed by the pipettor channel). Any or all of these operations can be performed in a continuous or stopped flow format.

[0084] The functions the chip performs typically include reaction assembly (assembly of reaction components), thermocycling, and acting as a "cuvette" for an optical system during an imaging step. In the reaction assembly, the reaction mixture components (particularly magnesium and the enzyme) which get combined at the last second before heating begins are assembled. This is called a "hot start" and provides advantages of specificity. During thermocycling, the system optionally provides both constant fluid movement and constant temperature change. During imaging, a high data rate CCD is useful in providing an adequate dynamic range using the dispersion/diffusion methods of quantification.

[0085] Commercial systems that perform all aspects of fluid handling and analysis that can be used in the practice of the present invention are available. Examples include the 250 HTS system and AMS 90 SE from Caliper Technologies (Mountain View, CA). These systems performs experiments in serial, continuous flow fashion and employ a "chip-to-world" interface, or sample access system, called a sipper through which materials in microwell plates are sipped into a capillary or capillaries attached to the chip and drawn into

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the channels of the chip. There they are mixed with components of interest and a processing and result detection steps are performed.

[0086] Whether conventional fluid handling or microfluidic approaches (or both) are used, the aliquotting and/or dilution events can be performed to achieve particular results. For example, a sample can be diluted equally in each aliquot, or, alternately, the aliquots can be differentially diluted (e.g., a dilution series can be made). The aliquots themselves are of a volume that is appropriate to the fluid handling approach being used by the system, e.g., on the order of a few microliters for microtiter plates to 100 nL, 10nL or even 1nL or less for microfluidic approaches.

[0087] The aliquots are selected to have high or low copy numbers of any relevant nucleic acid (e.g., for low copy number aliquots, 50 or fewer, generally 25 or fewer, usually 10 or fewer and often 5 or fewer, 2 or fewer or 1 or fewer copies of the relevant nucleic acid(s)). The number of aliquots generated will depend on the size of the sample and the amount of quantitative information desired by the practitioner. For example, where simple detection of a rare nucleic acid is desired, enough low copy number aliquots are made of the sample to detect the nucleic acid in one of the aliquots. Where more quantitative information is needed, enough copies are made to provide reliable statistical information, e.g., to a given confidence value. In either case, this can include anywhere from 1 aliquot to 10^9 or more aliquots, e.g., 10, 100, 1,000, 10,000, 100,000, 1,000,000, 1,000,000,000 or more aliquots. There is no theoretical limit on the number of aliquots that can be made and assessed for a nucleic acid of interest according to the present invention, though there are practical considerations with respect to the throughput of the system and the size of the sample (the lower the throughput, the fewer aliquots can be analyzed in a given time; the larger the sample size the more aliquots can be made of the sample). Using microfluidic approaches, reagent usage (and concomitant reagent costs) can be minimized. By formatting the system to provide for continuous flow of sample and reagents, including, optionally, during amplification, the systems of the invention can greatly speed the process of searching many different samples for a nucleic acid of interest. Similarly, if stopped flow approaches are used, simultaneous processing of signals from PCR reactions can be used to speed the process of searching samples for a nucleic acid of interest. In the examples below, about 150 aliquots for each dilution range was sufficient to provide reasonable quantitative information for Poisson statistics for model samples. Obviously, more or fewer aliquots can be used in the methods as well.

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[0088] In many of the embodiments herein, it is worth noting that many of the aliquots will have zero copies of the nucleic acid of interest, due to the rarity of the relevant nucleic acid in the sample (and the dilution that is chosen). This does not present a detection problem in a continuous flow analysis system—the flow rate can be used to calculate how many aliquots have passed (undetected) by a detector prior to detection of the nucleic acid of interest. In non-continuous flow systems (e.g., microwell plate based systems), one can simply count blank reactions (wells lacking amplification product) to determine the frequency of amplification of the nucleic acid of interest. In any event, anywhere from 1 to 10^6 or more zero copy reactions can be made and assessed by the system, e.g., about 10, 25, 50, 100, 500, 1,000, 10,000, 100,000, or 1,000,000 or more zero copy reactions can be detected in the process of detecting a nucleic acid of interest. Similarly, additional nucleic acids other than the nucleic acid of interest (e.g., controls, or alternate alleles of a nucleic acid of interest that are also amplified by the relevant locus specific reagent) can be detected (or not detected) by the system. The proportion of such alternate nucleic acids in the system to the nucleic acid of interest can range from less than 1 to 10^9 or more, e.g., 1x, 10x, 100x, 1,000x, 10,000x, 100,000x, 1,000,000x, 1,000,000,000x or more.

[0089] Furthermore, as demonstrated in the examples and figures herein, the continuous flow format is a surprisingly efficient system, meaning that a high proportion of single molecules that get into the system get amplified. This efficiency is useful in ensuring that very rare molecules are detected, if present, for example in a biowarfare or infectious disease detection applications. Evidence for high efficiency is in the examples, tables and figures herein. Typically, the systems of the invention can be used to amplify at least 90%, generally 95%, often 99% or more of the rare molecules that are present in sample of interest, or that are present in a collection of aliquots that are subjected to amplification.

AMPLIFYING THE ALIQUOTS

[0090] The methods of the invention include amplifying a nucleic acid of interest and, optionally, one or more additional nucleic acids. Any available amplification method can be used, including PCR, RT-PCR, LCR, and/or any of the various RNA mediated amplification methods. PCR, RT-PCR and LCR are preferred amplification methods for amplifying a nucleic acid of interest in the methods of the invention. Real time PCR and/or

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RT-PCR (e.g., mediated via TaqMan™ probes or molecular beacon-based probes) can also be used to facilitate detection of amplified nucleic acids.

[0091] It is expected that one of skill is generally familiar with the details of these amplification methods. Details regarding these amplification methods can be found, e.g., in Sambrook (2000); Ausubel (2002) and Innis (1990), *all above*. Additional details can be found in PCR: A Practical Approach (The Practical Approach Series) by Quirke et al. (eds.). (1992) by Oxford University Press.

[0092] Additional details can also be found in the literature for a variety of applications of PCR. For example, details regarding amplification of nucleic acids in plants can be found, e.g., in Plant Molecular Biology (1993) Croy (ed.) BIOS Scientific Publishers, Inc. Similarly, additional details regarding PCR for cancer detection can be found in any of a variety of sources, e.g., Bernard and Wittwer (2002) "Real Time PCR Technology for Cancer Diagnostics Clinical Chemistry 48(8):1178-1185; Perou et al. (2000) "Molecular portraits of human breast tumors" Nature 406:747–52; van't Veer et al. (2002) "Gene expression profiling predicts clinical outcome of breast cancer" Nature 415:530–6; Rosenwald et al. (2001) "Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia" J Exp Med 194:1639–47; Alizadeh et al. (2000) "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling" Nature 403:503–11; Garber et al. (2001) "Diversity of gene expression in adenocarcinoma of the lung" Proc Natl Acad Sci U S A 98: 13784–9; Tirkkonen et al. (1998) "Molecular cytogenetics of primary breast cancer by CGH" Genes Chromosomes Cancer 21:177–84; Watanabe et al. (2001) "A novel amplification at 17q21-23 in ovarian cancer cell lines detected by comparative genomic hybridization" Gynecol Oncol 81:172–7, and many others.

Molecular Beacons

[0093] In one aspect, real time PCR is performed on the various aliquots or reaction mixtures described herein, e.g., using molecular beacons or TaqMan™ probes. A molecular beacon (MB) is an oligonucleotide or PNA which, under appropriate hybridization conditions, self-hybridizes to form a stem and loop structure. The MB has a label and a quencher at the termini of the oligonucleotide or PNA; thus, under conditions that permit intra-molecular hybridization, the label is typically quenched (or at least altered in its fluorescence) by the quencher. Under conditions where the MB does not display intra-

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molecular hybridization (e.g., when bound to a target nucleic acid, e.g., to a region of an amplicon during amplification), the MB label is unquenched.

[0094] Details regarding standard methods of making and using MBs are well established in the literature and MBs are available from a number of commercial reagent sources. *See also*, e.g., Leone et al. (1995) "Molecular beacon probes combined with amplification by NASBA enable homogenous real-time detection of RNA." Nucleic Acids Res. 26:2150-2155; Tyagi and Kramer (1996) "Molecular beacons: probes that fluoresce upon hybridization" Nature Biotechnology 14:303-308; Blok and Kramer (1997) "Amplifiable hybridization probes containing a molecular switch" Mol Cell Probes 11:187-194; Hsuih et al. (1997) "Novel, ligation-dependent PCR assay for detection of hepatitis C in serum" J Clin Microbiol 34:501-507; Kostrikis et al. (1998) "Molecular beacons: spectral genotyping of human alleles" Science 279:1228-1229; Sokol et al. (1998) "Real time detection of DNA:RNA hybridization in living cells" Proc. Natl. Acad. Sci. U.S.A. 95:11538-11543; Tyagi et al. (1998) "Multicolor molecular beacons for allele discrimination" Nature Biotechnology 16:49-53; Bonnet et al. (1999) "Thermodynamic basis of the chemical specificity of structured DNA probes" Proc. Natl. Acad. Sci. U.S.A. 96:6171-6176; Fang et al. (1999) "Designing a novel molecular beacon for surface-immobilized DNA hybridization studies" J. Am. Chem. Soc. 121:2921-2922; Marras et al. (1999) "Multiplex detection of single-nucleotide variation using molecular beacons" Genet. Anal. Biomol. Eng. 14:151-156; and Vet et al. (1999) "Multiplex detection of four pathogenic retroviruses using molecular beacons" Proc. Natl. Acad. Sci. U.S.A. 96:6394-6399. Additional details regarding MB construction and use is found in the patent literature, e.g., USP 5,925,517 (July 20, 1999) to Tyagi et al. entitled "Detectably labeled dual conformation oligonucleotide probes, assays and kits;" USP 6,150,097 to Tyagi et al (November 21, 2000) entitled "Nucleic acid detection probes having non-FRET fluorescence quenching and kits and assays including such probes" and USP 6,037,130 to Tyagi et al (March 14, 2000), entitled "Wavelength-shifting probes and primers and their use in assays and kits."

[0095] MBs are robust reagents for detecting and quantifying nucleic acids, including in real time, e.g., during PCR, LCR or other nucleic acid amplification reactions (c.g., MBs can be used to detect targets as they are formed). A variety of commercial suppliers produce standard and custom molecular beacons, including Cruachem (cruachem.com), Oswel Research Products Ltd. (UK; oswel.com), Research Genetics (a

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division of Invitrogen, Huntsville AL (resgen.com)), the Midland Certified Reagent Company (Midland, TX mcrc.com) and Gorilla Genomics, LLC (Alameda, CA). A variety of kits which utilize molecular beacons are also commercially available, such as the Sentinel™ Molecular Beacon Allelic Discrimination Kits from Stratagene (La Jolla, CA) and various kits from Eurogentec SA (Belgium, eurogentec.com) and Isogen Bioscience BV (The Netherlands, isogen.com).

[0096] MB components (e.g., oligos, including those labeled with fluorophores or quenchers) can be synthesized using conventional methods. For example, oligos or peptide nucleic acids (PNAs) can be synthesized on commercially available automated oligonucleotide/PNA synthesis machines using standard methods. Labels can be attached to the oligos or PNAs either during automated synthesis or by post-synthetic reactions which have been described before *see, e.g.,* Tyagi and Kramer (1996) "Molecular beacons: probes that fluoresce upon hybridization" Nature Biotechnology 14:303-308 and USP 6,037,130 to Tyagi et al (March 14, 2000), entitled "Wavelength-shifting probes and primers and their use in assays and kits." and U.S. Pat. No. 5,925,517 (July 20, 1999) to Tyagi et al. entitled "Detectably labeled dual conformation oligonucleotide probes, assays and kits." Additional details on synthesis of functionalized oligos can be found in Nelson, et al. (1989) "Bifunctional Oligonucleotide Probes Synthesized Using A Novel CPG Support Are Able To Detect Single Base Pair Mutations" Nucleic Acids Research 17:7187-7194. Labels/ quenchers can be introduced to the oligonucleotides or PNAs, e.g., by using a controlled-pore glass column to introduce, e.g., the quencher (e.g., a 4-dimethylaminoazobenzene-4'-sulfonyl moiety (DABSYL). For example, the quencher can be added at the 3' end of oligonucleotides during automated synthesis; a succinimidyl ester of 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL) can be used when the site of attachment is a primary amino group; and 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI) can be used when the site of attachment is a sulphydryl group. Similarly, fluorescein can be introduced in the oligos, either using a fluorescein phosphoramadite that replaces a nucleoside with fluorescein, or by using a fluorescein dT phosphoramadite that introduces a fluorescein moiety at a thymidine ring via a spacer. To link a fluorescein moiety to a terminal location, iodoacetoamidofluorescein can be coupled to a sulphydryl group. Tetrachlorofluorescein (TET) can be introduced during automated synthesis using a 5'-tetrachloro-fluorescein phosphoramadite. Other reactive fluorophore derivatives and their respective sites of attachment include the succinimidyl ester of 5-carboxyrhodamine-6G

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(RHD) coupled to an amino group; an iodoacetamide of tetramethylrhodamine coupled to a sulphydryl group; an isothiocyanate of tetramethylrhodamine coupled to an amino group; or a sulfonylchloride of Texas red coupled to a sulphydryl group. During the synthesis of these labeled components, conjugated oligonucleotides or PNAs can be purified, if desired, e.g., by high pressure liquid chromatography or other methods.

TaqMan™ Probes

[0097] PCR quantification using dual-labeled fluorogenic oligonucleotide probes, commonly referred to as "TaqMan™" probes, can be performed according to the present invention. These probes are composed of short (e.g., 20-25 base) oligodeoxynucleotides that are labeled with two different fluorescent dyes. On the 5' terminus of each probe is a reporter dye, and on the 3' terminus of each probe a quenching dye is found. The oligonucleotide probe sequence is complementary to an internal target sequence present in a PCR amplicon. When the probe is intact, energy transfer occurs between the two fluorophores and emission from the reporter is quenched by the quencher by FRET. During the extension phase of PCR, the probe is cleaved by 5' nuclease activity of the polymerase used in the reaction, thereby releasing the reporter from the oligonucleotide-quencher and producing an increase in reporter emission intensity.

[0098] Accordingly, TaqMan™ probes are oligonucleotides that have a label and a quencher, where the label is released during amplification by the exonuclease action of the polymerase used in amplification. This provides a real time measure of amplification during synthesis. A variety of TaqMan™ reagents are commercially available, e.g., from Applied Biosystems (Division Headquarters in Foster City, CA) as well as from a variety of specialty vendors such as Biosearch Technologies (e.g., black hole quencher probes).

General Probe Synthesis Methods

[0099] In general, synthetic methods for making oligonucleotides, including probes, molecular beacons, PNAs, LNAs (locked nucleic acids), etc., are well known. For example, oligonucleotides can be synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), Tetrahedron Letts., 22(20):1859-1862, e.g., using a commercially available automated synthesizer, e.g., as described in Needham-VanDevanter et al. (1984) Nucleic Acids Res., 12:6159-6168. Oligonucleotides, including modified oligonucleotides can also be ordered from a variety of commercial sources known to persons of skill. There are many commercial providers of oligo synthesis services, and thus this is a broadly accessible technology. Any nucleic acid

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can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrc@oligos.com), The Great American Gene Company (www.genco.com), ExpressGen Inc. (www.expressgen.com), Operon Technologies Inc. (Alameda, CA) and many others. Similarly, PNAs can be custom ordered from any of a variety of sources, such as PeptidoGenic (pkim@ccnet.com), HTI Bio-products, inc. (www.htibio.com), BMA Biomedicals Ltd (U.K.), Bio·Synthesis, Inc., and many others.

Amplification in Microfluidic Systems

[0100] A number of high throughput approaches to performing PCR and other amplification reactions have been developed, e.g., involving amplification reactions in microfluidic devices, as well as methods for detecting and analyzing amplified nucleic acids in or on the devices. Details regarding such technology is found, e.g., in the technical and patent literature, e.g., Kopp *et al.* (1998) "Chemical Amplification: Continuous Flow PCR on a Chip" Science, 280 (5366):1046; USP 6,444,461 to Knapp, et al. (September 3, 2002) MICROFLUIDIC DEVICES AND METHODS FOR SEPARATION; USP 6,406,893 to Knapp, et al. (June 18, 2002) MICROFLUIDIC METHODS FOR NON-THERMAL NUCLEIC ACID MANIPULATIONS; USP 6,391,622 to Knapp, et al. (May 21, 2002) CLOSED-LOOP BIOCHEMICAL ANALYZERS; USP 6,303,343 to Kopf-Sill (October 16, 2001) INEFFICIENT FAST PCR; USP 6,171,850 to Nagle, et al. (January 9, 2001) INTEGRATED DEVICES AND SYSTEMS FOR PERFORMING TEMPERATURE CONTROLLED REACTIONS AND ANALYSES; USP 5,939,291 to Loewy, et al. (August 17, 1999) MICROFLUIDIC METHOD FOR NUCLEIC ACID AMPLIFICATION; USP 5,955,029 to Wilding, et al. (September 21, 1999) MESOSCALE POLYNUCLEOTIDE AMPLIFICATION DEVICE AND METHOD; USP 5,965,410 to Chow, et al. (October 12, 1999) ELECTRICAL CURRENT FOR CONTROLLING FLUID PARAMETERS IN MICROCHANNELS; Service (1998) "Microchips Arrays Put DNA on the Spot" Science 282:396-399), Zhang et al. (1999) "Automated and Integrated System for High-Throughput DNA Genotyping Directly from Blood" Anal. Chem. 71:1138-1145 and many others.

[0101] For example, USP 6,391,622 to Knapp, et al. (May 21, 2002) CLOSED-LOOP BIOCHEMICAL ANALYZERS and the references cited therein describes systems comprising microfluidic elements that can access reagent storage systems and that can perform PCR or other amplification reactions by any of a variety of methods in the microfluidic system. For example, the microfluidic system can have one or more capillaries

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