extending outwards from the body structure of the microfluidic system for drawing materials into the body structure. Within the body structure are microfluidic cavities (channels, chambers, or the like having at least one dimension smaller than about 500 microns, and, typically smaller than about 100 microns) in which the amplification reactions are performed. The capillaries that extend out from the body structure can access standard reagent storage elements (microtiter plates, or the like) by drawing fluid into the capillary, e.g., due to application of a vacuum or electroosmotic force. Similarly, the capillaries can access dried reagent libraries on substrates (e.g., the LibraryCard™ reagent array made by Caliper Technologies) by partly or completely expelling fluid to rehydrate library members and then by drawing the rehydration fluid back into the capillary. For example, the capillary can partly expel fluid to form a hanging drop on the capillary, which is then contacted to the material to be hydrated. The material in the hanging drop is then drawn back into the capillary. In any case, molecular beacons or TaqMan™ probes can be incorporated into the relevant amplification reaction and detected in the microfluidic device to provide for real time PCR detection. Alternately, PCR amplicons can be detected by conventional methods, such as hybridization to a labeled probe, e.g., prior to or following a separation operation that separates unhybridized probe from hybridized probe. For example, an electrophoretic separation can be performed in a channel of the microscale device.

Conventional High Throughput Systems

[0102] In an alternative embodiment, standard fluid handling approaches are used in place of, or in conjunction with, microfluidic approaches. PCR can be performed in standard reaction vessels (e.g., microtiter plates), as can dilutions or other operations relevant to the present invention. Various high-throughput systems are available for non-microfluidic approaches to fluid handling (typically involving plates comprising several reaction chambers, e.g., 96 well, 384 well or 1536 well microtiter plates). These approaches can utilize conventional robotics to perform fluid handling operations and can use conventional commercially available thermocyclers to perform amplification reactions. *See above*, for a discussion of automated fluid handling systems.

DETECTING THE AMPLIFIED NUCLEIC ACIDS

[0103] Any available method for detecting amplified nucleic acids can be used in the present invention. Common approaches include real time amplification detection with

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molecular beacons or TaqMan™ probes, detection of intercalating dyes (ethidium bromide or sybergreen), detection of labels incorporated into the amplification probes or the amplified nucleic acids themselves, e.g., following electrophoretic separation of the amplification products from unincorporated label), and/or detection of secondary reagents that bind to the nucleic acids. Details on these general approaches is found in the references cited herein, e.g., Sambrook (2000), Ausubel (2002), and the references in the sections herein related to real time PCR detection. Additional labeling strategies for labeling nucleic acids and corresponding detection strategies can be found, e.g., in Haugland (1996) Handbook of Fluorescent Probes and Research Chemicals Sixth Edition by Molecular Probes, Inc. (Eugene OR); or Haugland (2001) Handbook of Fluorescent Probes and Research Chemicals Eigth Edition by Molecular Probes, Inc. (Eugene OR) (Available on CD ROM).

[0104] Amplified nucleic acids (amplicons) can be detected in solution (e.g., using molecular beacons or TaqMan™ probes) or during or after separation (e.g., by electrophoresis). Details on these strategies can be found in the preceding references.

[0105] Amplification and detection are commonly integrated in a system comprising a microfluidic device in the present invention. Available microfluidic systems that include detection features for detecting nucleic acids include the 250 HTS system and AMS 90 SE from Caliper Technologies (Mountain View, CA), as well as the Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA). Additional details regarding systems that comprise detection (and separation/ detection) capabilities are well described in the patent literature, e.g., the references already noted herein and in Parce et al. "High Throughput Screening Assay Systems in Microscale Fluidic Devices" WO 98/00231.

[0106] In general, the devices herein optionally include signal detectors, e.g., which detect fluorescence, phosphorescence, radioactivity, pH, charge, absorbance, luminescence, temperature, magnetism or the like. Fluorescent detection is especially preferred and generally used for detection of amplified nucleic acids (however, upstream and/or downstream operations can be performed on amplicons, which can involve other detection methods).

[0107] The detector(s) optionally monitors one or a plurality of signals from an amplification reaction. For example, the detector can monitor optical signals which correspond to "real time" amplification assay results.

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[0108] Example detectors include photo multiplier tubes, spectrophotometers, CCD arrays, scanning detectors, microscopes, galvo-scanns and/or the like. Amplicons or other components which emit a detectable signal can be flowed past the detector, or, alternatively, the detector can move relative to the site of the amplification reaction (or, the detector can simultaneously monitor a number of spatial positions corresponding to channel regions, or microtiter wells e.g., as in a CCD array).

[0109] The detector can include or be operably linked to a computer, e.g., which has software for converting detector signal information into assay result information (e.g., presence of a nucleic acid of interest), or the like.

[0110] Signals are optionally calibrated, e.g., by calibrating the microfluidic system by monitoring a signal from a known source.

[0111] A microfluidic system can also employ multiple different detection systems for monitoring a signal in the system. Detection systems of the present invention are used to detect and monitor the materials in a particular channel region (or other reaction detection region). Once detected, the flow rate and velocity of cells in the channels are also optionally measured and controlled as described above.

[0112] Examples of detection systems include optical sensors, temperature sensors, pressure sensors, pH sensors, conductivity sensors, and the like. Each of these types of sensors is readily incorporated into the microfluidic systems described herein. In these systems, such detectors are placed either within or adjacent to the microfluidic device or one or more channels, chambers or conduits of the device, such that the detector is within sensory communication with the device, channel, or chamber. The phrase "within sensory communication" of a particular region or element, as used herein, generally refers to the placement of the detector in a position such that the detector is capable of detecting the property of the microfluidic device, a portion of the microfluidic device, or the contents of a portion of the microfluidic device, for which that detector was intended. For example, a pH sensor placed in sensory communication with a microscale channel is capable of determining the pH of a fluid disposed in that channel. Similarly, a temperature sensor placed in sensory communication with the body of a microfluidic device is capable of determining the temperature of the device itself.

[0113] Particularly preferred detection systems include optical detection systems for detecting an optical property of a material within the channels and/or chambers of the microfluidic devices that are incorporated into the microfluidic systems described herein.

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Such optical detection systems are typically placed adjacent to a microscale channel of a microfluidic device, and are in sensory communication with the channel via an optical detection window that is disposed across the channel or chamber of the device. Optical detection systems include systems that are capable of measuring the light emitted from material within the channel, the transmissivity or absorbance of the material, as well as the materials spectral characteristics. In preferred aspects, the detector measures an amount of light emitted from the material, such as a fluorescent or chemiluminescent material. As such, the detection system will typically include collection optics for gathering a light based signal transmitted through the detection window, and transmitting that signal to an appropriate light detector. Microscope objectives of varying power, field diameter, and focal length are readily utilized as at least a portion of this optical train. The light detectors are optionally spectrophotometers, photodiodes, avalanche photodiodes, photomultiplier tubes, diode arrays, or in some cases, imaging systems, such as charged coupled devices (CCDs) and the like. The detection system is typically coupled to a computer, via an analog to digital or digital to analog converter, for transmitting detected light data to the computer for analysis, storage and data manipulation.

[0114] In the case of fluorescent materials such as labeled amplicons, the detector typically includes a light source that produces light at an appropriate wavelength for activating the fluorescent material, as well as optics for directing the light source through the detection window to the material contained in the channel or chamber. The light source can be any number of light sources that provides an appropriate wavelength, including lasers, laser diodes and LEDs. Other light sources are used in other detection systems. For example, broad band light sources are typically used in light scattering/ transmissivity detection schemes, and the like. Typically, light selection parameters are well known to those of skill in the art.

[0115] The detector can exist as a separate unit, but can also be integrated with the system or microfluidic device, into a single instrument. Integration of these functions into a single unit facilitates connection of these instruments with the computer, by permitting the use of few or a single communication port(s) for transmitting information between the controller, the detector and the computer.

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COUNTING AND STATISTICALLY ANALYZING THE NUCLEIC ACID OF INTEREST

[0116] One feature of the present invention is that it provides for robust quantitation of rare (and other) nucleic acids in a sample. This robust quantitation provides the ability to perform statistical or probabilistic analysis of the sample. For example, Poisson analysis, Monte Carlo analysis, application of genetic algorithms, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, partial least squares (PLS) analysis, or principle component analysis (PCA) can all be applied to data generated by the present invention. These statistical evaluations can be used to determine the concentration or abundance of a given nucleic acid in a sample and to correlate abundance to diagnosis or prognosis associated with the diagnosis or prognosis.

[0117] General references that are useful in understanding how to generate and analyze data, as well as other relevant concepts include: Neil Weiss (1999) Introductory Statistics & Elementary Statistics Edition: 5th ISBN:0201434490; Berinstein (1998) Finding Statistics Online: How to Locate the Elusive Numbers You Need. Medford, NJ: Information Today; Everitt, (1998) The Cambridge Dictionary of Statistics New York: Cambridge University Press; Kotz (1988). Encyclopedia of Statistical Sciences, vol. 1-9 plus supplements New York: Wiley; Dillon and Goldstein (1984). Multivariate Analysis: Methods and Applications New York: Wiley; Tabachnick and Fidell (1996) Using Multivariate Statistics New York: HarperCollins College Publishers; Box et al. (1978) Statistics for Experimenters New York: Wiley; Cornell (1990) Experiments with Mixtures New York: Wiley; John, P. W. M. (1998) Statistical Design and Analysis of Experiments Philadelphia: SIAM; Gibas and Jambeck (2001) Bioinformatics Computer Skills O'Reilly, Sebastipol, CA; Pevzner (2000) Computational Molecular Biology and Algorithmic Approach, The MIT Press, Cambridge MA; Durbin et al. (1998) Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids, Cambridge University Press, Cambridge, UK; and Rashidi and Buehler (2000) Bioinformatic Basics: Applications in Biological Science and Medicine CRC Press LLC, Boca Raton, FL.

CALCULATING DIFFUSION AND DISPERSION

[0118] One feature of the invention is the discovery that the highly reproducible peak shape, e.g., amplitude and/or width and/or other shape features of a signal from an amplification reaction can be correlated to the starting copy number for the reaction and/or

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used to discriminate signals of interest from background fluctuations. This correlation can be performed at the theoretical level, taking thermal diffusivity and Taylor Aris diffusion into account, or it can be performed by comparison to standards (e.g., comparisons to peak shapes, e.g., heights, widths, or general shape profiles for amplification reactions that have known copy numbers for starting materials).

[0119] For theoretical calculation approaches, a label is typically initially confined in a region $-h<x<h$, as a function of time (t) and spatial position (x) with respect to the peak center (x=0) and the concentration (C) of the label, or of a component corresponding to the label (e.g., the nucleic acid of interest), is equal to $\frac{1}{2} C_0 \{\text{erf}[(h-x)/(2Dt)^{1/2})]\}$, where C_0 is the initial concentration at time t=0, erf is an error function, and D is a coefficient of overall dispersion. D is equal to the sum of thermal diffusion and Taylor dispersion (D_T) in the system. In turn, the Taylor dispersion (D_T) is dependent on the dimensions and shape of the microfluidic cavity through which the label is flowed, the flow velocity (u) and the thermal diffusivity (D). Typically, $D = K(d^2u^2)/D$, where K is a proportionality factor which is a function of the microfluidic cavity through which the label is flowed and d is a characteristic microfluidic cavity length. For example, where the microfluidic cavity is a circular channel and K=1/192, d is the diameter of the circular channel and $D = D + D_T$. Further details on thermal diffusivity and Taylor Aris dispersion can be found in MICROFLUIDIC SYSTEMS AND METHODS FOR DETERMINING MODULATOR KINETICS, USSN 09/609,030 By Andrea Chow, Filed June 30, 2000.

ADDITIONAL SYSTEM DETAILS

[0120] The systems of the invention can include microfluidic devices, detectors, sample storage elements (microtiter plates, dried arrays of components, etc.), flow controllers, amplification devices or microfluidic modules, computers and/or the like. These systems can be used for aliquoting, amplifying and analyzing the nucleic acids of interest. The microfluidic devices, amplification components, detectors and storage elements of the systems have already been described in some detail above. The following discussion describes appropriate controllers and computers, though many configurations are available and one of skill would be expected to be familiar in their use and would understand how they can be applied to the present invention.

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Flow Controllers

[0121] A variety of controlling instrumentation is optionally utilized in conjunction with the microfluidic devices described herein, for controlling the transport and direction of fluids and/or materials within the devices of the present invention, e.g., by pressure-based or electrokinetic control.

[0122] For example, in many cases, fluid transport and direction are controlled in whole or in part, using pressure based flow systems that incorporate external or internal pressure sources to drive fluid flow. Internal sources include microfabricated pumps, e.g., diaphragm pumps, thermal pumps, Lamb wave pumps and the like that have been described in the art. See, e.g., U.S. Patent Nos. 5,271,724, 5,277,556, and 5,375,979 and Published PCT Application Nos. WO 94/05414 and WO 97/02357. The systems described herein can also utilize electrokinetic material direction and transport systems.

[0123] Preferably, external pressure sources are used, and applied to ports at channel termini. These applied pressures, or vacuums, generate pressure differentials across the lengths of channels to drive fluid flow through them. In the interconnected channel networks described herein, differential flow rates on volumes are optionally accomplished by applying different pressures or vacuums at multiple ports, or preferably, by applying a single vacuum at a common waste port and configuring the various channels with appropriate resistance to yield desired flow rates. Example systems are described in USSN 09/238,467 filed 1/28/99.

[0124] Typically, the controller systems are appropriately configured to receive or interface with a microfluidic device or system element as described herein. For example, the controller and/or detector, optionally includes a stage upon which a microfluidic device is mounted to facilitate appropriate interfacing between the controller and/or detector and the device. Typically, the stage includes an appropriate mounting/alignment structural element, such as a nesting well, alignment pins and/or holes, asymmetric edge structures (to facilitate proper device alignment), and the like. Many such configurations are described in the references cited herein.

[0125] The controlling instrumentation discussed above is also optionally used to provide for electrokinetic injection or withdrawal of material downstream of the region of interest to control an upstream flow rate. The same instrumentation and techniques described above are also utilized to inject a fluid into a downstream port to function as a flow control element.

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Computer

[0126] As noted above, either or both of the controller system and/or the detection system are coupled to an appropriately programmed processor or computer which functions to instruct the operation of these instruments in accordance with preprogrammed or user input instructions, receive data and information from these instruments, and interpret, manipulate and report this information to the user. As such, the computer is typically appropriately coupled to one or both of these instruments (e.g., including an analog to digital or digital to analog converter as needed).

[0127] The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the operation of the fluid direction and transport controller to carry out the desired operation. The computer then receives the data from the one or more sensors/detectors included within the system, and interprets the data, either provides it in a user understood format, or uses that data to initiate further controller instructions, in accordance with the programming, e.g., such as in monitoring and control of flow rates (including for continuous flow), temperatures, applied voltages, and the like.

[0128] 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. For example, the system optionally includes system software that correlates a shape, length, width, volume and/or area occupied by amplified copies of the nucleic acid of interest, as detected by the 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. Similarly, the system optionally 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.

[0129] The statistical functions noted above can also be incorporated into system software, e.g., embodied in the computer, in computer memory or on computer readable media. For example, the computer can include statistical or probabilistic system software that performs one or more statistical or probabilistic analysis of signals received from one or

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more of the aliquots subjected to amplification (e.g., via 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 software optionally quantitatively determines a concentration, proportion, or number of the nucleic acids of interest in the sample.

[0130] In the present invention, the computer typically includes software for the monitoring of materials in the channels. Additionally, the software is optionally used to control electrokinetic or pressure modulated injection or withdrawal of material. The injection or withdrawal is used to modulate the flow rate as described above, to mix components, and the like.

Example System

[0131] Figures 6 and 7 provide a schematic illustration of a model system of the invention. As shown in Figure 6, system **600** includes microfluidic device **601**. Device **601** includes main channel **604** fabricated therein. Amplification components are flowed, e.g., from reservoir **606**, e.g., by applying a vacuum at vacuum source **608** (and/or at any of the reservoirs or wells noted below) through main channel **604**. Amplification components can also be flowed from wells **610** or **612** and into main channel **604**. Materials can be also flowed from wells **606** or **608**, or materials can be flowed into these wells, e.g., when they are used as waste wells, or when they are coupled to a vacuum source. Flow from wells **614**, **612**, **610**, **606**, or **608** can be performed by modulating fluid pressure, or by electrokinetic approaches. Instead of the arrangement of channels depicted in Figures 6 and 7, an arrangement such as the device of Figure 1 can be substituted. A variety of other appropriate microfluidic configurations are set forth in the references noted herein.

[0132] Materials relevant to performing the amplification reactions can be flowed from the enumerated wells, or can be flowed from a source external to Device **601**. As depicted, the integrated system can include pipettor channel **620**, e.g., protruding from device **601**, for accessing an outside source of reagents. For example, as depicted, pipettor channel **620** can access microwell plate **622** which includes samples or sample aliquots, or locus specific reagents, or other reagents useful in the practice of the invention in the wells of the plate. Aliquots or reagents relevant to amplification can be flowed into channel **604** through pipettor channel **620**. Detector **624** is in sensory communication with channel **604**, detecting signals resulting, e.g., from the interaction of a label with an amplicon as

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described above. Detector **624** is operably linked to Computer **626**, which digitizes, stores and manipulates signal information detected by detector **624**.

[0133] Voltage/pressure controller **628** controls voltage, pressure, or both, e.g., at the wells of the system, or at vacuum couplings fluidly coupled to channel **604** (or the other channels noted above). Optionally, as depicted, computer **626** controls voltage/pressure controller **628**. In one set of embodiments, computer **626** uses signal information to select further reaction parameters. For example, upon detecting amplification of a nucleic acid of interest in a well from plate **622**, the computer optionally directs withdrawal of additional aliquots from the well for analysis through pipettor channel **620**, e.g., to deliver different concentrations of the aliquot to the amplification reaction. Similarly, upon determining that no nucleic acid is present (a zero copy reaction) computer **626** can direct controller **628** to process another aliquot. If statistical information is desired, computer **626** directs controller **628** to perform appropriate fluid manipulations to generate enough data for the statistical analysis. Computer **626** is optionally coupled to or comprises a user viewable display, permitting control of the computer by the user and providing a readout for the user to view results detected by the system.

[0134] Figure 7 depicts an alternate embodiment, in which a solid phase array of reagents or samples is accessed by a microfluidic system. As shown in Figure 7, system **700** includes microfluidic device **701**. Device **701** includes pipettor channel **720** and a microfluidic network fabricated within the device. Amplification components are flowed through device **701**, typically by applying pressure (positive or negative) and/or electrokinetic pressure in the microfluidic network.

[0135] As depicted, the integrated system can include pipettor channel **720**, e.g., protruding from device **701**, for accessing an outside source of reagents. For example, as depicted, pipettor channel **720** can access solid phase array **725** which includes samples or sample aliquots, or locus specific reagents, or other reagents useful in the practice of the invention. Fluids are partly or completely expelled from channel **720** to rehydrate materials on array **725**. For example, channel **720** can comprise a hanging drop that is used to rehydrate materials, with the drop being withdrawn into channel **720** for distribution into microfluidic device **701**. Detector **724** is in sensory communication with device **701** and computer/controller **726**. Computer/ controller **726** can be operated in a manner similar to computer **626** of Figure 6. In either case, computer **626** or computer controller **726** optionally control movement of tray **622** or array **725**, and/or microfluidic device **601** or

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701 to permit the relevant pipettor channel to process samples or other materials on the array or in the wells of the tray.

[0136] Many variations of the above system are also appropriate. For example, many types of heating systems can be used in the present invention. For example, winding the channel around fixed heating areas can be performed. Robotics or fluid system elements can be used to heat fluids in multiple different temperature water baths (e.g., 3 baths for a typical amplification reaction at typical annealing, reaction and dissociation conditions).

ADDITIONAL KITS DETAILS

[0137] The present invention also provides kits for carrying out the methods described herein. In particular, these kits typically include system components described herein, as well as additional components to facilitate the performance of the methods by an investigator.

[0138] The kit also typically includes a receptacle in which the system component is packaged. The elements of the kits of the present invention are typically packaged together in a single package or set of related packages. The package optionally includes reagents used in the assays herein, e.g., buffers, amplification reagents, standard reagents, and the like, as well as written instructions for carrying out the assay in accordance with the methods described herein. In the case of prepackaged reagents, the kits optionally include pre-measured or pre-dosed reagents that are ready to incorporate into the methods without measurement, e.g., pre-measured fluid aliquots, or pre-weighed or pre-measured solid reagents that may be easily reconstituted by the end-user of the kit.

[0139] Generally, the microfluidic devices described herein are optionally packaged to include reagents for performing the device's preferred function. For example, the kits can include any of microfluidic devices described along with assay components, reagents, sample materials, control materials, or the like. Such kits also typically include appropriate instructions for using the devices and reagents, and in cases where reagents are not predisposed in the devices themselves, with appropriate instructions for introducing the reagents into the channels and/or chambers of the device. In this latter case, these kits optionally include special ancillary devices for introducing materials into the microfluidic systems, e.g., appropriately configured syringes/pumps, or the like (in one preferred embodiment, the device itself comprises a pipettor element, such as an electropipettor for introducing material into channels and chambers within the device). In the former case,

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such kits typically include a microfluidic device with necessary reagents predisposed in the channels/chambers of the device. Generally, such reagents are provided in a stabilized form, so as to prevent degradation or other loss during prolonged storage, e.g., from leakage. A number of stabilizing processes are widely used for reagents that are to be stored, such as the inclusion of chemical stabilizers (i.e., enzymatic inhibitors, microcides/bacteriostats, anticoagulants), the physical stabilization of the material, e.g., through immobilization on a solid support, entrapment in a matrix (i.e., a gel), lyophilization, or the like.

EXAMPLES

[0140] The following examples are offered to illustrate, but not to limit the claimed invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

SINGLE MOLECULE AMPLIFICATION AND DETECTION OF DNA IN A MICROFLUIDIC FORMAT

Introduction

[0141] The amplification of a desired region of DNA by polymerase chain reaction (PCR) has revolutionized the field of molecular biology. In conventional formats of PCR, which use many microliters of fluids during amplification, the starting DNA copy number is typically at least hundreds to tens of thousands of molecules. Recent advances in microfluidics have demonstrated that it is feasible to miniaturize PCR down by a thousand fold to a nanoliter-reaction volume range. When the sample concentration remains constant, the starting number of DNA template in such a small volume can drop below a cutoff copy number that could be considered statistically unacceptable in some applications. For instance, in single nucleotide polymorphism (SNP) analysis, if the starting copy number is too low (below about a few tens of copies), the amplification of the two different alleles from a heterozygous sample may not amplify equally in quantity due to statistical fluctuations, possibly causing uncertainty in a correct SNP identification for that sample.

[0142] In the theoretical limit, only one DNA copy is necessary as a starting template for a PCR reaction. From such a reaction, the amplified products is a pure "clone" of a single parent DNA template, instead of a mixture of many DNA parent templates.

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Single molecule amplification and detection results in some interesting applications that are not achievable otherwise. One such application is the detection of cancer genes. This example describes (1) a method to perform single molecule PCR using microfluidic technology, (2) analysis and detection of single molecule amplification, and (3) example applications using single molecule PCR detection for cancer detection.

[0143] We have experimentally demonstrated that single molecule PCR is possible in a microfluidic channel. In experiments in the absence of flow, there is evidence in support of single molecule PCR in that localized "clouds" of fluorescent probes (corresponding to amplification products) were observed along the heated channel. The evidence for single molecule PCR is more definitive in a sipper chip continuous flow format, in which a very large number of experiments can easily be conducted to obtain adequate statistics to support experimental observations.

Continuous Flow Protocol

[0144] Using a sipper chip as shown in the chip design schematic of Figure 1, a DNA sample (e.g., a genomic DNA) was brought onto chip **100** through a sipper using a pressure gradient into distribution channel **105**. Under continuous flow, in an assembly-line fashion, the sample was first mixed with a common reagent from an on-chip reagent reservoir through common reagent channel **106**, then split into 8 equal aliquots into 8 independent analysis channels **110-118**. Each aliquot was mixed with locus-specific reagents supplied from a channel-specific chip reservoir, then flowed through heated region **130** comprising metal traces proximal to channels **110-118** to provide controlled heated regions of chip **100**. Reagent addition for channel specific reagents into channels **110-118** provides an elegant microfluidic method of providing for an on-chip "hot start," in which all of the reagents are added to analysis channels just before amplification. The temperature of the region was cycled appropriately (temperature set points and respective dwell times are controlled) for PCR conditions in the channels in heated region **130**. Heated channel lengths and fluid velocity are chosen such that the total PCR cycles meet a desired number, usually between 25 to 40 cycles (though inefficient PCR approaches that have short cycle times and high cycle numbers can also be used; *See also*, USP 6,303,343 to Kopf-Sill (October 16, 2001) entitled INEFFICIENT FAST PCR). 8 channel detection region **135** comprises an appropriate detector for detecting PCR amplicons in channels **110-118**.

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Amplification and Detection of Rare Molecules

[0145] We used the PCR sipper chip illustrated in Figure 1 to demonstrate single molecule PCR amplification, experimentally, in a continuous flow format. DNA samples, with increasing dilution, in concentrations down to less than 1 molecule per nL, were prepared in a microtiter plate which supplies the samples to the sipper (on chip dilution could be performed in alternate embodiments). Due to statistical fluctuations in sampling very low concentration DNA down to below one molecule per channel, it is expected that some channels will show amplification signals and some will not. The fraction of tests at which amplification is observed is best described by Poisson statistics.

[0146] Table 1 summarizes results of a set of PCR experiments when the average copy number of DNA in each of the 8 channels varied from 0.02 to 48. For each DNA concentration, 8 PCR experiments were done simultaneously. The number of occurrences of a measurable PCR fluorescent signal for each sample was recorded in Table 1, with the maximum occurrence being 8 and the minimum being 0. The percent of occurrence of PCR was calculated and compared with a Poisson statistics prediction. A very good agreement between the measured and predicted percent occurrence of PCR was found. Table 2 summarizes a replication of similar sets of experiments on another day. Figure 2 is a graphical comparison of predicted (Poisson) and measured statistics (Run 1 and 2) for both sets of experiments. Predicted and actual measurements show close agreement.

TABLE 1

	AVERAGE NUMBER OF COPIES IN THE CHANNEL											
PLATE PASS	0.02	0.05	0.1	0.2	0.4	0.8	1.5	3	6	12	24	48
1	0	0	0	2	2	3	5	6	6	8	8	8
2	0	0	3	0	0	5	7	8	8	8	8	8
3	0	0	1	1	2	2	5	7	8	8	8	8
4	2	0	0	1	3	2	5	7	8	8	8	8
5	0	0	2	0	1	4	3	7	8	8	8	8
6	1	0	2	2	3	5	5	7	7	8	8	8
7	2	1	0	0	3	3	5	7	8	8	8	8
8	0	3	1	4	1	2	6	7	8	8	7	8
9	1	2	0	3	1	5	8	7	7	8	8	8
10	0	1	0	1	2	5	7	8	8	8	8	8
11	0	0	0	1	4	4	5	7	8	8	8	8
12	0	0	0	0	3	8	7	8	8	8	8	8
13	0	0	0	1	3	3	5	7	8	8	8	8
14	0	0	1	1	2	3	6	6	8	8	8	8
15	0	0	0	0	2	6	6	8	8	8	8	8
16	0	0	0	0	1	5	3	8	8	8	8	8
17	0	0	0	0	0	2	5	6	8	8	8	8

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18	0	1	0	2	2	4	4	7	8	8	8	8
19	0	0	0	2	1	2	5	8	8	8	8	8
TOTAL	6	8	10	21	36	73	102	136	148	152	151	152
POSSIBLE	152	152	152	152	152	152	152	152	152	152	152	152
(actual/possible) %	4%	5%	7%	14%	24%	48%	67%	89%	97%	100%	99%	100%
POISSON PREDICT	2%	5%	10%	18%	33%	55%	78%	95%	100%	100%	100%	100%

TABLE 2

AVERAGE NUMBER OF COPIES IN THE CHANNEL

PLATE PASS	0.02	0.05	0.1	0.2	0.4	0.8	1.5	3	6	12	24	48
1	0	0	1	1	5	4	5	7	8	8	8	8
2	1	1	0	2	2	3	5	6	7	8	8	8
3	0	0	2	2	2	3	2	8	8	8	8	8
4	1	0	0	0	3	3	5	5	8	8	8	8
5	0	0	1	1	1	2	5	8	8	8	8	8
6	0	0	0	2	2	3	4	8	7	8	8	8
7	0	0	0	2	1	4	5	7	8	8	8	8
8	0	1	2	0	0	3	5	6	8	8	8	8
9	0	0	0	2	1	2	3	6	8	8	8	8
10	1	0	0	0	3	1	4	6	7	8	8	8
11	0	0	1	0	4	5	5	6	8	8	8	8
12	0	0	0	2	3	2	3	7	7	7	8	8
13	3	1	4	0	1	1	4	6	7	8	8	8
14	0	1	2	1	1	4	7	6	7	8	8	8
15	0	0	2	1	0	5	4	8	8	8	8	8
16	0	0	0	1	1	4	3	7	8	8	8	8
17	0	0	0	2	3	4	4	4	8	8	8	8
18	0	0	1	0	4	0	6	7	8	8	8	8
19	0	1	0	1	1	3	2	8	8	8	8	8
20	1	0	1	3	2	2	4	7	8	8	8	8
TOTAL	7	5	17	23	40	58	85	133	154	159	160	160
POSSIBLE	160	160	160	160	160	160	160	160	160	160	160	160
(actual/possible) %	4%	3%	11%	14%	25%	36%	53%	83%	96%	99%	100%	100%
POISSON PREDICT	2%	5%	10%	18%	33%	55%	78%	95%	100%	100%	100%	100%

[0147] In a continuous flow mode, sipped samples broaden in plug length due to molecular diffusion and flow-induced dispersion. For a sipped sample containing tens or hundreds of copies of starting DNA templates, the effect of diffusion and dispersion on the width of the fluorescence PCR probe region can be predicted by considering Taylor-Aris dispersion. For single molecule PCR, similar calculations can be performed, and the peak

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shape of the fluorescent product is less broad than a large sample plug counterpart. The narrower peak is mainly due to the starting region from which DNA is amplified being narrower in the single molecule case (a few nm instead of hundreds of μm). Figure 3 summarizes an analysis of peak area and peak width as a function of starting copy number of DNA in channels. The lower copy number amplifications in fact showed narrow peaks as expected (and vice-versa).

[0148] Evidence for the system amplifying and typing single molecules also includes the fact that when the sample is a heterozygote, all peaks are positive for one or the other TaqMan probe, but not both.

[0149] There is another use for single molecule typing that can be performed according to the present invention as well. For example, two TaqMan™ or molecular beacon assays can be developed for sequences that are located close together in the genome. Those assays can be used to determine whether the proximal sequences are present on the same amplified molecule. This is an indirect way of doing a sizing assay: one can ask whether individual molecules have both TaqMan™/beacon sites, providing an indication of how often molecules are of a size that encompasses both sites. One can also type the two sites, providing a haplotyping method.

Experiment to monitor pcr amplification on-chip by measuring fluorescence generated by taqman probe cleavage.

[0150] This example provides an experiment to monitor PCR amplification on-chip by measuring fluorescence generated by TaqMan probe cleavage. Figure 5 shows the peak width at ½ max vs. calculated input copy number per channel (on-chip).

[0151] For this experiment, all necessary PCR reagents were loaded on-chip. One DNA sample was diluted in assay buffer in a 384-well plate (0.72ng/μL to 11.5ng/μL). The amplification cycle time was 17 seconds (5 seconds at 90°C, 7 seconds at 58°C and 5 seconds at 72°C) All injected samples were subjected to a total of 35 amplification cycles. Samples were injected for a total of 200 seconds, with a buffer wash (between samples) of 350 seconds. Width of PCR signal (peak) was measured at ½ the peak maximum for each microfluidic channel on-chip (8 total). Data shows that amplification of a single molecule in any channel produces the same width, in time (approximately 40-50 seconds). As more molecules (copies) are injected onto the chip, they begin to overlap, causing the width of the peak to increase in time. However, with long injection times, some single molecules show up on the edges of the injected slug of DNA.

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Application of Methods to Allele Typing

[0152] In cancer research, detection of cancer genes is very difficult because the mutated gene usually occurs at a much lower concentration then the wild type in a sample. The ability to detect amplification from a single molecule would solve the problem of detecting a low concentration of a mutated gene with a high concentration of wild type in the background since one can now study a single clone at a time. The use of the microfluidic sipper chip format with parallelized PCR on the chip speeds up the rate at which a single clone is examined at a time, to the point where it is practical to do a massive number of PCRs to find the few mutated genes responsible for cancer that exist in a given sample. Figure 4 illustrates raw fluorescence intensity measurements for SNP analysis at very low starting copy number to below 1 copy per channel on average. These data show the possibility of detecting SNP at single molecule PCR conditions.

[0153] Figures 10-12 show additional data from additional experimental runs, demonstrating single molecule amplification. As shown in Figures 10-11, a first set of experimental data with 100% of a first SNP allele is shown on the left, while a second set of experimental data with 1% of a second SNP allele (and 99% of the first allele) is shown on the right. The top signal line on the figure is data using a first dye detection system (which provides a "red" signal) for detecting amplification of the first allele, while the bottom line is data from a second dye detection system (a "blue" signal) for detecting amplification of the second allele. The data represents about 700 total detected DNA molecules in one sample slug. As shown, only the right side shows signal peaks corresponding to amplification of the second SNP. The data prove that a system of the invention can accurately amplify and detect rare molecules within a large population. That is, as a model, two DNA samples were mixed, each homozygous for the two alleles of a SNP. In this experiment, single DNA molecules for one allele that were present in a large population of DNA molecules of the other allele were detected (5-7 low copy alleles in about 700 for this case). Figure 11 provides results for 6 separate experimental runs, demonstrating that characteristic peak shapes from molecule to molecule is constant, providing experimental evidence that both PCR and dispersion of the resulting amplicons are very reproducible. In fact, a LabChip®-based system, as in the present invention, allows unlimited sensitivity to rare molecules in that: 1) it is practical, in a microfluidic system, to spread the sample out through the channel such that rare molecules are present amongst smaller numbers of wild-type molecules (reducing the problems created by proportional amplification of starting

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materials in each aliquot); and 2) reproducible fluidic handling and analysis gives a predictable single molecule peak shape that can be used to discriminate between molecular signals and random signal fluctuations.

[0154] Figure 12 provides a titration of a first SNP against a second SNP, showing that the signal from the amplicons corresponding to the first SNP ("FAM DNA," in the upper trace) and the second SNP ("VIC DNA," lower trace) show an appropriate signal correlation. The given percentages correspond to the percentage of DNA from a first homozygous sample (both alleles in the first homozygous sample are "FAM", that is, the material from the homozygous sample is "FF" homozygous) and a second homozygous sample (both alleles in the are VIC DNA sample, "VV"). In this context, "FAM DNA" stands for a DNA sequence that is probed for by a specific oligo sequence with a FAM dye label, while "VIC DNA" stands for a DNA sequence that is probed for by a specific oligo sequence with a VIC dye label. "FF" stands for a homozygous DNA sample for the "FAM" (oligo) sequence and "VV" stands for a homozygous DNA sample for the "VIC" (oligo) sequence.

Demonstration of Detection of Cancer Markers

[0155] Figure 13 provides an example of detection of 2 mutation sites relevant to cancer detection developed on-chip, using TaqMan probes. To demonstrate the relevance of the system of the invention to cancer diagnostics, it was used to test a number of cancer (e.g., colorectal cancer) markers using TaqMan probes. Two of those assays are shown in Figure 13: one for the K-RAS gene and one for the p53 gene, both diagnostic markers for a variety of cancers, such as colon cancer. The data trace shows fluorescence at two wavelengths vs. time for one microfluidic channel. Two TaqMan probes, one specific for the normal allele, and one specific for the mutant allele, were designed and tested in this on-chip assay format. The presence of normal DNA is detected with the wild-type probe (a "red" signal, designated in the black and white reproduction on the top data trace) and mutant DNA molecules are detected with the mutant probe (a "blue" signal, represented in the black and white reproduction as the bottom data trace). Most of the DNA molecules (approximately 500) in the sample slug are normal, shown by the high "red" top fluorescent signal and low "blue" bottom fluorescent signal. This signal is produced by the allele-specific (red, top) and background (blue, bottom) TaqMan probe cleavage surrounding the amplification products of normal genomic DNA molecules. When a mutant molecule

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(synthetic DNA template with the appropriate point mutation) traverses the system, it is amplified and recognized as a large blue (bottom) peak (with red (top) background peaks).

A DEVICE AND METHOD OF SINGLE MOLECULE AMPLIFICATION BY MICROFLUIDICS THAT PERMITS ACCURATE ANALYSIS OF HETEROGENEOUS NUCLEIC ACID MIXTURES.

[0156] Continuous flow PCR systems allow for spatial separation of different amplification reactions in a microfluidic processing environment. Normally, spatial separation is used to separate different reactions, where the starting template concentration is high enough to ensure accurate representation of alleles coming from both parents (e.g., about 50 genome equivalents are often used). In the present invention, the same task is accomplished by diluting the DNA enough such that individual template molecules are separated such that the amplification and detection products for each one are fluidically separated. If the detection product is allele specific, a signal for only one of the two alleles is detected. One can the count the results for each allele, giving the genotype quite accurately. The disadvantage for genotyping by this method is that the throughput decreases: one needs many reactions to get a genotype, instead of just one. Genotyping is typically performed with one reaction because the starting concentrations in a two allele system is usually about 50/50 (or at least on the same order of magnitude) and the signal-to-noise ratio of the genotyping biochemistry is good.

[0157] If, however, the representation of different alleles in the starting sample differs enormously, the genotyping biochemistry is not good enough to give an accurate quantitation of the under-represented allele. In fact, as a practical matter, it is very difficult to use many typical detection biochemistries for detection of alleles that are present in few as 1 in 5 copies. In cancer, the mutant/normal ratio can be quite low (1 in thousands) and therefore undetectable by conventional biochemical methods. On the other hand, if one amplifies single molecules, the reactions can be repeated and flowed in a continuous system for as long as desired-- and there is no theoretical limit of detection (just a practical one: if the mutant genotype is very rare, many reactions will have to be performed in the continuous flow format).

[0158] This also provides a strategy for quantifying infectious agents by PCR. Today, that is done by PCR or RT-PCR which depends on a cycle-by-cycle quantification and comparison to a standard curve of template molecules amplified under similar conditions. In the present invention, we flowed the sample at a known flow rate and

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measured the amplifications per unit volume as a more precise and quantitative determination of the template concentration. One can accomplish the same thing by amplifying dilute concentrations of the sample in wells. When the total number of positive wells equaled $e^0 = 0.37$, there was a high statistical probability that each well had only a single template molecule in it. One could also have more than one molecule present in the flow stream at any given time if an independent and reliable way of measuring the copy number is used.

SINGLE MOLECULE PCR IN A MICROFLUIDIC DEVICE UNDER STOP-FLOW CONDITIONS FOR VIRUS DETECTION & ANALYSIS.

[0159] The desired sensitivity for virus detection (e.g., about 50 – 100 copies / ml) make it a challenging application for detection using a microfluidic platform, due to the mismatch between processing volume on the chip and the initial sample volume. However, one of the features of PCR in a microfluidic device demonstrated in this application is the ability to quantitate single copies of nucleic acids. This allows one to count the number of infected cells, or virus particles in a sample of interest, at biologically relevant concentrations of cells or virus particles. In this example, we describe quantitative single molecule PCR from a starting volume on the order of 10 microliters (an initial pre-concentration step taking the sample from ~ 1 ml to about 10 ul is performed by standard techniques, e.g. immunoprecipitation in the presence of magnetic beads).

[0160] The ~10 ul of concentrated solution containing e.g., > 50 virus particles can be completely processed (or a substantial fraction of the volume) on a microfluidic chip in the following manner. The sample is mixed on-chip with the reagents necessary for PCR (at, e.g., a 1:1 ratio), e.g. primers, probes, dNTPs, etc. The mixture is pressure loaded into a microfluidic network that has a holdup volume on the order of 10 ul (*see*, Figure 8), and the flow is stopped. As shown, the schematic device of Figure 8 comprises PCR reagent well **801**, sample well **802** vacuum/waste well **803**, imaging area **804** and microfluidic network **805**. The contents of the network are then thermocycled by applying heat externally to the chip, or, optionally, via resistive or Joule heating. Upon completion of thermocycling, the chip is imaged to located all of the "clouds" of fluorescence (*see*, Figure 9), each corresponding, typically, to a single copy of DNA from a virus particle.

[0161] Figure 9 is a schematic of the fluidic network of Figure 8 after thermocycling. Spots **806** represent the fluorescence "clouds" from single amplicons (e.g.,

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in one example, virus particle amplicons). Spots **806** can be counted for quantitative PCR. For this particular application, it is likely that it is most efficient to image the entire fluidic network of the chip simultaneously, rather than in a continuous flow mode with the detector at fixed points. However, continuous flow can, alternatively, be used. The active area of the chip for imaging is typically on the order of 20 x 30 mm (but can, optionally, be smaller or larger). This area is compatible with high resolution imaging (~ 1 –2 um resolution) via techniques commonly used for DNA array applications. These can include CCD imaging, confocal laser scanning, and/or the like.

[0162] The dynamic range for quantification is typically at least 2 – 3 orders of magnitude, depending, in part, on the size of the chip. For a typical size of 20 x 30 mm, the dynamic range is about 2 orders of magnitude. One way to estimate the dynamic range is to examine the average separation between copies, and then compute the expected diffusion distances over the course of an experiment. A rough calculation to demonstrate that these types of volumes can be processed on a microfluidic chip is presented below.

[0163] To determine whether further concentration of a 10 uL sample down to the nL range was necessary, the following calculation was performed. The conclusion reached was that further concentration is not necessary.

[0164] If one loads a series of parallel channels (e.g., 64) that are 30 um deep, 120 um wide, and 30 mm long, the total volume in these channels is 6.2 μL. If it is further assumed that in the 6.2 μL, half of the volume comes from PCR reagents and the other half comes from the original 10 μL sample, then roughly 3 μL out of 10 μL are sampled per run, which is a reasonable volume from a statistical sampling and a practical ease of use standpoint. Furthermore, if the 10 μL concentrated sample contains 100 particles from an initial starting 1 mL volume of plasma, then one can detect about 30 PCR clouds per run, if the PCR efficiency is close to 100%. These clouds would be 62 mm apart, on average, from each other along the channel, or about 1 cloud in every 2 channels.

[0165] The next issue addressed is chip size and detection practicality. If the 64 (2^n binary split) parallel channels are packed together with 200 um landing area between, they will occupy 21 mm. So one images (or scans) an area of 30mm X 21mm to find the 30 PCR clouds (in stop flow mode) that should appear in the channels. This is similar to the size of a typical DNA chip, meaning that available chip scanners can be used for the detection.

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[0166] In summary, if 1 mL is concentrated to 10 μL and placed into a chip well, further concentration is unnecessary for detection. If anything is done to increase the volume (such as the addition of neutralization chemical(s) to an elution buffer, addition of lysing agents, etc.) , a further concentration step can be desirable. To avoid adding lysing agents, it can be desirable to do an ultrasonic lysing of particles in the 10 μL solution in the well.

[0167] The following is one example protocol for quantitative analysis by the above methods: 1. Off chip concentration, e.g., by affinity capture (a standard technique) and elution to reduce the sample from 1 mL to 10 μL; 2. Place 10 μL in chip well, apply ultrasonic power to lyse particles; 3. Load DNA sample into parallel channels with on chip addition of PCR reagents by pressure, then stop flow; 4. Activate external heater to perform PCR in stop flow mode for all channels; and 5. Image or scan the channel to detect signs of single molecule PCR.

[0168] One aspect of the invention provides methods to ensure stopped flow conditions on a chip. There are a number of methods that can be employed. For example, one can use temperature sensitive materials (e.g. polymers), to create the stop-flow condition. A simple method to achieve stopped flow is to use standard chip-capillary or chip-tubing connections combined with a valve.

[0169] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art, from a reading of this disclosure, that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above can be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

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WHAT IS CLAIMED IS:

1. A method of detecting a nucleic acid of interest, the method comprising:

aliquotting a sample comprising the nucleic acid of interest and one or more additional nucleic acid into a plurality of reaction mixtures, wherein at least two of the reaction mixtures are single copy reaction mixtures, each comprising a single copy of the nucleic acid of interest, wherein the plurality of reaction mixtures additionally comprise at least one additional reaction mixture comprising at least one copy of the additional nucleic acid;

subjecting the plurality of reaction mixtures to one or more amplification reaction while flowing the reaction mixtures; and,

detecting the nucleic acid of interest in the single copy reaction mixtures.

2. A method of detecting a low copy nucleic acid of interest in a sample that comprises one or more higher copy additional nucleic acid that is different from the low copy nucleic acid, the method comprising:

aliquotting the sample into a plurality of reaction mixtures, which reaction mixtures comprise a plurality of zero copy reaction mixtures comprising 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;

subjecting the zero and single copy reaction mixtures to an amplification reaction; and,

detecting the nucleic acid of interest in the single copy reaction mixture.

3. A method of quantifying a nucleic acid of interest in a sample, the method comprising:

aliquotting a sample comprising a copy of the nucleic acid of interest, or a complement thereof, into at least one reaction mixture;

subjecting the reaction mixture to at least one amplification reaction, thereby amplifying the copy of the nucleic acid of interest;

detecting a shape, volume, width, length, height, or area in which the nucleic acid of interest, or a signal corresponding thereto, is present; and,

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correlating the shape, volume, width, height, length, or area 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.

4. A method of quantifying a nucleic acid of interest in a sample, the method comprising:

aliquoting the sample into at least 25 reaction mixtures comprising 2 or fewer copies of the nucleic acid of interest each;

subjecting the reaction mixtures to one or more amplification reactions; and,

detecting the nucleic acid of interest in a plurality of the reaction mixtures.

5. A method of detecting a low copy nucleic acid of interest, the method comprising:

aliquotting a sample comprising the low copy nucleic acid of interest into a plurality of reaction mixtures, wherein 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;

subjecting a at least 10 of the plurality of zero copy reaction mixtures in a microfluidic device comprising at least one microchamber or microchannel to one or more amplification reaction;

determining that the nucleic acid of interest is not present in the zero copy reaction mixtures;

subjecting at least one additional zero copy reaction mixture and the reaction mixture comprising the nucleic acid of interest to one or more amplification reaction; and,

detecting the nucleic acid of interest in the reaction mixture comprising the nucleic acid of interest.

6. A method of detecting a nucleic acid of interest, the method comprising:

aliquotting a sample comprising the nucleic acid of interest into a plurality of reaction mixtures, wherein at least two of the reaction mixtures are single copy reaction mixtures, each comprising a single copy of the nucleic acid of interest;

flowing the reaction mixtures throughout a network of microchannels;

subjecting the plurality of reaction mixtures to one or more amplification reaction under stopped flow conditions in the network of microchannels; and,

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detecting the nucleic acid of interest in the single copy reaction mixtures under said stopped flow conditions.

7. The method of claim 1, 2, 3, 4, 5 or 6, wherein the reaction mixture comprises the nucleic acid of interest and a plurality of additional nucleic acids, wherein the method comprises detecting the nucleic acid of interest in the reaction mixture.

8. The method of claim 7, wherein the method comprises detecting the plurality of additional nucleic acids.

9. The method of claim 7, wherein the plurality of additional nucleic acids comprises one or more experimental nucleic acid.

10. The method of claim 7, wherein the plurality of additional nucleic acids comprises one or more unknown nucleic acid.

11. The method of claim 7, wherein the plurality of additional nucleic acids comprises one or more control nucleic acid.

12. The method of claim 7, wherein the method comprises adding the number of nucleic acids of interest, or the plurality of additional nucleic acids, or both, in the reaction mixture.

13. The method of claim 12, wherein the method comprises determining 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 the plurality of additional nucleic acids in the reaction mixture.

14. The method of claim 13, wherein the method comprises determining a concentration of the nucleic acid of interest in the reaction mixture.

15. The method of claim 13, wherein the sum of the nucleic acid of interest and the plurality of additional nucleic acids provides an indication of the total number of nucleic acids in the reaction mixture.

16. The method of claim 1, 2, 3, 4, 5 or 6, wherein the aliquotting comprises diluting the sample into a plurality of reaction containers.

17. The method of claim 16, wherein the reaction containers are wells in a microtiter plate.

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18. The method of claim 16, wherein the reaction containers are microchannels in a microchannel network.

19. The method of claim 1, 2, 3, 4 or 5, wherein the nucleic acid of interest is continuously flowed during a plurality of steps of the method.

20. The method of claim 1, 2, 3, 4, 5 or 6 , wherein the aliquotting comprises flowing the sample into a microfluidic dilution channel or chamber and diluting the sample in the microfluidic dilution channel or chamber, whereby the sample is aliquotted into multiple diluted aliquots in the microfluidic dilution channel or chamber.

21. The method of claim 20, wherein a plurality of samples are flowed into the device simultaneously.

22. The method of claim 20, wherein the samples are flowed into the device under pressure.

23. The method of claim 20, wherein the sample is diluted by flowing material from a common reaction component reservoir.

24. The method of claim 23, wherein the sample is diluted or concentrated to a concentration of about 1 nucleic acid of interest molecule per nanoliter, or less, in each reaction mixture.

25. The method of claim 23, wherein the reaction component reservoir comprises a locus specific amplification reagent.

26. The method of claim 20, wherein the multiple diluted aliquots are each diluted to the same degree.

27. The method of claim 20, wherein the multiple diluted aliquots are differentially diluted.

28. The method of claim 27, wherein the diluted aliquots form a dilution series.

29. The method of claim 20, wherein the aliquots are less than about 100 nl in volume.

30. The method of claim 20, wherein the aliquots are less than about 10 nl in volume.

31. The method of claim 20, wherein the aliquots are about 1 nl in volume or less.

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32. The method of claim 1, 2, 3, 4 or 5, wherein at least one of the reaction mixtures is in an aqueous solution.

33. The method of claim 1, 2, 3, 4 or 5, wherein at least one of the reaction mixtures is formulated in an aqueous phase of an emulsion comprising aqueous droplets suspended in an immiscible liquid.

34. The method of claim 33, wherein the amplification is performed on the reaction mixture when it is formulated in the emulsion.

35. The method of claim 34, wherein the nucleic acid of interest is present as a single copy in at least one aqueous droplet of the aqueous phase prior to performing the amplification reaction.

36. The method of claim 34, wherein the nucleic acid of interest is detected in the emulsion after the amplification reaction is performed.

37. The method of claim 34, wherein 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.

38. The method of claim 37, wherein a ratio of the additional nucleic acids in the emulsion to the nucleic acid of interest in the emulsion is determined.

39. The method of claim 37, wherein a total number of the additional nucleic acids and the nucleic acids of interest in the emulsion are determined and wherein the concentration of the nucleic acids of interest in the emulsion are also determined.

40. The method of claim 1, 2, 3, 5 or 6, wherein at least 10 of the reaction mixtures are low copy reaction mixtures comprising 2 or fewer copies of the nucleic acid of interest or the additional nucleic acid.

41. The method of claim 40, wherein the at least 10 low copy reaction mixtures comprise at least 10 single or zero copy reaction mixtures comprising 1 or fewer copies of the nucleic acid of interest.

42. The method of claim 1, 2, 3, 5, or 6, wherein at least 25 of the reaction mixtures are low copy reaction mixtures comprising 2 or fewer copies of the nucleic acid of interest.

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43. The method of claim 42, wherein the at least 10 low copy reaction mixtures comprise at least 10 single or zero copy reaction mixtures comprising 1 or fewer copies of the nucleic acid of interest.

44. The method of claim 1, 2, 3, 4, 5, or 6 wherein at least 50 of the reaction mixtures are low copy reaction mixtures comprising 2 or fewer copies of the nucleic acid of interest.

45. The method of claim 44, wherein the at least 10 low copy reaction mixtures comprise at least 10 single or zero copy reaction mixtures comprising 1 or fewer copies of the nucleic acid of interest.

46. The method of claim 1, 2, 3, 4, 5, or 6, wherein at least 100 of the reaction mixtures are low copy reaction mixtures comprising 2 or fewer copies of the nucleic acid of interest.

47. The method of claim 46, wherein the at least 100 low copy reaction mixtures comprise at least 100 single or zero copy reaction mixtures comprising 1 or fewer copies of the nucleic acid of interest.

48. The method of claim 1, 2, 3, 4, 5, or 6 , wherein at least 150 of the reaction mixtures are low copy reaction mixtures comprising 2 or fewer copies of the nucleic acid of interest.

49. The method of claim 48, wherein the at least 150 low copy reaction mixtures comprise at least 150 single or zero copy reaction mixtures comprising 1 or fewer copies of the nucleic acid of interest.

50. The method of claim 1, wherein the reaction mixtures comprise a plurality of zero copy reaction mixtures that comprise no copies of the nucleic acid of interest.

51. The method of claim 3, wherein the reaction mixtures comprise a plurality of zero copy reaction mixtures that comprise no copies of the nucleic acid of interest.

52. The method of claim 4, wherein the reaction mixtures comprise a plurality of zero copy reaction mixtures that comprise no copies of the nucleic acid of interest.

53. The method of claim 2, 5, 50, 51 or 52, wherein the plurality of zero copy reaction mixtures comprise at least about 10 zero copy reaction mixtures.

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54. The method of claim 2, 5, 50, 51 or 52, wherein the plurality of zero copy reaction mixtures comprise at least about 25 zero copy reaction mixtures.

55. The method of claim 2, 5, 50, 51 or 52, wherein the plurality of zero copy reaction mixtures comprise at least about 50 zero copy reaction mixtures.

56. The method of claim 2, 5, 50, 51 or 52, wherein the plurality of zero copy reaction mixtures comprise at least about 100 zero copy reaction mixtures.

57. The method of claim 2, 5, 50, 51 or 52, wherein the plurality of zero copy reaction mixtures comprise at least about 500 zero copy reaction mixtures.

58. The method of claim 2, 5, 50, 51 or 52, wherein the plurality of zero copy reaction mixtures comprise at least about 1,000 zero copy reaction mixtures.

59. The method of claim 2, 5, 50, 51 or 52, wherein the plurality of zero copy reaction mixtures comprise at least about 10,000 zero copy reaction mixtures.

60. The method of claim 3, wherein the sample comprises at least one additional nucleic acid that is different than the nucleic acid of interest.

61. The method of claim 4, wherein the sample comprises at least one additional nucleic acid that is different than the nucleic acid of interest.

62. The method of claim 5, wherein the sample comprises at least one additional nucleic acid that is different than the nucleic acid of interest.

63. The method of claim 6, wherein the sample comprises at least one additional nucleic acid that is different than the nucleic acid of interest.

64. The method of claim 1, 2, 60, 61 62 wherein the additional nucleic acid comprises a higher copy in the sample than the nucleic acid of interest.

65. The method of claim 1, 2, 60, 61 62 wherein the method comprises detecting the additional nucleic acid independent of the nucleic acid of interest.

66. The method of claim 65, comprising determining a ratio of the nucleic acid of interest to the additional nucleic acid.

67. The method of claim 65, comprising adding the number of nucleic acids in the reaction mixture.

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68. The method of claim 67, comprising determining the concentration of the nucleic acid of interest in the reaction mixture.

69. The method of claim 64, wherein the additional nucleic acid comprises at least about 100X as many copies as the nucleic acid of interest in the sample.

70. The method of claim 64, wherein the additional nucleic acid comprises at least about 1,000X as many copies as the nucleic acid of interest in the sample.

71. The method of claim 64, wherein the additional nucleic acid comprises at least about 10,000X as many copies as the nucleic acid of interest in the sample.

72. The method of claim 64, wherein the additional nucleic acid comprises at least about 100,000X as many copies as the nucleic acid of interest in the sample.

73. The method of claim 64, wherein the additional nucleic acid comprises at least about 1,000,000X as many copies as the nucleic acid of interest in the sample.

74. The method of claim 1, 2, 3, 4 or 5, wherein the nucleic acid of interest is a SNP.

75. The method of claim 1 or 3, wherein the nucleic acid of interest is a low copy nucleic acid.

76. The method of claim 1, 2, 3, 4 or 5, wherein the nucleic acid of interest is a cancer associated nucleic acid.

77. The method of claim 1, 2, 3, 4 or 5, wherein the nucleic acid of interest is a colon cancer associated nucleic acid.

78. The method of claim 1, 2, 3, 4 or 5, wherein the sample comprises DNA derived from a plurality of cells, which plurality of cells comprises one or more cancer cells.

79. The method of clam 78, wherein the cancer cells comprise less than 1% of the cells in the plurality of cells from which the sample was derived.

80. The method of clam 78, wherein the cancer cells are colon cancer cells.

81. The method of clam 78, wherein the cancer cells are colon cancer cells derived from stool.

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82. The method of claim 1, 2, 3, 4, 5 or 6, wherein the nucleic acid of interest is a nucleic acid from an infective agent.

83. The method of claim 1, 2, 3, 4, 5 or 6, wherein the sample comprises DNA derived from a plurality of cells and one or more infectious agent.

84. The method of claim 83, wherein the infectious agent is a cell or a virus.

85. The method of claim 83, wherein DNA from the infectious agent comprises less than 1% of the DNA in the sample.

86. The method of claim 1, 2, 3, 4, 5 or 6,, wherein the nucleic acid of interest is a forensic nucleic acid.

87. The method of claim 1, 2, 3, 4, 5 or 6, wherein subjecting the reaction mixtures to one or more amplification reactions comprises thermocycling the reaction mixtures in one or more microscale amplification chamber or channel.

88. The method of claim 1, 2, 3, 4, 5 or 6 wherein subjecting the reaction mixtures to one or more amplification reactions comprises performing one or more non-thermal amplification reactions in one or more microscale amplification chamber or channel.

89. The method of claim 87, wherein the reaction mixtures are heated in the one or more microscale channels by applying electrical current to fluid in the microscale amplification chambers or channels.

90. The method of claim 87, wherein the reaction mixtures are heated in the one or more microscale amplification channels by resistively heating a heating element that contacts or is in proximity to the microscale amplification chambers or channels.

91. The method of claim 87, wherein the reaction mixtures are heated in the one or more microscale amplification channels by heating fluid in the microscale amplification chambers or channels with a Joule-Thompson or Peltier device.

92. The method of claim 1, 2, 3, 4, 5 or 6, wherein the detecting comprises real time PCR detection.

93. The method of claim 92, wherein the real time PCR detection comprises molecular beacon-mediated detection, or detection of the nucleic acid of interest by

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detecting a double-labeled probe before, during, or after polymerase-mediated digestion of the double labeled probe.

94. The method of claim 1, 2, 3, 4, 5 or 6, wherein the detecting comprises quantifying the nucleic acid of interest in the reaction mixtures, or the sample, or both.

95. The method of claim 94, wherein quantifying the nucleic acid of interest 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.

96. The method of claim 95, wherein the statistical or probabilistic analysis comprises one or more of: Poisson analysis, Monte Carlo analysis, application of a genetic algorithm, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, PLS analysis, or PCA analysis.

97. The method of claim 1, 2, 4, 5 or 6 wherein the detecting comprises measuring a shape, length, width, height, volume or area of signal in a given reaction mixture.

98. The method of claim 3 or 97, wherein detecting the shape, length, width, height, volume or area comprises detecting a label bound to the nucleic acid of interest.

99. The method of claim 3 or 97, wherein the shape, length, width, height, volume or area is correlated to a number of nucleic acids interest present in one of the reaction mixtures or a sample by comparing the length, width, height, volume or area to a predicted length, width, height, volume or area, wherein the predicted length, width, height, volume or area is based upon a Taylor Aris dispersion calculation, or a thermal diffusivity calculation, or both.

100. The method of claim 3 or 97, wherein the shape, length, width, height, volume or area is correlated to a number of nucleic acids interest present in one of the reaction mixtures or a sample by comparing the length, width, height, volume or area to an observed length, width, height, volume or area for a reference nucleic acid amplified from an aliquot comprising a known number of reference nucleic acids.

101. The method of claim 3 or 97, further comprising calculating diffusion, or dispersion, or both, of one or more amplified nucleic acids in the given reaction mixture,

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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 the subjecting step.

102. The method of claim 3 or 97, further comprising calculating diffusion, or dispersion, or both, of one or more amplified nucleic acids in the given reaction mixture, wherein calculating diffusion, or dispersion, or both, comprises calculating Taylor-Aris dispersion, or thermal diffusivity, or both.

103. The method of claim 1, 2, 4 or 5, wherein detecting the nucleic acid of interest comprises detecting a plurality of signals from a plurality of amplification reactions comprising the nucleic acid of interest in a single detection channel.

104. The method of claim 103, wherein the plurality of amplifications are continuously flowed during said detecting.

105. The method of claim 103, wherein the signals comprise a plurality of signal clouds in the detection channel.

106. The method of claim 3, wherein detecting the nucleic acid of interest comprises detecting a plurality of signals from a plurality of amplification reactions comprising the nucleic acid of interest in a single detection channel.

107. The method of claim 106, wherein the plurality of amplifications are continuously flowed during said detecting.

108. The method of claim 106, wherein the signals comprise a plurality of signal clouds in the detection channel.

109. The method of claim 6, wherein detecting the nucleic acid of interest comprises simultaneously detecting a plurality of signals from a plurality of amplification reactions comprising the nucleic acid of interest in one or more detection channels.

110. The method of claim 109, wherein the signals comprise a plurality of signal clouds in the detection channel.

111 . The method of claim 110, wherein the signal clouds are simultaneously detected in the one or more detection channels with a CCD array or an image processor.

112. The method of claim 1, further comprising quantifying the nucleic acid of interest in the sample, or in one of the reaction mixtures, or both.

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113. The method of claim 2, further comprising quantifying the nucleic acid of interest in the sample, or in one of the reaction mixtures, or both.

114. The method of claim 5, further comprising quantifying the nucleic acid of interest in the sample, or in one of the reaction mixtures, or both.

115. The method of claim 3, 4, 112, 113 or 114, wherein quantifying the nucleic acid of interest comprises detecting the nucleic acid of interest in a plurality of reaction mixtures and further comprises performing statistical or probabilistic analysis to determine a percentage or distribution of reaction mixtures comprising a single copy of the nucleic acid of interest.

116. The method of claim 115, wherein the statistical or probabilistic analysis comprises one or more of: Poisson analysis, Monte Carlo analysis, application of a genetic algorithm, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, PLS analysis, or PCA analysis.

117. The method of claim 1, 2, 3, 4, 5 or 6, further comprising treating one or more amplification region with an acid or base to reduce contamination of the sample in the region.

118. A system for detecting low copy nucleic acids of interest in a sample, the system comprising:

a dilution module that dilutes the sample into multiple aliquots;

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, which detector is configured to detect one or more amplified copies of the nucleic acid of interest in or on the microfluidic device; and,

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.

119. The system of claim 118, further comprising system software that correlates a shape, length, width, volume or area occupied by amplified copies of the nucleic acid of interest, as detected by the detector, to the number of copies of the nucleic acid of interest

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present in one of the aliquots, or to the number of copies of the nucleic acid of interest present in the sample, or both.

120. The system of claim 118, wherein the microfluidic device is configured to permit flow of the multiple aliquots though the amplification chamber while performing one or more amplification reaction on at least one of the multiple aliquots in the amplification channel or chamber.

121. The system of claim 118, wherein the microfluidic device is configured to provide stopped flow conditions while performing one or more amplification reaction on at least one of the multiple aliquots in the amplification channel or chamber.

122. A system for quantifying one or more low copy nucleic acid of interest in a sample, the system comprising:

a dilution module that dilutes the sample into multiple aliquots;

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, which detector is configured to detect a 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; and,

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.

123. The system of claim 122, wherein the microfluidic device is configured to permit flow of the multiple aliquots though the amplification chamber while performing one or more amplification reaction on at least one of the multiple aliquots in the amplification channel or chamber.

124. The system of claim 122, wherein the microfluidic device is configured to provide stopped flow of the multiple aliquots in the amplification chamber while performing one or more amplification reaction on at least one of the multiple aliquots in the amplification channel or chamber.

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125. The system of claim 122, further comprising 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.

126. The system of claim 118 or 122, wherein the dilution module is integral with the microfluidic device.

127. The system of claim 118 or 122, wherein the microfluidic device comprises one or more electrodes positioned to flow electrical current into the microchamber or channel, whereby the flow of current into the microchamber or channel heats fluid in the microchamber or channel.

128. The system of claim 118 or 122, wherein the microfluidic device comprises or is coupled to one or more heating element positioned within or proximal to the microchamber or channel, which heating element heats fluid in the microchamber or channel.

129. The system of claim 128, wherein the heating element comprises a resistive heating element.

130. The system of claim 128, wherein the heating element comprises a Peltier or Joule-Thompson device.

131. The system of claim 118 or 122, wherein the detector is configured to detect one or more electromagnetic energy signal in or on the microfluidic device.

132. The system of claim 131, wherein the detector detects fluorescence, luminescence or fluorescence polarization.

133. The system of claim 118 or 122, wherein the system comprises 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.

134. The system of claim 133, wherein the statistical or probabilistic analysis comprises one or more of: Poisson analysis, Monte Carlo analysis, application of a genetic algorithm, neural network training, Markov modeling, hidden Markov modeling, multidimensional scaling, PLS analysis, or PCA analysis.

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135. The system of claim 133, wherein the statistical or probabilistic analysis comprises quantitatively determining a concentration, proportion, or number of the nucleic acids of interest in the sample.

136. The system of claim 118 or 122, further comprising a sample storage module that stores the sample until it is diluted by the dilution module.

137. The system of claim 136, further comprising a sample retrieval module that retrieves the sample from the sample storage module and delivers it to the dilution module.

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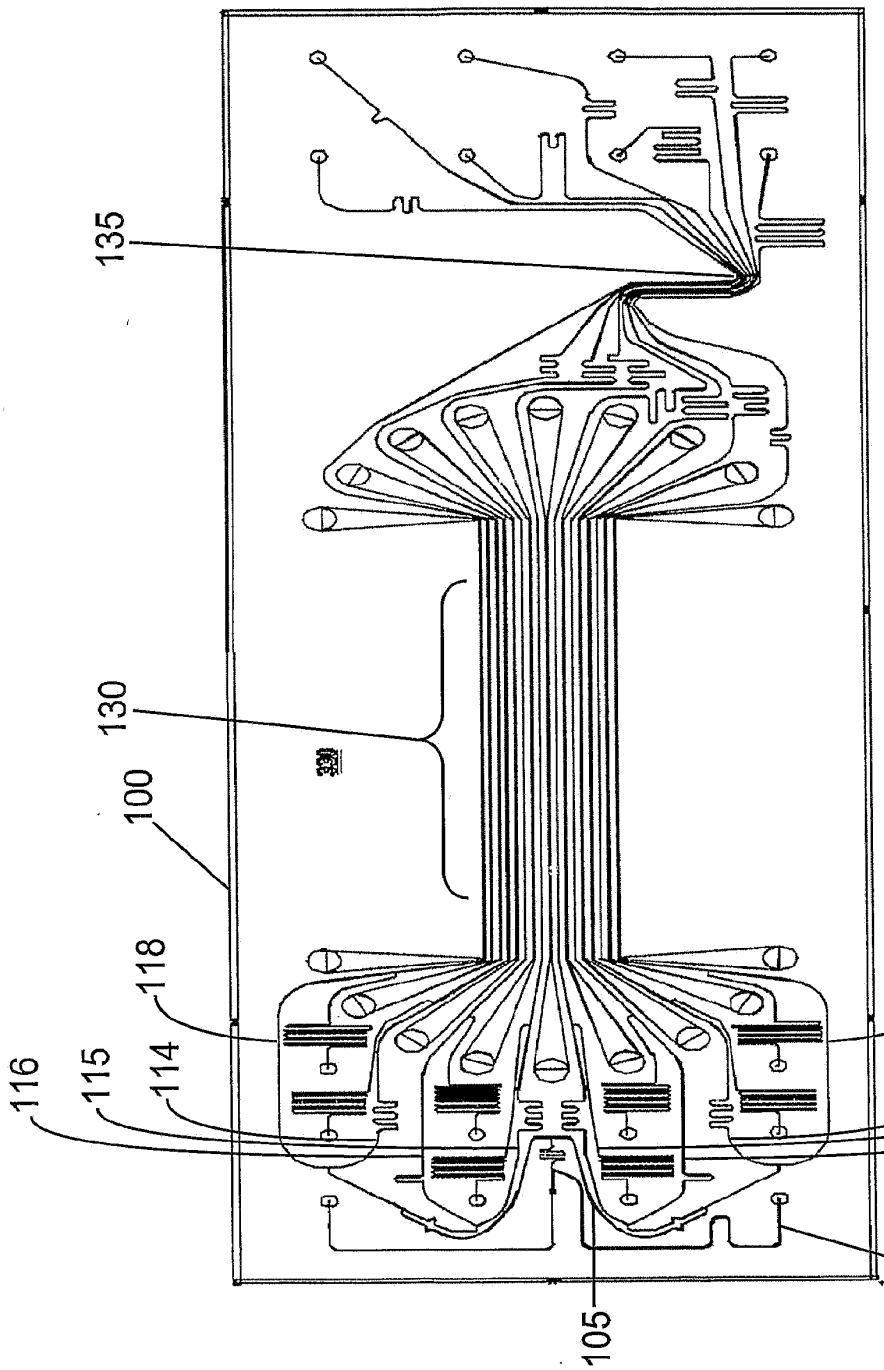


Fig. 1

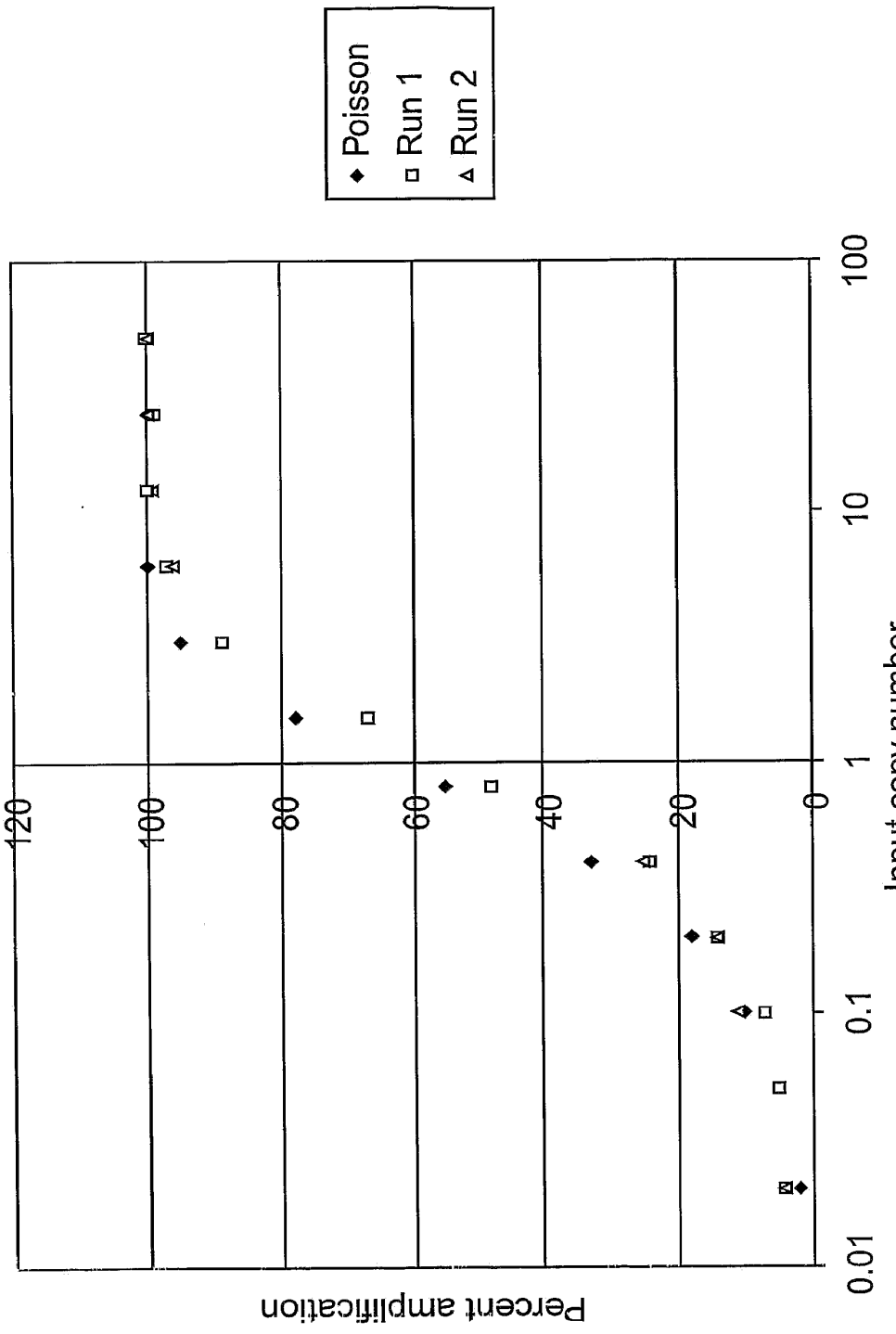

Poisson
Run 1
Run 2
Percent amplification
120
100
80
60
40
20
0
0.01
0.1
1
10
100
Input copy number

Fig. 2

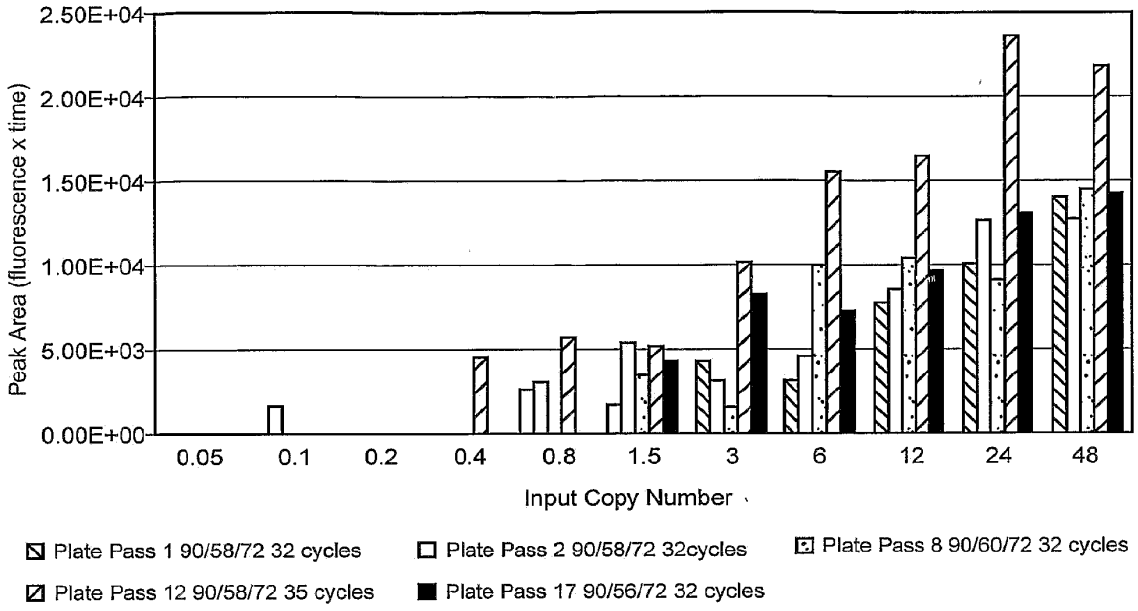

2.50E+04
2.00E+04
1.50E+04
1.00E+04
5.00E+03
0.00E+00
Peak Area (fluorescence x time)
0.05
0.1
0.2
0.4
0.8
1.5
3
6
12
24
48
Input Copy Number
Plate Pass 1 90/58/72 32 cycles
Plate Pass 2 90/58/72 32cycles
Plate Pass 8 90/60/72 32 cycles
Plate Pass 12 90/58/72 35 cycles
Plate Pass 17 90/56/72 32 cycles

Fig. 3A

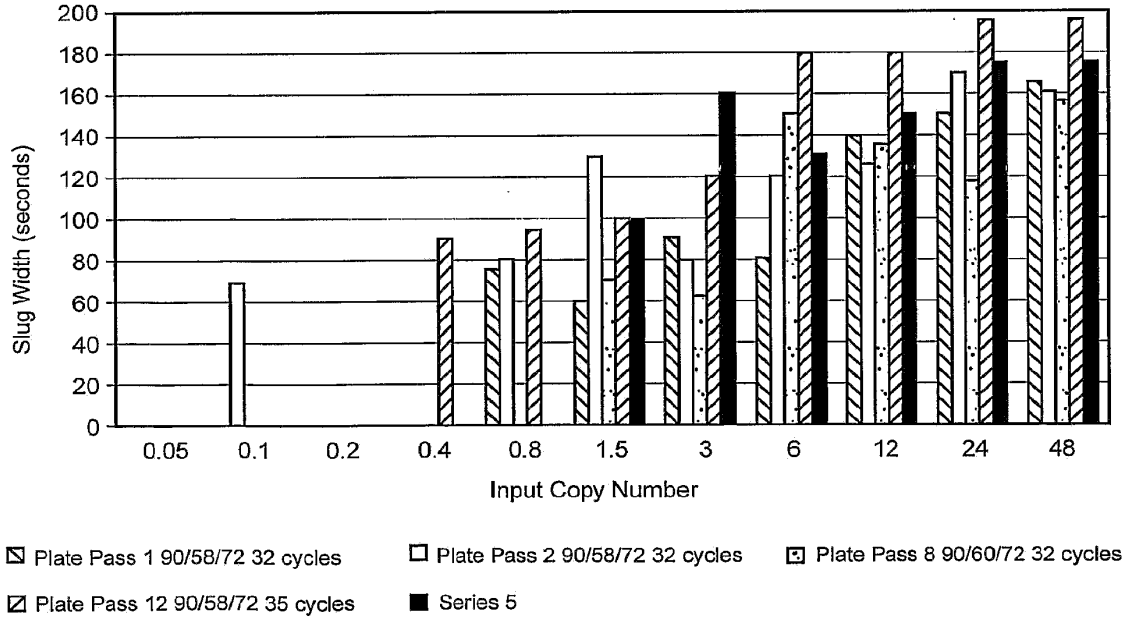

200
180
160
140
120
100
80
60
40
20
0
Slug Width (seconds)
0.05
0.1
0.2
0.4
0.8
1.5
3
6
12
24
48
Input Copy Number
Plate Pass 1 90/58/72 32 cycles
Plate Pass 2 90/58/72 32 cycles
Plate Pass 8 90/60/72 32 cycles
Plate Pass 12 90/58/72 35 cycles
Series 5

Fig. 3B

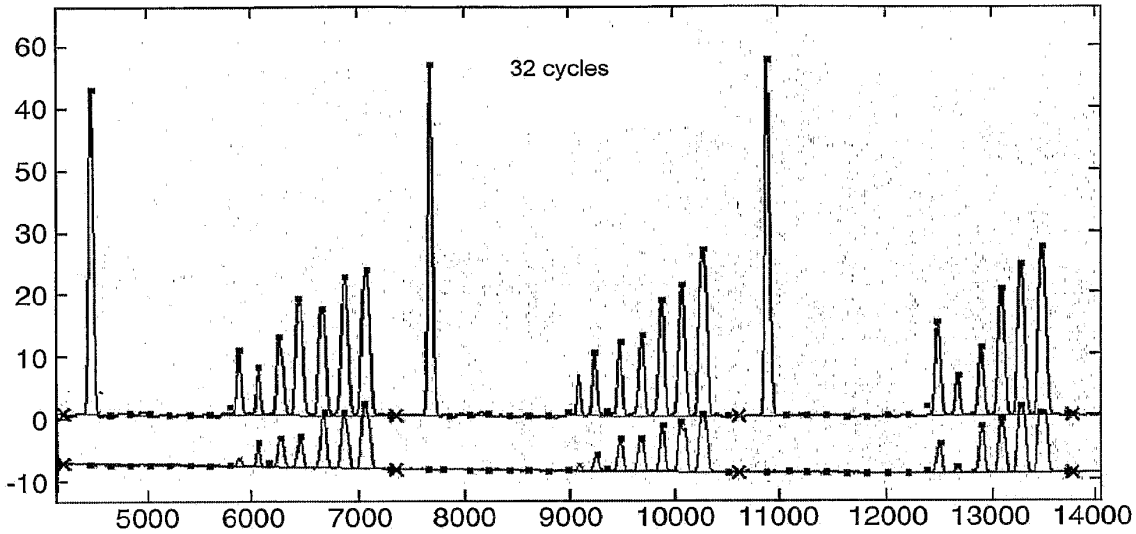

32 cycles
60
40
50
30
20
10
0
-10
5000
6000
7000
8000
9000
10000
11000
12000
13000
14000

Fig. 4A

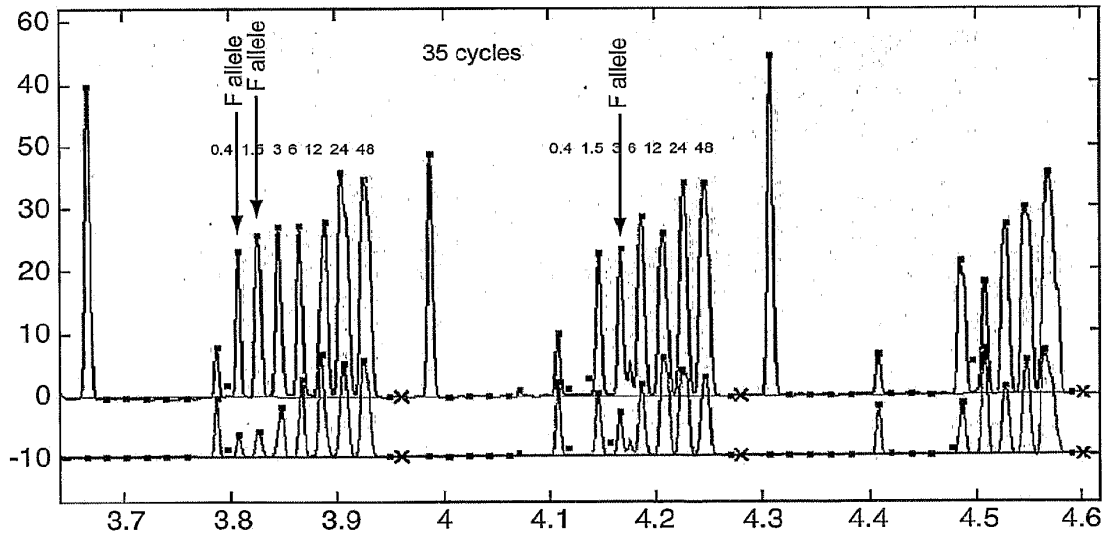

35 cycles
F allele
F allele
F allele
0.4 1.5 3 6 12 24 48
0.4 1.5 3 6 12 24 48
60
40
50
30
20
10
0
-10
3.7
3.8
3.9
4
4.1
4.2
4.3
4.4
4.5
4.6

Fig. 4B

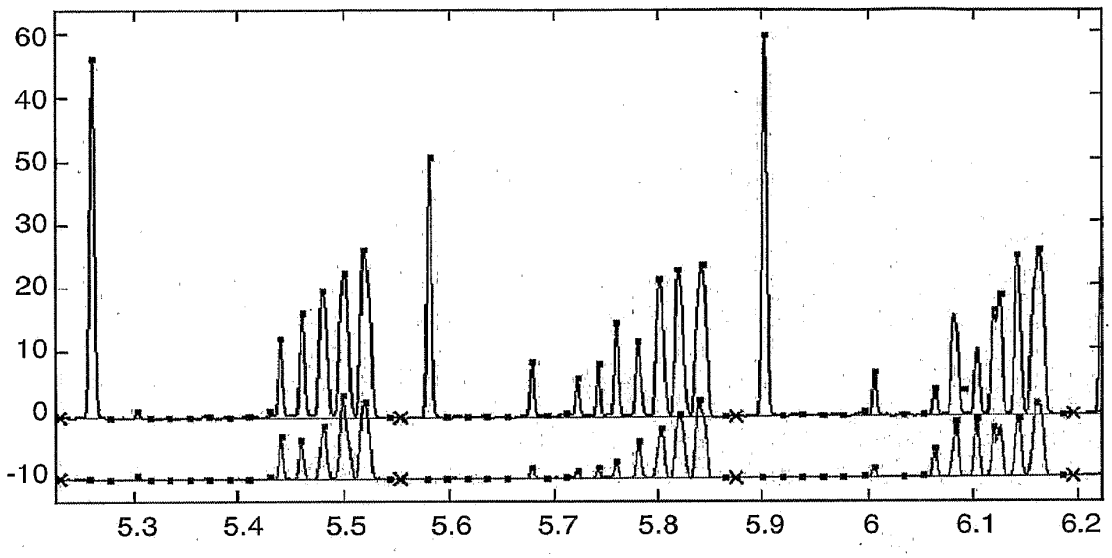

60
40
50
30
20
10
0
-10
5.3
5.4
5.5
5.6
5.7
5.8
5.9
6
6.1
6.2

Fig. 4C

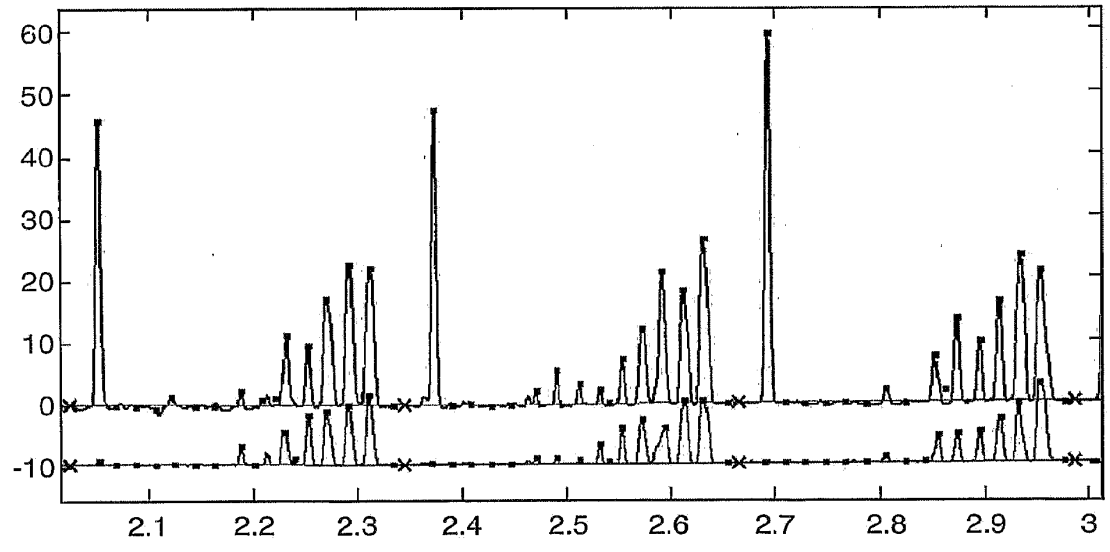

60
50
40
30
20
10
0
-10
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9
3

Fig. 4D

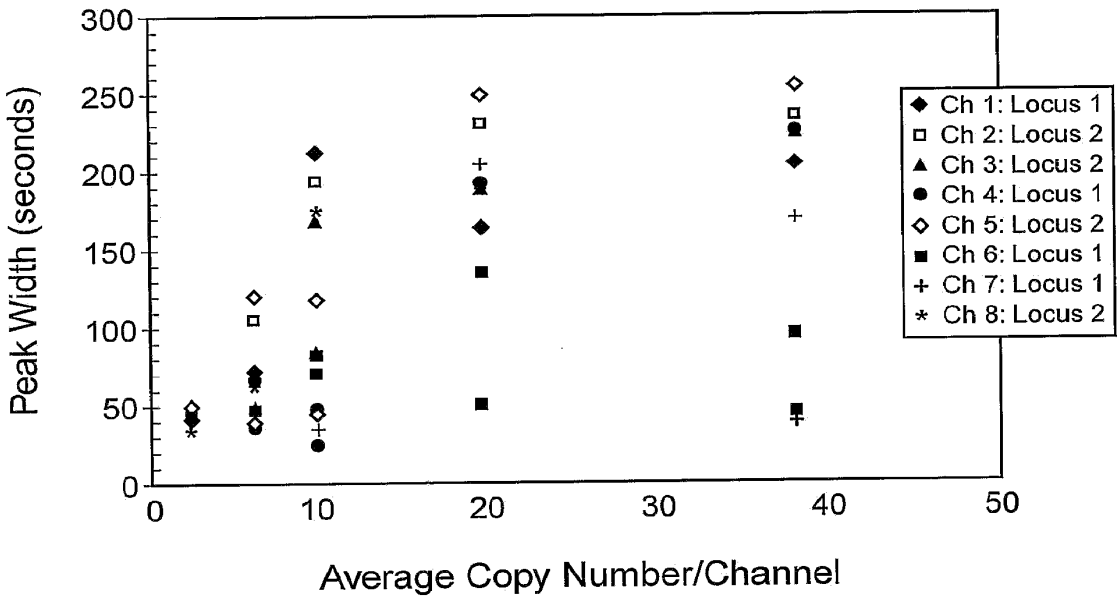

Peak Width (seconds)
300
250
200
150
100
50
0
0
10
20
30
40
50
Average Copy Number/Channel
Ch 1: Locus 1
Ch 2: Locus 2
Ch 3: Locus 2
Ch 4: Locus 1
Ch 5: Locus 2
Ch 6: Locus 1
Ch 7: Locus 1
Ch 8: Locus 2

Fig. 5

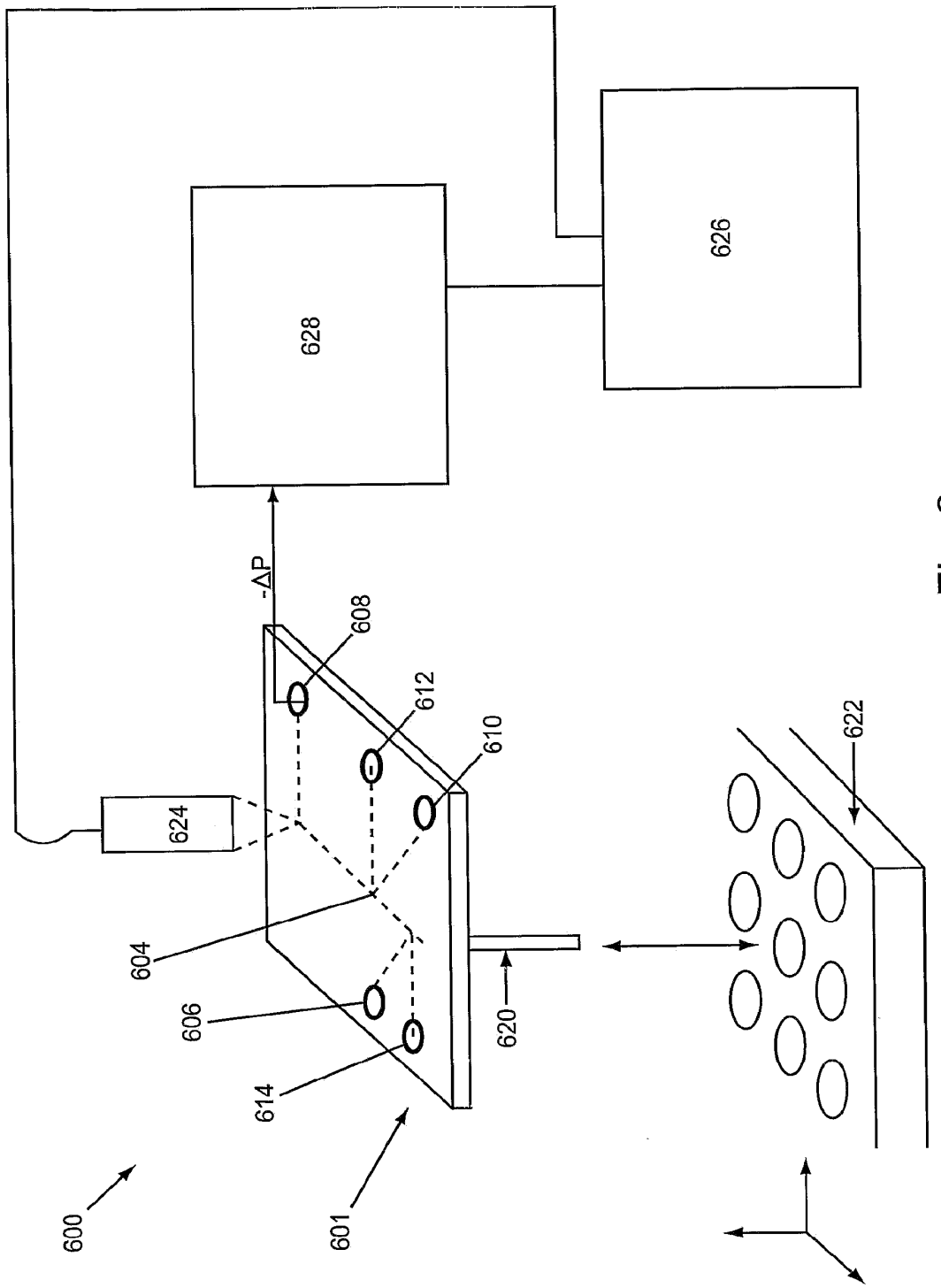


Fig. 6

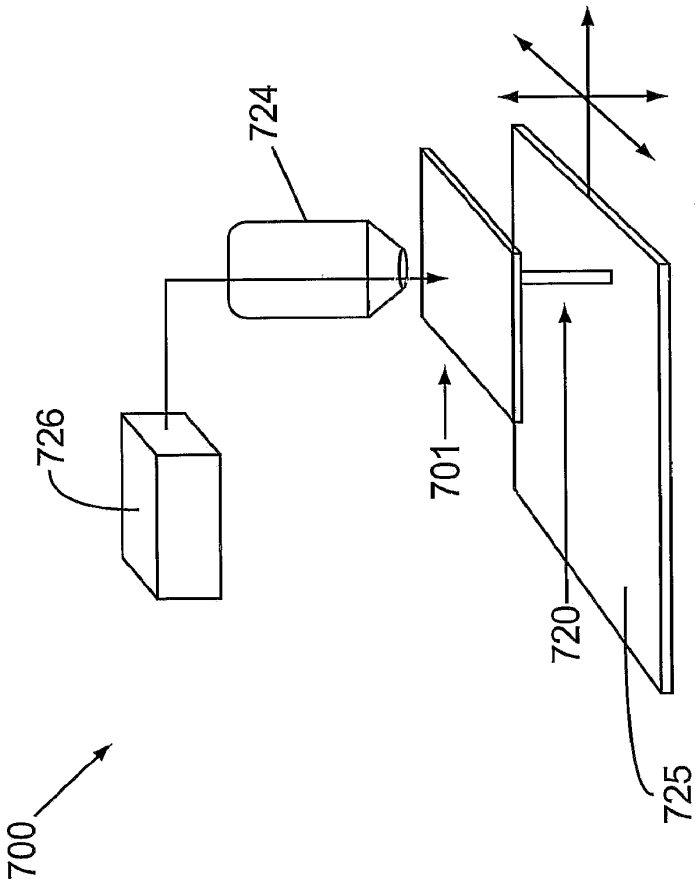


Fig. 7

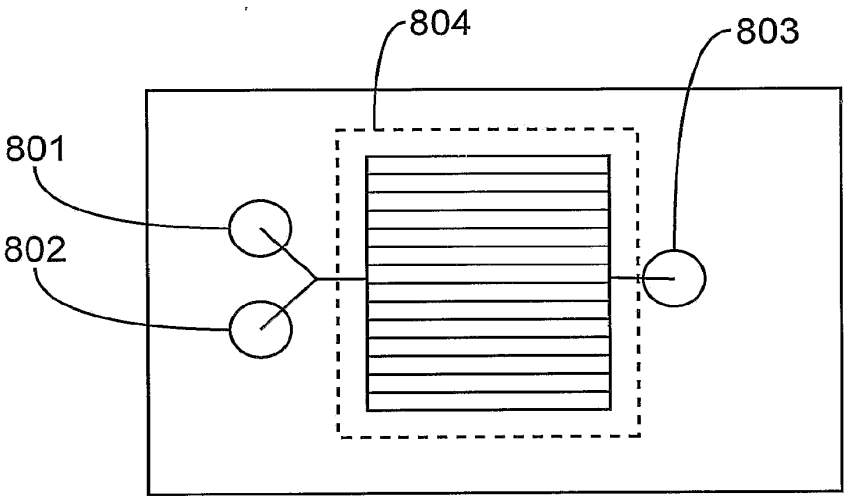


Fig. 8

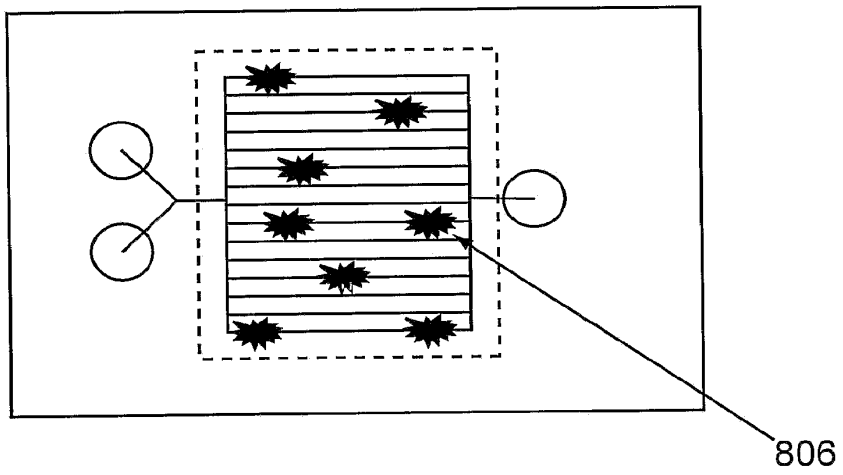


Fig. 9

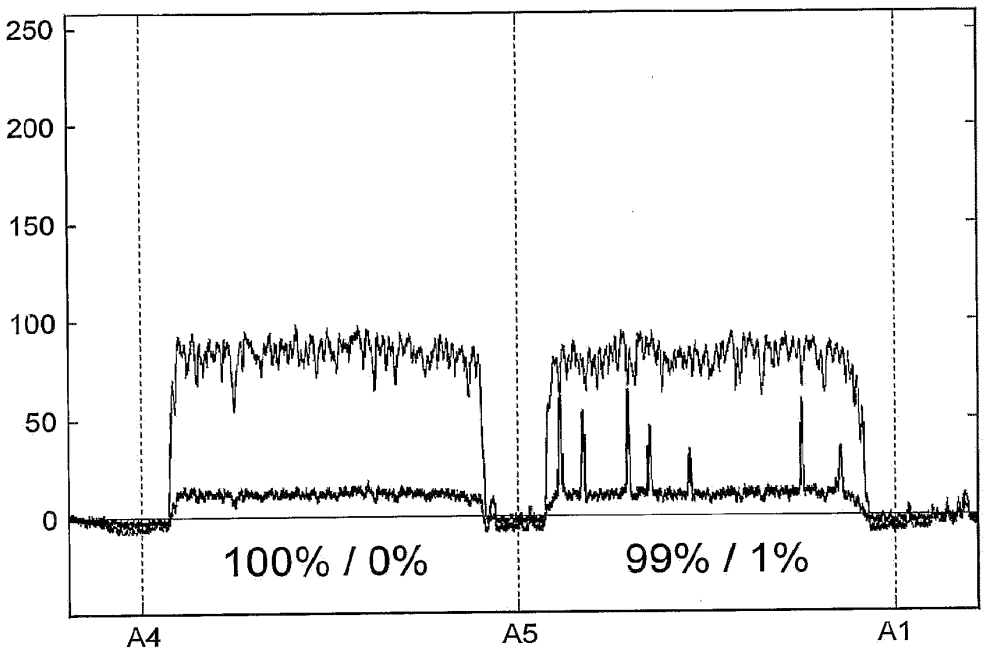


Fig. 10

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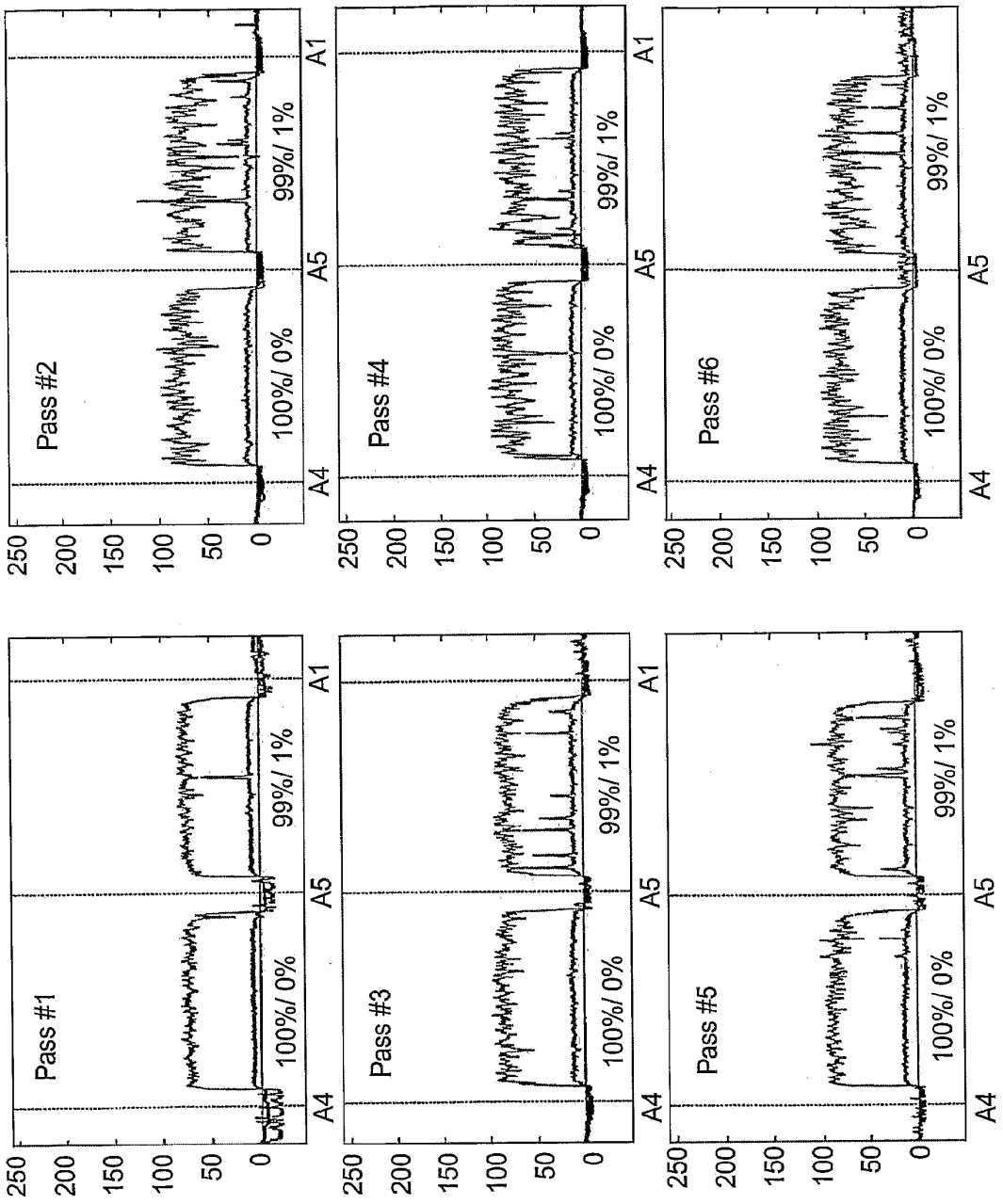

Pass #1
Pass #2
Pass #3
Pass #4
Pass #5
Pass #6
100%/ 0%
99%/ 1%
A1
A4
A5
0
50
100
150
200
250

Fig. 11

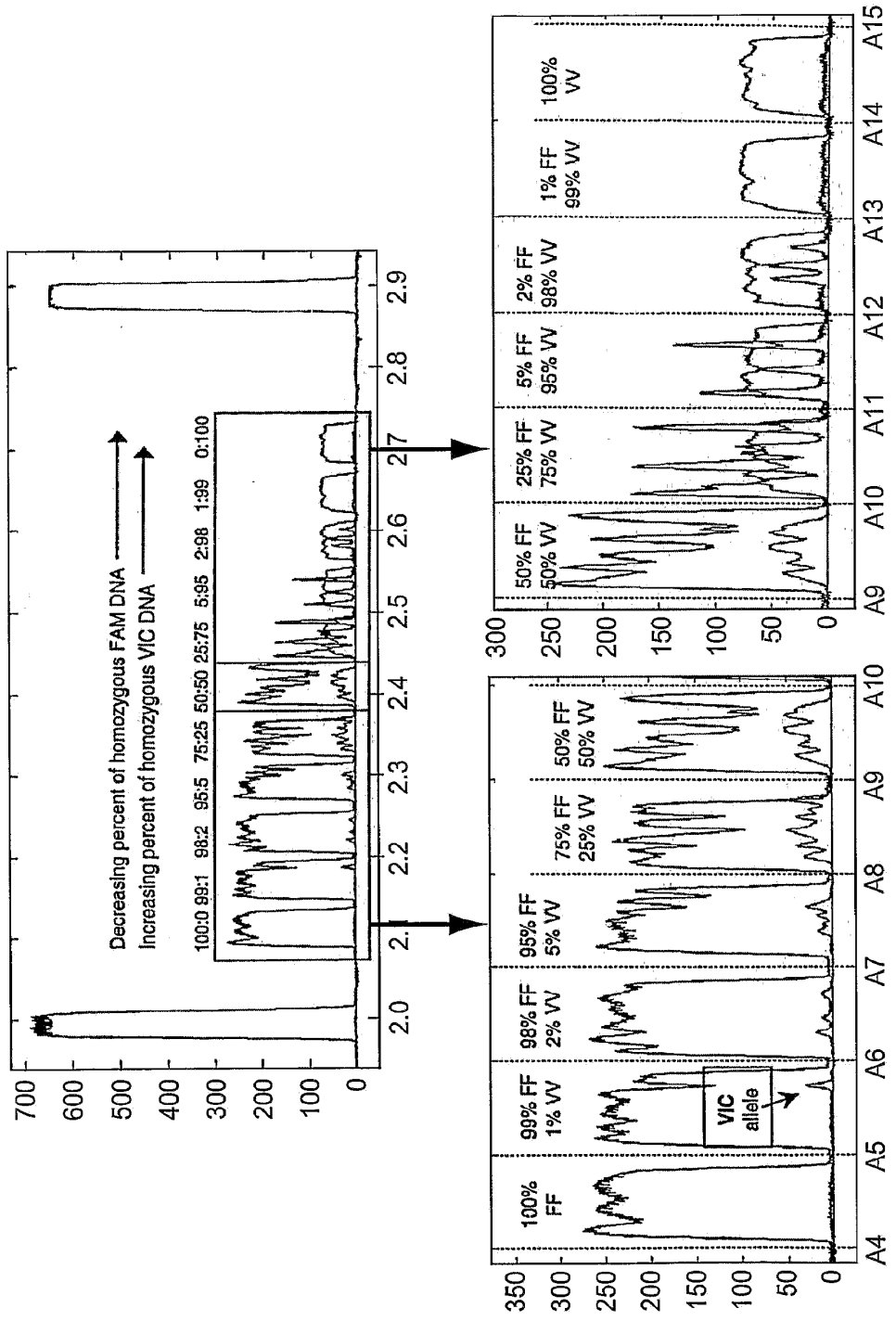

Decreasing percent of homozygous FAM DNA
Increasing percent of homozygous VIC DNA
100:0 99:1 98:2 95:5 75:25 50:50 25:75 5:95 2:98 1:99 0:100
100% FF
99% FF 1% VV
98% FF 2% VV
95% FF 5% VV
75% FF 25% VV
50% FF 50% VV
25% FF 75% VV
5% FF 95% VV
2% FF 98% VV
1% FF 99% VV
100% VV
VIC allele
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15

Fig. 12

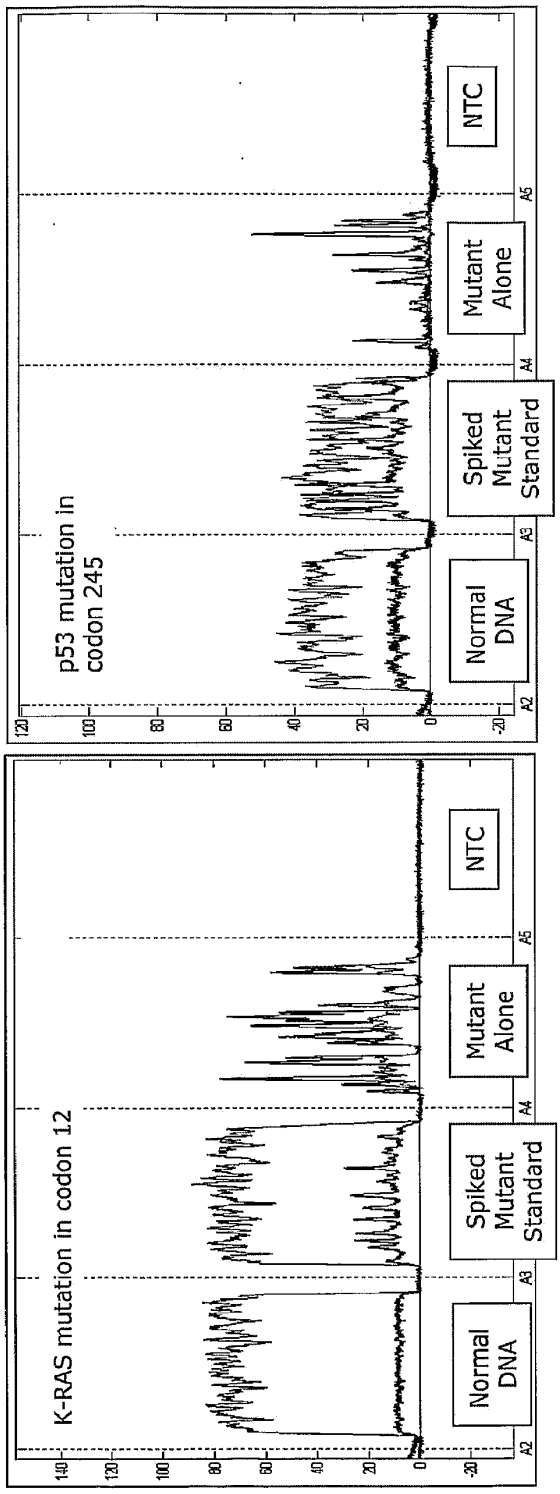

K-RAS mutation in codon 12
Normal DNA
Spiked Mutant Standard
Mutant Alone
NTC
p53 mutation in codon 245
Normal DNA
Spiked Mutant Standard
Mutant Alone
NTC

Fig. 13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/40599

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 19/34
US CL : 435/91.2

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)
U.S. : 435/91.2

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,314,809 A (ERLICH et al.) 24 May 1994 (24.05.1994).	1-97, 103-137
A	US 5,824,516 A (COLLU et al.) 20 October 1998 (20.10.1998).	1-97, 103-137

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 August 2004 (02.08.2004)	19 AUG 2004
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Kenneth R Horlick Telephone No. 571-272-1600

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/40599

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 98-102
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

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Continuation of B. FIELDS SEARCHED Item 3:
USPAT, DERWENT WPI, MEDLINE, BIOSIS
search terms: single copy, amplification, dilution, PCR, flow, microchannel, microfluidic

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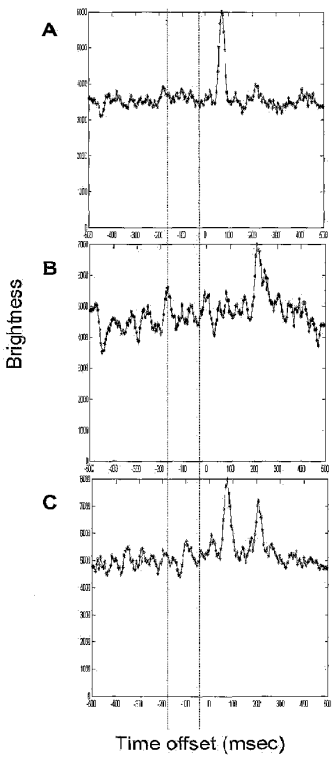
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[Continued on next page]

(54) Title: CO-DETECTION OF SINGLE POLYPEPTIDE AND POLYNUCLEOTIDE MOLECULES



(57) Abstract: The invention relates to a method for co-detecting and distinguishing individual polypeptide and polynucleotide molecules in a single sample analysis. The polypeptide and polynucleotide targets which emit electromagnetic radiation are labeled with probes that emit electromagnetic radiation at the same emission wavelength, are suspended in fluid and moved through an interrogation volume allowing co-detection of low concentrations of individual molecules and discrimination based on characteristics other than emission wavelength.

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TITLE OF THE INVENTION

Co-Detection of Single Polypeptide and Polynucleotide Molecules

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application Serial No. 60/492137 filed on July 31, 2003, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not Applicable.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ON A COMPACT DISC

Not Applicable.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to co-detecting single polypeptide and polynucleotide molecules in a single sample analysis.

2. Description of Related Art

The ability to co-detect proteins and nucleic acids is important for determining reactions and interactions during complex processes such as those that occur in biological systems. Most organisms contain both of these biomolecules, and the biological processes often involve measurable changes in both proteins and nucleic acids that help to elucidate their function. Known *in vitro* methods for co-detection of polypeptide and polynucleotide molecules are limited in that they require large amounts of sample, complex and expensive equipment, or a significant amount of time to perform. Many of these known methods measure large groups or ensembles of biomolecules, which contain perhaps hundreds or thousands of individual molecules within a single sample. These measurements obscure valuable information about properties of individual molecules. For example, if two properties are measured, ensemble measurement cannot determine whether both properties occur on one molecule or whether the first property and the second property each occur on only one half of the molecules. The current invention provides a method for co-detection of single polypeptide and polynucleotide molecules at very low concentrations that is fast, simple and inexpensive.

Numerous techniques have been developed for co-detection of polypeptides and polynucleotides using ensemble measurement of biomolecules. Co-detection can be

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performed using gel electrophoresis, flow cytometry, micro array technology, immunohistochemistry, in-situ hybridization and single molecule detection ("SMD").

The gel electrophoresis technique often detects one of the molecules indirectly. For example, a nucleic acid binding protein can be detected indirectly by a shift in electrophoretic velocity when the protein is bound to the nucleic acid. In these assays, the nucleic acid is detected by staining while the presence of the protein is inferred by a shift in electrophoretic velocity. Similar mobility shift assays have also been performed on single molecules with varying degrees of accuracy (LeCaptain, 2001). Other gel electrophoresis techniques distinguish the ensemble proteins and nucleic acids based on differences in emission characteristics of the molecules, either directly or after applying labels that emit electromagnetic radiation at different wavelengths. Jing *et al.* used SYBR Green and SYPRO Ruby labels, and Alba, *et al.* used SYPRO Red and YOYO labels (Molecular Probes, Eugene OR) (Jing, 2003; Alba, 2001-2) . One of the major disadvantages of these techniques is the need for large (pg to ng) quantities and high concentrations of target molecules (Haugland, 2002). Also, the run and analysis takes a long time (from a few hours to overnight). Further, quantitation requires densitometry measurements that are generally not precise, making it difficult to obtain accurate information.

Flow cytometry technology requires particles that scatter light using either cells or beads, and identifies fluorescent targets bound to the particles. The targets are suspended in fluid and flow past laser illumination sources and photo multiplier tube detectors. Proteins and nucleic acids are labeled with distinct fluorescent tags and the molecules are distinguished based on the different emission wavelengths of the labels. Flow cytometry methods that measure both proteins and nucleic acids include cellular assays for apoptosis (Waters, 2002; Hasper, 2000), cell cycle (Crissman, 1990; Niculescu, 1998), and cell replication (Faretta, 1998; Holm, 1998). In addition, molecular interactions between proteins and nucleic acids have been measured using beads detected by flow cytometry (Brodsky, 2002). Assays using beads to capture and detect molecules are sensitive in the high femtogram range. (Kellar, 2002; Dunbar, 2003). Although these techniques are fast to perform and a large number of events can be analyzed, detectable fluorescence intensity from single molecules cannot be achieved because it requires multiple bound target molecules and only single cells or particles are detected. Another disadvantage of this technology is that it does not allow for measurement of isolated molecules but requires they be attached to cells or beads. Precise quantitation is also difficult to accomplish.

Micro array technology is another method used for ensemble measurement of polypeptides and polynucleotides, and a co-detection assay has been developed (Perrin, 2003). In this co-detection experiment, antigens and oligonucleotides bound to a plate are used to capture antibodies and nucleic acids respectively from clinical samples using a micro

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titer plate format and colorimetric readout. Target proteins and nucleic acids are distinguished based on their locations on the array. The assay is configured as a diagnostic tool for virus infection and requires samples with molecule concentrations in the range 18-570 pM depending on the specific target (Perrin, 2003). However, this technique's usefulness is limited due to its low sensitivity. In addition, the equipment is very expensive and a significant amount of time is required (from hours to days) to build the arrays and to run and analyze samples.

Immunohistochemistry and in-situ hybridization can be used for co-detection of polypeptide and polynucleotide molecules in or on cells or tissues. Again, proteins and nucleic acids are generally distinguished based on different emission wavelengths from their respective labels. The preferred method uses fluorescently labeled tags to identify and locate specific polypeptides or polynucleotides. Probes can be labeled antibodies, oligonucleotides, or reactive metals, intercalating dyes, and fluorescent beads (Haugland, 2002). In these assays, cells or tissues are fixed on a surface (usually a glass slide or plastic dish), and illuminated with a UV light source. Microscopic imaging is used for the detection (Nagaso, 2001; Fischer, 2002). Co-detection using other distinguishing non-fluorescent labels such as radioactive and colorimetric labels has also been described (Chiu, 1996). However, again, detectable fluorescence intensity cannot be achieved with single molecules because this technique requires multiple molecules and only individual cell substructures are detected. Other disadvantages include relatively slow performance time (days), analysis of generally low numbers of cells or images and the requirement of expensive and fragile equipment. Also, a high level of skill and expertise is required to perform the techniques and additional sophisticated software is required for quantitation and analysis.

In the past 10 years, single molecule detection (SMD) has become a viable approach to the sensitive detection of biomolecules (Ambrose, 1999; Keller, 2002; Schwille, 2002; Wrotnowski, 2002). With SMD, individual molecules can be detected, quantitated, and discriminated in a mixture of molecules. Discrimination commonly uses measurement of electromagnetic characteristics, electrophoretic velocity and target-specific labels, such as oligomeric labels for nucleic acids and antibodies for proteins. SMD typically is used to identify or assay a single molecular species. Ma *et al.* have recently described co-detection of a protein and nucleic acid; however, the method described is very limited in that it requires labeling each of the analytes with a fluorescent dye that has emission wavelengths different from that of the other analyte (Ma, 2000). A CCD camera imaging system is used to identify complexes of protein bound to DNA, as well as individual labeled protein and DNA molecules in a mixture. Discrimination is based on the different emission wavelengths of the labeled molecules using a transmission grating. There are many disadvantages with these

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methods in that they require very specific materials. Sets of dyes that are compatible with both the analytes and the detection system are necessary. Further, the imaging system is very complex and there is a lack of automated data analysis.

Therefore, what is needed is a simple method to quickly and accurately measure individual molecules that would require smaller quantities of molecules and no attachment to a particle.

BRIEF SUMMARY OF THE INVENTION

Accordingly, it is an object of the invention to overcome these and other problems associated with the related art. These and other objects, features and technical advantages are achieved by providing methods of detecting single molecules by interaction with labels.

This invention provides a method for co-detecting a polynucleotide molecule and a polypeptide molecule contained within one sample comprising, in any order: (a) moving or disposing said polynucleotide molecule and said polypeptide molecule through at least one interrogation volume; and (b) measuring at least one electromagnetic characteristic of said polynucleotide molecule and measuring at least one electromagnetic characteristic of said polypeptide molecule, wherein said polynucleotide molecule and said polypeptide molecule are co-detected. In one aspect, the polynucleotide molecule and the polypeptide molecule are discriminated. In another aspect, the electromagnetic characteristic of the polynucleotide molecule and polypeptide molecule are measured simultaneously or sequentially.

In yet another aspect, a plurality of the same polynucleotide molecule and a plurality of the same polypeptide molecule are co-detected. In one alternative, a plurality of different polynucleotide molecules and a plurality of different polypeptide molecules are co-detected. In another alternative, a plurality of the same or different polynucleotide molecules and a plurality of the same or different polypeptide molecules are co-detected. Preferably, the polynucleotide is selected from the group consisting of a single-stranded DNA, a double-stranded DNA, an oligonucleotide, an RNA, a dendrimer, a nucleic acid hybrid and any combination thereof. Preferably, the polypeptide is selected from the group consisting of an oligopeptide and a protein.

In accordance with a further aspect of the invention, the polypeptide electromagnetic characteristic and the polynucleotide electromagnetic characteristic are measured within at least one interrogation volume, which may be optionally fluidly connected to one or more other interrogation volumes, said interrogation volume being in electromagnetic communication with at least one detector and at least one excitation source.

In another aspect, the sample comprises a plurality of the same or different polynucleotide molecules and a plurality of the same or different polypeptide molecules, and

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act (b) further comprises: measuring at least one of a first or a second electromagnetic characteristic of at least one polynucleotide molecule as the at least one polynucleotide molecule interacts with an excitation source within a first interrogation volume; measuring at least one of a first or a second electromagnetic characteristic of the at least one polynucleotide molecule as the at least one polynucleotide molecule interacts with an excitation source within a second interrogation volume; comparing the measured electromagnetic characteristics measured within the first and the second interrogation volumes of the at least one polynucleotide; measuring at least one of a first or a second electromagnetic characteristic of at least one polypeptide molecule as the at least one polypeptide molecule interacts with an excitation source within the first, the second or a third interrogation volume; measuring at least one of a first or a second electromagnetic characteristic of the at least one polypeptide molecule as the at least one polypeptide molecule interacts with an excitation source within the first, the second or a fourth interrogation volume; and comparing the measured electromagnetic characteristics measured within the first, the second, the third and the fourth interrogation volumes of the at least one polypeptide.

In another aspect, the act of comparing comprises distinguishing by statistical analysis the measured electromagnetic characteristics from the at least one polypeptide molecule and the at least one polypeptide molecule from background electromagnetic characteristics. In one alternative, the act of comparing comprises cross-correlating the measured electromagnetic emissions determined from the at least one polypeptide molecule and the at least one polypeptide molecule to determine the velocity of the molecules. In another alternative, the act of comparing comprises cross-correlating the measured electromagnetic emissions to determine the velocity of the molecules.

In another aspect, act (a) comprises subjecting the sample to a motive force selected from the group consisting of electro-kinetic, pressure, vacuum, surface tension, gravity, centrifugal, and any combination thereof. In yet another aspect, the act of moving the molecules between a first interrogation volume and a second interrogation volume further comprises subjecting the molecules to a separation method selected from the group consisting of capillary gel electrophoresis, micellar electro-kinetic chromatography, isotachophoresis, and any combination thereof.

In accordance with a further aspect of the invention, at least one of the measurable characteristics of the polynucleotide molecule and the measurable characteristics of the polypeptide molecule is produced by one of an intrinsic parameter of the molecule and an extrinsic parameter of the molecule. Preferably, the extrinsic parameter is provided by marking the polynucleotide molecule and the polypeptide molecule with at least one label to provide the extrinsic parameter. In one alternative, the polynucleotide molecule and

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polypeptide molecule are labeled prior to performing act (a). In another alternative, at least one polynucleotide and at least one polypeptide are labeled in separate reactions and are combined to create the sample prior to performing act (a). In yet another alternative, at least one polynucleotide and at least one polypeptide are labeled in the same reaction prior to performing act (a).

In accordance with a further aspect of the invention, the label affects the mobility of at least one of the polynucleotide molecule and the polypeptide molecule and is selected from the group consisting of a charge tag, a mass tag, a charge/mass tag, and any combination thereof. In one alternative, the method further comprises moving the polynucleotide molecule in a direction generally opposite to a direction of the polypeptide molecule. In another alternative, the sample comprises a plurality of different polynucleotide molecules, and further comprises moving a first polynucleotide molecule in a direction generally opposite to a direction of a second polynucleotide molecule. In another alternative, the sample comprises a plurality of different polypeptide molecules, further comprising moving a first polypeptide molecule in a direction generally opposite to a direction of a second polypeptide molecule.

In another aspect, the method further comprises moving the polynucleotide molecule in a direction generally perpendicular to a direction of the polypeptide molecule. In one alternative, the sample comprises a plurality of different polynucleotide molecules, further comprising moving a first polynucleotide molecule in a direction generally perpendicular to a direction of a second polynucleotide molecule. In another alternative, the sample comprises a plurality of different polypeptide molecules, further comprising moving a first polypeptide molecule in a direction generally perpendicular to a direction of a second polypeptide molecule.

In accordance with a further aspect of the invention, the label emits electromagnetic radiation selected from the group consisting of fluorescence, chemiluminescence, elastic light scattering, inelastic light scattering, and any combination thereof. In another aspect, the measured electromagnetic characteristic is selected from the group consisting of emission wavelength, emission intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime and any combination thereof.

In accordance with a further aspect of the invention, the polynucleotide label and the polypeptide label comprise indistinguishable emission wavelengths. Preferably, the polynucleotide label or polypeptide label further comprises a characteristic other than emission wavelength which is distinguishable. Preferably, the characteristic other than emission wavelength is selected from the group consisting of mobility, emission intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime, and any combination thereof. More preferably, the electromagnetic characteristics of the polynucleotide label and

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the polypeptide label are indistinguishable and wherein the polynucleotide and the polypeptide are distinguishable by their mobility. Still more preferably, the electromagnetic characteristics of the polynucleotide label and the polypeptide label are indistinguishable and wherein a predetermined range of labels is attached to each polynucleotide molecule and polypeptide molecule, and further wherein the polynucleotide molecule and polypeptide molecule are distinguishable by a characteristic signal produced by the difference in the range of labels attached to each molecule.

In accordance with a further aspect of the invention, the polynucleotide molecule and polypeptide molecule are labeled directly by means of specific or nonspecific interactions selected from a group consisting of covalent binding, ionic binding, hydrophobic binding, affinity binding, and any combination thereof. In one alternative, the polynucleotide and polypeptide are labeled indirectly by means of incubating with at least one binding partner to form a specific complex and wherein at least one binding partner comprises at least one label. Preferably, the binding partners are selected from the group consisting of polynucleotide/polynucleotide interactions, polynucleotide/polypeptide interactions and polypeptide/polypeptide interactions, and any combination thereof. More preferably, the interaction between binding partners is mediated through intermolecular forces selected from the group consisting of hydrophobic interactions, hydrogen bonding, ionic bonding, van der Waals attraction, covalent bonding, and any combination thereof.

In accordance with a further aspect of the invention, at least one polynucleotide molecule is detected and at least one polypeptide molecule is detected, and the method further comprises counting the molecules. Preferably, said counting comprises determining a concentration of polynucleotide molecules within the sample and a concentration of polypeptide molecules within the sample, further comprising comparing the counted polynucleotide molecules with a polynucleotide standard of a known concentration; and comparing the counted polypeptide molecules with a polypeptide standard of a known concentration. In one alternative, said counting comprises determining a concentration of polynucleotide molecules within the sample and a concentration of polypeptide molecules within the sample, further comprising comparing the counted polynucleotide molecules with an internal polynucleotide standard of a known concentration; and comparing the measured molecules of the polypeptide with an internal polypeptide standard of a known concentration. In yet another alternative, said counting comprises determining a concentration of polynucleotide molecules and polypeptide molecules without use of an external standard or an internal standard.

This invention also provides a method for co-detecting a polynucleotide molecule and a polypeptide molecule within one sample comprising: (a) labeling at least one polynucleotide molecule and at least one polypeptide molecule with at least one directly or

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indirectly detectable label in a mixture of polynucleotide molecules, polypeptide molecules and excess labels; (b) separating or rendering undetectable, or otherwise distinguishing unbound labels from the labeled polynucleotide molecules and labeled polypeptide molecules; (c) interacting the labeled polynucleotide molecules and labeled polypeptide molecules with an agent causing the release of directly or indirectly bound labels; and (d) detecting at least one polynucleotide molecule released label and at least one polypeptide molecule released label, thereby rendering the polynucleotide molecule and a polypeptide molecule co-detectable.

In accordance with a further aspect of the invention, a method is provided wherein act (d) comprises: moving or disposing the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label through at least one interrogation volume; and measuring at least one electromagnetic characteristic of the at least one polynucleotide molecule released label and measuring at least one electromagnetic characteristic of the at least one polypeptide molecule released label. In one aspect, the polynucleotide molecule released label and the polypeptide molecule released label are discriminated.

In another aspect, the electromagnetic characteristic of the polynucleotide molecule released label and polypeptide molecule released label are measured simultaneously or sequentially. In yet another aspect, a plurality of the same polynucleotide molecule and a plurality of the same polypeptide molecule are indirectly co-detected. In one alternative, a plurality of different polynucleotide molecules and a plurality of different polypeptide molecules are indirectly co-detected. In another alternative, a plurality of the same or different polynucleotide molecules and a plurality of the same or different polypeptide molecules are indirectly co-detected.

In accordance with a further aspect of the invention, the polypeptide molecule released label electromagnetic characteristic and the polypeptide molecule released label electromagnetic characteristic are measured within at least one fluidly connected interrogation volume, said interrogation volume being in electromagnetic communication with at least one detector and at least one excitation source.

In another aspect, the sample comprises a plurality of the same or different polynucleotide molecule released labels and a plurality of the same or different polypeptide molecule released labels, and wherein the act of measuring further comprises: measuring at least one of a first or a second electromagnetic characteristic of at least one polynucleotide molecule released label as the at least one polynucleotide molecule released label interacts with an excitation source within a first interrogation volume; measuring at least one of a first or a second electromagnetic characteristic of the at least one polynucleotide molecule released label as the at least one polynucleotide molecule released label interacts with an

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excitation source within a second interrogation volume; comparing the measured electromagnetic characteristics measured within the first and the second interrogation volumes of the at least one polynucleotide molecule released label; measuring at least one of a first or a second electromagnetic characteristic of at least one polypeptide molecule released label as the at least one polypeptide molecule released label interacts with an excitation source within the first, the second or a third interrogation volume; measuring at least one of a first or a second electromagnetic characteristic of the at least one polypeptide molecule released label as the at least one polypeptide molecule released label interacts with an excitation source within the first, the second or a fourth interrogation volume; and comparing the measured electromagnetic characteristics measured within the first, the second, the third and the fourth interrogation volumes of the at least one polypeptide molecule released label.

In another aspect, the act of comparing comprises distinguishing by statistical analysis the measured electromagnetic characteristics of the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label from background electromagnetic characteristics. In one alternative, the act of comparing comprises cross-correlating the measured electromagnetic emissions determined to be of the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label to determine the velocity of the labels. In another alternative, the act of comparing comprises cross-correlating the measured electromagnetic emissions to determine the velocity of the labels.

In yet another aspect, the act of moving comprises subjecting the sample to a motive force selected from the group consisting of electro-kinetic, pressure, vacuum, surface tension, gravity, centrifugal, and any combination thereof. In another aspect, the act of moving the released labels between a first interrogation volume and a second interrogation volume further comprises subjecting the released labels to a separation method selected from the group consisting of capillary gel electrophoresis, micellar electro-kinetic chromatography, isotachophoresis, and any combination thereof.

In accordance with a further aspect of the invention, at least one measurable characteristic of the polynucleotide molecule released label and at least one measurable characteristic of the polypeptide molecule released label is produced by one of an intrinsic parameter of the released labels and an extrinsic parameter of the released labels. Preferably, the extrinsic parameter is provided by attaching a secondary label to the released label to provide the extrinsic parameter. In one alternative, the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are marked with the secondary label prior to performing the act of moving. In another alternative, the secondary label is attached to the polynucleotide molecule released label

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and the polypeptide molecule released label in separate reactions and are combined to create the sample prior to performing the act of moving. In yet another alternative, the secondary label is attached to the polynucleotide molecule released label and the polypeptide molecule released label in the same reaction prior to performing the act of moving.

In another aspect, the secondary label affects the mobility of the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label, and wherein the secondary label is selected from the group consisting of a charge tag, a mass tag, a charge/mass tag, and any combination thereof. In one alternative, the method further comprises moving the marked polynucleotide molecule released label in a direction generally opposite to a direction of the marked polypeptide molecule released label. In another alternative, the sample comprises a plurality of different polynucleotide molecule released labels, further comprising moving a first marked polynucleotide molecule released label in a direction generally opposite to a direction of a second marked polynucleotide molecule released label. In another alternative, the sample comprises a plurality of different polypeptide molecule released labels, further comprising moving a first marked polypeptide molecule released label in a direction generally opposite to a direction of a second marked polypeptide molecule released label.

In accordance with a further aspect of the invention, the method further comprises moving the marked polynucleotide molecule released label in a direction generally, at an angle, preferably perpendicular to a direction of the marked polypeptide molecule released label. In one alternative, the sample comprises a plurality of different polynucleotide molecule released labels, further comprising moving a first marked polynucleotide molecule released label in a direction generally perpendicular to a direction of a second marked polynucleotide molecule released label. In another alternative, the sample comprises a plurality of different polypeptide molecule released labels, further comprising moving a first marked polypeptide molecule released label in a direction generally perpendicular to a direction of a second marked polypeptide molecule released label.

In accordance with a further aspect of the invention, the polynucleotide molecule released label, polypeptide molecule released label or secondary label emits electromagnetic radiation selected from the group consisting of fluorescence, chemiluminescence, light scattering, and any combination thereof. In another aspect, the measured electromagnetic characteristic is selected from the group consisting of emission wavelength, emission intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime and any combination thereof.

In accordance with a further aspect of the invention, the polynucleotide molecule released label and the polypeptide molecule released label comprise indistinguishable

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emission wavelengths. Preferably, the secondary label comprises a characteristic other than emission wavelength which is distinguishable. Preferably, the characteristic other than emission wavelength is selected from the group consisting of mobility, emission intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime and any combination thereof. More preferably, the electromagnetic characteristics of the polynucleotide molecule released label and the polypeptide molecule released label are indistinguishable, and wherein the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are indirectly distinguishable by the secondary label mobility.

In another aspect, the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are marked directly with the secondary label by means of specific or nonspecific interactions selected from a group consisting of covalent binding, ionic binding, hydrophobic binding, affinity binding, and any combination thereof. In one alternative, the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are marked indirectly with the secondary label by means of incubating with at least one binding partner to form a specific complex and wherein at least one binding partner comprises at least one secondary label. Preferably, the binding partners are selected from the group consisting of protein/protein interactions, protein/nucleic acid interactions, nucleic acid/nucleic acid interactions, and any combination thereof. More preferably, the interaction between binding partners is mediated through intermolecular forces selected from the group consisting of hydrophobic interactions, hydrogen bonding, ionic bonding, van der Waals attraction, covalent bonding, and any combination thereof.

In accordance with a further aspect of the invention, at least one released label is detected, and the method further comprises counting the released labels. Preferably, said counting comprises determining a concentration of at least one polynucleotide molecule released label and at least one polypeptide molecule released label within the sample, further comprising: comparing the counted polynucleotide molecule released labels with a polynucleotide molecule label standard of a known concentration; and comparing the counted polypeptide molecule released labels with a polypeptide molecule label standard of a known concentration. In one alternative, said counting comprises determining a concentration of at least one polynucleotide molecule released label and at least one polypeptide molecule released label within the sample, further comprising comparing the counted polynucleotide molecule released labels with an internal polynucleotide molecule label standard of a known concentration; and comparing the counted polypeptide molecule released labels with an internal polypeptide molecule label standard of a known concentration. In another alternative, said counting comprises determining a concentration

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of at least one polynucleotide molecule released label and at least one polypeptide molecule released label without use of external or internal standards. In yet another alternative, the number of the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label counted are proportional to the concentration of the polynucleotide molecules and polypeptide molecules in the original sample.

In accordance with a further aspect of the invention, a method is provided wherein at least one secondary label is detected, and the method further comprises counting the secondary labels. In one alternative, said counting comprises determining a concentration of at least one secondary label within the sample, further comprising comparing the counted secondary labels released from the polynucleotide molecule with a secondary label standard of a known concentration; and comparing the counted secondary labels released from the polypeptide molecule with a secondary label standard of a known concentration. In another alternative, said counting comprises determining a concentration of at least one secondary label within the sample, further comprising comparing the counted secondary labels released from the polynucleotide molecule with an internal secondary label standard of a known concentration; and comparing the counted secondary labels released from the polypeptide molecule with an internal secondary label standard of a known concentration. In another alternative, said counting comprises determining a concentration of at least one secondary label without use of external or internal standards. In yet another alternative, the number of the at least one secondary labels counted are proportional to the concentration of the polynucleotide molecules and polypeptide molecules in the original sample.

These and other features, aspects and advantages of the present invention will become better understood with reference to the following description, examples and appended claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1. Electrophoretic discrimination of a protein and nucleic acid, both labeled with Alexa Fluor® 647 with linear polyacrylamide buffer. A) Labeled protein alone. B) Labeled nucleic acid alone. C) Two peaks resolved in a mixture of labeled protein and labeled nucleic acid.

Figure 2. Electrophoretic discrimination of a protein and nucleic acid, both labeled with Alexa Fluor® 647. A) Labeled protein alone. B) Labeled nucleic acid alone. C) Two peaks resolved in a mixture of labeled protein and labeled nucleic acid.

Figure 3. Discrimination of a protein and a nucleic acid based on fluorescence intensity. A) Fluorescence intensity is plotted as a function of the time offset for detection of fluorescence at the two detectors. Each spot represents measurements taken on a single molecule. A value of 500 photons/msec was used to divide the "bright molecule" window

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from the "dim molecule" window. PBXL-3 molecules (left) emit at a higher average intensity than the pUC19 molecules (right). B) The concentration of PBXL-3 and pUC19 components in mixtures measured via counting fluorescent molecules is compared to the predicted values.

Figure 4. Electrophoretic velocity of protein complex (PBXL-3) shifts when bound to nucleic acid. A) PBXL-3 alone migrates as a peak at 368 ms. B) PBXL-3 bound to nucleic acid migrates as a peak at 294 ms. C) PBXL-3 in the presence of, but not bound to nucleic acid migrates at 409 ms.

Figure 5. Discrimination of labels released from protein target and nucleic acid targets. A) Detection of label released from a protein target. A linear relationship was observed between the net molecules of Alexa Fluor®647 measured and the original TSH concentration. B) & C) Examples of discrimination of released labels based on their electrophoretic mobility or fluorescence intensity.

Figure 6. Examples of FRET affects on protein (P) or nucleic acid (NA) labels. A) Oligonucleotide molecule can be labeled with a FRET donor (D) and a second oligonucleotide can be marked with a FRET acceptor (A). Protein molecules can be labeled with an antibody marked with the FRET donor only. B) Nucleic acid and protein molecules are labeled with the same FRET donor. Protein molecules can also be labeled with an antibody marked with a FRET acceptor. In both cases, protein molecules are distinguished from nucleic acid molecules by virtue different wavelengths of emitted radiation.

Figure 7. Discrimination of a protein and nucleic acid based on molecular mobility in a two dimensional separation system. An electrical field applied perpendicular to the axis of the capillary can cause molecules to move sideways within the capillary. The perpendicular electrical field can be combined with flow through the capillary created by pumping or gravitational force (A) or electrophoresis (B).

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Definitions

To facilitate understanding of the invention, a number of terms and abbreviations as used herein are defined below as follows:

Binding partner(s): As used herein the term "binding partners" refers to macromolecules that combine through molecular recognition to form a complex. Molecular recognition involves topological compatibility or the matching together of interacting surfaces on each partner. The partners can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other. Binding forces can be hydrophobic, hydrophilic, ionic, hydrogen, van der Waals, and/or covalent. Examples of binding partners include: epitope/antibody, oligonucleotide/nucleic acid, and ligand/receptor.

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Charge tags: As used herein, the term "charge tag" refers to any entity bearing a charge that when bound to or associated with the target distinguishes the charge tag+target from the target alone based on detection of the mass, charge, or charge to mass ratio. A charge tag can be a label.

Charge/mass tags: As used herein, the term "charge/mass tag" refers to any charge and mass added to the target that serves to distinguishes the charge/mass tag+target from the probe alone based on detection of the mass, charge, or charge to mass ratio. A charge/mass tag can be a label.

Chemiluminescence: As used herein, the term "chemiluminescence" refers to luminescence produced by the direct transformation of chemical energy into light energy. Also called chemoluminescence.

Co-detection: As used herein, the term "co-detection" refers to the detection of polynucleotide molecules and polypeptide molecules within a single sample. Preferably, the co-detection is provided by detecting a polynucleotide and polypeptide simultaneously, but may also include sequential detection so long as any single molecule is detected at any given time. Co-detected molecules may also be discriminated unless detection of the second molecular species is above a known threshold of the first molecular species.

Cross-correlation: As used herein, the term 'cross-correlation' involves subjecting two raw data sets g_j and h_k to analysis, whereby data sets from each detector (preferably photon detectors) are subjected to the following formula:

$$Corr(g,h)_j \equiv \sum_{k=0}^{N-1} g_{j+k} h_k ,$$

for j= -(N-1), -(N-2), ...,-1,0,1, ..., N-1

where N is the total number of data points. The data cross-correlations will be large at values of j where the first data set from a detector [preferably photon counts above a background level] (g) resembles the data set (h) from a second detector [preferably above a background level] at some lag time (j) that corresponds to the time for specific molecules to pass from the first detector to the second detector (preferably in a single molecule analytical system).

Dye: As used herein, the term "dye" refers to a substance used to color materials or to enable generation of luminescent or fluorescent light. A dye may absorb light or emit light at specific wavelengths. A dye may be intercalating, noncovalently bound or covalently bound to a target. Dyes themselves may constitute labels that detect minor groove structures, cruciforms, loops or other conformational elements of molecules. Dyes may include BODIPY and ALEXA dyes, Cy[n] dyes, SYBR dyes, ethidium bromide and related dyes, acridine orange, ethidium bromide, SYBR, dimeric cyanine dyes such as TOTO,

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YOYO, BOBO, TOPRO POPRO, and POPO and their derivatives, bis-benzimide, OliGreen, PicoGreen and related dyes, cyanine dyes, fluorescein, LDS 751, DAPI, AMCA, Cascade Blue, CL-NERF, Dansyl, Dialkylaminocoumarin, 4',5'-Dichloro-2',7'-dimethoxyfluorescein, 2',7'-Dichlorofluorescein, DM-NERF, Eosin, Erythrosin, Fluoroscein, Hydroxycourmarin, Isosulfan blue, Lissamine rhodamine B, Malachite green, Methoxycoumarin, Naphthofluorescein, NBD, Oregon Green, PyMPO, Pyrene, Rhodamine, Rhodol Green, 2',4',5',7'-Tetrabromosulfonefluorescein, Tetramethylrhodamine, Texas Red, X-rhodamine. Additional fluorophore families include Dyomics series, Atto tec series, coumarins, macromolecular, phycobilliproteins (including phycoerythrins, phycocyanins, and allophycocyanins), green, yellow, red, and other fluorescent proteins, and Quantum Dots. Those skilled in the art will recognize other dyes which may be used within the scope of the invention. This list includes but is not limited to all dyes now known or known in the future which could be used to allow detection of the labeled poly peptide or polynucleotide of the invention.

Electrophoretic Velocity: As used herein, the term "electrophoretic velocity" refers to the velocity of a charged or uncharged target under the influence of an electric field relative to the background electrolyte. Electrophoretic velocity in a capillary system may be a composite measure of electrokinetic velocity and electroosmotic force.

Emission: As used herein, the term "emission" refers to radiation generated by a molecule or particle in processes such as fluorescence, chemiluminescence and elastic or inelastic (*e.g.*, Raman) light scattering.

Emission wavelength: As used herein, the term "emission wavelength" refers to the spectrum of the photons that are released during emission and measured by the detectors used in the analysis instrument. For polyatomic molecules in solution, photon emissions occur over a rather broad spectrum, typically of 100-150 nm. A selected subset of the spectrum is allowed to pass to the detectors by the optical filters used in the instruments. Labels that are detected in the same spectral range are considered to have the same emission wavelength.

Fluid: As used herein the term "fluid" is a medium wherein molecules are suspended and move. It can be aqueous, non-aqueous, or a combination of both that can conduct an electrical current. It may further contain salts, ions, polymers, macromolecules, or other agents that can interact with the polypeptides or polynucleotides and influence their movement.

Fluorescence: As used herein, the term "fluorescence" refers to the photons of energy that are emitted as an excited fluorophore returns to its ground state. The energy of the emitted photon is lower, and therefore of longer wavelength, than the excitation photon.

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Fluorescence Burst Duration: As used herein, the term "fluorescence burst duration" refers to the period of time during which an emission event is detected. Fluorescence Intensity: As used herein, the term "fluorescence intensity" refers to the output of a detection system that measures the radiation from a fluorescing sample. It also refers to the number of photons detected per unit time (preferably milliseconds and preferably above a background threshold).

Fluorescence Lifetime: As used herein, the term "fluorescence lifetime" refers to the time required by a population of N excited fluorophores to decrease exponentially to N/e by losing excitation energy through fluorescence and other deactivation pathways.

Fluorescence Polarization: As used herein, the term "fluorescence polarization" refers to the property of fluorescent molecules in solution that are excited with plane-polarized light and emit light back into a fixed plane (*i.e.*, the light remains polarized) if the molecules remain stationary during the excitation and emission cycle of the fluorophore.

Interrogation volume: As used herein the term "interrogation volume" is the space illuminated by the illumination source and "seen," sensed or otherwise detected by the detectors, through which at least one polypeptide or polynucleotide may traverse.

Label: As used herein, the term "label" refers to a moiety that, when attached to the polypeptide or polynucleotide of the invention, alters detectable parameters of the polypeptide or polynucleotide such as its electromagnetic emission or its electrophoretic velocity. Exemplary labels include but are not limited to fluorophores, chromophores, radioisotopes, spin labels, enzyme labels, chemiluminescent labels, mass tags, charge tags, and charge/mass tags. Such labels allow detection of labeled compounds by a suitable detector. In addition, such labels include components of multi-component labeling schemes, *e.g.*, a system in which a target binds specifically and with high affinity to a detectable binding partner, *e.g.*, a labeled antibody binds to its corresponding antigen. Herein, label and "tag" are used synonymously.

Light Absorption: As used herein, the term "light absorption" refers to the light energy (wavelengths) not reflected by an object or substance.

Light Scattering: As used herein, the term "light scattering" refers to processes by which photons change directions. It includes both elastic light scattering where photons change direction without changing their wavelength and inelastic scattering where the scattered radiation has a different (normally lower) energy from the incident radiation.

Mass tags: As used herein, the term "mass tag" refers to any mass added to the target that serves to distinguish the mass tag+target from the target alone based on detection of the mass, or charge to mass ratio. A mass tag can be a label.

Nucleic acid hybrid: As used herein, the term "nucleic acid hybrid" refers to double-stranded nucleic acid molecule which is made by hybridizing two complementary single-

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stranded nucleic acid molecules. That is, forming hydrogen bonds between complementary base pairs of the two single-stranded nucleic acid molecules.

PE: As used herein, the term "PE" refers to the dye phycoerythrin.

Sample: As used herein, the term "sample" shall mean a contiguous volume containing at least one detectable polynucleotide and at least one detectable polypeptide. This term shall include, but shall not be limited to, detecting the polynucleotide and polypeptide in one sample run. Also within this definition, the polynucleotide is preferably detected within one hour of detecting the polypeptide from a contiguous volume, more preferably within 15 minutes, still more preferably within 1 minute, and still more preferably 10 microseconds. The term "sample" as described above also refers to the volume that contains only the detectable labels in the case when they are released from the original target molecules, and are analyzed in the released state.

SMD: As used herein, the term "SMD" refers to single molecule detection.

Target: As used herein, the term "target" refers to the entity to be detected in an assay, either a polypeptide or polynucleotide. This term is also known in the art as an analyte.

Co-Detection of Single Polypeptide and Polynucleotide Molecules

Co-detection of polypeptides and polynucleotides at very low concentrations or at the individual molecule level provides a new powerful tool for medical and biothreat applications. Most infectious organisms contain both of these biomolecules. Detecting the presence of both allows one to not only confirm the presence of the pathogen, but also to assess the course of an infection. For example, a viral pathogen may have a specific gene sequence that identifies the strain of the pathogen and may also code for specific protein toxins or virulence factors that vary with the strain. Detecting both components gives added assurance of the identification and may provide an evaluation of the stage of the disease and prognosis for disease progression. In another example, a pathogen may elicit an immune response resulting in production of antibodies directed against that pathogen. Co-detection of a pathogen's genetic component and the antibody suggests an active infection is in progress, while detecting only the pathogen or only the antibody suggests, respectively, that the infection is recent or has cleared. Similarly, biothreat applications allow for double confirmation of the identity of an organism at very low concentrations. Co-detection of polypeptides and polynucleotides also allows one to assess the risk involved by knowing specifically which organism is present.

Numerous applications for this co-detection technology also exist in biomedical research. The presence of a certain mRNA in a cell does not guarantee that the protein product of the gene is actually made, although this assumption is often made. Many factors

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including differences in translation efficiency, turnover rates, extracellular expression or compartmentalization, and post-translational modification affect protein levels independent of transcriptional controls. Co-detection would allow one to determine whether mRNA is translated on a cell-by-cell basis and possibly how much is translated. Another application is for comparison of normal and diseased cells. Mutations in genes often result in altered protein translation or degradation. With co-detection one can discover that relationship and, after comparing it to the normal relationship, learn its contribution to the disease state. Another application is for studying the direct interaction of proteins with nucleic acids. Proteins that bind DNA or RNA often have an effect on transcription or translation. Such proteins are often called cofactors, regulatory, or effector molecules. Co-detection technology allows one to identify both the protein and nucleic acid involved in the binding. In addition, proximity assays can be used with co-detection to further characterize the binding.

The advantages of the current technology over previous methods are numerous: Individual molecules can be detected directly without amplification or bead attachment. Multiple parameters of the molecules can be detected simultaneously. The amount of sample required is small. This is important, for example, for forensic biosensor or biomarker applications. The technology also is rapid and requires minimal sample handling. These considerations play a role in situations where changes in molecules are known or suspected to occur during sample preparation. In biological systems, many changes in molecules can occur if hours are needed for sample processing and analysis. Analysis times measured in minutes offer a significant advantage.

The invention described herein overcomes limitations of the previously described technologies. It enables detection of individual polypeptide and polynucleotide molecules without requiring amplification or attachment to a particle. Quantification of molecules only requires counting, which is simple and precise. Multiple parameters can be used to accomplish discrimination between polypeptides and polynucleotides including the electromagnetic characteristics of the labels such as intensity or lifetime of the emission. In addition, the sample can be flowing, or subjected to electrophoretic separation which enables discrimination based on the mobility of the polypeptide and polynucleotide. The method is fast (seconds to minutes) and able to measure large numbers of events. Low protein and nucleic acid concentrations (femtomolar or less) can be detected. Simple and inexpensive instrumentation is used that is composed of a single illumination source, simple detectors and electronics. Data analysis can also be automated using the present invention.

Low protein and nucleic acid concentrations (femtomolar or less) can be detected and molecules can readily be quantitated. The preferred instrumentation is simple and inexpensive with a single illumination source, simple detectors and electronics and the capacity to automate data analysis.

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In a preferred embodiment, samples are analyzed in an instrument that consists of a laser (the beam of which may be split into two or more beams), focusing light-collection optics, two single photon detectors, and detection electronics under computer control. Examples of such instruments may be found in U.S. Patent Nos. 4,793,705, and 5,209,834, each of which is incorporated herein by reference in its entirety. Additional features of the instruments may also be found in U.S. Patent Application Nos. 10/720047, 10/718194 and 10/720044, each of which is incorporated herein by reference in its entirety. Two lasers or detectors are not required for co-detection.

This invention provides a method for co-detecting a polynucleotide molecule and a polypeptide molecule contained within one sample comprising (a) moving said polynucleotide molecule and said polypeptide molecule through at least one interrogation volume; and (b) measuring an electromagnetic characteristic of said polynucleotide molecule and measuring an electromagnetic characteristic of said polypeptide molecule, wherein said polynucleotide molecule and polypeptide molecule are co-detected.

In one embodiment, two distinct electromagnetic properties are measured at each interrogation volume. In some cases this can be accomplished though additional analysis of the signal from a single detector as in the case of emission intensity and burst duration. In another example, two detectors can be used at each interrogation volume, such that distinct properties are measured, such as measuring the total photon signal with one set of detectors at each volume and measuring the fluorescence polarization with a second set of detectors.

Co-detection refers to the detection of two or more species within a single sample. In one embodiment, the protein and nucleic acid are detected in a system where the sample fluids are driven by mechanical means to flow past a detector. Examples of mechanical means are pressure (and vacuum) that can be applied to the sample by any controllable fluid delivery system, such as gravity feed, or pump. In another embodiment, the sample is subjected to electrophoresis, such as by placing the sample in an electrophoretic sample channel.

In one embodiment, the interrogation volume can be defined by a dimension of electromagnetic radiation received from the electromagnetic radiation source and/or a range of the electromagnetic radiation detector. In addition, a dimension of the interrogation volume can be adjustable, variable or both adjustable and variable. Alternatively, the interrogation volume can be defined by a wall of a solid material. Alternatively, the interrogation volumes can be defined by a fluid boundary.

In one embodiment, the electromagnetic characteristic is selected from the group consisting of emission wavelength, emission intensity, burst size, burst duration, fluorescence polarity, light scattering, fluorescence lifetime and any combination thereof. In one embodiment, the polynucleotide and polypeptide molecules are both contained within a

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single sample and the co-detection of the polypeptide and polynucleotide molecules is simultaneous, that is, the electromagnetic characteristics of the two molecules are measured at the precisely same time within the same interrogation volume. In a preferred embodiment, the polypeptide and polynucleotide molecules are both contained within a single sample, and electromagnetic characteristics of polynucleotide and polypeptide molecules are detected sequentially as they traverse the interrogation volume as part of a single sample analysis.

In one aspect of the invention, if the number of molecules of one of the polynucleotide or polypeptide in the sample is known prior to analysis, and a greater number of molecules is detected, the molecules are co-detected, but not discriminated. In a preferred embodiment, the molecules are co-detected and discriminated in the same sample. Discrimination is accomplished by detecting differences in the electromagnetic characteristics of the target molecules. In yet a further aspect, differences in the electrophoretic velocity of the polynucleotide molecules and polypeptide molecules are detected. In one embodiment of the invention, sieving media can be employed to affect the separation of molecules. Thus it will be obvious to one skilled in the art the art that multiple polynucleotides and polypeptides can be distinguished in a mixture by employing a combination of the electromagnetic characteristics of the molecules and the mobility of the molecules. The methods described herein enable at least one polynucleotide and at least one polypeptide molecule to be distinguished individually in a sample comprising multiple molecules. The terms discriminated, detected and distinguished are used interchangeably herein.

In another embodiment, a plurality of polynucleotide molecules and a plurality of polypeptide molecules are co-detected. This plurality includes multiple molecules of the same polynucleotide and multiple molecules of the same polypeptide, single molecules of many different polynucleotides and single molecules of many different polypeptides, multiple molecules of the same polynucleotide and single molecules of many polypeptides, or single molecules of many polynucleotide and multiple molecules of the same polypeptide. The plurality of molecules has a theoretical and function range of limitations. The theoretical and functional lower limit is one molecule of a polynucleotide and one molecule of a polypeptide. The theoretical upper limit depends on the physical limitations of the detectors and the computer software. For example, if 200 molecules can be detected in 10 us (one of the preferred arbitrary time segments with freely selectable channel widths, see below), then 2 million molecules (2 x 106) are detected per second. In the future, with improved detectors and software, this limit could increase. One skilled in the art will recognize that the upper limit will also depend on how long one analyzes the sample, how much sample is available, and the mode of analysis used, and that these factors will define a functional upper limit.

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Functional upper limits preferably fall in the range of thousands (103) of molecules, and more preferably fall in the range of hundreds of molecules.

The target molecules of the invention are polypeptides and polynucleotides. In one embodiment of the invention, the polypeptides are equivalent to proteins. The preferred target proteins are functional components of cells such as structural proteins that are components of the cellular cytoskeleton, enzymes, receptors or signaling factors. Polypeptides also include components of proteins such as peptides, epitopes, or binding domains and complexes of proteins such as dimers, trimers, or heteromeric assemblies of discrete protein subunits. Target molecules also include oligopepetides such as hormones or antibiotics.

In one embodiment, polynucleotides are equivalent to nucleic acids. The preferred target nucleic acids are functional components of cells such as DNA contained within chromosomes and messenger, ribosomal or transfer RNA. Polynucleotides further include fragments of DNA or RNA, single stranded or double stranded molecules, or complexes of nucleic acids such as dendrimers or nucleic acid hybrids. In a further embodiment, polynucleotides include oligonucleotides containing naturally occurring and/or modified nucleotides. Further, a polynucleotide can be a polymer comprised of linked nucleotides, and includes DNA or RNA. DNA is a polymer comprised of a phosphodiester backbone composed of monomers of purines and pyrimidines such as adenine, cytosine, guanine, thymine, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties. RNA is a polymer comprised of a phosphodiester backbone composed of monomers of purines and pyrimidines such as those described for DNA except that uracil is substituted for thymidine. DNA monomers may be linked to each other by their 5' or 3' hydroxyl group thereby forming an ester linkage. RNA monomers may be linked to each other by their 5', 3' or 2' hydroxyl group thereby forming an ester linkage. Alternatively, DNA or RNA monomers having a terminal 5', 3' or 2' amino group may be linked to each other by the amino group thereby forming an amide linkage. In some instances, the polymer is a peptide nucleic acid (PNA), or a locked nucleic acid (LNA).In one embodiment, sensitive co-detection of proteins and nucleic acids utilizes molecules which intrinsically produce, or are extrinsically labeled, to emit electromagnetic radiation. Preferred mechanisms of electromagnetic radiation emission include fluorescence, chemiluminescence, and light scattering. In a further embodiment, the molecule may be detected through a combination of an intrinsic property and an extrinsic property. The preferred electromagnetic radiation is fluorescence. In a further embodiment, polypeptides and polynucleotides can be distinguished based on non-electromagnetic characteristics such

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as mobility. These measurable characteristics may be produced by an intrinsic or extrinsic property of the molecule.

Many naturally occurring units of a polymer are light emitting compounds or quenchers. For instance, nucleotides of native nucleic acid molecules have distinct absorption spectra, *e.g.*, A, G, T, C, and U have absorption maximums at 259 nm, 252 nm, 267 nm, 271 nm, and 258 nm respectively. Modified units which include intrinsic labels may also be incorporated into polymers. A nucleic acid molecule may include, for example, any of the following modified nucleotide units which have the characteristic energy emission patterns of a light emitting compound or a quenching compound: 2,4-dithiouracil, 2,4-Diselenouracil, hypoxanthine, mercaptopurine, 2-aminopurine, and selenopurine.

Protein containing the aromatic amino acids (tryptophan, tyrosine and phenylalanine) may exhibit intrinsic florescence. In addition, there are specific cases of intrinsically fluorescent proteins or protein complexes such as green fluorescent protein (from the jellyfish *Aequorea victoria*) and phycobiliproteins produced by cyanobacteria and red algae. In one embodiment, the extrinsic property is provided by a label. The methods for labeling the molecule with a means of detection or discrimination are within the ordinary skill in the art. Attaching labels to molecules can employ any known means. In some cases, the method of labeling is non-specific, for example, a method that labels all nucleic acids regardless of their specific nucleotide sequence. In other cases, the labeling is specific, as in where a labeled oligonucleotide binds specifically to a target nucleic acid sequence. Specific and non-specific labeling techniques will be discussed in more detail in the following sections.

Non-specific labeling of nucleic acids generally employs functional groups which label all nucleic acid regardless of the particular nucleotide sequence. One skilled in the art is familiar with various techniques for general labeling of nucleic acids. Methods include: intercalating dyes such as propidium iodide, acridine orange, and Hoechst dyes; labeling the N-7 position of guanine bases (ULYSIS kit); incorporating a chemically reactive nucleotide analog to which a label can be readily attached (Ares kit); incorporation of a biotin containing nucleotide analog for attachment of a strepavidin-bound label. Enzymatic labeling incorporates labeled nucleotide analogs during strand replication. In a preferred embodiment, the non-specific label is directly attached to the target molecule.

Techniques to non-specifically label proteins are also well known to those skilled in the art. For example, the free amine at the N-terminus of the protein, and chemically reactive amino acids, such as lysine, on the surface of proteins can be used to attach a label (Molecular Probes Handbook pp. 7-93). In additions, labels can be added to carbohydrate moieties on glycoproteins (Molecular Probes Handbook pp. 99-112). In some applications, the label can be added to the protein in a two step process where first one member of a high

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affinity binding system, *e.g.*, biotin is added to the protein, and the label is attached to the other member, *e.g.*, avidin. Isotype specific reagents have also been developed for labeling antibodies (Zenon, Molecular Probes). In a preferred embodiment, the non-specific label is directly attached to the target molecule. In another embodiment, target proteins can be directly labeled by expressing them as a fusion protein with a fluorescent protein such as green fluorescent protein (GFP).

In a preferred embodiment, only specific proteins or nucleic acids within a mixture are labeled. In one embodiment, the label is added indirectly to the target molecules though a labeled binding partner which interacts with the target molecule. Specific labeling can be accomplished by combining a protein or nucleic acid with a labeled binding partner, where the binding partner interacts specifically with the protein or nucleic acid molecule through complementary binding surfaces. Binding forces can be covalent interactions or non-covalent interactions such as hydrophobic interactions, ionic, or hydrogen bonds, van der Waals attraction. Examples of proteins and binding partners includes: proteins and antibodies, antigens and specific antibodies, hormone and hormone receptor, ligands and receptors, and enzymes and enzyme substrates or cofactors. Examples of nucleic acid and binding partners include: polynucleotide and complementary polynucleotide, oligonucleotide and complementary polynucleotide or two complementary oligonucleotides where either the oligonucleotide or polynucleotide can be DNA, RNA, or include analogs or modified bases such as LNAs or PNAs.

In preferred embodiment target polynucleotides can be labeled using an oligonucleotide which specifically hybridizes to the nucleic acid as a primer and nucleic acid synthesis techniques using either polymerase chain reaction (PCR) or run-off transcription (*See* U.S. Patent Application No. 10/718194 incorporated herein by reference in its entirety). The labels can consist of either fluorescent nucleotide analogs such as ChromaTide dUTP (molecular probes) which are incorporated into the product or reactive nucleotides with functional groups such as aminoallyl dUTP to which fluorescent tags can readily be added.

In a further embodiment, the protein and nucleic acid species are distinguished based on their molecule interaction with one or more binding partners. These interactions may be protein/protein binding, nucleic acid hybridization or protein/nucleic acid binding. Immunoassays based on specific antibody/protein pairs are one example of binding partners. In one case, the interaction may alter the electromagnetic emission properties of either the target protein or nucleic acid species. Examples of this include binding the target molecule to a labeled binding partner or a large species to alter the fluorescence polarization. In another embodiment, the labeled target molecule can be bound to a molecular species containing a fluorescence quencher or a molecular species containing a FRET partner. In a further embodiment, the interaction may alter the mobility of either the

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protein or nucleic acid species. Examples of this include: mass, charge or mass/charge tags to alter the electophoretic mobility.

One skilled in the art will recognize that labels can be applied before, after, or simultaneously with positioning the molecule into the interrogation fluid. In a preferred embodiment, the molecules are labeled prior to moving through the interrogation volume. In one embodiment, the protein and nucleic acid are labeled in the same reaction mix prior to analysis. In another embodiment, the protein and the nucleic acid are labeled in separate reactions and combined prior to analysis. Once a molecule is detectably labeled, any suitable means of detection that is known in the art can be used.

Labels include dye tags, radioactive tags, charge tags, mass tags, charge/mass tags, light scattering tags, chemiluminescent tags, quantum dots, or beads, polymeric dyes, dyes attached to polymers. Dyes encompass a very large category of compounds that add color to materials or enable generation of luminescent or fluorescent light. A dye may absorb light or emit light at specific wavelengths. A dye may be intercalating, or be noncovalently or covalently bound to a polypeptide or polynucleotide. Dyes themselves may constitute probes as in probes that detect minor groove structures, cruciforms, loops or other conformational elements of molecules. Dyes may include BODIPY and ALEXA dyes, Cy[n] dyes, SYBR dyes, ethidium bromide and related dyes, acridine orange, dimeric cyanine dyes such as TOTO, YOYO, BOBO, TOPRO POPRO, and POPO and their derivatives, bis-benzimide, OliGreen, PicoGreen and related dyes, cyanine dyes, fluorescein, LDS 751, DAPI, AMCA, Cascade Blue, CL-NERF, Dansyl, Dialkylaminocoumarin, 4',5'-Dichloro-2',7'-dimethoxyfluorescein, 2',7'-Dichlorofluorescein, DM-NERF, Eosin, Erythrosin, Fluoroscein, Hydroxycourmarin, Isosulfan blue, Lissamine rhodamine B, Malachite green, Methoxycoumarin, Naphthofluorescein, NBD, Oregon Green, PyMPO, Pyrene, Rhodamine, Rhodol Green, 2',4',5',7'-Tetrabromosulfonefluorescein, Tetramethylrhodamine, Texas Red, X-rhodamine, Dyomic series, Atto tec series, Coumarins, macromolecular, phycobilliproteins (phycoerythrins, phycocyanins, allophycocyanins), green, yellow, red and other fluorescent proteins, metallic crystals and other dyes that interact with or may be attached to molecules. Those skilled in the art will recognize other dyes which may be used within the scope of the invention. This list includes but is not limited to all dyes now known or known in the future which could be used to allow detection of the labeled polypeptide or polynucleotide of the invention. By having fluorescent markers, such as fluorescent molecules, fluorescent conjugated antibodies, or the like, the sample may be irradiated with light absorbed by the fluorescent molecules and the emitted light measured by light measuring devices. Dyes can be employed as the label or produced as a result of a reaction, e.g., an enzymatically catalyzed reaction. Chemiluminescence is the generation of electromagnetic radiation as light by the release of energy as the result of a chemical reaction. While the light can, in

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principle, be emitted in the ultraviolet, visible or infrared region, those processes emitting visible light are the most common. Types of chemiluminescent reactions include the following: Chemical reactions using synthetic compounds and usually involving a highly oxidized species such as a peroxide are commonly termed chemiluminescent reactions. Light-emitting reactions arising from a living organism, such as the firefly or jellyfish, are commonly termed bioluminescent reactions.

Target molecules can be labeled directly or indirectly with enzyme labels such as alkaline phosphatase, G-6-P dehydrogenase, horseradish peroxidase, luciferase or xanthine oxidase. The labeled target molecules are then combined with chemiluminescent molecules such as luminol, isoluminol, and acridinium esters, prior to entering the interrogation volume. For light scattering, the emission is at the same wavelength as the incident light, but has been dispersed by the molecule itself. Useful light scattering labels include metals, such as gold, silver, platinum, selenium and titanium oxide.

In yet another aspect, the labels affect the electrophoretic analysis and/or separation of target polypeptides or polynucleotides. These labels are referred to as charge/mass tags. Attachment of such a label alters the ratio of charge to translational frictional drag of the target polypeptides or polynucleotides in a manner and to a degree sufficient to affect their electrophoretic velocity and/or separation.

In another embodiment, the label alters the charge, or the mass, or a combination of charge and mass. The charge/mass tag bound to a polypeptide or polynucleotide can be discriminated from the unbound polypeptide or polynucleotide or unbound tag by virtue of spatial differences in their behavior in an electric field, and by virtue of velocity differences in their behavior in an electric field.

Direct coupling attaches the binding partners to the charge/mass tags. Indirect coupling can be accomplished by several methods. The binding partners may be coupled to one member of a high affinity binding system, *e.g.*, biotin, and the tags attached to the other member, *e.g.*, avidin. For binding partners which are antibodies, one may also use second stage antibodies that recognize species-specific epitopes of the antibodies, *e.g.*, anti-mouse Ig, anti-rat Ig, and the like. Indirect coupling methods allow the use of a single charge/mass tag, *e.g.*, antibody, avidin, and the like, with a variety of binding partners. Those of skill in the art will recognize several methods for providing labeled secondary antibodies against primary antibodies.

Especially useful binding partners are antibodies specific for the target. Whole antibodies may be used, or fragments, *e.g.*, Fab, $F(ab')_2$, and light or heavy chain fragments. Such antibodies may be polyclonal or monoclonal and are generally commercially available or alternatively, readily produced by techniques known to those skilled in the art. Antibodies selected for use will have a low level of non-specific binding. In a further embodiment,

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binding partners that are oligonucleotides or polynucleotides which specifically bind to the target are useful reagents.

To accurately detect a labeled target molecule, the labeled molecule must be distinguished from unbound label. Many ways to accomplish this are familiar to those skilled in the art. For example, in heterogeneous assays, unbound label is separated from labeled molecules prior to analysis. In a preferred embodiment, the sample, including unbound label, is analyzed by a combination of electrophoresis and individual molecule fluorescence detection. In this case, electrophoretic conditions are chosen which provide distinct velocities for the labeled molecule versus the unbound label.

In one embodiment, the protein and nucleic acid are detected in a system where the sample fluids are driven by mechanical means to flow past a detector. Examples of mechanical means are pressure (and vacuum) that can be applied to the sample by any controllable fluid delivery system, such as gravity feed, or pump. Generally these methods demonstrate parabolic flow and all molecules move at a constant velocity as detected in suitable detection systems.

In another embodiment, the sample is subjected to electrophoresis, such as by placing the sample in an electrophoretic sample channel. Mobility of polypeptides or polynucleotides within the sample fluid varies with the properties of the polypeptide or polynucleotide. The velocity of movement produced by electrokinetic force is determined by the relative charge and mass of the individual polypeptide or polynucleotide and the electroosmotic force. Movement of a polypeptide or polynucleotide can be altered by the type of label that has been attached to the molecule, such as a charge/mass tag. The electrophoretic velocity of the molecules can also be altered by changes in the electroosmotic flow. In an additional embodiment, when two or more polypeptides or polynucleotides are present, at least one may move through at least one interrogation volume in a direction opposite to that of the other molecule.

In one embodiment, the electrophoretic velocity of each detectably labeled molecule is determined. Based on the determination of the electrophoretic velocity of each detectably labeled molecule, individual molecules in a sample comprising multiple molecules can be distinguished. Any electrophoretic separation technique together with immunoassay or nucleic acid hybridization labeling can be, in principle, adapted for use in the context of the present invention. The electrophoretic velocity of the molecule can be modified through additional forces such as pressure or vacuum, surface tension or gravitational flow.

In a preferred embodiment, the sample comprises a buffer. While any suitable buffer can be used, the preferable buffer has low fluorescence background, is inert to the detectably labeled molecule, can maintain the working pH and has suitable ionic strength for electrophoresis. The buffer concentration can be any suitable concentration, such as in the

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range from 1-200 mM. Preferably, the buffer is selected from the group consisting of Tris, Gly-Gly, bicine, tricine, MES, MOPS and AMP. Preferred buffers include: Tris/borate, Tris/glycine and Tris/HCl.

For some applications, the buffer desirably further comprises a sieving matrix for use in this method. While any suitable sieving matrix can be used, desirably the sieving matrix has low fluorescence background and can specifically provide size-dependent retardation of detectably labeled molecules. The sieving matrix can be present in any suitable concentration; from about 0.1% to about 10% is preferred. Any suitable molecular weight can be used; from about 100,000 to about 10 million is preferred. Examples of sieving matrixes include poly(ethylene oxide) (PEO), poly(vinylpyrrolidine) (PVP), linear polyacrylamide and derivatives (LPA), hydroxypropylmethylcellulose (HPMC) and hydroxyethylcellulose (HEC), all of which are soluble in water and have exceptionally low viscosity in dilute concentration (0.3% wt/vol). In addition, these polymer solutions are all below their entanglement threshold and are easy to prepare, filter and fill into capillary tubes.

Electrokinetic force can be combined with other motive forces such as pressure, vacuum, gravity, surface tension, and centrifugal to discriminate between protein and nucleic acid molecules. In one embodiment, these forces can be chosen so as to have differential effects on different molecules within a sample so that when two or more molecules are present, at least one molecule moves through the interrogation volume with a velocity that differs from the other molecule(s). The velocities of the target molecules can be aligned with the fluid flow or at least one molecule can move antiparallel to the fluid flow. In another embodiment, at least one molecule has an antiparallel velocity exceeding the velocity of the fluid flow. In another embodiment, at least one molecule is in motion perpendicular to the fluid flow. In another embodiment, at least one molecule is in motion with a combination of motions that are antiparallel and perpendicular to the fluid flow.

Several approaches can be used to distinguish between labeled (or intrinsically fluorescent) proteins and nucleic acids. In one embodiment, the protein and nucleic acid are distinguished based on electromagnetic properties of their respectively labels. In another embodiment, physical properties such as velocity in an electric field are used to distinguish between the protein and nucleic acid.

In one embodiment, electromagnetic emission refers to the release of photons from a molecule in response to a stimulus. In the case of fluorescent emission, the stimulus is absorbed light. In the case of chemiluminescence and bioluminescence, the stimulus is a chemical reaction or biochemical reaction.

For elastic light scattering, the emission is at the same wavelength as the incident light, but has been dispersed by the molecule itself. In other cases, there is a scattered light is of a different wavelength than the incident light. For example, when nano-sized metal

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colloid particle are illuminated with a standard while light source the scattering produces intense monochromatic light.

Light, particularly light in the ultra-violet, visible or infrared range, is the preferred electromagnetic radiation to detect. The detectors of the instrument are capable of capturing the amplitude and duration of photon bursts from fluorescent particles and converting them to electronic signals. Detection devices such as CCD cameras, video input module cameras, and Streak cameras can be used to produce images with contiguous signals. In another embodiment, devices such as a bolometer, a photodiode, a photodiode array, avalanche photodiodes, and photomultipliers which produce sequential signals can be used. In a preferred embodiment, avalanche photodiodes are used for the very sensitive detection of photons. Using specific optics between the interrogation volume and the detector, several distinct characteristics of the emitted electromagnetic radiation can be detected including: emission wavelength, emission intensity, burst size, burst duration, fluorescence lifetime, and fluorescence polarization. It will be obvious to one skilled in the art that one or more detectors can be configured at each interrogation volume and that the individual detectors may be configured to detect any of the characteristics of the emitted electromagnetic radiation listed above.

The preferred illumination sources are continuous wave lasers for wavelengths of >200-1000 nm. These illumination sources have the advantage of being small, durable and relatively inexpensive. In addition, they generally have the capacity to generate larger fluorescent signal than other light sources. Specific examples of suitable lasers include: lasers of the argon, krypton, helium-neon, helium-cadmium types as well as tunable diode lasers (red to infrared regions), each with the possibility of frequency doubling. The lasers provide continuous illumination with no accessory electronic or mechanical devices such as shutters, to interrupt their illumination. Light emitting diodes (LEDs) are another low-cost, high reliability illumination source. Recent advances in ultra-bright LEDs coupled with dyes with high absorption cross-section and quantum yield, support their applicability to single molecule detection. Such lasers could be used alone or in combination with other light sources such as mercury arc lamps, elemental arc lamps, halogen lamps, arc discharges, plasma discharges, light-emitting diodes, or combination of these. The optimal laser intensity depends on the photo bleaching characteristics of the individual dyes and the length of time required to traverse the interrogation volume (including the speed of the particle, the distance between interrogation volumes and the size of the interrogation volumes). To obtain a maximal signal, it is desirable to illuminate the sample at the highest laser intensity which will not result in photo bleaching a high percentage of the dyes. The preferred laser intensity is one such that no more that 5% of the dyes are bleached by the time the particle has traversed the final interrogation volume.

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Alternatively, pulsed lasers can be used as illumination sources. Pulsed lasers together with time-gated detectors can be used for determining the fluorescence lifetime of labeled polypeptides and polynucleotides as one option for discrimination and co-detection. In the case of fluorescent emissions, the photon signal detected depends both on the wavelength spectra of the fluorescent emission and the filters used with the detectors in the instrument. Therefore, labels with different but overlapping emission spectra may appear indistinguishable if the filter range encompasses both spectra.

In addition, several distinct characteristics of electromagnetic radiation can be measured including properties such as emission intensity, burst size, burst duration, fluorescence lifetime or fluorescence polarization. Fluorescence intensity is quantitatively dependent on the fluorescence quantum yield of the dye, the excitation source intensity, polarity and wavelength, and the detection efficiency of the instrument. It is also affected by the components of the solution, including solvents, ions (such as those that determine pH) and the concentration of the dye, Dye intensity can change if the molecule is exposed to a light source that causes photobleaching.

In one embodiment, both the protein and nucleic acid are labeled with labels having indistinguishable emission wavelengths. It is clear to one skilled in the art that fluorescent emission spectra from typical dyes range over approximately 100 – 150 nm. Therefore a given dye can be detected at a variety of different wavelengths In this case, the protein and nucleic acid can be distinguished by detection of other electromagnetic properties such as emission intensity, burst size, burst duration, fluorescence lifetime or fluorescence polarization. In the case of fluorescent emissions, the photon signal detected depends both on the wavelength spectra of the fluorescent emission and the optics used with the detectors in the instrument. Alternatively, the protein and nucleic acid can be distinguished based on non-electromagnetic properties such as mobility including electrophoretic velocity.

In one embodiment, the electromagnetic labels on the protein and nucleic acid molecules cannot be distinguished on any of the electromagnetic characteristics listed above. In these cases, other methods can be used to co-detect the protein and the nucleic acid. For example, an additional binding interaction can introduce a second label to alter the electromagnetic properties of the target. Examples of such labels include fluorescence quencher and FRET pairs. In another embodiment, a protein and nucleic acid can be co-detected by differences in fluorescent intensity resulting from different numbers of labels bound to the two species (*See* U.S. Patent Application No. 10/720044 incorporated herein by reference in its entirety). In other cases, the protein and nucleic acid can be distinguished based on their mobility. This can be the result of intrinsic mobility differences of the molecules in an electric field or due to extrinsic charge/mass tags.

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In one embodiment, the polynucleotide molecule can be labeled at a high density, with a range of 50 labels/molecule, and the polypeptide molecule can be labeled with a range of 5 labels/molecule. In this case, the polynucleotide can be distinguished from the polypeptide based on the fluorescence intensity. In one embodiment, the protein and nucleic are distinguished based on different electrophoretic velocities. As discussed above, the mobility of molecules within the sample fluid varies with the properties of the molecule. Specifically, the velocity of movement produced by electrokinetic force is determined by the relative charge and mass of the individual molecule. In some cases, electrophoretic conditions can be readily identified where the protein and nucleic acid molecules have different velocities. In a further embodiment, a mass/charge tag may be added to either the protein or nucleic acid to alter its electrophoretic velocity and allow the two species to be distinguished. It is clear to one skilled in the art that multiplexing to characterize multiple species of protein and nucleic acid in a single sample can be accomplished by combining both differences in electrophoretic velocity and differences in electromagnetic emission characteristics to generate a unique pattern for each species in the sample.

The methods described herein allow individual polypeptides or polynucleotides to be enumerated as they pass through the interrogation volumes and thereby elucidating the concentrations of the target protein and nucleic acid molecules in the sample. Therefore, the concentration of a test sample can be determined without a reference to a standard curve by counting the detected molecules passing through the interrogation volume. The concentration of the sample can be determined from the number of molecules counted and the volume of sample passing though the interrogation volume in a set length of time. In the case where the interrogation volume encompasses the entire cross-section of the sample stream; only the number of molecules counted and the volume passing through a cross-section of the sample stream in a set length of time are needed to calculate the concentration the sample. In another embodiment, the concentration of a polypeptide or polynucleotide in a sample can be determined by interpolating from a standard curve generated with a control sample of known concentration. In a further embodiment, the concentration of a polypeptide or polynucleotide in a sample can be determined by comparing the measured polynucleotide and polypeptide molecules to an internal molecular standard.

In one aspect of the invention, the velocity of the molecules is determined by the time needed to pass through or between two discrete interrogation volumes and the polypeptide and polynucleotide molecules can be distinguished by differences in velocity. Specifically, the sample comprises polynucleotide molecules and polypeptide molecules, and co-detection comprises: measuring an electromagnetic characteristic of a polynucleotide molecule as the polynucleotide molecule interacts with an excitation source within a first

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interrogation volume; and measuring the electromagnetic characteristic of the polynucleotide molecule as the polynucleotide molecule interacts with an excitation source within a second interrogation volume; then comparing the measured first and measured second electromagnetic characteristics of the polynucleotide; in addition measuring an electromagnetic characteristic of a polypeptide molecule as the polypeptide molecule interacts with an excitation source within a first interrogation volume; measuring the electromagnetic characteristic of the polypeptide molecule as the polypeptide molecule interacts with an excitation source within a second interrogation volume; and comparing the measured first and measured second electromagnetic characteristics of the polypeptide.

By comparing the electromagnetic characteristics measured at the two detectors, the velocities of the labeled components of the sample are determined and the polypeptide molecule and polynucleotide molecule co-detected. In one embodiment, the analysis of data from protein and nucleic acid detection includes cross-correlation. In another embodiment, photon signals are cross-correlated directly. In this case the fluorescent signals (photons) emitted by the sample which come from at least two interrogation volumes are detected by at least two detectors. The signals respectively detected in the detectors are cut into uniform arbitrary, time segments with freely selectable time channel widths. Preferred channel widths (bins) are in the range of 10 us to 5 ms. The number of signals contained in each segment is established. For each time segment from the first detection unit, a cross-correlation analysis with at each segment of the second detection unit is performed. At least one statistical analysis of the results of the coincidence analysis is performed, and/or the results are subjected to a threshold analysis. Said statistical analysis or at least one combination of several statistical analyses is evaluated for the presence of molecules. In this way, a molecule is discriminated from stochastic and background noise based on the presence of correlated signal(s) in at least two detector channels.

In a preferred embodiment, the detected signal is first analyzed to determine the noise level and signals are selected above a threshold prior to cross correlating the data. In one embodiment, the noise level is determined by averaging the signal over a large number of bins. In other embodiments, the background level is determined from the mean noise level, or the root-mean-square noise. In other cases, a typical noise value is chosen or a statistical value. In most cases, the noise is expected to follow a Poisson distribution.

A threshold value is determined to discriminate true signals (peaks, bumps, molecules) from noise. Care must be taken for choosing a threshold value such that the number of false positive signals from random noise is minimized while the number of true signals which are rejected is minimized. Methods for choosing a threshold value include: determining a fixed value above the noise level and calculating a threshold value based on the distribution of the noise signal. In a preferred embodiment, the threshold is set at a fixed

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number of standard deviations above the background level. Assuming a Poisson distribution of the noise, using this method one can estimate the number of false positive signals over the time course of the experiment. Then cross-correlation analysis is performed on the signals identified from the two detectors.

The time-offset of the cross-correlated signals provides the transit time between the corresponding detectors and therefore based on the distance between the detectors, the electrophoretic velocity of the polypeptide or polynucleotide is determined.

In some cases, a polypeptide or polynucleotide is detected by the fact that the time off-set corresponds to a known time offset. In other cases, a polypeptide or polynucleotide is detected via unknown offset which is determined via population distribution. Therefore, polypeptide or polynucleotides in a mixture labeled with labels with identical electromagnetic properties can be distinguished based on their electrophoretic velocity.

In another embodiment, labels with different electromagnetic characteristics may be used to discriminate between a protein and a nucleic acid. Further, it will be recognized by those of skill in the art that employing labels with different electromagnetic characteristics together with measuring electrophoretic mobility will allow for the increased detection and discrimination of polypeptides or polynucleotides.

In a further embodiment, the cross-correlation analysis can be performed on data from more than two detectors, such as 3, 4, 5, and 6 detectors, or any other desired number that are distinct either in relative location of the interrogation volume or in the wavelength detected. In this case, the cross-correlation analysis can performed on data from any combination of detectors that are distinct. For example, in a case where three detectors, each detecting a distinct wavelength emission (R, G & B) are at each of two interrogation volumes (1 & 2), R1 is correlated with R2, G1 is correlated with G2 and B1 is correlated with B2; resulting in time offsets for molecules with wavelength emission detected by the individual detectors. Other combinations of cross-correlation analysis can also be performed, such as overlapping sets where R1 is correlated with G1; R1 is correlated with B1 and G1 is correlated with B1. Results of these cross-correlation analyses would indicate the frequency of double-labeled polypeptides or polynucleotides. Different combinations of cross-correlation analyses can be used with one another to distinguish molecules based on velocity and labeling (color). In addition, using multiple pairs of cross-correlation analysis will result in more accurate determination of the properties of the individual polypeptide or polynucleotides with in the mixture. In a further embodiment, analysis methods are employed wherein cross-correlation analysis is performed on data from detectors in any combinations of locations and/or wavelengths that are distinct.

Methods for attaching labels to polypeptide molecules and polynucleotide molecules described in detail above in the broad embodiment can also be used in this case. In one

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embodiment the labels are released from the polynucleotide and polypeptide molecules prior to the analysis. Methods of release are well known to those skilled in the art. For example, labels attached by protein/protein interactions can typically be disrupted using agents such as low pH solutions, such as 100 mM glycine-HCl pH 2.8), by addition of chaotropic agents such as urea or detergents. Labels attached by nucleic acid hybridization can be released using low ionic strength solutions and/or agents such as increased temperature. Those skilled in the art would recognize these and other agents to be effective in removing labels attached to polynucleotide or polypeptide molecules. In addition, labels can be removed by enzymatic cleavage of the molecule/target complex.

Also well know in the art are methods for removing, or rendering non-detectable, excess label not bound to target molecules. These methods include physical separation such as washing to remove unbound label not attached to a surface, or filtering to remove the smaller unbound label from a large target/label complex. In addition, there are methods of rendering excess label non-detectable such as binding a fluorescent label to a complementary molecule which contains a fluorescence quencher. Those of skill in the art will recognize other methods of rendering excess labels non-detectable.

In a further embodiment, the number of labels attached to the polynucleotide and polypeptide molecules and subsequently released and detected is proportional to the number of polynucleotide and polypeptide molecules in the original sample. The relationship between the labels counted and the target molecules can be a linear or non-linear correlation. This relationship can be predefined or determined as a result of analyzing the sample.

EXAMPLES

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following specific examples are offered by way of illustration and not by way of limiting the remaining disclosure.

A fragment of DNA, a protein, and a protein complex bound to a nucleic acid were subjected to electrophoresis. Data was analyzed in each of the following examples by analyzing grouped adjacent 1 msec detection blocks derived from molecules using the instrument software. Molecule-derived photon bursts were then cross-correlated to determine their electrophoretic velocities (time offset for detection at the two detectors). Examples of the histogram plots of the molecule cross correlations are shown in Figs. 1-7.

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Example 1 - Discrimination of a protein and nucleic acid directed labeled with the same fluorescent dye – electrophoresis with linear polyacrlyamide

Samples of Alexa Fluor® 647-labeled IgG and 1.1 kb PCR product were prepared in 18 mM tris, 18 mM glycine, pH 8.6 with 0.2% linear polyacrylamide (LPA, 5,000,000 – 6,000,000 mw), 0.01% sodium dodecyl sulfate and 1 μg/ml each bovine serum albumin, Ficoll®, and polyvinylpyrrolidone. Samples were pumped into the SMD capillary, the pump was stopped, and an electric field was applied (300 V/cm). Cross-correlation of the molecules was determined as a function of time offset. One minute data sets were collected and analyzed.

Examples of the histogram plots of the molecule cross correlations are shown in Figure 1. A. 26 fM Alexa Fluor 647 labeled IgG (cross-correlation peak at 75 msec). B. 10 fM Alexa Fluor labeled PCR product (peak at 220 msec). C. 13 fM Alexa Fluor 647 labeled IgG and 5 fM Alexa Fluor labeled PCR product (peaks at 75 and 215 msec).

Example 2 - Discrimination of a protein and nucleic acid directed labeled with the same fluorescent dye – electrophoresis

SMD electrophoretic separation of a protein and a nucleic acid. Samples of Alexa Fluor® 647 labeled IgG and 1.1 kb PCR product were prepared in 18 mM tris, 18 mM glycine, pH 8.6 with 0.01% sodium dodecyl sulfate and 1 μg/ml each bovine serum albumin, Ficoll®, and polyvinylpyrrolidone. Samples were pumped into the SMD capillary, the pump was stopped, and an electric field was applied (150 V/cm). Cross-correlation of the molecules between channels 1 and 2 was determined as a function of time offset. One minute data sets were collected and analyzed. a: 52 fM Alexa Fluor 647 labeled IgG (cross-correlation peak at 210 msec). b: 20 fM Alexa Fluor labeled PCR product (peak at 175 msec). c: 26 fM Alexa Fluor 647 labeled IgG and 10 fM Alexa Fluor labeled PCR product (peaks at 170 and 215 msec).

Example 3 - Differences in the characteristic intensity of fluorescence emission were used to distinguish a protein complex and a nucleic acid within a mixture.

This example also demonstrates that the concentration of sample components can be determined by comparing the counts detected to a standard curve. The protein, PBXL-3, emits at a generally high intensity, and the nucleic acid, linearized pUC19, emits at a generally lower intensity. PBXL-3 is an intrinsically fluorescent protein complex. The pUC19 DNA was labeled with Alexa Fluor® 647 following the protocol of a ULYSIS® nucleic acid labeling kit (Molecular Probes, Inc. Eugene, OR). PBLX-3-strepavidin was purchased from Martek Biosciences Corp. (Columbia, MD). Phosphate Buffered Solution (PBS) (10 mM $NaPO_4$, 150 mM NaCl, pH 7.2) was supplemented with 0.01% Casein Acid Hydrolysate and

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used to make dilution series (2.5, 5, 7.5, 10 and 20 fM) of protein alone, nucleic acid alone or mixtures of both. Samples were moved through the analyzer by pumping at 1 μL/min for 4 min. The data is shown in Figure 3.

Brightness windows were separated at the intensity of 500 photons. This separation intensity was determined from the plots of intensity for PBXL-3 alone and pUC19 alone at 20 fM (Figure 3A). Standard curves were plotted for the protein and nucleic acid in both brightness windows and the slopes of the curves were determined. The protein and nucleic acid showed distinct patterns of fluorescence intensities in these plots allowing for discrimination between them. Furthermore, the number of molecules detected in the mixtures of PBXL-3 and pUC19 were used to calculate the concentrations of each component based on the slopes of the standard curves. The measured concentrations for the protein and nucleic acid were compared to the predicted values in Figure 3B. Counting molecules determined concentrations equivalent to concentrations determined by macro spectrophotometric measurement of undiluted stock solutions.

Example 4 - The mobility of a protein complex was shifted after binding to a mobility label consisting of a nucleic acid molecule, a method which can be used to distinguish protein and nucleic acid by electrophoretic mobility.

Streptavidin labeled PBXL-3 (PBXL-3/SA) (Martek Biosciences Corp., Columbia, MD) was combined with a biotin-labeled 1 kb PCR fragment (b-NA) to demonstrate detection of binding interactions. Equi-molar concentrations of PBSL-3/SA and b-NA (800 pM) were incubated at room temperature for at least 1 hr in 10 mM Tris, 0.5 mM EDTA pH 8.1 with 0.1% casein hydrolysate as a carrier. Control incubations were PBXL-3/SA alone and PBXL-3/SA with the same 1 kb fragment without biotin (NA). Following the incubation, samples were diluted 10,000x to final concentration of 8 fM in 2 mM Tris, 0.1 mM EDTA pH 8.1. Samples were loaded into an SMD similar to that disclosed in U.S. Patent No. 4,793,705 and subjected to electrophoresis for 4 min.

Examples of the histogram plots of the molecule cross correlations are shown in Figure 4. In the absence of nucleic acid, the organelle (PBXL-3/SA) migrated as a peak at 368 (A). Bound to the nucleic acid, it migrated more slowly, as seen by the shift of the peak to 294 ms (B). The shift only occurred when the nucleic acid was bound to the organelle, since its presence (without the biotin tag) in the reaction resulted in the organelle migrating as a peak at 409 ms (C). The standard deviation of electrophoretic velocities is 15 ms.

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Example 5. Utility in Multiple Apparatus

Example 5A

This example describes how a bead-based florescence detection instrument can be used for the co-detection of proteins and nucleic acids. Proteins and nucleic acid can be co-detected in a bead-based fluorescence detection instrument. To accomplish this one color of beads can be labeled with an antibody specific for the target protein and beads with a distinct spectral profile can be labeled with an oligonucleotide specific for the target nucleic acid. In addition, labeled detection reagents specific for the target protein and nucleic acid (an antibody and an oligonucleotide, respectively) labeled with PE can be constructed. Sample containing the target protein and nucleic acid can be incubated with beads and washed then incubated with the detection reagents and washed. The sample can then be run on the instrument with two illumination sources, one which excites the bead dyes and another for the detection reagent (PE). For each particle, the emission from the bead dyes will identify the target and the emission from the detection reagent can be used for quantitation of the target.

Example 5B

This example describes how a capillary electrophoresis instrument with laser induced fluorescence detection can be used for the co-detection of proteins and nucleic acids. Samples containing target protein and nucleic acid molecules labeled with indistinguishable labels (and containing ~10 nM of each target molecule) can be electrophoresed though a capillary. Laser induced fluorescence was detected using an avalanche photodiode detector. With different electrophoretic velocities, the protein and nucleic acid will reach the detector at different times, and therefore can be detected based on their characteristic migration time.

Example 5C

This example describes how single molecule electrophoresis instrument with pulsed laser and time-gated detection can be used for the co-detection of proteins and nucleic acids. For example the target protein can be labeled with a dye such as Alexa 647 (with a short fluorescence lifetime (nanoseconds)) and the target nucleic acid with a long lifetime dye (microseconds) such as those described by Herman *et al.*, (2001) and Maliwal *et al.*, (2001)). Sample containing the target molecules can be pumped (if using only lifetime discrimination) or electrophoresed (if using lifetime and velocity discrimination) through the interrogation volume. In this instrument, a pulsed laser can be used as an illumination source. Time-gated avalanche photodiode detectors can be used to detect photons from individual molecules which traverse the interrogation volume. Time-resolved data can then

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be analyzed using methods such as maximum likelihood estimators. By comparing the measured lifetime measurements with know standards the protein and nucleic acid can be co-detected.

Example 5D

This example describes how a single molecule electrophoresis instrument with CCD detection can be used for the co-detection of proteins and nucleic acids. The electrophoretic mobility differences of individual proteins and nucleic acids labeled with dyes emitting at the same wavelength can also be detected using imaging with a CCD camera. In this case, the illumination laser is configured to illuminate a plane within the volume electrophoretic path of the laser. Labeled protein and nucleic acid molecules can be electrophoresed through a capillary. The fluorescent signal can be detected with a CCD camera where a sequence of consecutive images are taken and the velocity of the molecule calculated by the distance moved per unit time.

Example 5E

This example describes how a fluorescence correlation spectroscopy together with electrophoresis can be used for the co-detection of proteins and nucleic acids. Samples containing target protein and nucleic acid molecules labeled with indistinguishable labels (and containing ~1 nM of each target molecule) can be electrophoresed though a capillary with or without sieving medium. In this example, detection is accomplished in a single interrogation volume using a confocal illumination and detection geometry. Data from the avalanche photodiode detectors can be collected in successive 500 us bins and autocorrelation analysis performed to determine the average transit time of molecules through the interrogation volume. Based on their characteristic electrophoretic velocities protein and nucleic acid molecules can be co-detected.

Example 5F

This example describes how a two beam fluorescence cross-correlations spectroscopy together with electrophoresis can be used for the co-detection of proteins and nucleic acids. Samples containing target protein and nucleic acid molecules labeled with indistinguishable labels (and containing ~1 nM of each target molecule) can be electrophoresed though a capillary. In this example, detection can be accomplished in two closely spaced interrogation volumes using a confocal illumination and detection geometry. Data from the two avalanche photodiode detectors can be collected in successive 500 us bins and cross-correlation analysis performed to determine the average transit time between

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the interrogation volumes for each molecule. Based on their characteristic electrophoretic velocities protein and nucleic acid molecules can be co-detected.

Example 5G

This example describes how a protein and a nucleic acid can be co-detected using an instrument where the molecule is pulled, drawn or otherwise passes through a pore and individual units of the biopolymer are detected sequentially. For example, the target nucleic acid can be labeled with a fluorescent tag on all of the adenine residues, resulting in multiply substituted molecules. The N-terminus of the target protein can be labeled with the same tag. The samples can then be combined and analyzed by traversing individual molecules through nanopores and measuring the fluorescent signals. Only a single photon burst will be detected for each protein molecule, which passed by the detection station. In contrast, multiple photon bursts will be detected for each nucleic acid as each labeled adenosine passes through the pore. Therefore, based on the relative number of fluorescent labels detected per molecule, the protein and nucleic acid can be co-detected.

Example 6. Discrimination of a protein and nucleic acid is based on detection of their respective labels after release from the original target molecule.

Biotinylated anti-thyroid stimulating hormone (TSH) antibody was immobilized on a streptavidin-coated 96 well plate, and the excess unbound antibody was washed away. TSH antigen and Alexa Fluor®647 labeled anti-TSH antibody were added to the wells in phosphate buffered saline with 1% bovine serum albumin and 0.1% Tween® 20. The plate was incubated with agitation. The liquid was removed by aspiration, and the wells were washed three times . The Alexa Fluor 647 labeled antibody was dissociated from the TSH sandwich by incubation with 0.1 M glycine-HCl, pH 2.8. The free Alexa Fluor 647 labeled antibody was collected, diluted and analyzed by SMD. The linear relationship between released label and the original target molecule concentration is seen in Fig. 5A.

It is obvious to one skilled in the art that similar methods are available for labeling and release of labels from nucleic acids. Matray *et al.* teaches methods for labeling and releasing labels from both proteins and nucleic acids (Matray, 2004). One skilled in the art will also recognize that separation and discrimination of a mixture of labels released from the target proteins and nucleic acids is essentially the same as for the original targets, such as was demonstrated in Examples 1 and 2. Fig. 5B and C shows two possible ways to distinguish two released labels.

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Example 7. Fluorescence resonance energy transfer (FRET) examples

Example 7A

This example describes co-detection of a protein and nucleic acid from an organism using two oligonucleotides containing a FRET donor and acceptor pair to distinguish the protein (P) from the nucleic acid (NA). An oligonucleotide specific for the target nucleic acid is labeled with a donor component (D) of a FRET pair and a primary antibody to the target protein can be labeled with the same label. A second oligonucleotide probe which hybridizes near the first probe can be labeled with a FRET acceptor (A). The oligonucleotides and antibody can be incubated with a sample containing the target protein and nucleic acid. The sample can then be pumped through an interrogation volume and the fluorescent signal measured by two detectors (with filters to discriminate between the donor and acceptor fluorescence). Based on the relative signals from the two detectors, the protein and nucleic acid can be co-detected, Figure 6A.

Example 7B

This example describes the co-detection of a protein and nucleic acid using an antibody labeled with a FRET acceptor to distinguish the protein from the nucleic acid. Samples containing target protein and nucleic acid molecules can be labeled with indistinguishable labels (the donor component of a FRET pair) and an antibody specific for the target protein is labeled with FRET acceptor molecules. The antibody can be incubated with a sample containing the target protein and nucleic acid. The sample can then be pumped through an interrogation volume and the fluorescent signal measured by two detectors (with filters to discriminate between the donor and acceptor fluorescence). Based on the relative signals from the two detectors, the protein and nucleic acid can be co-detected, Figure 6B.

Example 8. Discrimination of a protein and nucleic acid based on molecular mobility in a two dimensional separation system.

Protein and nucleic acid discrimination by mobility in one dimension is demonstrated in Example 1. One skilled in the art will recognize that molecular mobility can be further influenced by applying forces in more than one dimension. For example, applying an electrical field perpendicular to the axis of the capillary can cause molecules to move sideways within the capillary. The perpendicular electrical field can be applied continuously or intermittently and its polarity can be constant or reversed. The perpendicular field can be combined with any other motive force, such as electrophoresis, pumping and gravitational force, which is applied parallel to the capillary axis. One skilled in the art will recognize that movement from the center of the capillary to the side, or vice versa, will change the influence

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of the electroosmotic forces, which increase with proximity to the capillary wall, and ultimately the rate of molecular movement through the capillary. To the extent that the protein and nucleic acid are differentially affected by the additional force, their separation will be enhanced. Figure 7 shows a diagram of an electric field applied perpendicular to the capillary axis while molecules are being moved along the capillary by pumping (7A) or electrophoresed (7B). In this example, the detector(s) must be downstream but near the location where the perpendicular force is applied. The detectors could also be located within the position of the perpendicular force. Alternatively, two detectors (and the interrogation volumes) could be stacked on the capillary, that is, oriented in a cross-sectional plane. In this case, the protein and nucleic acid may be co-detected as one species passes through one detector's interrogation volume, and the other species passes through the other detector's interrogation volume.

Other Embodiments

The detailed description set-forth above is provided to aid those skilled in the art in practicing the present invention. However, the invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed because these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description which do not depart from the spirit or scope of the present inventive discovery. Such modifications are also intended to fall within the scope of the appended claims.

References Cited

All publications, patents, patent applications and other references cited in this application are incorporated herein by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application or other reference was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. Citation of a reference herein shall not be construed as an admission that such is prior art to the present invention. Such references shall include:

1. Alba, F. J., A. Bermudez, *et al.* (2001-2). "Green-light transilluminator for the detection without photodamage of proteins and DNA labeled with different fluorescent dyes." Electrophoresis 22(3): 399-403.

2. Ambrose, W. P., P. M. Goodwin, *et al.* (1999). "Single molecule fluorescence spectroscopy at ambient temperature." Chem Rev 99(10): 2929-56.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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3. Brodsky, A. S. and P. A. Silver (2002). "A microbead-based system for identifying and characterizing RNA-protein interactions by flow cytometry." Mol Cell Proteomics 1(12): 922-9.

4. Chiu, K. P., K. A. Duca, *et al.* (1996). "A novel in situ double-labeling method for simultaneous detection of mRNA and expressed protein or two different mRNAs." J Neurosci Methods 66(2): 69-79.

5. Crissman, H. A., Z. Darzynkiewicz, *et al.* (1990). "Simultaneous fluorescent labeling of DNA, RNA, and protein." Methods Cell Biol 33: 305-14.

6. Dunbar, S. A., J. W. Jacobson, *et al.* (2003). "Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAPTM system." Journal of Microbiological Methods 53(2): 245-252.

7. Faretta, M., D. Bergamaschi, *et al.* (1998). "Characterization of cyclin B1 expression in human cancer cell lines by a new three-parameter BrdUrd/cyclin B1/DNA analysis." Cytometry 31(1): 53-9.

8. Fischer, T. C., Q. T. Dinh, *et al.* (2002). "Simultaneous detection of receptor mRNA and ligand protein in human skin tissues." J Cutan Pathol 29(2): 65-71.

9. Hasper, H. J., R. M. Weghorst, *et al.* (2000). "A new four-color flow cytometric assay to detect apoptosis in lymphocyte subsets of cultured peripheral blood cells." Cytometry 40(2): 167-71.

10. Haugland, R. (2002). Molecular Probes Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes Inc.

11. Holm, M., M. Thomsen, *et al.* (1998). "Optimization of a flow cytometric method for the simultaneous measurement of cell surface antigen, DNA content, and *in vitro* BrdUrd incorporation into normal and malignant hematopoietic cells." Cytometry 32(1): 28-36.

12. Jing, D., J. Agnew, *et al.* (2003). "A sensitive two-color electrophoretic mobility shift assay for detecting both nucleic acids and protein in gels." Proteomics: 1172-80.

13. Kellar, K. L. and M. A. Iannone (2002). "Multiplexed microsphere-based flow cytometric assays." Exp Hematol 30(11): 1227-37.

14. Keller, R. A., W. P. Ambrose, *et al.* (2002). "Analytical applications of single-molecule detection." Anal Chem 74(11): 316A-324A.

15. LeCaptain, D. J., M. A. Michel, *et al.* (2001). "Characterization of DNA-protein complexes by capillary electrophoresis-single molecule fluorescence correlation spectroscopy." Analyst 126(8): 1279-84.

16. Ma, Y., M. R. Shortreed, *et al.* (2000). "High-throughput single-molecule spectroscopy in free solution." Anal Chem 72(19): 4640-5.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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17. Matray, T., V. Hernandex, *et al.* (2004). ELECTROPHORETIC TAG REAGENTS COMPRISING FLUORESCENT COMPOUNDS. US, Aclara Biosciences, Inc. Mountain View, CA.

18. Nagaso, H., T. Murata, *et al.* (2001). "Simultaneous detection of RNA and protein by in situ hybridization and immunological staining." J Histochem Cytochem 49(9): 1177-82.

19. Niculescu, A. B., 3rd, X. Chen, *et al.* (1998). "Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication." Mol Cell Biol 18(1): 629-43.

20. Perrin, A., D. Duracher, *et al.* (2003). "A combined oligonucleotide and protein microarray for the codetection of nucleic acids and antibodies associated with human immunodeficiency virus, hepatitis B virus, and hepatitis C virus infections." Anal Biochem 322(2): 148-55.

21. Schwille, P. and E. Haustein (2002). Fluorescence Correlation Spectroscopy: An introduction to its concepts and applications. Single molecule techniques. P. Schwille. Bethesda, MD http://www.biophysics.org/biophys/society/btol, Biophysics Textbook On-Line, Biophysical Society.

22. Waters, W. R., K. R. Harkins, *et al.* (2002). "Five-color flow cytometric analysis of swine lymphocytes for detection of proliferation, apoptosis, viability, and phenotype." Cytometry 48(3): 146-52.

23. Wrotnowski, C. (2002). "Next-generation single molecule detection." Genetic Engineering News 22: 28-27.

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CLAIMS

What is claimed is:

1. A method for co-detecting a polynucleotide molecule and a polypeptide molecule contained within one sample comprising:

(a) moving said polynucleotide molecule and said polypeptide molecule through at least one interrogation volume; and

(b) measuring at least one electromagnetic characteristic of said polynucleotide molecule and measuring at least one electromagnetic characteristic of said polypeptide molecule,

wherein said polynucleotide molecule and said polypeptide molecule are co-detected.

2. A method according to claim 1, wherein the polynucleotide molecule and the polypeptide molecule are discriminated.

3. A method according to claim 1, wherein the electromagnetic characteristic of the polynucleotide molecule and polypeptide molecule are measured simultaneously or sequentially.

4. A method according to claim 1, wherein a plurality of the same polynucleotide molecule and a plurality of the same polypeptide molecule are co-detected.

5. A method according to claim 1, wherein a plurality of different polynucleotide molecules and a plurality of different polypeptide molecules are co-detected.

6. A method according to claim 1, wherein a plurality of the same or different polynucleotide molecules and a plurality of the same or different polypeptide molecules are co-detected.

7. A method according to claim 1, wherein the polynucleotide is selected from the group consisting of a single-stranded DNA, a double-stranded DNA, an oligonucleotide, an RNA, a dendrimer, a nucleic acid hybrid and any combination thereof.

8. A method according to claim 1, wherein the polypeptide is selected from the group consisting of an oligopeptide and a protein.

9. A method according to claim 1, wherein the polypeptide electromagnetic characteristic and the polynucleotide electromagnetic characteristic are measured within at least one interrogation volume, said interrogation volume being in electromagnetic communication with at least one detector and at least one excitation source.

10. A method according to claim 9, wherein the interrogation volume is fluidly connected to at least one other interrogation volume.

11. A method according to claim 9, wherein the sample comprises a plurality of the same or different polynucleotide molecules and a plurality of the same or different polypeptide molecules, and wherein act (b) further comprises:

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measuring at least one of a first or a second electromagnetic characteristic of at least one polynucleotide molecule as the at least one polynucleotide molecule interacts with an excitation source within a first interrogation volume;

measuring at least one of a first or a second electromagnetic characteristic of the at least one polynucleotide molecule as the at least one polynucleotide molecule interacts with an excitation source within a second interrogation volume;

comparing the measured electromagnetic characteristics measured within the first and the second interrogation volumes of the at least one polynucleotide;

measuring at least one of a first or a second electromagnetic characteristic of at least one polypeptide molecule as the at least one polypeptide molecule interacts with an excitation source within the first, the second or a third interrogation volume;

measuring at least one of a first or a second electromagnetic characteristic of the at least one polypeptide molecule as the at least one polypeptide molecule interacts with an excitation source within the first, the second or a fourth interrogation volume; and

comparing the measured electromagnetic characteristics measured within the first, the second, the third and the fourth interrogation volumes of the at least one polypeptide.

12. A method according to claim 11, wherein the act of comparing comprises distinguishing by statistical analysis the measured electromagnetic characteristics of the at least one polypeptide molecule and the at least one polypeptide molecule from background electromagnetic characteristics.

13. A method according to claim 12, wherein the act of comparing comprises cross-correlating the measured electromagnetic emissions determined from the at least one polypeptide molecule and the at least one polypeptide molecule to determine the velocity of the molecules.

14. A method according to claim 12, wherein the act of comparing comprises cross-correlating the measured electromagnetic emissions to determine the velocity of the molecules.

15. A method according to claim 1, wherein act (a) comprises subjecting the sample to a motive force selected from the group consisting of electro-kinetic, pressure, vacuum, surface tension, gravity, centrifugal, and any combination thereof.

16. A method according to claim 15, wherein the act of moving the molecules between a first interrogation volume and a second interrogation volume further comprises subjecting the molecules to a separation method selected from the group consisting of capillary gel electrophoresis, micellar electro-kinetic chromatography, isotachophoresis, and any combination thereof.

17. A method according to claim 1, wherein at least one of the measurable characteristics of the polynucleotide molecule and at least one of the measurable

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characteristics of the polypeptide molecule is produced by one of an intrinsic parameter of the molecule and an extrinsic parameter of the molecule.

18. A method according to claim 17, further comprising marking the polynucleotide molecule and the polypeptide molecule with at least one label to provide the extrinsic parameter.

19. A method according to claim 18, wherein the polynucleotide molecule and polypeptide molecule are labeled prior to performing act (a).

20. A method according to claim 19, wherein at least one polynucleotide and at least one polypeptide are labeled in separate reactions and are combined to create the sample prior to performing act (a).

21. A method according to claim 19, wherein at least one polynucleotide and at least one polypeptide are labeled in the same reaction prior to performing act (a).

22. A method according to claim 18, wherein the label affects the mobility of at least one of the polynucleotide molecule and the polypeptide molecule and is selected from the group consisting of a charge tag, a mass tag, a charge/mass tag, and any combination thereof.

23. A method according to claim 22, further comprising moving the polynucleotide molecule in a direction generally opposite to a direction of the polypeptide molecule.

24. A method according to claim 22, wherein the sample comprises a plurality of different polynucleotide molecules, further comprising moving a first polynucleotide molecule in a direction generally opposite to a direction of a second polynucleotide molecule.

25. A method according to claim 22, wherein the sample comprises a plurality of different polypeptide molecules, further comprising moving a first polypeptide molecule in a direction generally opposite to a direction of a second polypeptide molecule.

26. A method according to claim 22, further comprising moving the polynucleotide molecule in a direction generally perpendicular to a direction of the polypeptide molecule.

27. A method according to claim 22, wherein the sample comprises a plurality of different polynucleotide molecules, further comprising moving a first polynucleotide molecule in a direction generally perpendicular to a direction of a second polynucleotide molecule.

28. A method according to claim 22, wherein the sample comprises a plurality of different polypeptide molecules, further comprising moving a first polypeptide molecule in a direction generally perpendicular to a direction of a second polypeptide molecule.

29. A method according to claim 18, wherein the label emits electromagnetic radiation selected from the group consisting of fluorescence, chemiluminescence, elastic light scattering, inelastic light scattering, and any combination thereof.

30. A method according to claim 29, wherein the measured electromagnetic characteristic is selected from the group consisting of emission wavelength, emission

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intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime and any combination thereof.

31. A method according to claim 30, wherein the polynucleotide label and the polypeptide label comprise indistinguishable emission wavelengths.

32. A method according to claim 31, wherein the polynucleotide label or polypeptide label further comprises a characteristic other than emission wavelength which is distinguishable.

33. A method according to claim 32, wherein the characteristic other than emission wavelength is selected from the group consisting of mobility, emission intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime, and any combination thereof.

34. A method according to claim 31, wherein the electromagnetic characteristics of the polynucleotide label and the polypeptide label are indistinguishable and wherein the polynucleotide and the polypeptide are distinguishable by their mobility.

35. A method according to claim 32, wherein the electromagnetic characteristics of the polynucleotide label and the polypeptide label are indistinguishable and wherein a predetermined range of labels is attached to each polynucleotide molecule and polypeptide molecule, and further wherein the polynucleotide molecule and polypeptide molecule are distinguishable by a characteristic signal produced by the difference in the range of labels attached to each molecule.

36. A method according to claim 18, wherein the polynucleotide molecule and polypeptide molecule are labeled directly by means of specific or nonspecific interactions selected from a group consisting of covalent binding, ionic binding, hydrophobic binding, affinity binding, and any combination thereof.

37. A method according to claim 18, wherein the polynucleotide and polypeptide are labeled indirectly by means of incubating with at least one binding partner to form a specific complex and wherein at least one binding partner comprises at least one label.

38. A method according to claim 37, wherein the binding partners are selected from the group consisting of polynucleotide/polynucleotide interactions, polynucleotide/polypeptide interactions and polypeptide/polypeptide interactions, and any combination thereof.

39. A method according to claim 37, wherein the interaction between binding partners is mediated through intermolecular forces selected from the group consisting of hydrophobic interactions, hydrogen bonding, ionic bonding, van der Waals attraction, covalent bonding, and any combination thereof.

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40. A method according to claim 1, wherein at least one polynucleotide molecule is detected and at least one polypeptide molecule is detected, and the method further comprises counting the molecules.

41. A method according to claim 40, wherein said counting comprises determining a concentration of polynucleotide molecules within the sample and a concentration of polypeptide molecules within the sample, further comprising

comparing the counted polynucleotide molecules with a polynucleotide standard of a known concentration; and

comparing the counted polypeptide molecules with a polypeptide standard of a known concentration.

42. A method according to claim 40, wherein said counting comprises determining a concentration of polynucleotide molecules within the sample and a concentration of polypeptide molecules within the sample, further comprising

comparing the counted polynucleotide molecules with an internal polynucleotide standard of a known concentration; and

comparing the measured molecules of the polypeptide with an internal polypeptide standard of a known concentration.

43. A method according to claim 40, wherein said counting comprises determining a concentration of polynucleotide molecules and polypeptide molecules without use of an external standard or an internal standard.

44. A method for co-detecting a polynucleotide molecule and a polypeptide molecule within one sample comprising:

(a) labeling at least one polynucleotide molecule and at least one polypeptide molecule with at least one directly or indirectly detectable label in a mixture of polynucleotide molecules, polypeptide molecules and excess labels;

(b) separating or rendering undetectable unbound labels from the labeled polynucleotide molecules and labeled polypeptide molecules;

(c) interacting the labeled polynucleotide molecules and labeled polypeptide molecules with an agent causing the release of directly or indirectly bound labels; and

(d) detecting at least one polynucleotide molecule released label and at least one polypeptide molecule released label;

thereby rendering the polynucleotide molecule and a polypeptide molecule co-detectable.

45. A method according to claim 44, wherein act (d) comprises:

moving the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label through at least one interrogation volume; and

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measuring at least one electromagnetic characteristic of the at least one polynucleotide molecule released label and measuring at least one electromagnetic characteristic of the at least one polypeptide molecule released label.

46. A method according to claim 44, wherein the polynucleotide molecule released label and the polypeptide molecule released label are discriminated.

47. A method according to claim 45, wherein the electromagnetic characteristic of the polynucleotide molecule released label and polypeptide molecule released label are measured simultaneously or sequentially.

48. A method according to claim 44, wherein a plurality of the same polynucleotide molecule and a plurality of the same polypeptide molecule are indirectly co-detected.

49. A method according to claim 44, wherein a plurality of different polynucleotide molecules and a plurality of different polypeptide molecules are indirectly co-detected.

50. A method according to claim 44, wherein a plurality of the same or different polynucleotide molecules and a plurality of the same or different polypeptide molecules are indirectly co-detected.

51. A method according to claim 45, wherein the polypeptide molecule released label electromagnetic characteristic and the polypeptide molecule released label electromagnetic characteristic are measured within at least one fluidly connected interrogation volume, said interrogation volume being in electromagnetic communication with at least one detector and at least one excitation source.

52. A method according to claim 51, wherein the sample comprises a plurality of the same or different polynucleotide molecule released labels and a plurality of the same or different polypeptide molecule released labels, and wherein the act of measuring further comprises:

measuring at least one of a first or a second electromagnetic characteristic of at least one polynucleotide molecule released label as the at least one polynucleotide molecule released label interacts with an excitation source within a first interrogation volume;

measuring at least one of a first or a second electromagnetic characteristic of the at least one polynucleotide molecule released label as the at least one polynucleotide molecule released label interacts with an excitation source within a second interrogation volume;

comparing the measured electromagnetic characteristics measured within the first and the second interrogation volumes of the at least one polynucleotide molecule released label;

measuring at least one of a first or a second electromagnetic characteristic of at least one polypeptide molecule released label as the at least one polypeptide molecule released

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label interacts with an excitation source within the first, the second or a third interrogation volume;

measuring at least one of a first or a second electromagnetic characteristic of the at least one polypeptide molecule released label as the at least one polypeptide molecule released label interacts with an excitation source within the first, the second or a fourth interrogation volume; and

comparing the measured electromagnetic characteristics measured within the first, the second, the third and the fourth interrogation volumes of the at least one polypeptide molecule released label.

53. A method according to claim 52, wherein the act of comparing comprises distinguishing by statistical analysis the measured electromagnetic characteristics of the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label from background electromagnetic characteristics.

54. A method according to claim 52, wherein the act of comparing comprises cross-correlating the measured electromagnetic emissions determined from the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label to determine the velocity of the labels.

55. A method according to claim 51, wherein the act of comparing comprises cross-correlating the measured electromagnetic emissions to determine the velocity of the labels.

56. A method according to claim 45, wherein the act of moving comprises subjecting the sample to a motive force selected from the group consisting of electro-kinetic, pressure, vacuum, surface tension, gravity, centrifugal, and any combination thereof.

57. A method according to claim 56, wherein the act of moving the released labels between a first interrogation volume and a second interrogation volume further comprises subjecting the released labels to a separation method selected from the group consisting of capillary gel electrophoresis, micellar electro-kinetic chromatography, isotachophoresis, and any combination thereof.

58. A method according to claim 45, wherein at least one measurable characteristic of the polynucleotide molecule released label and at least one measurable characteristic of the polypeptide molecule released label is produced by one of an intrinsic parameter of the released labels and an extrinsic parameter of the released labels.

59. A method according to claim 58, further comprising attaching a secondary label to the released label to provide the extrinsic parameter.

60. A method according to claim 59, wherein the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are marked with the secondary label prior to performing the act of moving.

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61. A method according to claim 60, wherein the secondary label is attached to the polynucleotide molecule released label and the polypeptide molecule released label in separate reactions and are combined to create the sample prior to performing the act of moving.

62. A method according to claim 60, wherein the secondary label is attached to the polynucleotide molecule released label and the polypeptide molecule released label in the same reaction prior to performing the act of moving.

63. A method according to claim 59, wherein the secondary label affects the mobility of the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label, and wherein the secondary label is selected from the group consisting of a charge tag, a mass tag, a charge/mass tag, and any combination thereof.

64. A method according to claim 63, further comprising moving the marked polynucleotide molecule released label in a direction generally opposite to a direction of the marked polypeptide molecule released label.

65. A method according to claim 63, wherein the sample comprises a plurality of different polynucleotide molecule released labels, further comprising moving a first marked polynucleotide molecule released label in a direction generally opposite to a direction of a second marked polynucleotide molecule released label.

66. A method according to claim 63, wherein the sample comprises a plurality of different polypeptide molecule released labels, further comprising moving a first marked polypeptide molecule released label in a direction generally opposite to a direction of a second marked polypeptide molecule released label.

67. A method according to claim 63, further comprising moving the marked polynucleotide molecule released label in a direction generally perpendicular to a direction of the marked polypeptide molecule released label.

68. A method according to claim 63, wherein the sample comprises a plurality of different polynucleotide molecule released labels, further comprising moving a first marked polynucleotide molecule released label in a direction generally perpendicular to a direction of a second marked polynucleotide molecule released label.

69. A method according to claim 63, wherein the sample comprises a plurality of different polypeptide molecule released labels, further comprising moving a first marked polypeptide molecule released label in a direction generally perpendicular to a direction of a second marked polypeptide molecule released label.

70. A method according to claim 58, wherein the polynucleotide molecule released label, polypeptide molecule released label or secondary label emits

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electromagnetic radiation selected from the group consisting of fluorescence, chemiluminescence, light scattering, and any combination thereof.

71. A method according to claim 58, wherein the measured electromagnetic characteristic is selected from the group consisting of emission wavelength, emission intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime and any combination thereof.

72. A method according to claim 71, wherein the polynucleotide molecule released label and the polypeptide molecule released label comprise indistinguishable emission wavelengths.

73. A method according to claim 72, wherein the secondary label comprises a characteristic other than emission wavelength which is distinguishable.

74. A method according to claim 73, wherein the characteristic other than emission wavelength is selected from the group consisting of mobility, emission intensity, burst size, burst duration, fluorescence polarity, fluorescence lifetime and any combination thereof.

75. A method according to claim 72, wherein the electromagnetic characteristics of the polynucleotide molecule released label and the polypeptide molecule released label are indistinguishable, and wherein the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are indirectly distinguishable by the secondary label mobility.

76. A method according to claim 59, wherein the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are marked directly with the secondary label by means of specific or nonspecific interactions selected from a group consisting of covalent binding, ionic binding, hydrophobic binding, affinity binding, and any combination thereof.

77. A method according to claim 59, wherein the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label are marked indirectly with the secondary label by means of incubating with at least one binding partner to form a specific complex and wherein at least one binding partner comprises at least one secondary label.

78. A method according to claim 77, wherein the binding partners are selected from the group consisting of polynucleotide/polynucleotide interactions, polynucleotide/polypeptide interactions and polypeptide/polypeptide interactions, and any combination thereof.

79. A method according to claim 77, wherein the interaction between binding partners is mediated through intermolecular forces selected from the group consisting of

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hydrophobic interactions, hydrogen bonding, ionic bonding, van der Waals attraction, covalent bonding, and any combination thereof.

80. A method according to claim 45, wherein at least one released label is detected, and the method further comprises counting the released labels.

81. A method according to claim 80, wherein said counting comprises determining a concentration of at least one polynucleotide molecule released label and at least one polypeptide molecule released label within the sample, further comprising:

comparing the counted polynucleotide molecule released labels with a polynucleotide molecule label standard of a known concentration; and

comparing the counted polypeptide molecule released labels with a polypeptide molecule label standard of a known concentration.

82. A method according to claim 80, wherein said counting comprises determining a concentration of at least one polynucleotide molecule released label and at least one polypeptide molecule released label within the sample, further comprising

comparing the counted polynucleotide molecule released labels with an internal polynucleotide molecule label standard of a known concentration; and

comparing the counted polypeptide molecule released labels with an internal polypeptide molecule label standard of a known concentration.

83. A method according to claim 80, wherein said counting comprises determining a concentration of at least one polynucleotide molecule released label and at least one polypeptide molecule released label without use of external or internal standards.

84. A method according to claim 80, wherein the number of the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label counted are proportional to the concentration of the polynucleotide molecules and polypeptide molecules in the original sample.

85. A method according to claim 59, wherein at least one secondary label is detected, and the method further comprises counting the secondary labels.

86. A method according to claim 85, wherein said counting comprises determining a concentration of at least one secondary label within the sample, further comprising

comparing the counted secondary labels released from the polynucleotide molecule with a secondary label standard of a known concentration; and

comparing the counted secondary labels released from the polypeptide molecule with a secondary label standard of a known concentration.

87. A method according to claim 85, wherein said counting comprises determining a concentration of at least one secondary label within the sample, further comprising

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comparing the counted secondary labels released from the polynucleotide molecule with an internal secondary label standard of a known concentration; and

comparing the counted secondary labels released from the polypeptide molecule with an internal secondary label standard of a known concentration.

88. A method according to claim 85, wherein said counting comprises determining a concentration of at least one secondary label without use of external or internal standards.

89. A method according to claim 85, wherein the number of the at least one secondary labels counted are proportional to the concentration of the polynucleotide molecules and polypeptide molecules in the original sample.

90. A method for co-detecting a polynucleotide molecule and a polypeptide molecule contained within one sample comprising:

(a) disposing said polynucleotide molecule and said polypeptide molecule in at least one interrogation volume; and

(b) measuring at least one electromagnetic characteristic of said polynucleotide molecule and measuring at least one electromagnetic characteristic of said polypeptide molecule,

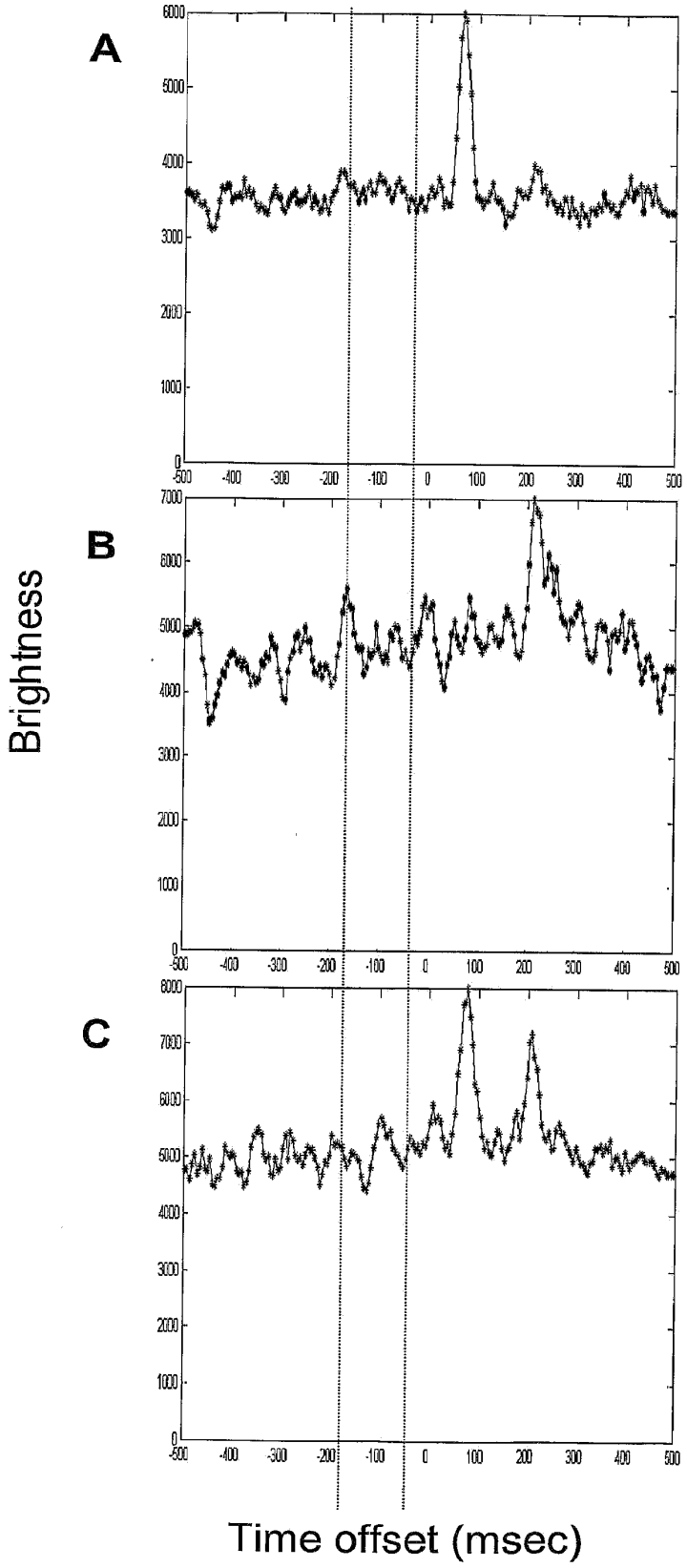
wherein said polynucleotide molecule and said polypeptide molecule are co-detected.

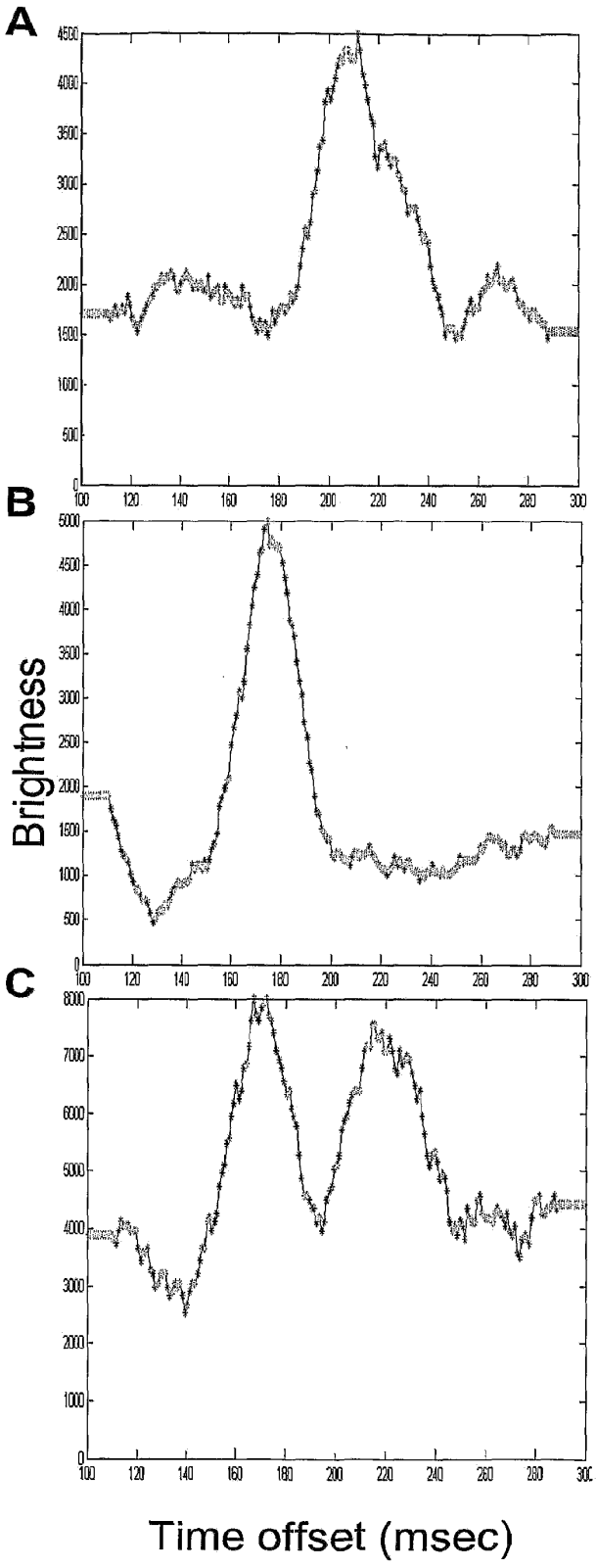
91. A method according to claim 44, wherein act (d) comprises:

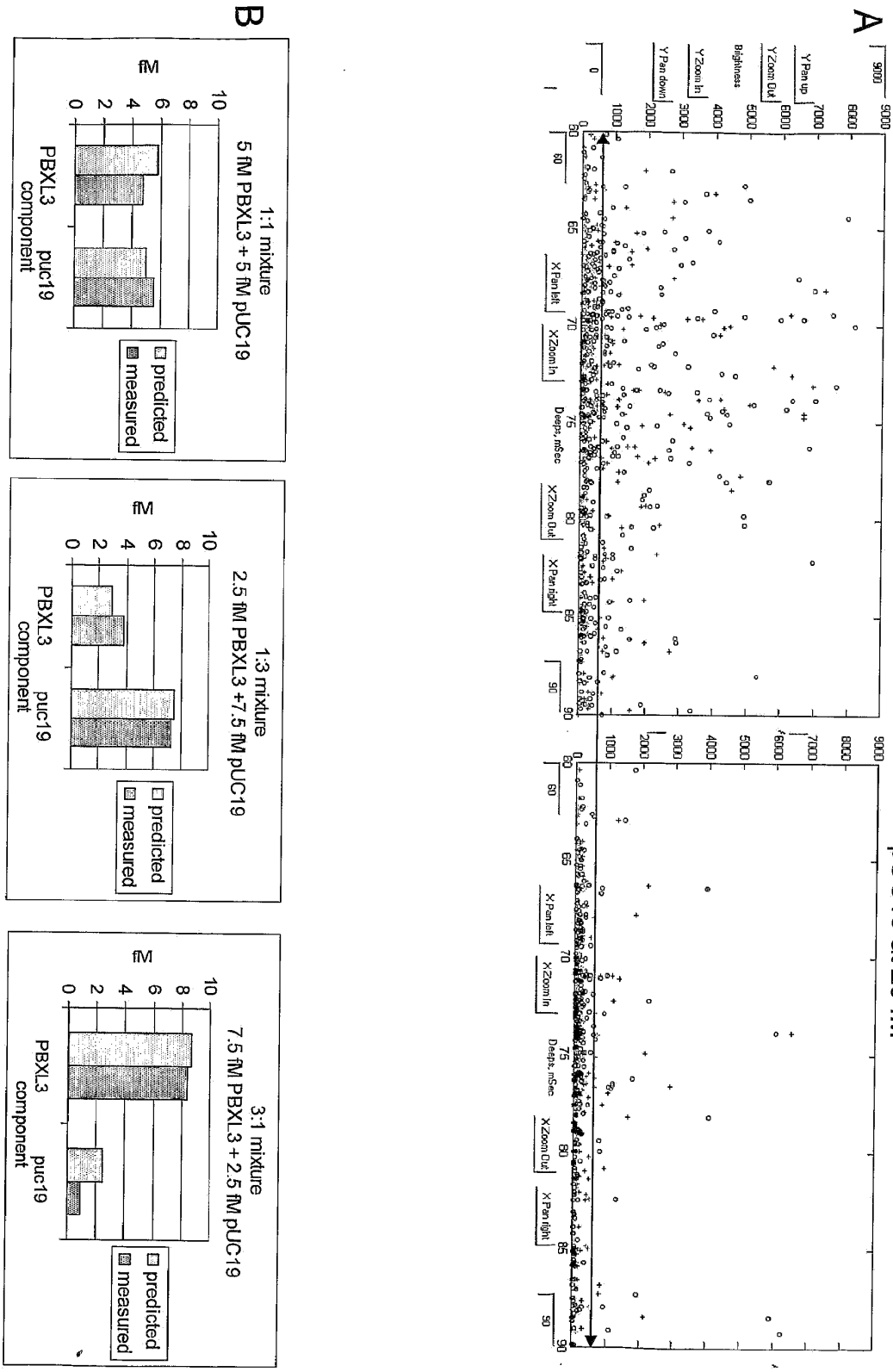
disposing the at least one polynucleotide molecule released label and the at least one polypeptide molecule released label through at least one interrogation volume; and

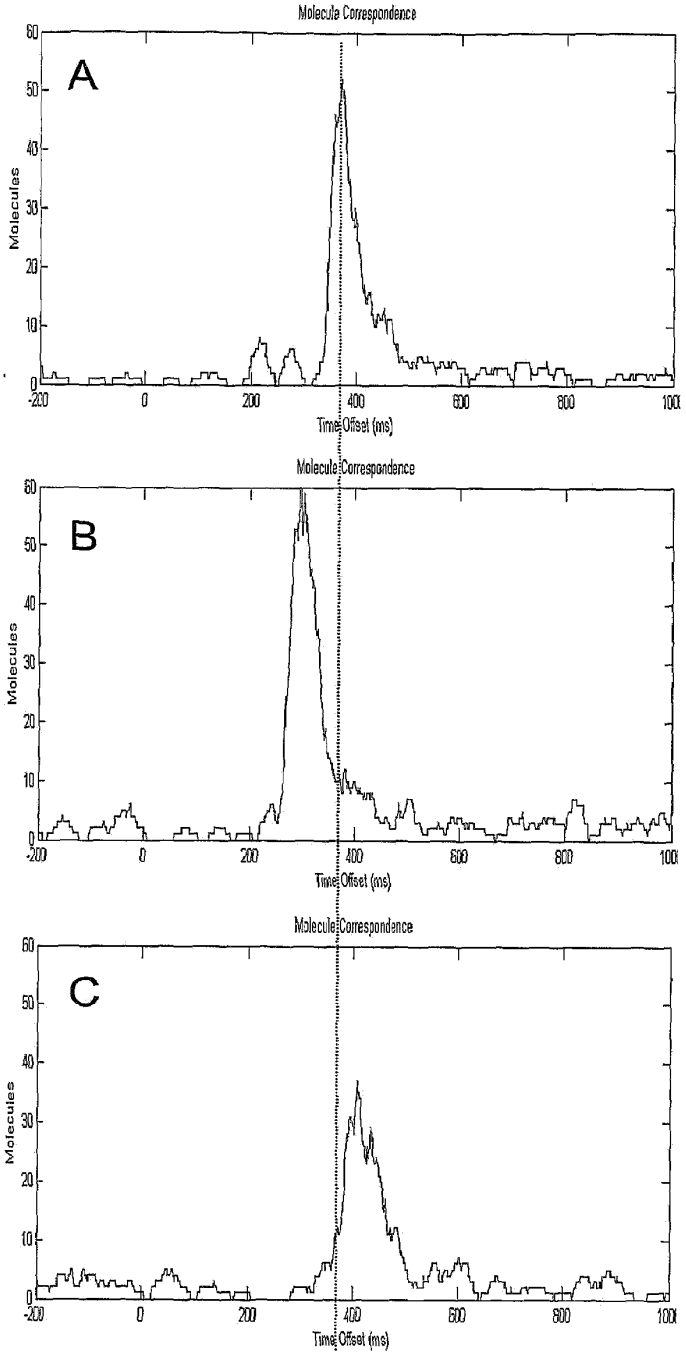
measuring at least one electromagnetic characteristic of the at least one polynucleotide molecule released label and measuring at least one electromagnetic characteristic of the at least one polypeptide molecule released label.

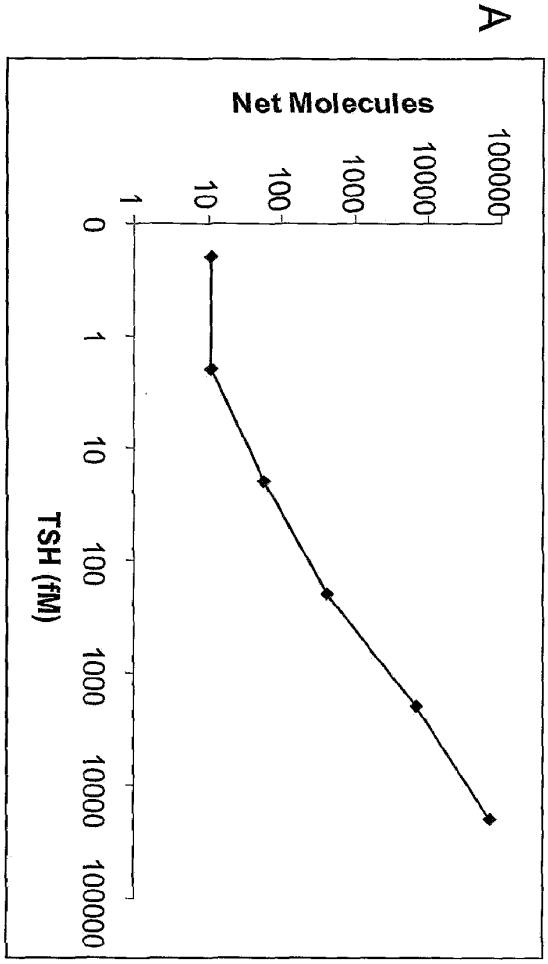
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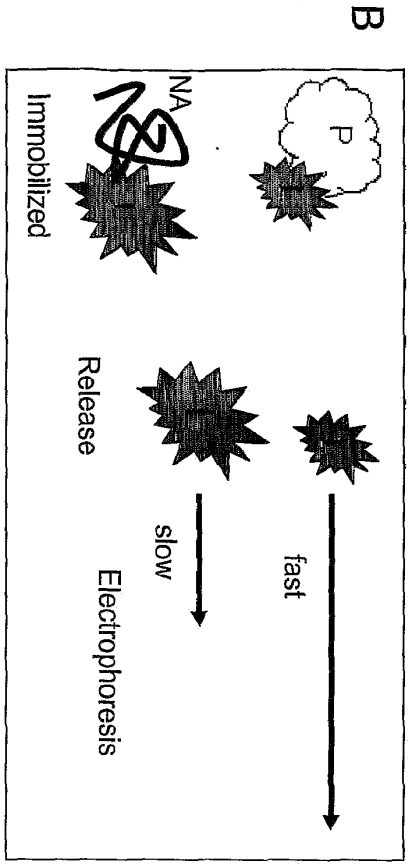

A
B
C
Brightness
Time offset (msec)

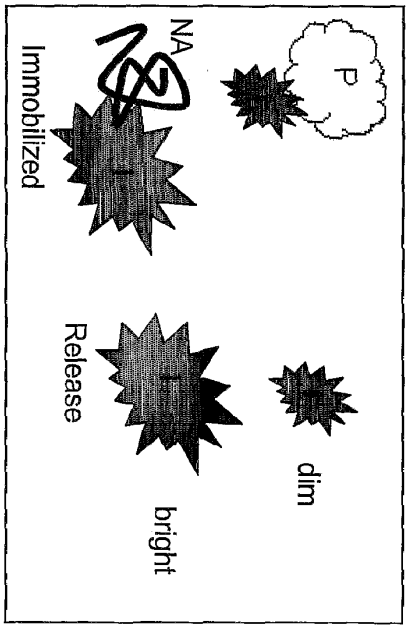

A
B
C
Brightness
Time offset (msec)

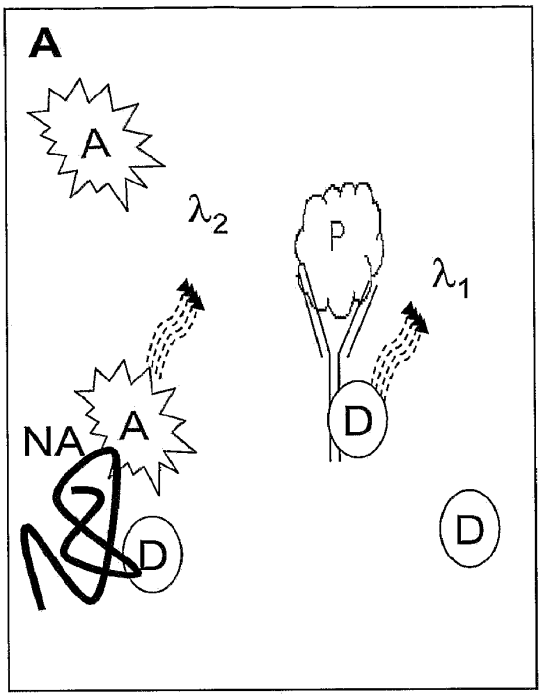

A
PBXL-3 at 20 fM
pUC19 at 20 fM
Y Pan up
Y Zoom Out
Brightness
Y Zoom In
Y Pan down
X Pan left
X Zoom In
Deepz, mSec
X Zoom Out
X Pan right
B
1:1 mixture
5 fM PBXL3 + 5 fM pUC19
1:3 mixture
2.5 fM PBXL3 +7.5 fM pUC19
3:1 mixture
7.5 fM PBXL3 + 2.5 fM pUC19
fM
PBXL3 component
puc19 component
predicted
measured

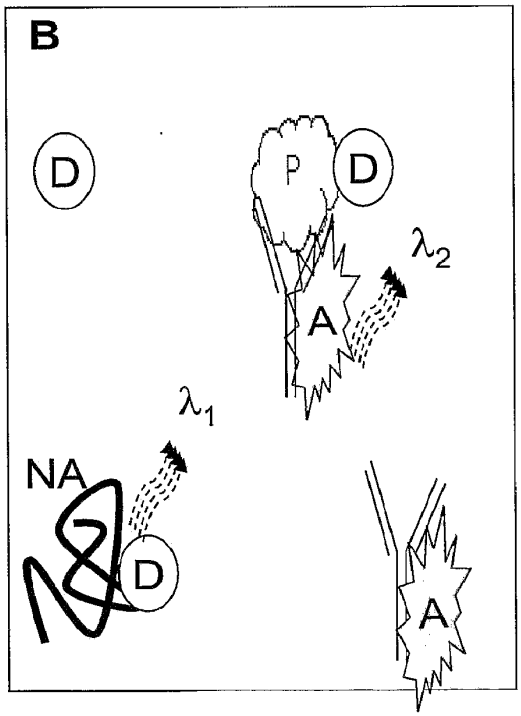

Molecule Correspondence
A
Molecules
Time Offset (ms)
Molecule Correspondence
B
Molecules
Time Offset (ms)
Molecule Correspondence
C
Molecules
Time Offset (ms)

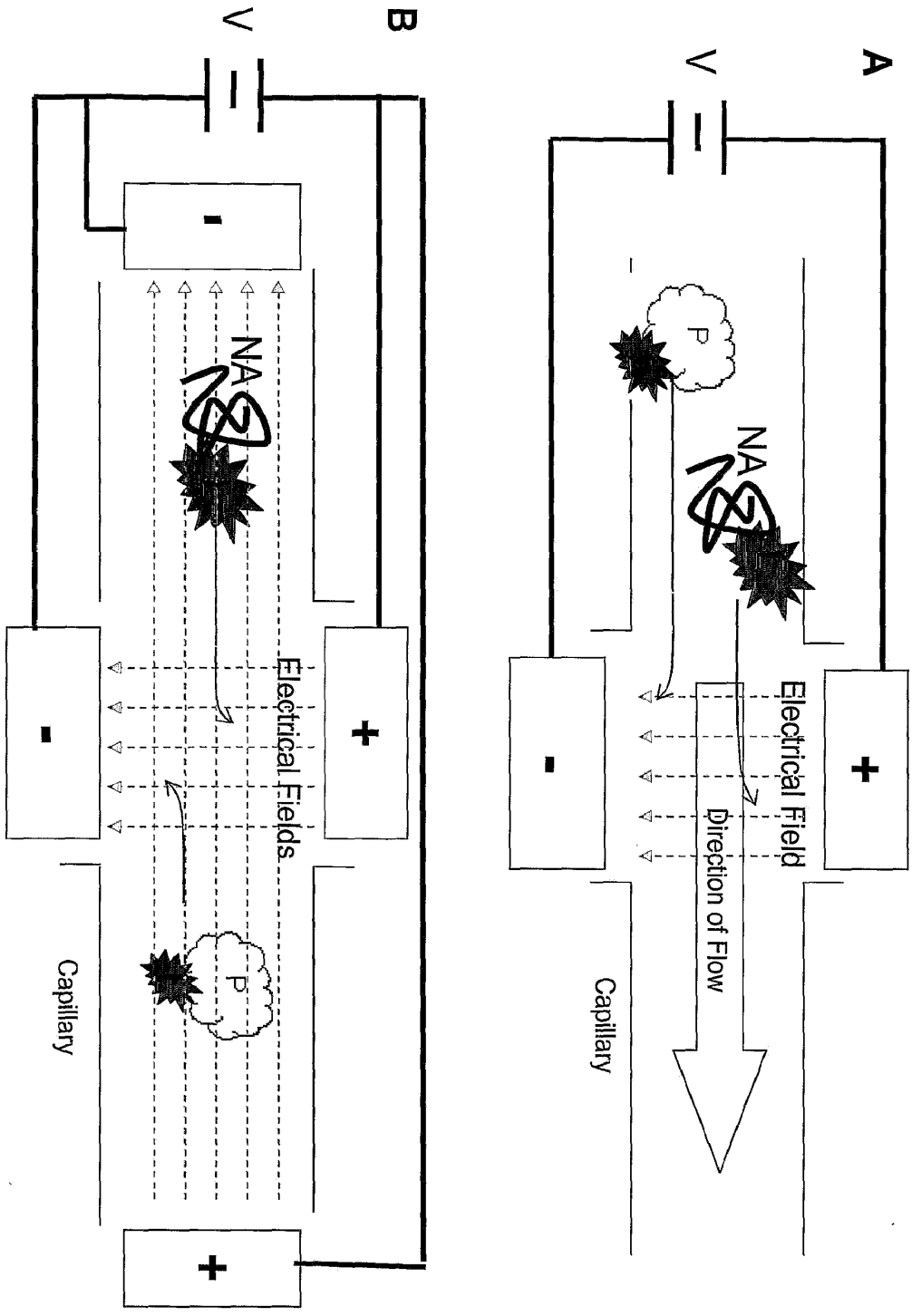

A
Net Molecules
100000
10000
1000
100
10
1
0
1
10
100
1000
10000
100000
TSH (fM)


B
P
NA
Immobilized
Release
slow
fast
Electrophoresis


C
P
NA
Immobilized
Release
dim
bright


A
A
λ_2
P
λ_1
NA
A
D
D
D


B
D
P
D
λ_2
A
λ_1
NA
D
A


A
V
P
NA
Electrical Field
Direction of Flow
Capillary
B
V
NA
Electrical Fields
P
Capillary

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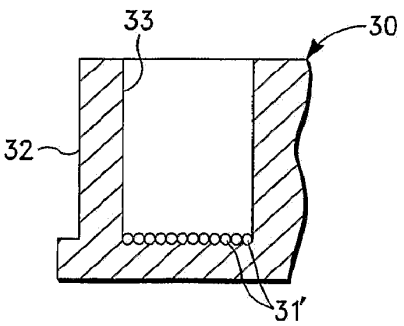
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(54) Title: METHOD OF FORMING AND USING SOLID-PHASE SUPPORT



(57) Abstract: A method of forming a solid-phase support (30), the method including the steps of providing a substrate (32) having a reaction vessel (33), dispensing a particle (31) in the reaction vessel (33), and permanently bonding the particle in the substrate (32) within the reaction vessel (33). The particle (31) may include a microbead. The particle (31) may include controlled pore glass. A method of synthesis is also disclosed that includes including the steps of providing a solid-phase support (30) including a particle (31) embedded to the substrate (32) adjacent a surface of substrate, the particle (31) being functionalized to covalently attach an intermediate compound of a synthetic reaction, dispensing a liquid including a reagent to the solid-phase support (31) to effect the synthetic reaction, and removing the liquid from the solid-phase support (31) by centrifugation, whereby the intermediate compound remains attached to the substrate (32) by the particle (31).

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Attorney Docket No. 33493/RMS/VEJ

METHOD OF FORMING AND USING SOLID-PHASE SUPPORT

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] This invention was made with government support under Grant No. 9 R44 HG02805-02 awarded by National Human Genome Research Institute. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] This invention relates, in general, to a solid-phase support for high-throughput synthesis, and more particularly to substrates having particles attached thereto, the solid-phase support being capable of withstanding centrifugal forces generated during high-throughput synthesis.

Description of Related Art

[0003] Solid-phase synthesis of organic molecules is the method of choice for preparation of libraries and compound megaarrays, which are currently being applied for screening in the quest to find new drugs or pharmaceutical lead compounds, i.e., compounds which exhibit a particular biological activity of pharmaceutical interest. These leads can serve as a starting point for the selection and synthesis of a drug compound, which in addition to the particular biological activity of interest have pharmacologic and toxicologic properties suitable for administration to animals, including humans.

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[0004] Solid-phase synthesis requires repetitions of several relatively simple operations of addition of reagents, incubation and separation of liquid-phases. In solid-phase synthesis, final compounds are synthesized attached to solid-phase supports that permit the use of mechanical means to separate intermediate, partially-synthesized compounds between synthesis steps. Various designs of manual and automated instruments for combinatorial synthesis utilizing solid-phase synthesis have appeared in patent and non-patent literature. For example, U.S. Patent Nos. 5,202,418 and 5,338,831, both to Lebl et al., each describe a method of performing multiple synthesis of peptides on a solid carrier. U.S. Patent No. 5,342,585, also to Lebl et al., describes an apparatus for multiple syntheses of peptides on solid support. U.S. Patent No. 6,045,755, also to Lebl, et al., describes an apparatus and a method for combinatorial chemistry synthesis. The entire contents of the above patents are incorporated herein by this reference.

[0005] Some known instruments have employed centrifugation for the separation of solid and liquid-phases in order to provide devices that are capable of high-throughput synthesis. Such devices can utilize solid-phase particles, for example, macrobeads or microbeads, as a solid-phase support to which intermediate and final compounds are attached. Such solid-phase particles are generally suspended within a reaction vessel, which reaction vessel is subjected to centrifugal forces to separate the liquid and solid-phases. For example, U.S. Patent No. 6,121,054 to Lebl, describes a method for separation of liquid and solid-phases for solid-phase organic synthesis in which a slurry of beads are suspended in a solvent. Similarly, U.S. Patent Application Publication No. US 2002/0044894 A1 to Lebl et al. describes an oligonucleotide synthesizer that utilizes centrifugation to separate the liquid-phase from beads. Generally, such instruments employ mechanical means to retain the solid-phase beads within a reaction vessel, for example, a well of a microtiter plate, during centrifugation. The entire contents of the above patent and publication are incorporated herein by this reference.

[0006] One means to retain the solid-phase beads is to tilt the reaction vessel to form pockets from which the solid-phase cannot be removed by centrifugal force. See, e.g.,

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U.S. Patent No. 6,121,054. Alternatively, mesh or other frit materials can be employed to enclose and restrain the beads within the reaction vessel. See, e.g., U.S. Patent Application Publication No. US 2002/0044894 A1. Disadvantageously, such retention means increase the mechanical complexity of the instruments and/or the reaction vessels.

[0007] What is needed is a solid-phase support for high-throughput synthesis that overcomes the above and other disadvantages of known instruments employing solid-phase particles suspended within reaction vessels.

BRIEF SUMMARY OF THE INVENTION

[0008] In summary, one aspect of the present invention is directed to a method of forming a solid-phase support, the method including the steps of providing a substrate having a reaction vessel, dispensing a particle in the reaction vessel, and permanently bonding the particle in the substrate within the reaction vessel. The particle may include a microbead. The particle may include controlled pore glass. The dispensing step may include brushing the particle into a recess of a dispenser plate. The substrate may include a microtiter plate including a plurality of microtiter wells. The dispensing plate may include a plurality of the recesses and each of the microtiter the wells may define a respective reaction vessel that is aligned with a respective the recess. The dispensing step may further include contacting the dispensing plate and the microtiter plate such that the recess is aligned with the reaction vessel. The dispensing step may further include pivoting the dispensing plate into engagement with the microtiter plate. The dispensing step may further include providing a first dispensing pressure to the recess facilitating transfer of the particle from the recess to the reaction vessel.

[0009] In one embodiment, bonding can include embedding a particle in a substrate. Bonding can also include heating the substrate. The embedding step may further include heating the substrate, thereby increasing the temperature of a portion of the substrate, and in the case of multi-well plate at least a portion of each the reaction

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vessels, to the melting point of the substrate. The bonding step may further include providing a second bonding pressure to the reaction vessel biasing the particle against the reaction vessel. The bonding step may further include heating the substrate, thereby increasing the temperature of a portion of the substrate adjacent a bottom surface of each the reaction vessel to the melting point of the substrate. The bonding step may further include providing a second bonding pressure to the reaction vessel biasing the particle against the bottom of the reaction vessel. The method may include dispensing a plurality of the particles to the reaction vessel, thereby bonding one or more of the particles into the substrate within the reaction vessel. The one or more of the particles may remain nonbonded to the substrate. The method may further include the step of removing the one or more nonbonded particles from the reaction vessel. The providing step may include providing a bottomless-well plate and a rigid sheet, the method further including providing a layer of hot-melt adhesive to the rigid sheet to form a laminate sheet, applying the laminate sheet to a bottom surface of the bottomless-well plate thereby forming the substrate having a reaction vessel. The bonding step may include embedding the particle in the layer of hot-melt adhesive.

[0010] Another aspect of the present invention is directed to a method of synthesis including the steps of providing a solid-phase support including a particle bonded to the substrate adjacent a surface of substrate, the particle being functionalized to covalently attach an intermediate compound of a synthetic reaction, dispensing a liquid including a reagent to the solid-phase support to effect the synthetic reaction, and removing the liquid from the solid-phase support by centrifugation, whereby the intermediate compound remains attached to the substrate by the particle. The solid-phase support may include a reaction vessel and wherein the particle may be bonded the substrate and in the reaction vessel. The solid-phase support may include a microtiter plate. The particle may include controlled pore glass. The providing step may include heating the substrate and bonding the particle in the substrate, thereby making the solid-phase support. The removing step may include centrifugation around an axis of rotation, wherein the surface of the substrate may be oriented away

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from the axis of rotation and wherein the particles may remain bonded in the surface. The particle may include 1400 Angstrom controlled pore glass.

[0011] A further aspect of the present invention is directed to a method of making a solid-phase support, including heating a substrate, thereby forming a molten substrate, contacting the molten substrate with a particle, and allowing the molten substrate to cool in the presence of the particle, thereby making a solid-phase support including the particle bonded in the substrate.

[0012] Yet another aspect of the present invention is directed to a method of separating a liquid and solid-phase, including providing a solid-phase support, the solid phase support including a particle embedded in a surface of a substrate, and removing the liquid from the solid-phase support by centrifugation around an axis of rotation, wherein the surface of the substrate may be oriented away from the axis of rotation and wherein the particles remain embedded in the surface.

[0013] An object of the present invention is to provide a method of forming and using a solid-phase support.

[0014] Another object of the present invention is to provide a substrate having a solid-phase support attached thereto, in which the substrate and support are capable of withstanding centrifugal forces generated during high-throughput synthesis.

[0015] Yet another object of the present invention is to provide a method of forming a substrate of the above character.

[0016] The solid-phase support 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.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIG. 1 is a perspective view of a solid-phase support for high-throughput synthesis in accordance with the present invention.

[0018] FIG. 2 is an enlarged, fragmented, cross-sectional view of the solid-phase support of FIG. 1 schematically illustrating particles disposed in the well of the substrate prior to bonding of the particles to the substrate.

[0019] FIG. 3 is an enlarged, fragmented, cross-sectional view of the solid-phase support of FIG. 1 schematically illustrating particles disposed in the well of the substrate following bonding but prior to removal of excess particles from the well.

[0020] FIG. 4 is an enlarged, fragmented, cross-sectional view of the solid-phase support of FIG. 1 schematically illustrating particles bonded to the well of the substrate.

[0021] FIG. 5 is a schematic view of a workstation for applying particles to a substrate, which figure shows particles which have been loaded on an applicator.

[0022] FIG. 6 is a schematic view of the workstation of FIG. 5 in which a reclaim tray has been disengaged from the applicator.

[0023] FIG. 7 is a schematic view of the workstation of FIG. 5 in which a substrate has been mounted on a heating element.

[0024] FIG. 8 is a schematic view of the workstation of FIG. 5 in which the applicator has been pivoted from a loading position to a bonding position resting against the substrate.

[0025] FIG. 9 is a schematic view of the workstation of FIG. 5 in which the applicator has been returned to its loading position and the substrate has been removed from the heating element.

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[0026] FIG. 10 is a cross-sectional view of another substrate for high-throughput synthesis in accordance with the present invention.

[0027] FIG. 11 is an enlarged perspective view of particles embedded in a substrate in accordance with the present invention.

[0028] FIG. 12 is a perspective view of the solid-phase support of FIG. 1 mounted on rotor assembly of a high-throughput synthesis apparatus.

DETAILED DESCRIPTION OF THE INVENTION

[0029] Reference will now be made in detail to particular embodiments of the invention, examples of which are illustrated in the accompanying drawings. While the invention will be described in conjunction with particular 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 can be included within the spirit and scope of the invention as defined by the appended claims.

[0030] The present invention is directed to a solid-phase support for separation of liquid and solid phase components of a two-phase system such as a synthetic or analytical system. A solid-phase support of the invention can be used for high-throughput synthesis including, but not limited to, solid-phase combinatorial chemistry synthesis of organic molecules. In particular, the present invention is directed to substrates particularly suited for separation of components using a centrifuge. For example, the substrates are particularly suited for use with high-throughput synthesis ("HTS") apparatus which utilize a centrifugal rotor assembly for the step-wise addition and removal of solid-phase and liquid-phase solutions, and the separation and removal of the solid-phase synthetic reactions, as is described in U.S. Patent No. 6,121,054 to Lebl ("the Lebl '054 patent"), and in U.S. Patent Application Publication No. US 2002/0044894 A1 to Lebl et al. ("the Lebl '894 publication"), the entire contents of which patent and publication are incorporated by this reference.

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The present invention is also useful for other phase separation systems such as those based on removal of a liquid phase from a solid phase by pipetting or aspiration.

[0031] Accordingly, the invention provides a method of separating a liquid and solid-phase. The method includes the steps of (a) providing a solid-phase support having a particle embedded in a surface of a substrate; and (b) removing the liquid from the solid-phase support by centrifugation around an axis of rotation, wherein the surface of the substrate is oriented away from the axis of rotation and wherein the particles remain embedded in the surface.

[0032] By way of introduction, combinatorial chemistry synthesis protocols prescribe the stepwise, sequential addition of building blocks to intermediate and/or partially synthesized intermediate compounds in order to synthesize a final compound. In solid-phase synthesis, final compounds are synthesized attached to solid-phase supports that permit the use of simple mechanical means to separate intermediate, partially-synthesized intermediate compounds between synthetic steps.

[0033] In a particular embodiment, a solid-phase support of the present invention is used to synthesize nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that can have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem.

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Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone can be done to increase the stability and half-life of such molecules in physiological environments.

[0034] The nucleic acids (sometimes referred to herein as oligonucleotides) can be synthesized using a variety of possible synthetic reactions. In a particular embodiment, phosphoramidite chemistry is used, with enzymatic techniques and techniques based on photodeprotection useful as well. In addition, any number of nucleic acid analogs and labeled nucleic acids can be made and used. See for example Oligonucleotides and Analogs: A Practical Approach, Ed. F. Eckstein, IRL Press, 1991, hereby incorporated by reference in its entirety.

[0035] An exemplary solid-phase combinatorial protocol is that for the synthesis of peptides attached to polymer resin, which proceeds according to Lam et al., 1991, A new type of synthetic peptide library for identifying ligand-binding activity, Nature

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354:82-84. U.S. Pat. No. 5,510,240 to Lam et al. for Method of screening a peptide library; Lam et al., 1994, Selectide technology: Bead-binding screening. Methods: Companion to Methods in Enzymology 6:372-380. Another exemplary protocol is that for the synthesis of benzodiazepine moieties, which proceeds according to Bunin et al., 1992, A general and expedient method for the solid phase synthesis of 1,4-benzodiazepine derivatives, J. Amer. Chem. Soc., 114:10997-10998. U.S. Pat. No. 5,288,514 to Ellman for Solid phase and combinatorial synthesis of benzodiazepine compounds on a solid support. Also, for protocols for the addition of N-substituted glycines to form peptoids, see, e.g., Simon, et al., 1992, Peptoids: A modular approach to drug discovery. Proc. Natl. Acad. Sci. USA, 89:9367-9371; Zuckermann et al., 1992, Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis. J. Amer. Chem. Soc., 114:10646-10647; WO PCT94/06,451 to Moos et al. for Synthesis of N-substituted polyamide monomers, useful as solvents, additives for food, enzyme inhibitors etc. Approaches for synthesis of small molecular libraries were recently reviewed by, e.g., Krchnak and Lebl, 1996, Synthetic library techniques: Subjective (biased and generic) thoughts and views, Molecular Diversity, 1:193-216; Ellman, 1996, Design, synthesis, and evaluation of small-molecule libraries, Account. Chem. Res., 29:132-143; Armstrong et al., 1996, Multiple-component condensation strategies for combinatorial library synthesis, Account. Chem. Res., 29:123-131.; Fruchtel et al., 1996, Organic chemistry on solid supports, Angew. Chem. Int. Ed., 35:17-42; Thompson et al., 1996, Synthesis and application of small molecule libraries, Chem. Rev., 96:555-600; Rinnova et al., 1996, Molecular diversity and libraries of structures: Synthesis and screening, Collect. Czech. Chem. Commun., 61: 171-231; Hermkens et al., 1996, Solid-phase organic reactions: A review of the recent literature, Tetrahedron, 52:4527-4554. Exemplary building blocks and reagents are amino acids, other organic acids, aldehydes, alcohols, and so forth, as well as bifunctional compounds, such as those given in Krchnak and Lebl, 1996, Synthetic library techniques: Subjective (biased and generic) thoughts and views, Molecular Diversity, 1:193-216. All of these references are hereby expressly incorporated by reference.

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[0036] In one embodiment, peptides are synthesized. By "peptide" herein is meant at least two amino acids joined via a peptide bond. The peptide may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. The side chains may be in either the (R) or the (S) configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradations. The stepwise solid phase synthesis of peptides is well known. An exemplary solid-phase combinatorial protocol is that for the synthesis of peptides attached to polymer resin, which proceeds according to Lam et al., 1991, Nature 354:82-84; U.S. Patent 5,510,240; Lam et al., 1994, Selectide technology: Bead-binding screening. Methods: A Companion to Methods in Enzymoloqy 6:372-380. Another exemplary protocol is that for the synthesis of benzodiazepine moieties, which proceeds according to Bunin et al., 1992, J. Amer. Chem. Soc., 114:10997-10998 and U.S. Patent 5,288,514. Also, for protocols for the addition of N-substituted glycines to form peptoids, see, e.g., Simon, et al., 1992, Proc. Natl. Acad. Sci. USA, 89:9367-9371; Zuckermann et al., 1992, J. Amer. Chem. Soc., 114:10646-10647; WO PCT94/06,451 to Moos et al.; Approaches for synthesis of small molecular libraries were recently reviewed by, e.g., Krchnak and Lebl, 1996, Molecular Diversity, 1:193-216; Ellman, 1996, Account. Chem. Res., 29:132-143; Armstrong et al., 1996, Account. Chem. Res., 29:123-131.; Fruchtel et al., 1996, Anaew. Chem. Int. Ed., 35:17-42; Thompson et al., 1996, Chem. Rev., 96:555-600; Rinnova et al., 1996, Collect. Czech. Chem. Commun., 61: 171-231; Hermkens et al., 1996, Tetrahedron, 52:4527-4554. All of these references are hereby expressly incorporated by reference.

[0037] One should appreciate however that a solid-phase support of the present invention is similarly applicable to other chemical protocols having similar functional steps. For example, components of the present invention can be applied to appropriate liquid-phase, combinatorial chemistry synthesis protocols, to other solid- or liquid-phase chemical protocols, or to any combination thereof. Such synthesis

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protocols include, but are not limited to organic syntheses such as peptide synthesis, polymer synthesis, and small molecule synthesis.

[0038] A solid-phase support of the invention can also be used for an analytical system in which it is desired to separate a liquid-phase from a solid-phase. Exemplary analytical systems include, without limitation, a binding assay such as an assay for detecting nucleic acid hybridization, binding of a protein to a ligand, binding of an antibody to an epitope, binding of a cell to another cell, binding of a molecule to a cell or subcellular component or binding between cells or subcellular components; an enzyme activity assay such as a kinetic assay or inhibition assay; or a cellular activity assay such as detection of response of a cell to a particular molecule.

[0039] The invention provides a method of making a solid-phase support. The method includes the steps of (a) heating a substrate, thereby forming a molten substrate; (b) contacting the molten substrate with a particle; and (c) allowing the molten substrate to cool in the presence of the particle, thereby making a solid-phase support having the particle embedded in the substrate.

[0040] As used herein the term "molten" is intended to mean transformed from a solid state to a state that is at least partially liquid. A molten substrate can be one that is converted from a solid state to a fully liquid state or to a state that is partially liquid. A molten substrate in a partially liquid state includes, for example, a substrate that has a liquid surface and solid core. A substrate can be transformed from a solid state to a liquid state, for example, by application of heat, pressure, chemical agents or a combination thereof.

[0041] As used herein, the term "embedded" is intended to mean placed at least partially within a substrate, thereby interrupting the surface of the substrate. In accordance with the invention, a particle embedded in a substrate can be fully or partially surrounded by the substrate, so long as the particle interrupts the surface of the substrate and can be accessed by a liquid applied to the surface of the substrate.

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[0042] Turning now to the drawings, wherein like components are designated by like reference numerals throughout the various figures, attention is directed to FIG. 1, which illustrates a solid-phase support for high-throughput synthesis, generally designated by the numeral 30. In accordance with the present invention, one or more particles 31 are securely attached to a substrate 32 thus enabling the use of the particles in apparatus which utilize centrifuge technology to separate the liquid-phase from the solid-phase such as those used for high-throughput synthesis ("HTS").

[0043] As used herein, the term "substrate" is intended to mean a material capable of forming a solid base. A material included in the term can have any of a variety of properties depending upon the desired application of the invention including for example, porous, nonporous, rigid, elastic, pliable, malleable, low temperature melting, high temperature melting, and/or chemically resistant to one or more solvents commonly used in the reactions set forth herein. The substrate can be formed of a variety of materials including, but not limited to, plastic; polypropylene, polyethylene, polybutylene, polyurethane, nylon, polymer such as acrylic, acrylonitrile butadiene styrene (ABS), ULTEM (Polyetherimide), acetal copolymer, PROPYLUX HS (heat stabilized polypropylene), RADEL A (polyethersulfone), RADEL R (polyarylethersulfone), UDEL (polysulfone), NORYL PPO (polyphenylene oxide & styrene), Polycarbonate, UHMW-PE (ultra high molecular weight polyethylene), Polyetheretherketone (PEEK), polyphenylene sulfide (PPS, TECHTRON or RYTON), polyolefin or polystyrene; metal such as aluminum, iron, steel or an alloy; other materials such as glass or silicon, or derivatives or combinations of these or other suitable materials. The material of the substrate is typically formed of a material that is capable of achieving a state wherein it is malleable or pliable such that particles can be bonded to it, as discussed below. The material of the substrate can also be in a state where it is rigid such that it can withstand forces applied during synthetic or analytical processes. As set forth below, substrates useful in the invention include, for example, those that can be heated to a molten state that is malleable or pliable such that when loaded with particles and cooled to a rigid state a solid-phase substrate is formed.

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[0044] Substrate 32 can include one or more reaction vessels or wells 33. One or more particles 31 can be disposed within each well 33 and affixed to substrate 32. Such configuration allows synthetic reactions to be performed within one or more wells of the substrate. In particular, liquid reagents can be added to each well, reacted within each well, and then the residual liquid can be removed from each well, for example, via centrifugation, inversion of the wells, and/or aspiration. In one embodiment, the substrate is a multi-well plate that includes a plurality of wells disposed in arrays. 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).

[0045] One will appreciate that the substrate and the wells can be configured in a variety of ways. The shape of the wells can vary depending on the particular application and/or desired fluid dynamics. For example, the depth and diameter of the wells can be U-shaped, V-shaped, or flat bottomed. The wells can be substantially vertical, inclined inwardly (e.g., toward a centrifugal axis) and/or inclined outwardly (e.g., away from a centrifugal axis). Furthermore, the sidewall of the well can be cylindrical, conical, flat, tapered inwardly or outwardly, or have any other desired geometry. One should also appreciate that the shape of the substrate itself can also vary. For example, the shape of the substrate can be polygonal (e.g., rectangular), regular, irregular, arcuate or round.

[0046] As noted above, one or more particles can be affixed to the substrate within each well. The particles can be embedded into the substrate while the substrate is heated to an elevated temperature approximating the melting point of the substrate. In particular embodiments, external mechanical pressure can be applied to the particles while the substrate is heated. Alternatively, embedding can be allowed to occur due to gravity in the absence of externally applied mechanical pressure. As the substrate cools, the particles will typically be permanently bonded to the substrate, which bond

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is capable of withstanding forces typically used to remove liquids such as centrifugal force, aspiration, vacuum, or gravity. Alternatively, a particle can be adhesively bonded to the internal surface of the well utilizing a thermoplastic material having a relatively low melting point (i.e., lower than that of the substrate), a two-part epoxy, and/or other suitable means. One or more particles can be bonded to a well bottom as exemplified in FIG. 2 through FIG. 4. Those skilled in the art will recognize that particles can be bonded to all or part of a well surface including, without limitation, the bottom, sides, subsections thereof or combinations thereof.

[0047] Accordingly, the invention provides a solid-phase support having a particle bonded to a substrate capable of withstanding centrifugal forces directed opposite the surface of the substrate to which the particle is bonded including, for example, centrifugal forces of at least about 10 x *g*, 100 x *g*, 500 x *g*, 1,000 x *g*, 5,000 x *g*, 10,000 x *g*, or 100,000 x *g*.

[0048] Although the invention has been exemplified above with particles bonded to wells, those skilled in the art will recognize that a solid-phase support useful in the invention need not include wells. For example, a solid-phase substrate of the invention can include a flat or planar substrate to which particles are bonded. Alternatively, particles can be bonded to a raised area of a substrate or protrusion. For example, particles can be bonded to protruding "pins" that are configured in an array having dimensions similar to a microtiter plate such that the pins can be dipped into a microtiter plate for delivery of synthetic reactants or analytical reagents. An array of pins can be centrifuged or aspirated to remove liquid from the bonded beads using methods similar to those exemplified herein with respect to microtiter plate-based solid-phase supports.

[0049] As used herein, the term "particle" is also intended to mean a substance having a size or diameter small enough to fit within the area of a substrate to which it is bonded. Exemplary particles include, without limitation, a "microparticle" or "nanoparticle" or "bead" or "microbead" or "microsphere." Particles useful in the invention include, for example, beads such as microbeads measuring 30 microns to

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300 microns in diameter and/or macrobeads measuring larger than 300 microns in diameter. A particle can be made of glass, plastic, polystyrene, resin, gel, agarose, sepharose, and/or other suitable materials.

[0050] Typically, particles will have a melting temperature that is higher than that of the substrate to which they are being bonded when the bonding method includes melting of the substrate. In particular embodiments, a low-melting-point polymer can be placed in contact with particles and a substrate to which they are to be bonded, thus providing an adhesive. There are other types of beads on which chemical synthesis can be performed, which can also be used in the invention.

[0051] In particular embodiments, a particle can be controlled pore glass ("CPG"). CPG having any of a variety of particle sizes and pore sizes can be used in the invention including, for example, CPG with a median particle size falling within about 37 to 177 microns and a median pore size falling within about 65 to 3300 Angstroms. A particularly useful CPG has a median particle size and pore size of about 100 microns and about 1400 Angstroms, respectively. Those skilled in the art will recognize that CPG is typically obtained with a median particle size specification and there can be variation within a lot. For example, a lot of 100-micron CPG can include members be as small as about 75 or as large as about 125 microns. It will be understood that the above described ranges, as with all ranges described herein, are intended to include individual integer and non integer values therein.

[0052] CPG can have a surface modification or it can be unmodified. Unmodified CPG is typically used as a porous bed material in chromatography columns. Its surface can be derivatized to provide reactive functional groups for synthesis using surface modifications methods known in the art.

[0053] A particle can be functionalized in order to covalently attach molecules, cells, other particles or other materials. Exemplary molecules that can be attached include intermediate compounds (or final compounds) of a synthetic reaction. As will be appreciated by those in the art, functionalization of a particle will depend on the

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synthetic reactions to be performed. The functionalization of solid support surfaces such as certain polymers with chemically reactive groups such as thiols, amines, carboxyls, etc. is generally known in the art. Some examples of these surface chemistries for subsequent addition of building blocks during synthetic reactions include, but are not limited to, amino groups including aliphatic and aromatic amines, carboxylic acids, aldehydes, amides, chloromethyl groups, hydrazide, hydroxyl groups, sulfonates and phosphates.

[0054] These functional groups can be used to add any number of different building block moieties to the particles, generally using known chemistries, including, but not limited to the use of amino-functionalized supports, sulfhydryl linkers, etc. There are a number of sulfhydryl reactive linkers known in the art such as SPDP, maleimides, α-haloacetyls, and pyridyl disulfides (see for example the 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). Similarly, amino groups on the building blocks and on the surface can be attached using linkers; for example, a large number of stable bifunctional groups are well known in the art, including homobifunctional and heterobifunctional linkers (see Pierce Catalog and Handbook, pages 155-200). In an additional embodiment, carboxyl groups (either from the surface or from the building block) can be derivatized using well-known linkers (see the Pierce catalog). For example, carbodiimides activate carboxyl groups for attack by good nucleophiles such as amines (see Torchilin et al., Critical Rev. Therapeutic Drug Carrier Systems, 7(4):275-308 (1991), expressly incorporated herein). In addition, useful methods include systems that allow post-synthesis cleavage from the reaction vessels.

[0055] The configuration of the particles affixed to the substrate in accordance with the present invention allows the use of solid-phase support 32 with methods that utilize centrifuges for liquid-phase and solid-phase separation, such as the ones described by the Lebl '054 patent, and with apparatus that utilize centrifugal rotors for liquid-phase and solid-phase separation, such as the ones described by the Lebl '894 publication. For example, FIG. 12 illustrates a solid-phase support mounted on an apparatus 34 similar to that described in the Lebl '894 publication, which apparatus is

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configured for high-throughput, multi-protocol combinatorial syntheses. The apparatus includes a rotor assembly 35 for rotatably supporting one or more solid-phase supports. Preferably, the rotor assembly includes a plate holder 36 that releasably secures the solid-phase support to the rotor assembly.

[0056] Rotor assembly 35 is supported for rotation about a centrifugal axis 37 which extends substantially vertically. Apparatus 34 also includes a motor drivingly connected to rotor assembly 35 in order to spin the rotor assembly for centrifugation in a manner similar to that described in the Lebl '894 publication. Apparatus 34 is adapted for synthesizing oligomers in each of a plurality of the reaction vessels disposed in the substrate. Thusly, the apparatus can also be equipped with a liquid delivery system (not shown) that is capable of delivering one or more liquids to one or more wells of the microtiter plate, in a manner similar to that also described in the Lebl '894 publication. In addition to the components mentioned above, the apparatus can also comprise additional components. For example, the centrifuge can comprise a sensor to signal the computer and liquid distribution system when a set of reaction vessels is in a particular orientation, and a motor to rotate the rotor into the correct orientation for liquid delivery, also in control of the computer. Furthermore, in the case of adjustable tilt rotors, the centrifuge can utilize a control and a sensor to control the degree of tilt.

[0057] Turning now to centrifugation and liquid-phase removal, rotor assembly 35 typically is activated to centrifuge the solid-phase support, exemplified in the illustrated embodiment, microtiter plates 32. The microtiter plates can be held in a fixed angle with respect to the rotor and with respect to vertical, as described in U.S. Patent No. 6,045,755 to Lebl et al. ("the Lebl '755 patent"), the entire contents of which patent is incorporated herein by this reference, as well as the Lebl '054 patent. Alternatively, the microtiter plates can be dynamically adjusted such that the angle of the microtiter plates varies during centrifugation, as described in the Lebl '894 publication.

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[0058] As particles are permanently affixed to the substrate in accordance with the present invention, the solid-phase support can be centrifuged in any of a variety of orientations. For example, solid-phase support 32 allows a "zero-tilt" centrifugation, that is, centrifugal removal of the liquid-phase from microtiter plates that are horizontally oriented during centrifugation. Accordingly, the solid-phase support of the present invention need not be tilted in such a manner to create "pockets" within the wells to retain the particles, namely because particles are securely attached to the substrate. Since particles are bonded to the substrate, a solid-phase substrate of the invention can be centrifuged in a reverse-tilt orientation in which the opening of a reaction vessel is oriented away from the axis of rotation. Thus, substantially all of the liquid in the vessel can be expelled due to centrifugation, while the bonded particles are maintained in the vessel. Furthermore, a solid-phase support of the invention need not have vessels with walls for containing particles or liquid-phase during centrifugation. Thus, particles can be attached to a portion of a substrate that is not in a vessel such as on a flat surface or on a protruding feature. The solid-phase supports of the present invention beneficially allow for the use of less complex high-throughput synthesizers, for example, by reducing the number of components of the rotor assembly, and simplifying the maintenance of the rotor assembly.

[0059] A method of forming solid-phase support 32, in accordance with the present invention, will now be described. In one embodiment, solid-phase particles in the form of controlled pore glass ("CPG") are bonded to the well bottoms of the substrate. For example, the method of the present invention can be utilized to bond a relatively equal amount of 1000 or 1400 Angstrom CPG to the bottom well surfaces of a 384-well polypropylene microtiter plate, for example, 384-well plates of the type sold by Robbins Scientific of Sunnyvale, California.

[0060] In a particular embodiment, approximately one to three grams of CPG can be initially applied to each microtiter plate and approximately 0.2 mg per well is bonded to the substrate. Preferably, the well is overloaded with CPG, that is, more CPG is provided in each well than is necessary to cover the bottom surface of the well. Not all of the CPG will be bonded to the plate, however, most of the excess non-bonded

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CPG is recoverable, as will become apparent below. One will appreciate that the actual amount of CPG, or other particle used will vary depending upon various factors including, but not limited to, the geometry, size and number of the wells present in the substrate. Accordingly, an amount of particles that overload a well or other area of a substrate or, alternatively, an amount of particles that sparsely load or load only a portion of a well or other area of a substrate can be applied.

[0061] Turning now to FIG. 5, the method of the present invention can be performed at a workstation 38 which includes a heating assembly 39 for warming the substrate and a solid-phase particle applicator 40 for dispensing and applying the solid-phase particles to the substrate. In a particular embodiment, the applicator is complementary in shape to substrate 32, and in the embodiment illustrated in FIG. 5, has an array of recesses 41 that correspond to the arrangement of wells 33 of a 384-well microtiter plate.

[0062] Recesses 41 are fluidly connected to a vacuum source 42 for generating a negative pressure within the recesses, which negative pressure facilitates loading CPG into the recesses and temporarily restraining the CPG within the recesses, as will become apparent below. Preferably, the bottoms of the recesses, which are fluidly connected to the vacuum source, are lined with a filter mesh or frit material to retain the CPG within the recesses. Recesses 41 of the applicator are also fluidly connected to a pressurized fluid source 43 that supplies fluid into the recesses and generates a fluid pressure for dispensing the CPG from recesses 41 into wells 33 when applicator 40 is positioned over substrate 32, as well become apparent below. Applicator 40 further includes a pressure plate assembly 44 that is also fluidly connected to pressurized fluid source 43 and is configured to bias substrate 32 against heating assembly 39 the applicator is positioned over the substrate. In one embodiment, pressure plate assembly 44 is a single-acting cylinder assembly that is configured to seat against and bias substrate 32 when a bonding pressure is supplied to the pressure plate assembly. A control unit 45 is provided which is used to control the temperature of heating assembly 39, activation of vacuum source 42, as well as the dispensing and bonding pressures of applicator 40. Preferably, the pressurized fluid source is

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compressed air, however, one will appreciate that other pressurized fluids can be used.

[0063] In one embodiment, the control unit is adjusted to set the heating assembly at a desired temperature, that is, the approximate melting point of the substrate material. In the illustrated embodiment, the heating assembly is set to approximately 235°C to 240°C, however, one will appreciate that the desired temperature will vary depending upon the melting point of the particular substrate and particles being used. An infrared thermometer or other suitable means can be utilized to measure the true temperature of the heating assembly immediately adjacent the substrate such as the microtiter plate exemplified in FIG. 5. In the illustrated embodiment, the true temperature is approximately 270°C (+/-5°C). Again, one will appreciate that the actual temperature can vary depending upon various factors, including the material of the microtiter plate and/or the configuration of the heating assembly.

[0064] Next, control unit can 45 be adjusted to set the desired vacuum as well as the desired "Dispense" and "Bond" pressures of the applicator. In one embodiment, the vacuum is set to approximately 20 SCFH, the dispense pressure is set to approximately 30 psi and the bond pressure to approximately 60 psi, however, one will appreciate that the actual vacuum and pressures necessary will vary depending upon various factors including, but not limited to, the configuration and materials of the substrate.

[0065] In the illustrated embodiment, applicator 40 is pivotally mounted on the workstation and located immediately adjacent heating assembly 39. Applicator 40 pivots about a pivot axis 46 between a first loading position (FIG. 5) and a second bonding position (FIG. 8). Such a pivotal configuration readily allows CPG to be loaded on a dispenser plate 47 of the applicator in the loading position. By way of example, approximately one to three grams of CPG can be poured onto the dispenser plate and thoroughly spread over the recesses of the dispenser plate with a foam brush 48 or other suitable means. Excess CPG can be brushed from the dispenser plate into a CPG reclaim tray 49 with the foam brush (FIG. 5), thus minimizing waste of CPG.

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The reclaim tray can then be removed (FIG. 6) and the excess CPG can be placed in a reclaim container 50 for later use.

[0066] The amount of particles adhered to a substrate can be influenced by altering any of a variety of conditions used for bonding. Exemplary conditions that can be varied to alter particle bonding include, without limitation, the amount of time the substrate is heated, the amount of pressure applied to the particles and substrate or a combination thereof. In a particular embodiment, a polypropylene microtiter plate can be heated to its melting point for about 20 to 40 seconds and CPG pressed with sufficient force to deform well depth by about 0.02 to 0.125 inches.

[0067] As shown in FIG. 7, a release sheet 51 can be placed on the heating assembly in order to facilitate removal of the microtiter plate from the heating assembly after bonding has taken place. In one embodiment, a sheet of aluminum foil is placed on the heating element, however, one will appreciate that other suitable means can be used. Next, a microtiter plate can be placed on the heating element, as well as the release sheet in the event that one is used.

[0068] With microtiter plate 32 in place on heating assembly 39, applicator 40 can be pivoted from its loading position to the bonding position and latched in place, as shown in FIG. 8. The vacuum within recesses 41 applied by vacuum source 42 facilitate restraint of the CPG within the recesses when the applicator is pivoted and latched in place. In one embodiment, the control unit is activated causing the dispensing and bonding cycle to commence. First, control unit 45 will terminate the vacuum allowing the CPG to fall under the force of gravity from recesses 41 into wells 33. The control unit may further activate the dispensing pressure to further facilitate travel of the CPG from the recesses of the dispensing plate into the wells of the microtiter plate. Meanwhile, a hot plate 52 can move upwardly and immediately adjacent microtiter plate 32 in order to heat the bottom of the microtiter plate, and particularly, the bottom surfaces of wells 33 to a temperature approximating the melting point of the material of the microtiter plate. At this time, the control unit can be used to increase the fluid pressure within the pressure plate assembly 44 thus

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firmly seating the pressure plate assembly on the microtiter plate 32 and biasing the microtiter plate downwardly against the heating assembly to facilitate heating of the microtiter plate.

[0069] When the temperature of the substrate material adjacent the well bottoms increases, the well bottoms become malleable and pliable. Once the well bottoms are warm and pliable, CPG 31 is embedded into the bottoms of wells 33 under the force of gravity and bonded with microtiter plate 32, thus permanently bonding the CPG to the microtiter plate.

[0070] Typically, for a solid-phase support of the invention at least a portion of a particle is embedded in a substrate. The embedded portion can be less than one-half of the particle embedded within the substrate, and more preferably, less than 25%. The embedded portion can be at least 1%, 5%, 10%, 25% or 50% or more. In one embodiment, only a nominal portion of the CPG is embedded within the substrate, as shown in FIG. 11. In this embodiment, CPG 31 is embedded in the bottom of well 33 at a depth of less than approximately 10 microns but, nonetheless, provides a bond that is sufficient to withstand the forces that the particle is subjected to during centrifugation. Such configuration advantageously leaves the majority of the CPG surface exposed, thus maximizing the surface area upon which synthetic reactions may take place.

[0071] Once the bonding cycle is complete, the applicator can be unlatched and returned to the loading position. The microtiter plate, along with the release sheet, can then be removed from the heating assembly and allowed to cool on a work surface of the workstation (FIG. 9). Once the microtiter plate is cool, the release sheet can be readily removed, typically falling away from the microtiter plate with gravity alone.

[0072] Any excess CPG, that is, any CPG that has not been bonded to the microtiter plate can be removed from the microtiter plate and stored in a CPG reclaim container for later use. In this regard, the microtiter plate can be tapped against the reclaim

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container to dislodge excess CPG. If desired, the entire microtiter plate can also be vacuumed to further remove any remaining excess CPG from the microtiter plate. The microtiter plate can also be visually inspected to evaluate CPG coverage.

[0073] In operation and use, the CPG bonded microtiter plate 32 can be used upon various HTS apparatuses including, but not limited to, apparatus 34 for solid-phase combinatorial synthesis which typically proceeds according to the following steps. In a first step, the substrate can be mounted on the rotor assembly. As one or more solid-phase supports are already affixed to the substrate within each well, there is no need to charge the wells with a solid-phase support as is typically done with other microtiter plate-based methods (e.g., charging each well with a slurry of microbeads suspended in a solvent). The solid-phase can be preconditioned with an appropriate solvent, if desired, and the first of the plurality of building blocks or a linker moiety can be covalently linked to the solid-phase supports. Subsequently, a plurality of building block addition steps can be performed, all of which involve repetitive execution of the following or similar sub-steps, and in a sequence chosen to synthesize a desired compound. First, a sufficient quantity of a solution, which contains the building block moiety selected for addition, can be dispensed into the well so that the building block moiety is present in a molar excess to the intermediate compound present in the well. A sub-step reaction can be triggered and promoted by activating reagents and other reagents and solvents, which are also added to the well. The well can then be incubated at a controlled temperature for a time, typically between 5 second and 24 hours, sufficient for the building block addition reaction to go to substantial completion. Optionally, during this incubation, the well can be intermittently agitated, stirred, or the liquid contents otherwise moved to increase the volume of liquid contacting the surface area of the bonded particles. Finally, in a last sub-step of building block addition, the well containing the solid-phase support with attached intermediate compound can be prepared for addition of the next building block by removing the spent reaction fluid and thoroughly washing and reconditioning the solid-phase support.

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[0074] Washing typically involves one to seven cycles of adding and removing a wash solvent. Optionally, during the addition steps, multiple building blocks can be added to one well in order to synthesize multiple compounds attached to one solid-phase support. After the desired number of building block addition steps, the final compound is present in the well and attached to the solid-phase support. The final compounds can be utilized either directly attached to their synthetic solid-phase support, or alternatively, can be cleaved from their support. In the latter case, the linker moiety attaching the compound to the solid-phase support can be cleaved in a variety of ways, and the final compound, or library of compounds can be extracted from the reaction vessel into a liquid-phase.

[0075] The substrate and methods of the present invention are advantageously useful for the manual or automated preparation of combinatorial libraries or megaarrays of compounds by fluorous phase organic synthesis. As is well known to those skilled in the art, such combinatorial libraries or megaarrays have numerous uses, in particular, for the selection of pharmaceutical lead compounds, for the optimization of pharmaceutical lead compounds and for the identification and/or isolation of pharmaceutical drugs. The methods and apparatus of the invention for liquid/solid-phase separation can also advantageously be used for parallel extraction and purification of compound arrays synthesized or obtained by other methods. Other applications in analytical chemistry (extraction, desalting or other means of parallel preparations of samples), biochemistry (parallel processing of samples) are envisioned.

[0076] The affixed-support configuration of the substrate also enables centrifuge-HTS apparatus to run with higher accelerations and velocities compared to systems in which particles are free-floating in a reaction vessel, thus providing quicker cycle times. In particular, the solid-phase support of the present invention is capable of withstanding significant acceleration because any likelihood of particle loss is significantly reduced and/or prevented with the particles securely bonded to the substrate.

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[0077] The solid-phase support and method of the present invention also obviates the need to design a specific 384 microtiter plate having specific well geometries intended to contain loose liquid-phase particles, as is the case with other centrifugal HTS systems. Bonding the solid-phase supports directly to the substrate of the present invention has resulted in the increased and consistent production of high-quality oligonucleotides even when used in combination with existing centrifuge synthesis technology.

[0078] In one embodiment of the present invention, solid-phase support 32a is similar to solid-phase support 32 described above but includes a laminate sheet 53 bonded to a bottomless-well plate 54 as shown in FIG. 10. Like reference numerals have been used to describe like components of solid-phase support 32 and solid-phase support 32a. In this embodiment, the laminate sheet includes a rigid sheet 55 having a layer of hot melt adhesive 56 in which particles 31a are embedded. For example, the laminate sheet can be in the form of a "heat-sealing sheet" that is commonly used to close the contents of a microtiter plate. In operation and use, solid-phase support 32a can be used in substantially the same manner as solid-phase support 32 discussed above.

[0079] In another embodiment, the substrate can be formed of a porous polymeric material. Preferably, the porosity of the material allows the substrate to support the liquid-phase within the wells under the normal force of gravity but does not support the liquid-phase under the higher forces of centrifugation. For example, the substrate can be formed of a porous polymeric material including, but not limited to, polyethylene, polypropylene, and other suitable materials. Such porous polymeric materials are typically available in sheets, rods, tubes, and molded shapes and can be machined to form the wells. Porosity of the material typically depends on the specific material and can be as low as 7 μm. As the solid-phase supports are affixed to the substrate, the porosity of the substrate need not be less than the maximum physical dimension of a solid-phase support, as is the case with prior substrates in which the solid-phase supports are merely disposed within the wells. One will appreciate that, in the event the substrate bottom is heated to a temperature approximating the melting

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point of the substrate material in order to affix the solid-phase supports, the porosity of the well bottom can be substantially impaired or lost. Provided that the sides of the well do not reach the melting point, the porosity of the sides of the well remain porous in such a manner that would facilitate the removal of the liquid-phase.

[0080] Alternatively, a solid-phase support can be provided with one or more openings adjacent to the bottom of, and in fluid communication with, the wells to facilitate expulsion of the liquid-phase from the wells. For example, the opening can be a round aperture or one or more slits that would allow expulsion of the liquid-phase during centrifugation or other separation procedure including, but not limited to, vacuum, pressure, venturi vacuum, blotting, and other suitable means. Preferably, the opening or openings are dimensioned such that the bulk of the liquid-phase within the wells remains within the wells until a pressure differential is applied to the liquid-phase within the wells. Alternatively, larger openings can be utilized with a discrete filter mesh or frit extending across the opening, which mesh or frit is provided to retain the liquid-phase The filter mesh or frit is sufficiently fine to retain the liquid-phase within the well under the normal force of gravity but sufficiently course to allow the passage of the liquid-phase through the opening upon the application of a pressure differential.

[0081] For convenience in explanation and accurate definition in the appended claims, the terms "upwardly", "bottom", and "side" are used to describe features of the present invention with reference to the positions of such features as displayed in the figures.

[0082] 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.

[0083] 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

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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.

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WHAT IS CLAIMED IS:

1. A method of forming a solid-phase support, said method comprising the steps:

providing a substrate having a reaction vessel;

dispensing a particle in said reaction vessel; and

permanently bonding said particle to said substrate within said reaction vessel.

2. The method of claim 1, wherein said permanently bonding step includes embedding said particle in said substrate.

3. The method of claim 2, wherein less than one-half of said particle is embedded into said substrate.

4. The method of claim 1, wherein said particle comprises a microbead.

5. The method of claim 1, wherein said particle comprises controlled pore glass.

6. The method of claim 1, wherein said dispensing step includes brushing said particle into a recess of a dispenser plate.

7. The method of claim 6, wherein said substrate comprises a microtiter plate including a plurality of microtiter wells.

8. The method of claim 7, wherein said dispensing plate comprises a plurality of said recesses and each said microtiter well defining a respective reaction vessel that is aligned with a respective said recess.

9. The method of claim 7, wherein said dispensing step further comprises contacting said dispensing plate and said microtiter plate such that said recess is aligned with said reaction vessel.

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10. The method of claim 9, wherein said dispensing step further includes pivoting said dispensing plate into engagement with said microtiter plate.

11. The method of claim 10, wherein said dispensing step further includes providing a first dispensing pressure to said recess facilitating transfer of said particle from said recess to said reaction vessel.

12. The method of claim 11, wherein said permanently bonding step includes heating said substrate.

13. The method of claim 12, wherein said permanently bonding step further includes heating said substrate, thereby increasing the temperature of a portion of said substrate of each said reaction vessel to the melting point of said substrate.

14. The method of claim 13, wherein said permanently bonding step further includes providing a second bonding pressure to bias said substrate against a heating element..

15. The method of claim 12, wherein said permanently bonding step further includes heating said substrate, thereby increasing the temperature of a portion of said substrate adjacent a bottom surface of each said reaction vessel to the melting point of said substrate.

16. The method of claim 15, wherein said permanently bonding step further includes providing a second bonding pressure to said reaction vessel biasing said particle against the bottom of said reaction vessel.

17. The method of claim 1, comprising dispensing a plurality of said particles to said reaction vessel, whereby said permanently bonding further includes

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embedding one or more of said particles into said substrate within said reaction vessel.

18. The method of claim 17, wherein one or more of said particles remain nonbonded to said substrate.

19. The method of claim 18, wherein said method further comprises the step of removing said one or more nonbonded particles from said reaction vessel.

20. The method of claim 1, wherein said providing step comprises providing a bottomless-well plate and a rigid sheet, said method further including providing a layer of hot-melt adhesive to said rigid sheet to form a laminate sheet, applying said laminate sheet to a bottom surface of said bottomless-well plate thereby forming said substrate having a reaction vessel.

21. The method of claim 20, wherein said permanently bonding step comprises embedding.

22. The method of claim 20, wherein said permanently bonding step comprises embedding said particle in said layer of hot-melt adhesive.

23. A method of synthesis comprising:

providing a solid-phase support comprising a particle bonded to said substrate adjacent a surface of substrate, said particle being functionalized to covalently attach an intermediate compound of a synthetic reaction;

dispensing a liquid comprising a reagent to said solid-phase support to effect said synthetic reaction; and

removing said liquid from said solid-phase support by centrifugation, whereby said intermediate compound remains attached to said substrate by said particle.

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24. The method of claim 23, wherein said particle is embedded in said substrate.

25. The method of claim 23, wherein said solid-phase support comprises a reaction vessel and wherein said particle is embedded in said substrate and in said reaction vessel.

26. The method of claim 24, wherein less than one-half of said particle is embedded into said substrate.

27. The method of claim 24, wherein said solid-phase support comprises a microtiter plate.

28. The method of claims 27, wherein said particle comprises controlled pore glass.

29. The method of claim 23, wherein said providing step comprises heating said substrate and embedding said particle in said substrate, thereby making said solid-phase support.

30. The method of claim 29, wherein said removing step comprises 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 bonded in said surface.

31. The method of claim 30, wherein said particle comprises 1400 Angstrom controlled pore glass.

32. A method of making a solid-phase support, comprising:
heating a substrate, thereby forming a molten substrate;
contacting said molten substrate with a particle; and

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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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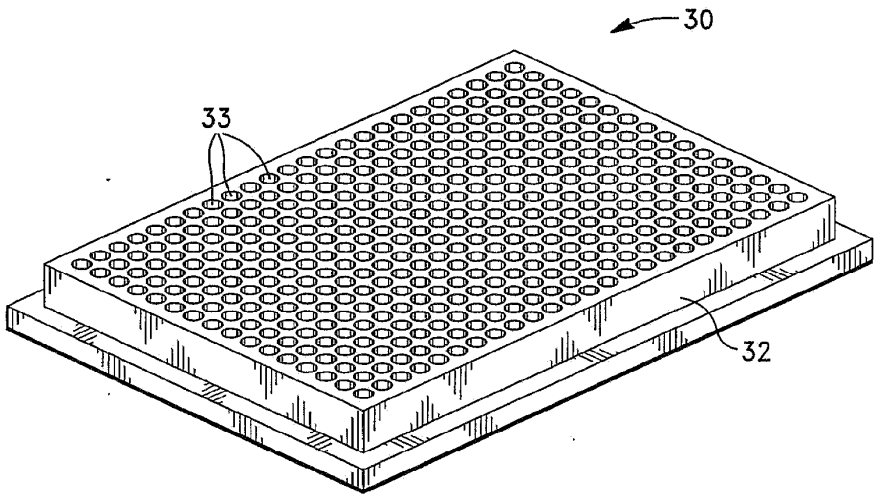


FIG.—1

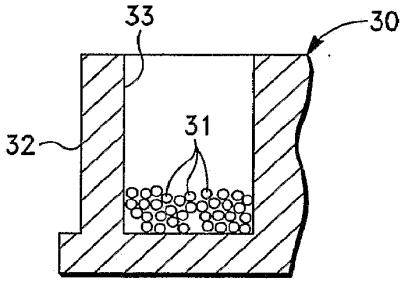


FIG.—2

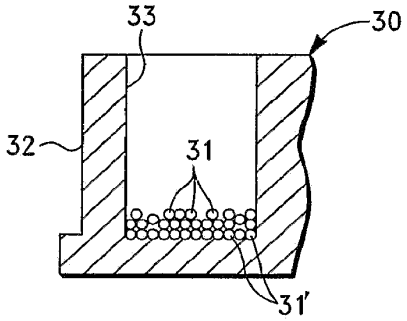


FIG.—3

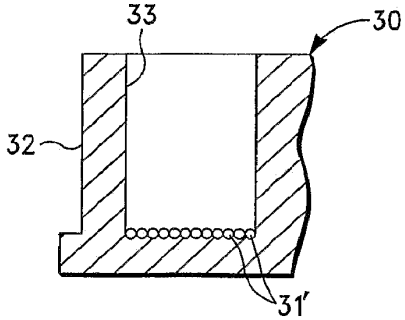


FIG.—4

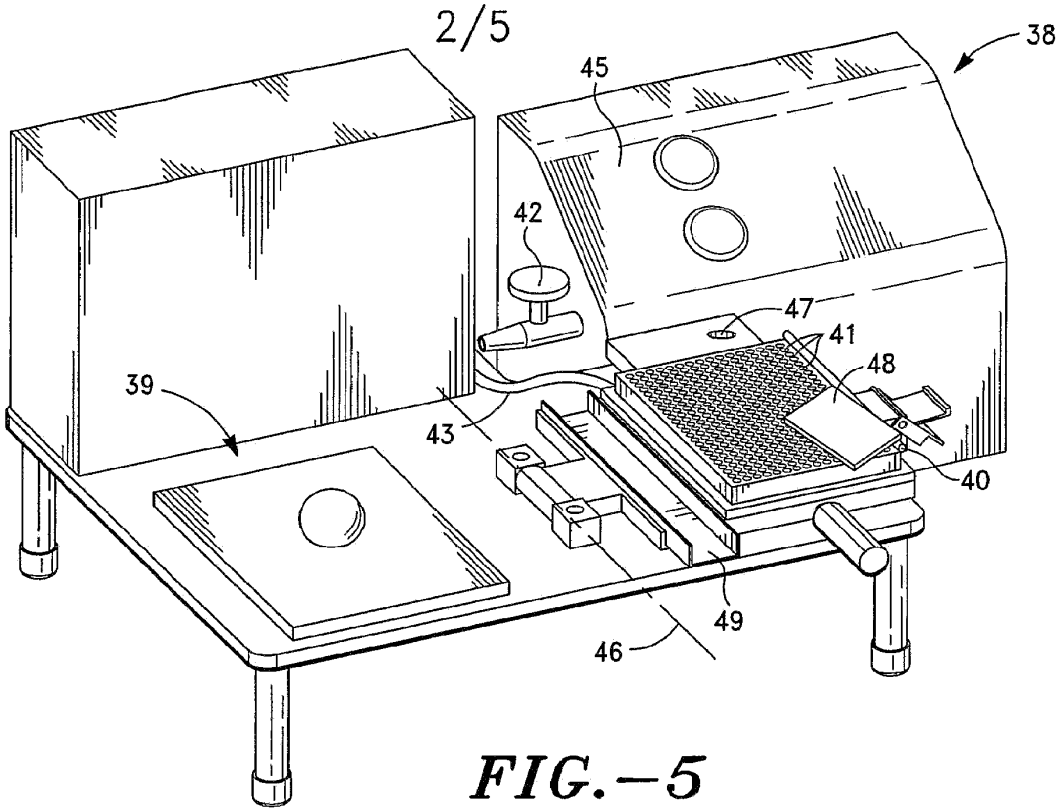


FIG.—5

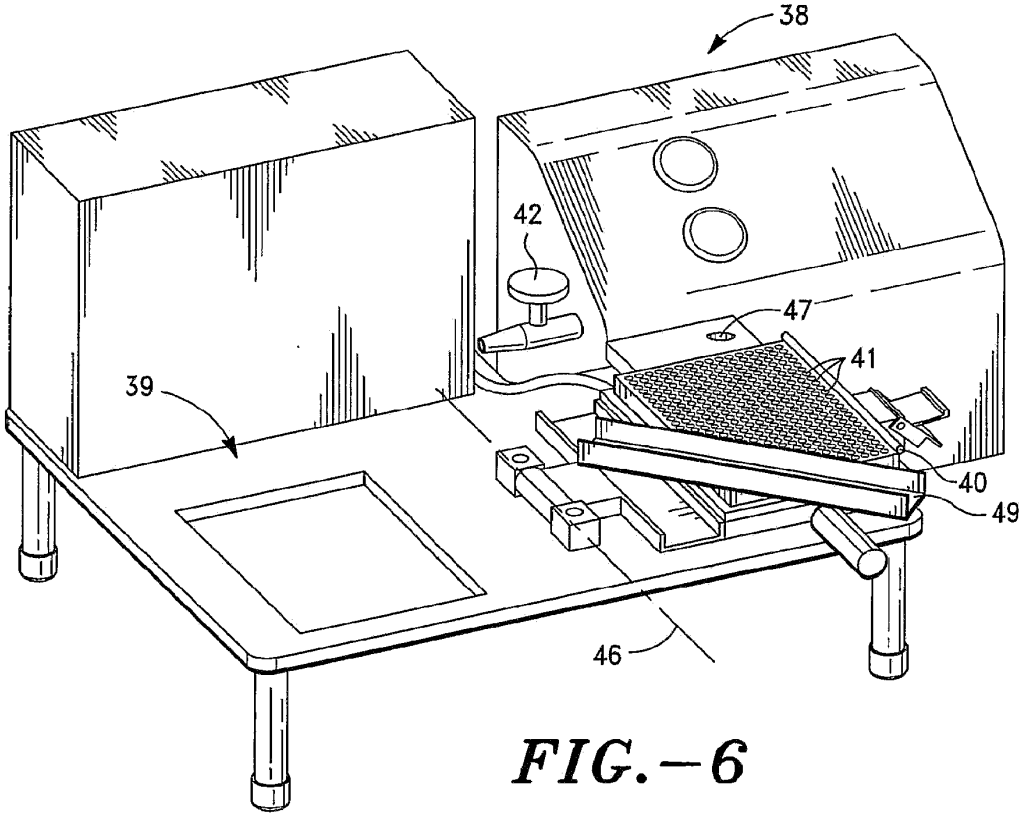


FIG.—6

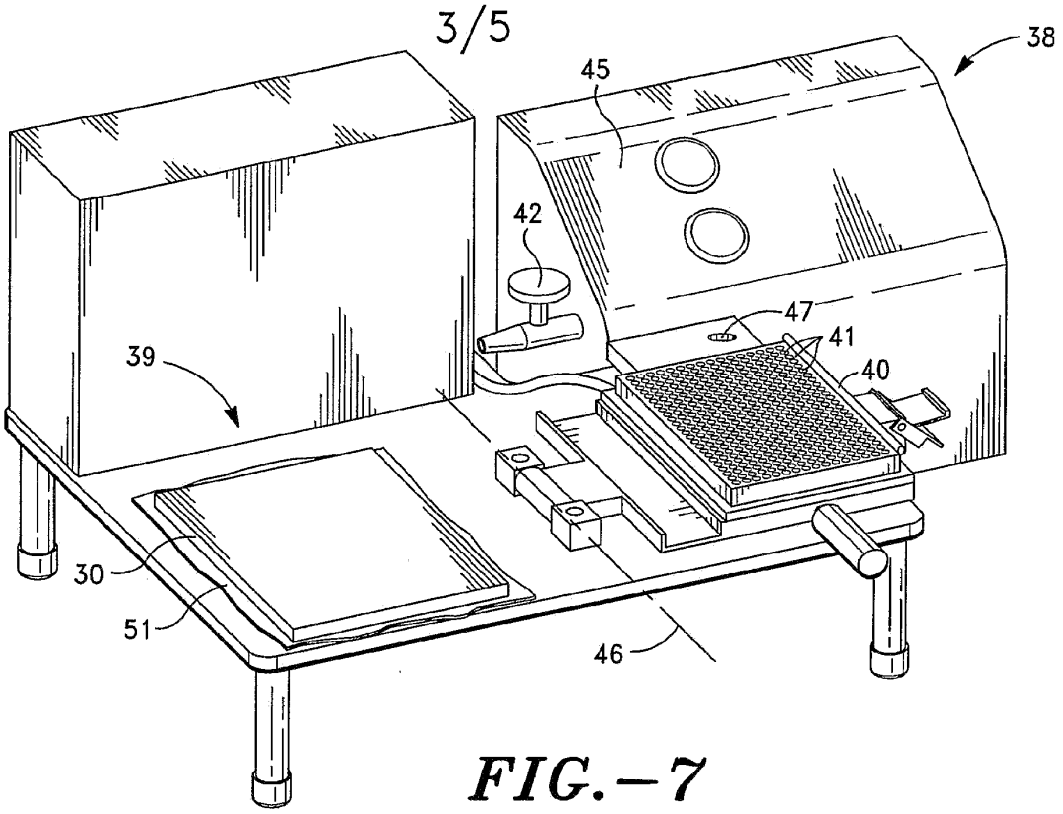


FIG.–7

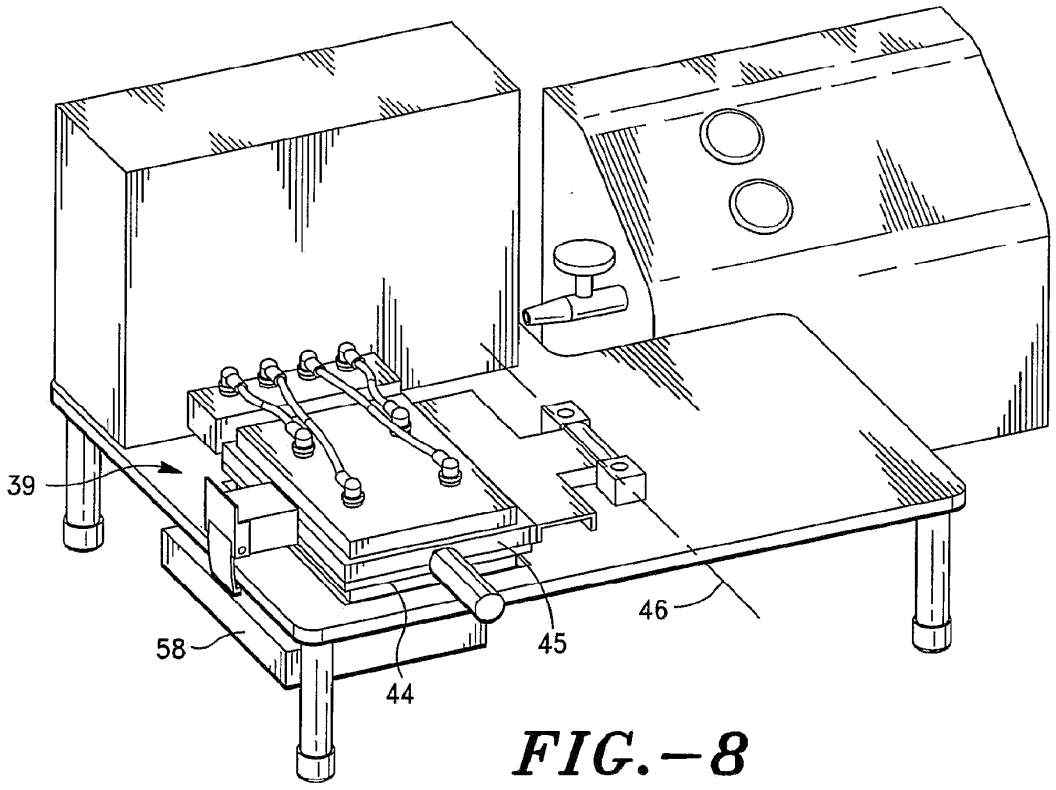


FIG.–8

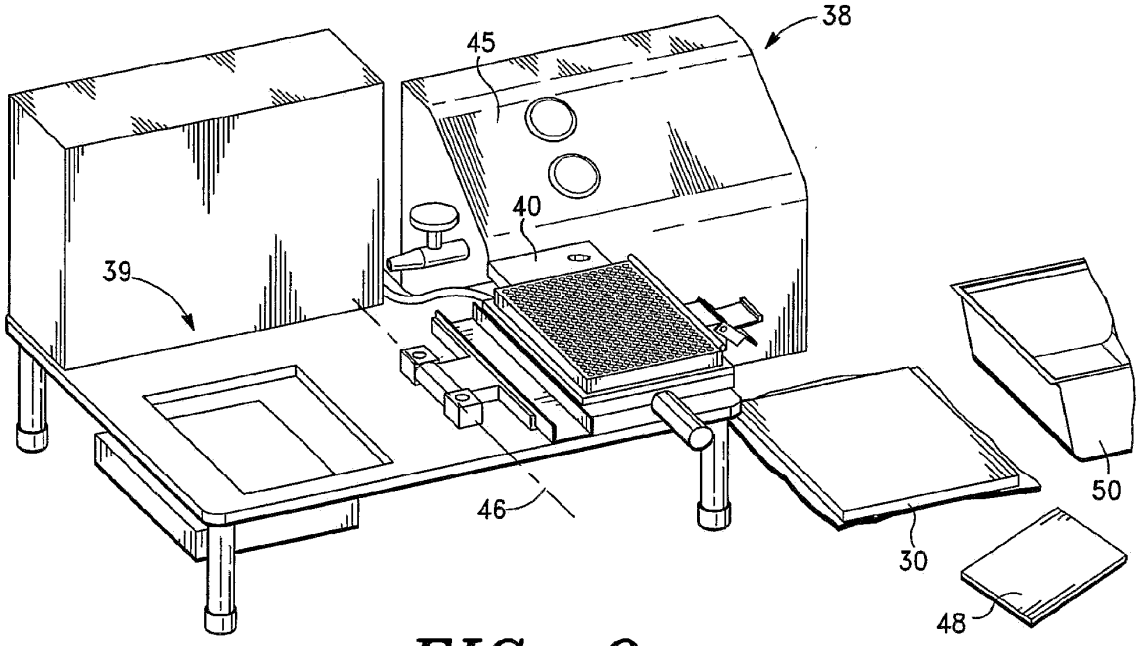


FIG.—9

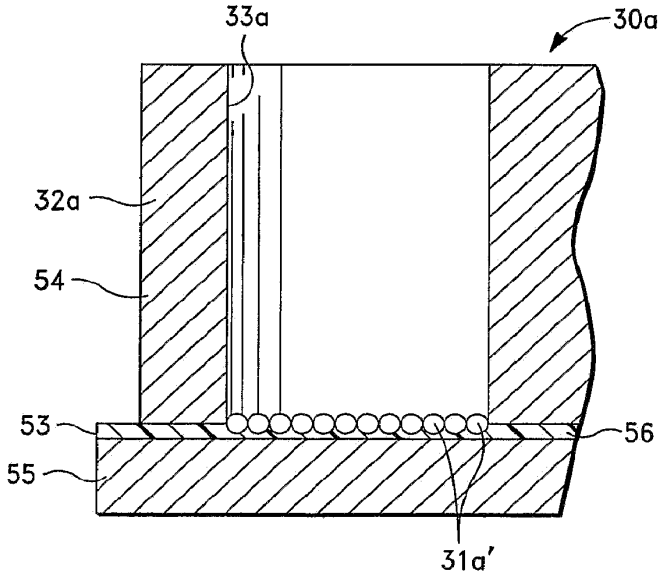


FIG.—10

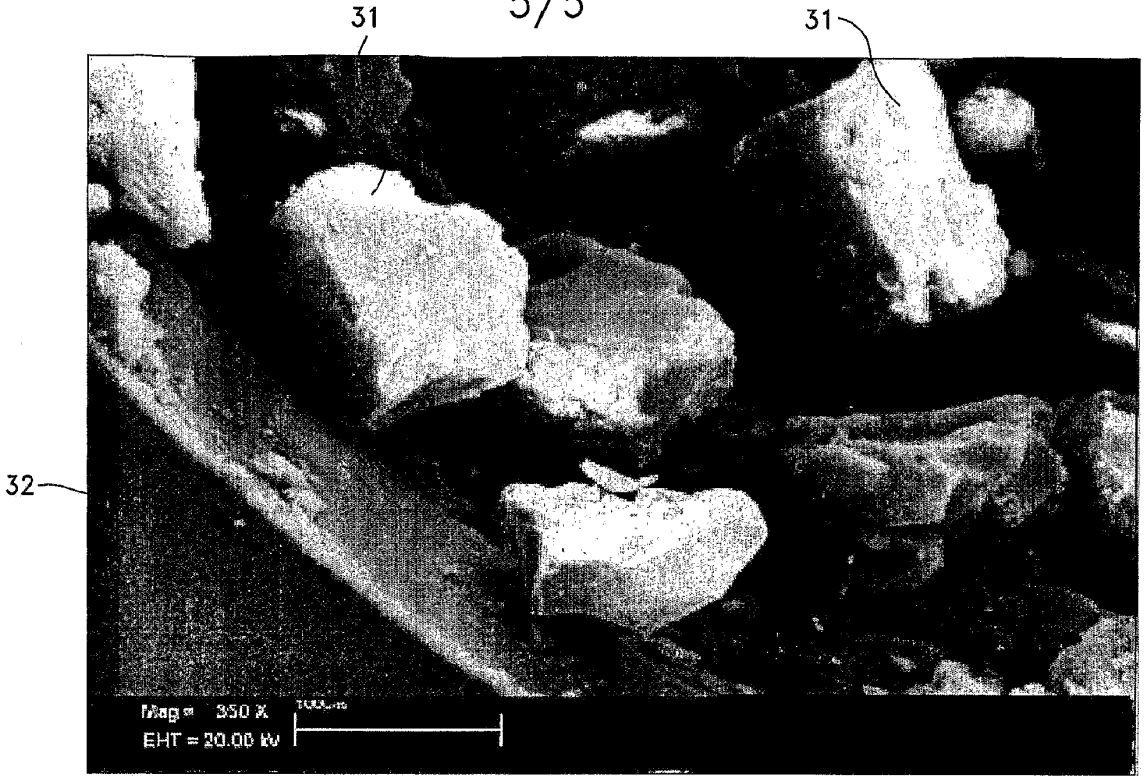


FIG.—11

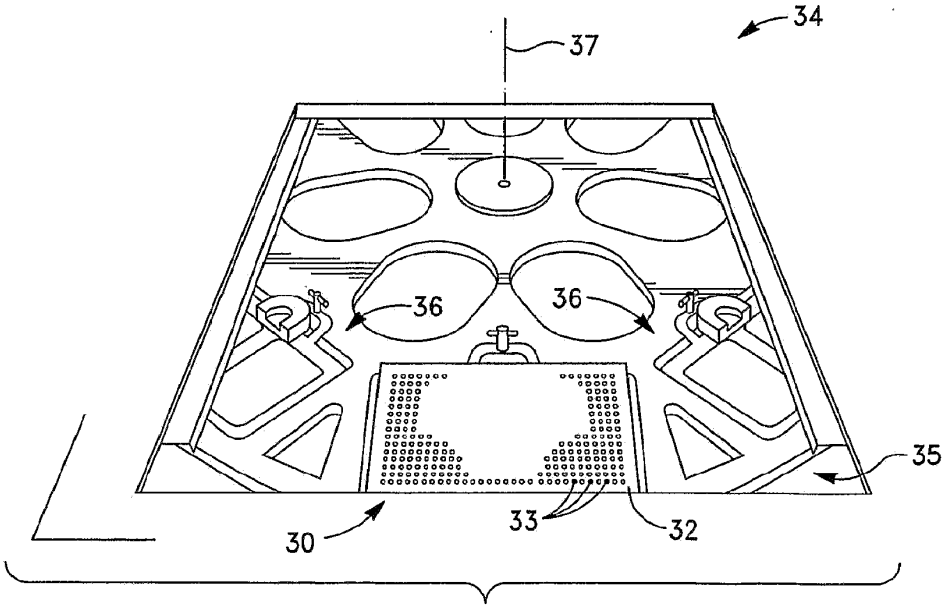


FIG.—12

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

[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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"E" earlier document but published on or after the international filing date

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

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

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

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

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

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

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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.
X	DE 199 20 156 A (GRAFFINITY PHARMACEUTICAL DESI) 20 January 2000 (2000-01-20)	1,2,4-11
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
PCT/US2004/027599

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03016868 A	27-02-2003	US 2002102578 A1	01-08-2002
		WO 03016868 A2	27-02-2003
		US 2004219590 A1	04-11-2004
DE 19920156 A	20-01-2000	DE 19920156 A1	20-01-2000
		AT 246959 T	15-08-2003
		AT 244068 T	15-07-2003
		AU 4139299 A	23-11-1999
		AU 4139399 A	23-11-1999
		DE 29907804 U1	07-10-1999
		DE 59906190 D1	07-08-2003
		DE 59906595 D1	18-09-2003
		DK 1075326 T3	27-10-2003
		WO 9956877 A1	11-11-1999
		WO 9956878 A1	11-11-1999
		EP 1073522 A1	07-02-2001
		EP 1075326 A1	14-02-2001
WO 03031054 A	17-04-2003	US 2003099949 A1	29-05-2003
		CA 2462868 A1	17-04-2003
		EP 1444034 A2	11-08-2004
		WO 03031054 A2	17-04-2003
WO 0160520 A	23-08-2001	AU 3683301 A	27-08-2001
		CA 2369690 A1	23-08-2001
		EP 1177044 A1	06-02-2002
		JP 2003522959 T	29-07-2003
		WO 0160520 A1	23-08-2001
WO 9925470 A	27-05-1999	US 6121054 A	19-09-2000
		AT 234149 T	15-03-2003
		AU 751424 B2	15-08-2002
		AU 1415499 A	07-06-1999
		CA 2309753 A1	27-05-1999
		DE 69812154 D1	17-04-2003
		DE 69812154 T2	08-01-2004
		EP 1032469 A1	06-09-2000
		JP 2001523550 T	27-11-2001
		WO 9925470 A1	27-05-1999

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30 September 2004 (30.09.2004)

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(26) Publication Language: English

(30) Priority Data:
60/507,243 30 September 2003 (30.09.2003) US

(71) Applicant *(for all designated States except US)*: **SINGULEX, INC.** [US/US]; 4041 Forest Park Avenue, St. Louis, Missouri 63108 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **PUSKAS, Robert, S.** [US/US]; 752 Sherwick Terrace, Manchester, Missouri 63021 (US).

(74) Agents: **BUCKLEY, Kevin, W.** et al.; Sonnenschein Nath & Rosenthal LLP, P.O. Box 061080, Wacker Drive, Station-Sears Tower, Chicago, IL 60606-1080 (US).

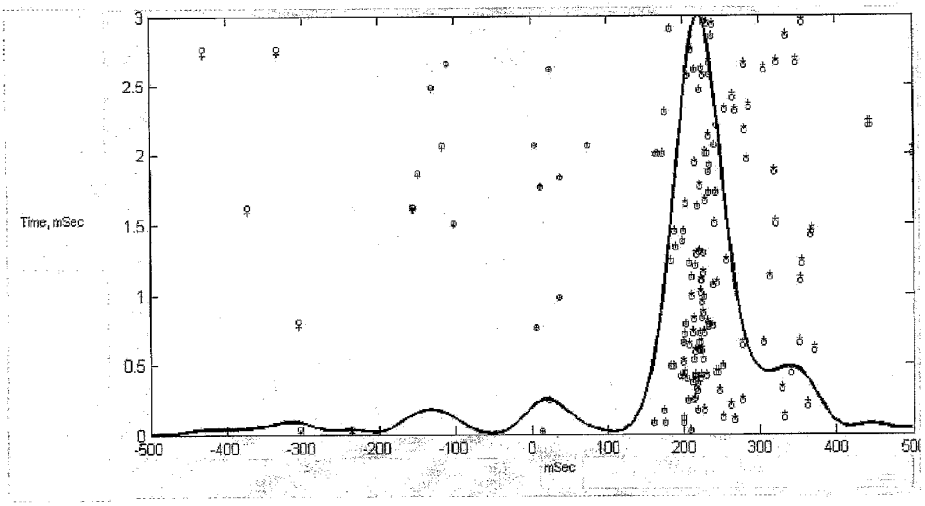
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(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

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:** METHODS FOR ENHANCING THE ANALYSIS OF PARTICLE DETECTION



(57) **Abstract:** Methods for enhancing the analysis of particle detection are provided comprising measuring a first electromagnetic radiation signal provided by a particle, comparing by cross-correlation the electromagnetic radiation signal emitted by the particle, and further applying an analytical filter to the cross-correlation events, thereby enhancing the analysis of the particle emission.

WO 2005/033283 A2

TITLE OF THE INVENTION

Methods for Enhancing the Analysis of Particle Detection

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application Serial No. 60/507,243 filed on September 30, 2003, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not Applicable.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ON A COMPACT DISC

Not Applicable.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates generally to detection and discrimination of individual particles at ultra-low concentrations in a flowing solution. Electromagnetic emission from the particles is detected as they move into two interrogation volumes and the data collected by detectors at each interrogation volume is analyzed by cross-correlation and application of analytical filters to distinguish particle signals from background.

2. Description of Related Art

Techniques for detecting lower and lower concentrations of biological molecules are needed as advances in biological sciences and medicine allow investigations to be made at the molecular level. For example, measurements of biomarkers that can drive clinical diagnosis often require ultra-sensitive detection of biomolecules. It is generally recognized that the most sensitive technology currently available for detection of molecules uses detection of fluorescence emission, and fluorescence-based single molecule detection ("SMD") has become a viable approach to the sensitive detection of biomolecules. The challenge of fluorescent SMD is to maximize the signal to background ratio while continuing to lower the detection limits. In addition to detection and quantification of single molecules, information to identify or discriminate between different molecules in a mixture is critical to the successful application of SMD technology. Using current SMD methods a number of artifacts or fluorescence from impurities in the sample can lead to erroneous interpretations. Enhanced methods of analysis are extremely useful for working with very small amounts of samples and for detecting low concentrations of substances.

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The first studies of fluorescence intensity fluctuations demonstrated the ability to measure fluctuations in the number of fluorescent molecules detected per unit time in a fixed volume. This established a technology called fluorescence correlation spectroscopy ("FCS"). FCS excels in providing information about dynamic processes over time in an ensemble of molecules, but can also be used for SMD. Basic instruments detect fluorescence emission from a small, open, static measurement volume. The measurement volume is defined by a focused laser beam, which excites the fluorescence, and by a pinhole in the image plane of the microscope collecting fluorescence. Fluorescence emissions are proportional to the number of fluorescent molecules present as they diffuse into and out of the measurement volume and as they are created or eliminated by the chemical reactions under observation. The detected fluorescence data are processed based on autocorrelation analysis. The disadvantage of autocorrelation is that random background is generally included in the analysis. In contrast, cross-correlation analysis, which requires data acquired from two detectors, allows signals from random background that are detected in only one detector but not both detectors to be eliminated.

Methods and apparatus for detecting and discriminating molecules have been described. In most cases, data analysis utilizes only one measurable characteristic of the target particles. While these analyses are sufficient for simple cases of detection and discrimination, they may not yield reliable results for samples composed of complex mixtures. Most targets possess multiple characteristics that can be measured and when those measurements are included in the data analysis, the power of the analysis will increase.

Instruments with dual detectors and flowing samples have been described, but while they enable use of cross-correlation analysis, the discrimination of target particles was based only on their velocity in the systems described. FCS generally distinguishes different particles according to their diffusion rate for static samples, or velocity for flowing samples. Fluorescence intensity distribution analysis ("FIDA"), uses the same type of data as FCS, but processes it in a different way to distinguish particles according to their specific brightness. Thus, both FCS and FIDA rely on measurement of only a single specific physical property.

Particle discrimination based only on fluorescence decay lifetime has been described. Methods and an apparatus that uses flowing samples are used to measure time-resolved fluorescence decay using a pulsed laser for particle illumination at a single location in the sample stream. While photon bursts are related to the laser pulse that created them, the discrimination of particles in this system is not related to the flow velocity of the particles.

In two-color fluorescence cross-correlation analysis discrimination is based on only emission wavelengths. These studies use a flowing sample and data collected by two detectors each measuring a different emission wavelength. Particles emitting one color, and

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detected by only one detector, are distinguished from particles emitting both colors and detected by both detectors. Similar techniques have been described using static samples and two detectors. Others have demonstrated that two detectors can be used to measure different emission wavelengths from a single interrogation volume. Sometimes referred to as coincidence analysis, these methods are rapid and accurate for target identification, but still utilize only one particle characteristic.

Methods that utilize more than one measurable characteristic for detecting and discriminating target molecules have also been described. Some have used methods for distinguishing between particles with similar spectroscopic properties based on one characteristic, burst size, but used another characteristic, intraburst fluorescence decay rate (fluorescence lifetime), to reduce background. A flowing sample with a single interrogation volume, detector, and pulsed laser use such methods. The resulting data is analyzed by correlating measurements of burst size and fluorescence lifetime to reduce background bursts and accidental coincidences. Fluorescence burst size and lifetime are similar spectroscopic properties that may both be subject to artifacts of the detection system which will limit the power of combining them for data analysis.

In another technology, fluorescence activated cell sorting (FACS) or flow cytometry, uses more than one parameter, such as fluorescence intensities at different wavelengths and light scattering in different directions, to distinguish target particles, but measurement of particle mobility cannot be utilized because particles move at uniform velocity.

Thus, what is needed are methods that utilize the measurement of multiple diverse properties of the target for increasing the reliability of the analysis of samples by accurately distinguishing between actual particles and general radiation background of the detection system.

BRIEF SUMMARY OF THE INVENTION

Accordingly, it is an object of the invention to overcome these and other problems associated with the related art. These and other objects, features and technical advantages are achieved by combining technologies that use two interrogation volumes through which a sample flows, cross-correlation analysis of the two streams of data collected from those interrogation volumes, and analytical filters to select events with a high probability of being produced by the target particles.

This invention provides a method for enhancing the analysis of particle detection comprising measuring a first electromagnetic radiation signal provided by a particle within a first interrogation volume and optionally applying a first analytical filter to the first electromagnetic radiation signal and measuring a second electromagnetic radiation signal emitted by the particle in a second interrogation volume and optionally applying a second

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analytical filter to the second electromagnetic radiation signal, comparing by cross-correlation the electromagnetic radiation signal emitted by the particle within the first interrogation volume to the electromagnetic radiation signal emitted by the particle within the second interrogation volume, and further applying a third analytical filter to the cross-correlation events, thereby enhancing the analysis of the particle detection.

In accordance with a further aspect of the invention, one of or both the first analytical filter and the second analytical filter are applied. In one alternative, both the first analytical filter and the second analytical filter are applied, and the first analytical filter and the second analytical filter are the same analytical filter. Preferably, the first and second analytical filters are selected from the group consisting of signals that are greater than a predetermined threshold level, signals within a predetermined number of adjacent time segments, and a combination thereof. In accordance with another aspect of the invention, applying the third analytical filter comprises detecting a particle characteristic selected from the group consisting of emission intensity, burst size, burst duration, fluorescence lifetime, fluorescence polarization, and any combination thereof.

In accordance with yet another aspect of the invention, the particle characteristic is provided by one of an intrinsic parameter of the particle or an extrinsic parameter of the particle. Preferably, the extrinsic parameter is provided by marking the particle with at least one label selected from the group consisting of a dye tag, a light-scattering tag, and any combination thereof.

In accordance with yet another aspect of the invention, the first analytical filter, the second analytical filter and the third analytical filter are applied before cross-correlating the first electromagnetic radiation signal and second electromagnetic radiation signal.

In accordance with a further aspect of the invention, the first and second interrogation volumes are in electromagnetic communication with at least one excitation source selected from the group consisting of a light-emitting diode, a continuous wave laser, and a pulsed laser.

In accordance with yet another aspect of the invention, the particle is selected from the group consisting of a polypeptide, a polynucleotide, a nanosphere, a microsphere, a dendrimer, a chromosome, a carbohydrate, a virus, a bacterium, a cell, and any combination thereof. In one alternative, the particle is selected from the group consisting of an amino acid, a nucleotide, a lipid, a sugar, a toxin, and any combination thereof. In another alternative, the particle is selected from the group consisting of an aggregate, a complex, an organelle, a micelle, and any combination thereof.

In accordance with a further aspect of the invention, the method comprises moving a target particle through the first interrogation volume and through the second interrogation volume by a force selected from the group consisting of electro-kinetic force, pressure

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difference, osmotic difference, ionic difference, gravity, surface tension, centrifugal force, a magnetic field, an optical field, and any combination thereof.

In accordance with a further aspect of the invention, the target particle is one of a population of different particles. In one alternative, the target particle is moved through the first interrogation volume and through the second interrogation volume with the population of different particles at a uniform velocity by a force selected from the group consisting of positive pressure, negative pressure, gravity, surface tension, inertial force, centrifugal force, and any combination thereof. In another alternative, the target particle is moved through the first interrogation volume and through the second interrogation volume with the population of different particles at a different velocity by a force selected from the group consisting of electro-kinetic force, centrifugal force, a magnetic force, an optical force, and any combination thereof. Preferably, the target particle mobility is determined by an intrinsic parameter of the particle or an extrinsic parameter of the particle. Still more preferably, the extrinsic parameter of the target particle is provided by a label selected from the group consisting of a charge tag, a mass tag, a charge/mass tag, a magnetic tag, an optical tag, and any combination thereof.

In accordance with yet another aspect of the invention, the electromagnetic radiation signal is selected from the group consisting of stimulated emission, fluorescence, elastic light scattering, inelastic light scattering, and any combination thereof.

In accordance with yet another aspect of the invention, the electromagnetic radiation signal passes through an optical band pass filter within an image plane of a detector. Preferably, the optical band pass filter enables differential detection of emission spectra.

In accordance with yet another aspect of the invention, the analysis comprises multiple passes through the processes of applying analytical filters and comparing the electromagnetic radiation signal emitted by the particle within the first interrogation volume to the electromagnetic radiation signal emitted by the particle within the second interrogation volume.

These and other features, aspects and advantages of the present invention will become better understood with reference to the following description, examples and appended claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1. Schematic diagram of the basic apparatus for single molecule detection using laser induced fluorescence.

Figure 2. Schematic diagram of the interrogation chamber for the single molecule analyzer.

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Figure 3. Panel shows linearized pUC19 at 7.5 fM in PBS with 0.01% casein hydrolysate pumped through the analyzer at 1 ml/min. Initial cross-correlation of the data revealed no discernable peaks.

Figure 4. Panel shows linearized pUC19 at 7.5 fM in PBS with 0.01% casein hydrolysate pumped through the analyzer at 1 ml/min. Restricting the cross-correlations to only those events with a brightness of 15-500 allowed for detection of a dominant peak at around 80 ms. Panels C and D shows the plot of elapsed time vs. time of sample run with a 7.2 kb DNA fragment labeled with Alexa Fluor®647 subjected to electrophoresis at 3000V for 60 seconds in 0.2x TB, 0.01% SDS.

Figure 5. Panel shows linearized pUC19 at 7.5 fM in PBS with 0.01% casein hydrolysate pumped through the analyzer at 1 ml/min. The shoulder on the peak is composed of events that occurred primarily in the last half of the sample run (dot density is higher near the top of the chart), suggesting a change in the electrophoresis system with time.

Figure 6. Panel shows linearized pUC19 at 7.5 fM in PBS with 0.01% casein hydrolysate pumped through the analyzer at 1 ml/min. Restricting the data set based on time shows the same data excluding the last 30 seconds of the run. The resulting histogram shows a single peak primarily without the shoulder that was an artifact of changes in the electrophoresis system.

Figure 7. Fluorescence brightness is plotted against the elapsed time between detectors. Each spot represents measurements taken on a single molecule. The scale of the x-axis (elapse time) was restricted to emphasize the events that occur within the peak. A separation value of 500 photons was used to divide the bright window from the dim window. PBXL-3 molecules (this figure) emit at a higher average intensity than the pUC19 molecules (Figure 8).

Figure 8. Fluorescence brightness is plotted against the elapsed time between detectors. Each spot represents measurements taken on a single molecule. The scale of the x-axis (elapse time) was restricted to emphasize the events that occur within the peak. A separation value of 500 photons was used to divide the bright window from the dim window. PBXL-3 molecules (Figure 7) emit at a higher average intensity than the pUC19 molecules (This figure).

Figure 9. The measured values of the concentration of PBXL-3 and pUC19 components in mixtures are compared to the predicted values.

Figure 10. Figure 5. A sample that contains a protein and nucleic acid, both labeled with Alexa Fluor®647. The broadest peak width analytical filter (0-5 bins) was optimal for detecting the two peaks, demonstrating discrimination based on both the electrophoretic velocity and the peak width.

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Figure 11. A sample that contains a protein and nucleic acid, both labeled with Alexa Fluor®647. When the analysis was performed with narrower peak width analytical filters, only one peak is seen, a faster moving peak corresponding to the protein (0-1 bins) and

Figure 12. A sample that contains a protein and nucleic acid, both labeled with Alexa Fluor®647. When the analysis was performed with narrower peak width analytical filters, a slower moving peak corresponding to the nucleic acid (1-5 bins).

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Definitions

To facilitate understanding of the invention, a number of terms and abbreviations as used herein are defined below as follows:

Analytical filter: As used herein, the term "analytical filter" refers to methods where the measured electromagnetic signals or events that are identified by cross-correlation are compared to criteria that are known to match the characteristics of the target particle. When the filter is applied, only those signals or events that meet the criteria of the filter are selected, counted, and used in the analysis results.

Background: As used herein, the term "background" refers to signals that are detected, but do not originate from a target particle in the sample. Sources of background signals include Raman and Rayleigh scattering from solutes, scattering from the capillary walls, and fluorescent contaminants within the sample.

Band pass filter: As used herein, the term "band pass filter" refers to an optical filter that allows transmission of a specific range of frequencies and rejects frequencies both above and below that range.

Binding partner(s): As used herein, the term "binding partners" refers to macromolecules that combine through molecular recognition to form a complex. Molecular recognition involves topological compatibility or the matching together of interacting surfaces on each partner. The partners can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other. Binding forces can be hydrophobic, hydrophilic, ionic, hydrogen, covalent, hybridization, induced fit, polarizing, induced polarization, and intercalation. Examples of binding partners are antigen/antibody, oligonucleotide/nucleic acid, inhibitor/enzyme, and ligand/receptor.

Bins: As used herein, the term "bins" refers to uniform arbitrarily chosen time segments that are used to divide the electromagnetic radiation signals that are recorded in each detector channel. Bin widths are typically in the range of 1 μs to 5 ms.

Brightness: As used herein, the term "brightness" refers to total number of photons detected within a peak of emission that consists of adjacent time segments (bins) where the

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number of photons is above the average background number of photons. A synonymous term is fluorescence burst size.

Charge tags: As used herein, the term "charge tag" refers to any entity bearing a charge that when bound to or associated with the target distinguishes the charge tag+target from the target alone based on detection of the mass, charge, or charge to mass ratio. A charge tag can be a label.

Charge/mass tags: As used herein, the term "charge/mass tag" refers to any charge and mass added to the target that serves to distinguish the charge/mass tag+target from the target alone based on detection of the mass, charge, or charge to mass ratio. A charge/mass tag can be a label.

Cross-correlation: As used herein, the term "cross-correlation" involves subjecting two data sets g_j and h_k to analysis, whereby data sets from each detector (preferably photon detectors) are subjected to the following formula:

$$Corr(g,h)_j \equiv \sum_{k=0}^{N-1} g_{j+k} h_k,$$

for j= -(N-1), -(N-2), ...,-1,0,1, ..., N-1

where N is the total number of data points. The data cross-correlations will be large at values of j where the first data set from a detector (preferably photon counts above a background level) (g) resembles the data set (h) from a second detector (preferably above a background level) at some lag time (j) that corresponds to the time for specific particles to pass from the first detector to the second detector (preferably in a single molecule analytical system). In a single molecule electrophoresis instrument with an electric field applied to the sample, the lag time (j) for detection between photon detectors arrayed along the length of capillary is related to the electrophoretic velocity of a detected particle.

Dye: As used herein, the term "dye" refers to a substance used to color materials or to enable generation of luminescent or fluorescent light. A dye may absorb light or emit light at specific wavelengths. A dye may be intercalating, noncovalently bound or covalently bound to a target. Dyes themselves may constitute labels that detect minor groove structures, cruciforms, loops or other conformational elements of molecules. Dyes may include BODIPY and ALEXA dyes, Cy[n] dyes, SYBR dyes, ethidium bromide and related dyes, acridine orange, dimeric cyanine dyes such as TOTO, YOYO, BOBO, TOPRO POPRO, and POPO and their derivatives, bis-benzimide, OliGreen, PicoGreen and related dyes, cyanine dyes, fluorescein, LDS 751, DAPI, AMCA, Cascade Blue, CL-NERF, Dansyl, Dialkylaminocoumarin, 4',5'-Dichloro-2',7'-dimethoxyfluorescein, 2',7'-Dichlorofluorescein, DM-NERF, Eosin, Erythrosin, Fluoroscein, Hydroxycourmarin, Isosulfan blue, Lissamine rhodamine B, Malachite green, Methoxycoumarin, Naphthofluorescein, NBD, Oregon Green,

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PyMPO, Pyrene, Rhodamine, Rhodol Green, 2',4',5',7'-Tetrabromosulfonefluorescein, Tetramethylrhodamine, Texas Red, X-rhodamine. Additional fluorophore families include Dyomics series, Atto tec series, coumarins, macromolecular, phycobilliproteins (including phycoerythrins, phycocyanins, and allophycocyanins), green, yellow, red, and other fluorescent proteins, up-converting phosphors, and Quantum Dots. Those skilled in the art will recognize other dyes which may be used within the scope of the invention. This list includes but is not limited to all dyes now known or known in the future which could be used to allow detection of the labeled polypeptide or polynucleotide of the invention.

Elapsed time: As used herein, the term "elapsed time" refers to the number of seconds, or partial seconds, *e.g.*, milliseconds (ms), required for particles to travel the distance between two interrogation volumes. Synonymous terms are transit time, time-offset, and inverse velocity.

Electrophoretic Velocity: As used herein, the term "electrophoretic velocity" refers to the velocity of a charged target under the influence of an electric field relative to the background electrolyte. Net velocity in a capillary system may be a composite measure of electrokinetic velocity and electroosmotic velocity.

Emission: As used herein, the term "emission" refers to radiation generated by a molecule or particle in processes such as fluorescence and elastic or inelastic (*e.g.*, Raman) light scattering.

Emission wavelength: As used herein, the term "emission wavelength" refers to the spectrum of the photons that are released during emission and measured by the detectors used in the analysis instrument. For polyatomic particles in solution, fluorescent photon emissions occur over a spectrum typically in the range of 100-150 nm. A selected subset of the spectrum is allowed to pass to the detectors by the optical band pass filters used in the instruments. Labels that are detected in the same spectral range are considered to have the same emission wavelength.

Event: As used herein, the term "event" refers to a cross-correlated signal. Events may or may not be the result of fluorescence from a target particle. Events are considered to be of interest if they meet additional criteria known to match the characteristics of the target particle.

Fluid: As used herein, the term "fluid" is a medium wherein particles are suspended and move. It can be gaseous, aqueous, non-aqueous, or any combination thereof. In some cases, it can have an electric field or conduct an electrical current. It may further contain salts, ions, polymers, macromolecules, or other agents that can interact with the polypeptides or polynucleotides and influence their movement.

Fluorescence: As used herein, the term "fluorescence" refers to the photons of energy that are emitted as an excited fluorophore returns to its ground state. The energy of

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the emitted photon is usually, but not always lower, and therefore of longer wavelength, than the excitation photon.

Fluorescence Burst Duration: As used herein, the term "fluorescence burst duration" refers to the period of time during which an emission event is detected. A synonymous term is peak width.

Fluorescence Intensity: As used herein, the term "fluorescence intensity" refers to the total number of photons measured during a single time segment (e.g., over a millisecond and above a background level).

Fluorescence Lifetime: As used herein, the term "fluorescence lifetime" refers to the time required by a population of N excited fluorophores to decrease exponentially to N/e by losing excitation energy through fluorescence and other deactivation pathways.

Fluorescence Polarization: As used herein, the term "fluorescence polarization" refers to the property of fluorescent particles in solution that are excited with plane-polarized light and emit light back into a fixed plane (*i.e.*, the light remains polarized) if the particles remain stationary during the excitation and emission cycle of the fluorophore.

Interrogation volume: As used herein, the term "interrogation volume" is the space, through which at least one particle may traverse, that is illuminated by the illumination source and observed, sensed or otherwise detected by the detectors.

Label: As used herein, the term "label" refers to an entity that, when attached to the target particle of the invention, alters measurable parameters of the particle such as its electromagnetic emission or its electrophoretic velocity. Exemplary labels include but are not limited to fluorophores, chromophores, radioisotopes, spin labels, enzyme labels, mass tags, charge tags, and charge/mass tags. Such labels allow detection of labeled compounds by a suitable detector. In addition, such labels include components of multi-component labeling schemes, *e.g.*, a system in which a target binds specifically and with high affinity to a detectable binding partner, *e.g.*, a labeled antibody binds to its corresponding antigen. Herein, label and "tag" are used synonymously.

Light Scattering: As used herein, the term "light scattering" refers to processes by which photons change directions. It includes both elastic light scattering where photons change direction without changing their wavelength and inelastic scattering where the scattered radiation has a different (normally lower) energy from the incident radiation.

Mass tags: As used herein, the term "mass tag" refers to any mass added to the target that serves to distinguish the mass tag+target from the target alone based on detection of the mass, or charge to mass ratio. A mass tag can be a label.

Particle: As used herein, the term "particle" means an entity that can be detected, counted and/or discriminated in the current invention. Examples of particles are proteins, nucleic acids, nanospheres, microspheres, aggregates, dendrimers, organelles,

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chromosomes, carbohydrates, micelles, viruses, bacteria, cells, prions, and chemical entities (such as amino acids, nucleotides, lipids, sugars, toxins, venoms, drugs, reaction products and substrates).

Sample: As used herein, the term "sample" shall mean a contiguous volume containing at least one detectable particle. This term shall include, but shall not be limited to, detecting the particle in one sample run. The term "sample" also refers to the volume that contains only the detectable labels in the case when they are released from the original target particles, and are analyzed in the released state.

Signal: As used herein, the term "signal" refers to the output of a detection system that measures the electromagnetic radiation from a fluorescing particle.

SMD: As used herein, the term "SMD" refers to single molecule detection.

Target: As used herein, the term "target" refers to the particle to be detected in an assay. This term is also known in the art as an analyte.

Methods for Enhancing the Analysis of Particle Detection

The invention provides methods for increasing the reliability of the analysis of samples by accurately distinguishing between actual particles and radiation background. This is accomplished by combining technologies that use two interrogation volumes through which a sample flows, cross-correlation analysis of the two streams of data collected from those interrogation volumes, and analytical filters to select events with a high probability of being produced by the target particles.

The methods of the invention strive to increase the reliability of SMD data analysis by distinguishing between target particles, contaminants, and general noise of the detection system. In addition, the invention allows for increased accuracy of discrimination between particles in a mixture by a novel series of steps taken for data analysis. These analyses result in determining a particle's mobility through cross-correlation of particle emissions measured independently in two interrogation volumes, and identifying true cross-correlation events by applying analytical filters based on electromagnetic emission characteristics together with the cross-correlation analysis.

The present invention is an important step in the advancement of SMD. Biological sciences and medicine are driving development of methods for detection of particles to lower and lower levels. The ultimate detection level desired is that of individual particles, interactions between individual particles, and individual complexes of particles. Applications for ultrasensitive detection include monitoring for bioterror agents, medical application such as in the detection of drugs of abuse, biomarkers for therapeutic dosage monitoring, health status, donor matching for transplantation purposes, pregnancy, and detection of disease,

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pathogens, and the like, and applications in environmental, ecological, and industrial monitoring, manufacturing process monitoring and food safety.

Achieving the goal of single particle detection is within the scope of laser-induced detection systems; however, the lower the detection level, the more challenging it is to maximize the signal to background ratio. Depending on the source of the background, various methods have been implemented to reduce background radiation such as using very small interrogation volumes, specific band pass filters, pulsed lasers with time-gated detection, and near-infra red emission and detection. Methods of data analysis can also be used to discriminate true signals from background. The current invention uses a data analysis process to enhance the sensitivity and accuracy of single particle detection. The process combines cross-correlation analysis with methods for filtering based on electromagnetic radiation characteristics to increase the discrimination power of the analysis.

The samples used in the invention contain target particles. Such particles include molecules and organisms. Examples of molecular particles include biopolymers such as proteins, nucleic acids, carbohydrates, and small molecule chemical entities. Chemical entities encompass small molecules such as amino acids, nucleotides, lipids, sugars, drugs, toxins, venoms, substrates, reaction products, pharmacophores, and any combination thereof. Other examples of particles include nanospheres, microspheres, dendrimers, chromosomes, organelles, micelles and carrier particles. Examples of organelles include subcelluar particles such as nuclei, mitochondria, and endosomes. Examples of organisms include viruses, bacteria, fungal cells, animal cells, plant cells, eukaryotic cells, prokaryotic cells, archeobacteria, prions, and any combination thereof. Also included are particles composed of complexes of molecules, organisms with labels bound, complexes of two or more nucleic acids, and complexes of target particles bound to one or more antibodies or antibody fragments. Also included are complexes where two or more types of single particles are detected, such as any particles selected from the list of protein, receptor, DNA, RNA, pNA, LNA, carbohydrate, organelle, virus, cell, bacterium, fungus, or fragments thereof, combined with any or all in the list and/or any or all combinations thereof.

In one embodiment, chemical entities includes naturally occurring hormones, naturally occurring drugs, synthetic drugs, pollutants, allergens, effecter molecules, growth factors, chemokines, cytokines, lymphokines, amino acids, oligopeptides, chemical intermediates, nucleotides, and oligonucleotides.

In another embodiment, particles include labels that were bound to target particles, separated from unbound labels, and interacted with an agent causing the release of the bound labels. These released labels can be considered as particles, and analyzed by the methods of the current invention, thereby indirectly detecting the original target particle.

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Of particular interest is detection of microorganisms and cells, including viruses, prokaryotic and eukaryotic cells, unicellular and multicellular organism cells, *e.g.*, fungi, animal, mammal, or fragments thereof. The methods of the invention may also be used for detecting pathogens. Pathogens of interest may be, but are not limited to, viruses such as *Herpesviruses*, *Poxviruses*, *Togaviruses*, *Flaviviruses*, *Picornaviruses*, *Orthomyxoviruses*, *Paramyxoviruses*, *Rhabdoviruses*, *Corona viruses*, *Arenaviruses*, and *Retroviruses*. They may also include bacteria including but not limited to *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Staphylococcus epidermidis*, *Serratia marcescens*, *Mycobacterium bovis*, methicillin resistant *Staphylococcus aureus* and *Proteus vulgaris*.

The examples of such pathogens are not limited to those listed above, and one skilled in the art will know which specific species of microorganisms and parasites are of particular importance. The non-exhaustive list of these organisms and associated diseases can be found for example in U.S. Patent Number 5,795,158 issued to Warinner and incorporated herein by reference in its entirety.

Particles of the invention can be obtained from biological specimens, including separated or unfiltered biological liquids such as urine, cerebrospinal fluid, pleural fluid, synovial fluid, peritoneal fluid, amniotic fluid, gastric fluid, blood, serum, plasma, lymph fluid, interstitial fluid, tissue homogenate, cell extracts, saliva, sputum, stool, physiological secretions, tears, mucus, sweat, milk, semen, seminal fluid, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and abscesses, and extracts of tissues including biopsies of normal, malignant, and suspect tissues or any other constituents of the body which may contain the target particle of interest. Other similar specimens such as cell or tissue culture or culture broth are also of interest. The test sample can be pre-treated prior to use, such as preparing plasma from blood, diluting viscous fluids, or the like; methods of treatment can involve filtration, distillation, concentration, inactivation of interfering compounds, and the addition of reagents.

Multiple particle assays

In one embodiment, several types of particles may be detected and discriminated in the same sample. Examples of combinations of particles that are of special interest for the applications of the invention include an infectious agent/ antibody to the agent, an infectious agent/nucleic acid/toxin, cancer cell/dysregulated protein, mRNA /corresponding protein transcript, gene(DNA)/message(RNA), gene(DNA)/protein, virus/toxin, bacterium/toxin, enzyme/substrate, and enzyme/product.

Reactive particles may be analyzed through their interaction with specific ligands, cofactors, agonists or antagonists. Furthermore, enzymes in solution may be detected by

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monitoring changes (electrophoretic velocity, brightness, or other properties) of the substrate of the enzyme or of a substance that interacts with the substrate. In another embodiment, multiple particles of the same type are discriminated in the same sample. For example, mixtures of nucleic acid fragments of varying length or sequence, or mixtures of proteins with different sizes or charge to mass ratio can be discriminated.

Particles must provide, directly or indirectly, electromagnetic radiation to be detected. The electromagnetic radiation may be an intrinsic property of the particle, an extrinsic property of the particle, or a combination thereof. Examples of intrinsic properties can include fluorescence, and light scattering. A particle may possess more than one intrinsic property that renders it detectable. Extrinsic properties are those that are provided by a label when it is attached to the particle. Labels are applied before, after, or simultaneously with positioning the particle into the interrogation fluid. Once a particle is detectably labeled, any suitable means of detection that are known in the art can be used. Different characteristics of the electromagnetic radiation may be detected including: emission wavelength, emission intensity, burst size, burst duration and fluorescence polarization. The only proviso is that the means of detection can be used in accordance with an SMD instrument such as that provided in U.S. Patent No. 4,793,705, incorporated herein by reference in its entirety. A particle may be detectable based on any combination of intrinsic and extrinsic properties. Preferably, the means of detection is a fluorescent label.

In one aspect of the invention, photons are counted from samples emitting fluorescent light. However, it may be desirable in some embodiments to monitor photon counts of origin other than fluorescence, such as light scattering or Raman radiation.

In a preferred embodiment, the emitted radiation is monitored in terms of the numbers of photons counted in consecutive time intervals. In another preferred embodiment emissions are monitored in terms of time of arrival of photons at the two detectors. In a further preferred embodiment, emissions are monitored in terms of time intervals between consecutive photon bursts.

The labeling of the particle with a means of detection is within the ordinary skill in the art. Attaching labels to particles can employ any known means including attaching directly or by means of binding partners. In some cases, the method of labeling is non-specific, for example, a method that labels all nucleic acids regardless of their specific nucleotide sequence. In other cases, the labeling is specific, as in where a labeled oligonucleotide binds specifically to a target nucleic acid sequence. Specific and non-specific labeling techniques will be discussed in more detail in the following sections.

Labels include dye tags, charge tags, mass tags, quantum dots, or beads, magnetic tags, light scattering tags, polymeric dyes, dyes attached to polymers. Dyes include a very large category of compounds that add color to materials or enable generation of luminescent

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or fluorescent light. A dye may absorb light or emit light at one or more wavelengths. A dye may be intercalating, or be noncovalently or covalently bound to a particle. Dyes themselves may constitute probes such as dye probes that detect minor groove structures, cruciforms, loops or other conformational elements of particles. In one embodiment, the label may be non-fluorescent in the unbound state, but become fluorescent through changes that occur in the molecule when it binds to the target particle.

By having fluorescent markers, such as fluorescent particles, fluorescent conjugated antibodies, or the like, the sample may be irradiated with light that is absorbed by the fluorescent particles and the emitted light measured by light measuring devices.

Useful light scattering tags include metals such as gold, selenium and titanium oxide, as well as nanoclusters of materials, such as ceramics or metals. Certain microspheres or beads can also be used as light scattering tags.

In yet another aspect, the labels affect the electrophoretic velocity and/or separation of target particles of identical or different sizes. These labels are referred to as charge/mass tags. Attachment of a label can alter the ratio of charge to translational drag of the target particles in a manner and to a degree sufficient to affect their electrophoretic mobility and separation in sieving or non-sieving media. In another embodiment, the label alters the charge, or the mass, or a combination of charge and mass. The charge/mass tag bound to a particle can be discriminated from the unbound particle or unbound tag by virtue of spatial differences in their behavior in an electric field or by virtue of velocity differences in their behavior in an electric field.

Polysaccharide coated paramagnetic microspheres or nanospheres are used to label particles. U.S. Pat. No. 4,452,773, incorporated herein by reference in its entirety, describes the preparation of magnetic iron-dextran beads and provides a summary describing the various means of preparing particles suitable for attachment to biological materials. A description of polymeric coatings for magnetic particles used in high gradient magnetic separation (HGMS) methods is found in DE 3720844 and U.S. Patent No. 5,385,707, both incorporated herein by reference in their entirety. Methods to prepare paramagnetic beads are described in U.S. Pat. No. 4,770,183, incorporated herein by reference in its entirety. The exact method for attaching the bead to the particle is not critical to the practice of the invention, and a number of alternatives are known in the art. The attachment is generally through interaction of the particle with a specific binding partner that is conjugated to the coating on the bead and provides a functional group for the interaction. Antibodies are examples of binding partners. Antibodies may be coupled to one member of a high affinity binding system, *e.g.*, biotin, and the particles attached to the other member, *e.g.*, avidin. One may also use secondary antibodies that recognize species-specific epitopes of the

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primary antibodies, *e.g.*, anti-mouse Ig, anti-rat Ig. Indirect coupling methods allow the use of a single magnetically coupled entity, *e.g.*, antibody and avidin, with a variety of particles.

In one application of this technique, the target particle is coupled to a magnetic tag and suspended in a fluid within a chamber. In the presence of a magnetic field supplied across the chamber, the magnetically labeled target is retained in the chamber. Materials which do not have magnetic labels pass through the chamber. The retained materials can then be eluted by changing the strength of, or by eliminating, the magnetic field. The chamber across which the magnetic field is applied is often provided with a matrix of a material of suitable magnetic susceptibility to induce a high magnetic field locally in the chamber in volumes close to the surface of the matrix. This permits the retention of weakly magnetized particles and the approach is referred to as high gradient magnetic separation.

Optical tags are well known to one skilled in the art and include any entity that augments the optical properties of a target particle when bound to that particle. Examples are beads, quantum dots, or other molecules that might affect properties such as reflectivity or absorbance.

In a further embodiment of the invention, the extrinsic properties that render the particle detectable are provided by at least two labels of characterized photon yield. For example, the target particle is labeled with two or more labels and each label is distinct due to detected emission at one or more wavelengths that is distinguishable from the emission of the other label(s). In this example, the particle is distinguished from free label by the ratio of detected emission at two or more wavelengths. In another example, the particle is labeled with two or more labels and at least two of the labels emit at the same wavelength. In this example particles are distinguished based on the difference in the intensity of the detected fluorescence produced by emission from the two, three, or more labels attached to each particle.

In another embodiment, the dyes have the same or overlapping excitation spectra, but possess distinguishable emission spectra. Preferably dyes are chosen such that they possess substantially different emission spectra, preferably having emission maxima separated by greater than 10 nm, more preferably having emission maxima separated by greater than 25 nm, even more preferably separated by greater than 50 nm. When it is desirable to differentiate between the two dyes using instrumental methods, a variety of optical filters and diffraction gratings allow the respective emission spectra to be independently detected. Instrumental discrimination can also be enhanced by selecting dyes with narrow bandwidths rather than broad bandwidths; however, such dyes should possess a high amplitude emission or be present in sufficient concentration that the loss of integrated signal strength is not detrimental to signal detection.

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In one embodiment, the second label may quench the fluorescence of the first label, resulting in a loss of fluorescent signal for doubly labeled particles. Examples of suitable fluorescencing/quenching pairs include 5' 6-FAMTM/3' Dabcyl, 5' Oregon Green® 488-X NHS Ester/3' Dabcyl, 5' Texas Red®-X NHS Ester/3' BlackHole QuencherTM-1 (Integrated DNA Technologies, Coralville, IA).

In one embodiment, two labels may be used for fluorescence resonance energy transfer (FRET), which is a distance-dependent interaction between the excited states of two dye molecules. In this case, excitation is transferred from the donor to the acceptor molecule without emission of a photon from the donor. The donor and acceptor molecules must be in close proximity (1-10 nm). Suitable donor, acceptor pairs include fluorescein/tetramethylrhodamine, IAEDANS/fluorescein, EDANS/dabcyl, fluorescein/ QSY7, (Haugland, 2002) and many others known to one skilled in the art.

Particles may be labeled with more than one kind of label, such as a dye tag and a mass tag, to facilitate detection and/or discrimination. For example, a protein may be labeled with two antibodies, one that is unlabeled and acts as a mass or mass/charge tag, and another that has a dye tag. That protein might then be distinguished from another protein of similar size that is bound only to an antibody with a dye tag by its difference in velocity (caused by the increased mass or altered mass/charge of the additional bound antibody).

Distinguishing labels

To accurately detect a labeled particle, the labeled particle must be distinguished from unbound label. Many ways to accomplish this are familiar to one skilled in the art. For example, in heterogeneous assays, unbound label is separated from labeled particles prior to analysis. In a preferred embodiment, the assay is a homogenous assay, and the sample, including unbound label, is analyzed by a combination of electrophoresis and single particle fluorescence detection. In this case, electrophoretic conditions are chosen which provide distinct velocities for the labeled particle and the unbound label.

Non-specific labeling of nucleic acids generally labels all nucleic acids regardless of the particular nucleotide sequence. One skilled in the art is familiar with various techniques for general labeling of nucleic acids. Methods include: intercalating dyes such as TOTO, ethidium bromide, and propidium iodide, ULYSIS kits for formation of coordination complexes, ARES kits for incorporation of a chemically reactive nucleotide analog to which a label can be readily attached, and incorporation of a biotin containing nucleotide analog for attachment of a streptavidin bound label. Enzymatic incorporation of labeled nucleotide analogs is another approach.

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Techniques to non-specifically label proteins are also well known to one skilled in the art. Several chemically reactive amino acids on the surface of proteins have been used, for example, primary amines such a lysine. In additions, labels can be added to carbohydrate moieties on proteins. Isotype specific reagents have also been developed for labeling antibodies, such as Zenon labeling.

In a preferred embodiment, only specific particles within a mixture are labeled. Specific labeling can be accomplished by combining the target particle with a labeled binding partner, where the binding partner interacts specifically with the target particle through complementary binding surfaces. Binding forces between the partners can be covalent interactions or non-covalent interactions such as hydrophobic, hydrophilic, ionic and hydrogen bonding, van der Waals attraction, or coordination complex formation. Examples of binding partners are agonists and antagonists for cell membrane receptors, toxins and venoms, antibodies and viral epitopes, hormones (*e.g.*, opioid peptides and steroids) and hormone receptors, enzymes and enzyme substrates, enzymes and enzyme inhibitors, binding cofactors and target sequences, drugs and drug targets, oligonucleotides and nucleic acids, proteins and monoclonal antibodies, antigen and specific antibody, polynucleotide and complementary polynucleotide, polynucleotide and polynucleotide binding protein; biotin and avidin or streptavidin, enzyme and enzyme cofactor; and lectin and specific carbohydrate. Illustrative receptors that can act as a binding partner include naturally occurring receptors, *e.g.*, thyroxine binding globulin, lectins, various proteins found on the cell surfaces (*e.g.*, cluster of differentiation or cluster designation, or CD molecules), and the like. An example is CD4, the molecule that primarily defines helper T lymphocytes. In a related embodiment, a binding partner may specifically bind to related particles. An example would be a peptide that binds to a family of related enzymes.

In one embodiment, a sample is reacted with beads or microspheres that are coated with a binding partner that reacts with the target particle. The beads are separated from any non-bound components of the sample, and the analyzer of the invention detects the beads containing bound particles. Fluorescently stained beads are particularly well suited for these methods. For example, fluorescent beads coated with oligomeric sequences will specifically bind to target complementary sequences, and after the appropriate separation steps, allow for detection of the target sequence.

In one embodiment, a method for detecting particles uses a sandwich assay with monoclonal antibodies as binding partners. An antibody is linked to a surface to serve as capture antibody. The sample is added and particles having the epitope recognized by the antibody would bind to the antibody on the surface. Unbound particles are washed away leaving substantially only those that are specifically bound. The bound particle/antibody can be reacted with a detection antibody that contains a detectable label. After incubating to

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allow reaction between the detection antibody and particles, unbound detection antibodies are washed away. The particle and detection antibody can be released from the surface and detected in the instrument of the invention. Alternatively, only the detection antibody might be released and detected, thereby indirectly detecting the particle.

A variation would be to employ a ligand recognized by a cell receptor. In this embodiment, the ligand is bound to the surface to capture the cells that express the specific receptor. For example, the receptor could be a surface immunoglobulin, and a labeled ligand used to label the cells. Therefore, having the ligand of interest complementary to the receptor bound to the surface, cells having the specific immunoglobulin for such ligand could be detected. In another embodiment, one could have antibodies to the ligand bound to the surface to non-covalently attach the ligand to the surface.

In another embodiment, binding partners include any entity that can produce a detectable particle such as an enzyme that converts a substrate to a fluorescent form, or a chemical that induces fluorescence in another molecule.

Motive Forces

The sample to be detected may be subjected to electrophoresis. Mobility of particles within the sample fluid varies with the properties of the particle. The velocity of movement produced by electrokinetic force is determined by the relative charge and mass of the individual particle and the fluid encasing it. Movement of a particle can be altered by the type of label that has been attached to the particle, such as a charge/mass tag. Therefore, the electrophoretic velocity of each detectably labeled particle is determined. Based on the determination of the electrophoretic velocity of each detectably labeled particle, individual particles in a sample comprising multiple particles can be distinguished. Any electrophoretic separation technique combined with an immunoassay or nucleic acid hybridization labeling technique can, in principle, be adapted for use in the context of the present invention.

In an additional embodiment, when two or more particles are present, at least one particle may move through at least two interrogation volumes in a direction opposite to that of the other particle.

Preferably, the sample comprises a buffer. While any suitable buffer can be used, the preferable buffer has low fluorescence background, is inert to the detectably labeled particle, can maintain the working pH and is at, or can be combined with suitable reagents to make an ionic strength suitable for electrophoresis. The buffer concentration can be any suitable concentration, such as in the range from 1-200 mM. Preferably, the buffer is selected from the group consisting of Gly-Gly, bicine, tricine, 2-morpholine ethanesulfonic acid (MES), 4-morpholine propanesulfonic acid (MOPS) and 2-amino-2-methyl-1-propanol

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hydrochloride (AMP). An especially preferred buffer is 2 mM Tris/borate at pH 8.1, but Tris/glycine and Tris/HCl are also acceptable. Preferred ionic strength is at least 50 mM.

For some applications, the buffer desirably further comprises a sieving matrix for use in the embodiment of the method. While any suitable sieving matrix can be used, desirably the sieving matrix has low fluorescence background and can specifically provide size-dependent retardation of the detectably labeled particle relative to other components in the fluid. The sieving matrix can be present in any suitable concentration; from about 0.1% to about 10% is preferred. Any suitable molecular weight can be used; from about 100,000 to about 10 million is preferred. Examples of sieving matrixes include poly(ethylene oxide) (PEO), poly(vinylpyrrolidone) (PVP), linear polyacrylamide and derivatives (LPA), hydroxypropyl methylcellulose (HPMC) and hydroxyethylcellulose (HEC), all of which are soluble in water and have exceptionally low viscosity in dilute concentration (0.3% wt/vol). These polymer solutions are easy to prepare, filter and fill into capillaries. To achieve sieving, the polymers are used at concentrations above their entanglement threshold. Addition of 0.2% LPA (5,000,000 – 6,000,000 MW) to a Tris/borate buffer enabled discrimination of IgG and a 1.1 kb nucleic acid fragment during a one minute electrophoretic separation (see Example 4 below).

Electrokinetic force can be combined with other motive forces such as pressure, vacuum, surface tension, gravitational force, and centrifugal force to discriminate between particles. In one embodiment, these forces can be chosen for their differential effects on different particles within a sample when two or more particles are present, resulting in at least one particle moving through at least two interrogation volumes with a velocity that differs from the other particle(s). The velocities of the particles can be aligned with the fluid flow or at least one particle can move antiparallel to the fluid flow. In another embodiment, at least one particle has an antiparallel velocity exceeding the velocity of the fluid flow. In another embodiment, at least one particle is in motion perpendicular to the fluid flow. In another embodiment, at least one particle is in motion with a combination of motions that are antiparallel and perpendicular to the fluid flow.

In yet another aspect, the act of moving the particles between a first interrogation volume and a second interrogation volume further comprises subjecting the particles to a separation method selected from the group consisting of capillary gel electrophoresis, micellar electro-kinetic chromatography, isotachophoresis, a magnetic field, an optical field, sorption, and any combination thereof.

One skilled in the art will recognize that capillary gel electrophoresis, micellar electro-kinetic chromatography, isotachophoresis and magnetic field separations are standard biochemical techniques. Use of optical fields for moving, scattering or trapping (as with optical tweezers) particles is described in U.S. Patent Nos. 6,784,420 and 6,744,038,

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incorporated herein by reference in their entirety. Optophoresis™ consists of subjecting particles to various optical forces, especially moving optical gradient forces. By moving the light relative to particles, typically through a medium having some degree of viscosity, particles are separated or otherwise characterized based at least in part upon the optical force asserted against the particle and the particle's dielectric constants. Generally, the light sources will be lasers, and the separations are accomplished in capillary or microchannel structures that are compatible with the instrumentation described for the current invention.

Instrumentation

In one embodiment of the invention, an SMD system described in Fig. 1 may be used. As shown in Fig. 1, an analyzer of one embodiment of the present invention is designated in its entirety by the reference numeral **10**. The analyzer **10** includes two electromagnetic radiation sources **12**, a mirror **14**, a lens **16**, capillary flow cells **18**, two microscope objective lenses **20**, two apertures **56**, two detector lenses **24**, two detector filters **26**, two single photon detectors **28**, and a processor **30** operatively connected to the detectors.

In operation, the radiation sources **12** are aligned so their beams **22**, **24** are reflected off a front surface of mirror **14**. The lens **16** focuses the beams **22, 24** into two separate interrogation volumes (*e.g.*, interrogation volumes **38**, **40** shown in Fig. 2 in the capillary flow cell **18**). The microscope objective lenses **20** collect light from sample particles and form images of the beams **22**, **24** onto the apertures **56**. The apertures **56** block out scattering from walls of the capillary flow cell **18**. The detector lenses **24** collect the light passing through the apertures **56** and focus the light onto an active area of the detectors **28** after it passes through the detector filters **26**. The detector filters **26** facilitate minimizing noise signals (*e.g.*, scattered light, ambient light) and maximizing the light signal from the particle. The processor **30** processes the light signal from the particle according to the methods described herein. In one embodiment, the microscope objective lenses **20** are high-numerical aperture microscope objectives.

The heart of the system is the glass capillary flow cell of the apparatus **18** shown in Figure 2. Two laser beams **22, 24** are optically focused about 100 µm apart and perpendicular to the length of the sample-filled capillary tube. The lasers **12** (Fig. 1) are operated at particular wavelengths depending upon the nature of the molecules to be excited. The interrogation volumes **38,40** of the detection system is determined by the cross sectional area of a laser beam **22** or **24** and by the segment of the laser beam selected by the optics that direct light to the detectors. The interrogation volume **38** or **40** is set such that, with an appropriate sample concentration, single particles are present in the interrogation volume during each time interval over which observations are made. When

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laser beams **22, 24** with Gaussian intensity distributions are employed as excitation sources, the strength of illumination is not uniform across the interrogation volume unless that volume is very small and confined to the center of the laser beam. As a result, particles experience different intensities of excitation and different numbers of photons are emitted depending on whether a particle passed through the beam near the center or the edge. Therefore, a population of identical particles will show a distribution of emission intensities.

Particles are moved through the capillary either in bulk fluid flow, via an electric field applied to the sample, or a combination thereof. Under electrophoretic conditions, like particles move through the tube in lockstep (plug flow). As particles pass through each laser beam, excitation of each fluorescent particle takes place via one-photon excitation. Within a fraction of a second, the excited particle relaxes, emitting a detectable burst of light. The excitation-emission cycle is repeated many times by each particle as it passes through the laser beam allowing the instrument to detect hundreds of particles per second. Photons emitted by fluorescent particles are registered in both detectors with a time delay indicative of the time for the particle to pass from the interrogation volume of one detector to the interrogation volume of the second detector.

Electromagnetic radiation is detected by at least two detectors, at least one detector for each of two interrogation volumes. In one embodiment, electromagnetic emission refers to the release of photons from a particle in response to a stimulus. In the case of fluorescent emission, the stimulus is absorbed light.

For elastic light scattering, the emission is at the same wavelength as the incident light, but has been dispersed by the particle itself. In other cases, the scattered light is of a different wavelength than the incident light. For example, when nano-sized metal colloid particle are illuminated with a standard white light source, the scattering produces intense monochromatic light.

Light is the preferred electromagnetic radiation to detect, particularly light in the ultra-violet, visible, or infrared ranges. The detectors of the instrument are capable of capturing the amplitude and the time segment adjacency of photon bursts from fluorescent particles and converting them to electronic signals. Detection devices such as CCD cameras, Foveon X3® sensors, video input module cameras, and Streak cameras can be used to produce images with contiguous signals. In another embodiment, devices such as a bolometer, a photodiode, a photodiode array, avalanche photodiodes, and photomultipliers can be used. In a preferred embodiment, avalanche photodiodes are used for the very sensitive detection of individual photons. Using specific optics between the interrogation volume and the detector, several distinct characteristics of the emitted electromagnetic radiation can be detected including: emission wavelength, emission intensity, burst size, burst duration, fluorescence lifetime, and fluorescence polarization. A preferred characteristic is emission

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intensity. Emission intensity is quantitatively dependent on the fluorescence quantum yield of the dye, the excitation source intensity, polarity, and wavelength, and the detection efficiency of the instrument. It is also affected by the components of the solution, including solvents, ions (such as those that determine pH) and the concentration of the dye. Dye intensity can change if the particle is exposed to a light source that causes photo-bleaching. One skilled in the art will recognize that one or more detectors can be configured at each interrogation volume and that the individual detectors may be configured to detect any of the characteristics of the emitted electromagnetic radiation listed above.

The preferred illumination sources are continuous wave lasers for wavelengths of >200-1100 nm. These illumination sources have the advantage of being small, durable and relatively inexpensive. In addition, they generally have the capacity to generate large fluorescent signals. Specific examples of suitable lasers include: lasers of the argon, krypton, helium-neon, helium-cadmium types as well as tunable diode lasers (red to infrared regions), each with the possibility of frequency doubling. The lasers provide continuous illumination with no accessory electronic or mechanical devices such as shutters, to interrupt their illumination. Light emitting diodes (LEDs) are another low-cost, high reliability illumination source. Recent advances in ultra-bright LEDs coupled with dyes with high absorption cross-section and quantum yield, support their applicability to single particle detection. Such lasers could be used alone or in combination with other light sources such as mercury arc lamps, elemental arc lamps, halogen lamps, arc discharges, plasma discharges, light-emitting diodes, or combination of these. The optimal laser intensity depends on the photo bleaching characteristics of the individual dyes and the length of time required to traverse the interrogation volume (including the speed of the particle, the distance between interrogation volumes and the size of the interrogation volumes). To obtain a maximal signal, it is desirable to illuminate the sample at the highest laser intensity that will not overly photo-bleach of the dyes. The preferred laser intensity is one such that no more that 5% of the dyes are bleached by the time the particle has traversed the final interrogation volume.

Alternatively, pulsed lasers can be used as illumination sources. Pulsed lasers together with time-gated detectors can be used for determining the fluorescence lifetime of particles as one option for detection and discrimination. In the case of fluorescent emissions, the photon signal detected depends both on the wavelength spectra of the fluorescent emission and the filters used with the detectors in the instrument. Therefore, particles with different but overlapping emission spectra may appear indistinguishable if the filter range encompasses both spectra.

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Data analysis

Data analysis may be conducted according to the following stepped embodiment:

1. measuring a first electromagnetic radiation signal emitted by a particle within a first interrogation volume and applying a first analytical filter to the first electromagnetic radiation signal and measuring a second electromagnetic radiation signal emitted by the particle in a second interrogation volume and applying a second analytical filter to the second electromagnetic radiation signal;
2. comparing by cross-correlation the filtered electromagnetic radiation signal emitted by the particle within the first interrogation volume to the filtered electromagnetic radiation signal emitted by the particle within the second interrogation volume; and
3. further applying another analytical filter to the cross-correlation events.

In a further embodiment, the stepped method may be accomplished according to the following:

1. Detect all electromagnetic radiation signals during a sample measurement period.
2. Subdivide data into arbitrary time segments and determine the background level of electromagnetic radiation over all the time segments (bins) independently for data collected in both detection channels.
3. Set a threshold level above the background level, and apply analytical filters to select signals that have electromagnetic radiation signals above the threshold and form a peak. Peaks are identified independently for data collected in each detection channels. The criteria for the analytical filters fits the criteria known to match the signals of similar particles.
4. Cross-correlating the peaks selected above that occur in channel one (from the first interrogation volume) with peaks in channel two (from the second interrogation volume) over a range of time.
5. Apply analytical filters to the cross-correlated events to select events that match the known characteristics of signal brightness or peak width of the target particles.
6. Plot each selected cross-correlated event and draw a histogram to show the density of the events at each unit of elapsed time.
7. Data sets can be further restricted by observing events that fall within only a portion of the elapsed time range or a portion of the duration of the measurement.

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More specifically, the signals detected by each of the first and second detectors are divided into arbitrary, time segments with freely selectable time channel widths. Preferred channel widths (bins) are in the range of 1 μs to 5 ms. The number of signals contained in each segment is then established. In a preferred embodiment, the detected signals are first analyzed to determine the background. The background is determined by averaging the signal over a large number of bins. In one embodiment the average signal is calculated using the entire number of bins in the sample. In a preferred embodiment, a second average is calculated where bins that contain photons 2-3 standard deviations above the original background calculation are eliminated. In other embodiments, the background level is determined from the mean noise level, or the root-mean-square noise. In other cases, a typical noise value or a statistical value is chosen. In the case of single photon counting detectors, the noise is expected to follow a Poisson distribution.

In a preferred embodiment, the detected signals are selected above a threshold prior to cross-correlating the data. A threshold value is determined to discriminate true signals (peaks, bumps, particles) from background. Care must be taken to choose a threshold value such that the number of false positive signals from random background is minimized and the number of true signals that are rejected is minimized. Methods for choosing a threshold value include: arithmetic methods, statistical methods, determining a fixed value above the background level, and calculating a threshold value based on the distribution of the background signal. In a preferred embodiment, the threshold is set at a fixed number of standard deviations above the background level. Assuming a Poisson distribution of the background and using this method, one can estimate the number of false positive signals detected during the experiment.

Analytical filters are applied to signals that are above threshold levels by comparing those signals to signals known to originate from similar particles and only those that match the criteria in terms of the number of photons above the threshold occurring in adjacent time segments are selected. For each signal selected from the first detection channel, a cross-correlation analysis is performed with the signals selected from the second detection channel within a predetermined time range. In this way, an event is discriminated from background based on the presence of correlated signal(s) in at least two detector channels. The elapsed time of the cross-correlated signals provides the transit time between the corresponding detectors and therefore based on the distance between the detectors, the velocity of the particle is determined. A particle can be detected when the elapsed time for the correlation corresponds to a known elapsed time. In other cases, a particle is detected via unknown elapsed time which is determined empirically by repeating the cross-correlation using broader or narrower ranges in the analysis until the optimum conditions for particle detection and discrimination are determined.

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In a further embodiment, the cross-correlation analysis can be performed on data from more than two detectors, such as 3, 4, 5, 6, 7, 8 and more detectors that are distinct either in relative location of the interrogation volume or in the wavelength detected. In this case, the cross-correlation analysis can be performed on data from any combination of detectors that are distinct. For example, in a case where three detectors, each detecting a distinct electromagnetic radiation characteristic (R, G & B) are at each of two interrogation volumes (1 & 2), R1 is correlated with R2, G1 is correlated with G2 and B1 is correlated with B2, resulting in elapsed times for particles with characteristic emission detected by the individual detectors. Other combinations of cross-correlation analysis can also be performed, such as overlapping sets where R1 is correlated with G1; R1 is correlated with B1 and G1 is correlated with B1. Results of these cross-correlation analyses would indicate the frequency of double-labeled particles. Different combinations of cross-correlation analyses can be used with one another to distinguish particles based on velocity and electromagnetic characteristic, for example, R1 is correlated to G1 and the combination is correlated with the correlation of R2 and G2. In addition, using multiple cross-correlation analyses will result in more accurate determination of the properties of the individual particles within the mixture.

In a further embodiment, analysis methods are employed wherein cross-correlation analysis is performed on data from detectors in any or all combinations of locations and/or characteristics that are distinct.

For samples where particles are moved at a uniform velocity, cross-correlated signals that have the expected velocity are determined to be events of interest. For samples where particles are moved at different velocities, cross-correlated signals are determined to be events of interest when, at a particular velocity, they have the expected (predetermined) photon burst attributes for that velocity in a particular instrumentation system configuration. Faster moving particles will have fewer bursts of photons in adjacent time segments than slower moving particles.

False cross-correlated events occur when particles do not have the expected velocity due to any one of several reasons: fluorescent impurities in the sample, particles passing through only one interrogation volume during their transit through the capillary or erroneous cross-correlations. Erroneous cross-correlation can result when photons from other particles move closely behind or ahead of the "correct" photon associated with the "correct" particle.

Following cross-correlation, at least one analytical filter is applied to the cross-correlated data that eliminates events that fall outside the known characteristics of the target particles. These filters can be based on electromagnetic characteristics such as fluorescent brightness (intensity), and the width of emission signal above the threshold value (bin number). These filters are different from those applied to the signals before cross-

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correlation. Events can also be restricted to a certain range of elapsed time that is evaluated or a portion of the time during which the sample is analyzed. More than one filter can be applied to a data set simultaneously.

Filtering is used to determine when a cross-correlated event was generated by a particle (*i.e.*, the emission was of the expected duration, intensity, and/or magnitude for a single particle under these conditions as predetermined). Other characteristics or combinations of characteristics also can be used to detect particle events. In this manner, filtering allows one to detect particles moving at the expected velocity and having the emission characteristics of particles moving at this velocity.

Finally, the computer produces a histogram of velocities that shows a peak for every fluorescent particle present in the sample. When the sample moves in a bulk fluid flow through the capillary, all particles move at the same velocity. When an electric field is applied to the sample, the transit time between the detectors for each particle is dependent upon the particle's characteristic charge, size and shape.

The methods described herein allow particles to be enumerated as they pass individually through the interrogation volumes. The concentration of the sample can be determined from the number of particles counted and the volume of sample passing though the interrogation volume in a known amount of time. In the case where the interrogation volume encompasses the entire cross-section of the sample stream, only the number of particles counted and the volume passing through a cross-section of the sample stream in a known amount of time are needed to calculate the concentration the sample. When the interrogation volume is smaller than the sample stream, the concentration of the particle can be determined by interpolating from a standard curve generated with a control sample of standard concentration. In a further embodiment, the concentration of the particle can be determined by comparing the measured particles to an internal or external particle standard. Knowing the sample dilution, one can calculate the concentration of particles in the starting sample.

EXAMPLES

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following specific examples are offered by way of illustration and not by way of limiting the remaining disclosure.

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Example 1. Detection of nucleic acid targets moving at uniform rate using cross-correlation and analytical filters.

1a. Linearized pUC19 was labeled with Alexa Fluor®647 using a ULYSIS® nucleic acid labeling kit (Molecular Probes, Inc., Eugene, Oregon) according to the manufacturer's instructions. Unbound label was removed and the sample was suspended at 7.5 fM in phosphate buffered saline with 0.01% casein hydrolysate and pumped through the interrogation volumes of the analyzer at 1 µl/min.

1b. Another sample consisted of a 7.2 kb DNA fragment labeled as above and subjected to electrophoresis at 3000V for 60 seconds in 0.2x TB, 0.01% SDS. Analyzed data is shown in Figure 3. Panels 3A and 3B show data from 7.5 mM linearized pUC19 moved through the analyzer by pumping. The detected signals were filtered to select those that were greater than two standard deviations above the average background. The filtered signals were cross-correlated and plotted. Dot plots show brightness (y-axis) vs. elapsed time (x-axis) for each individual cross-correlated pair of events, circles representing events originating in channel 1 and pluses representing events originating in channel 2. The solid line is a histogram of dot density. A) Initial cross-correlation of the filtered signals revealed no discernable peaks. B) Applying another analytical filter to the cross-correlation events enabled selection of events with brightness between 15-500 photons that moved as a dominant peak at around 80 ms.

Panels 3C and 3D show data from a 7.2 kb DNA fragment moved through the analyzer by electrophoresis. The detected signals were filtered to select those that were greater than six standard deviations above the average background. The filtered signals were cross-correlated and plotted. Dot plots of time (y-axis) vs. elapsed time (x-axis) for each individual cross-correlated pair of events, circles representing events originating in channel 1 and plusses representing events originating in channel 2. The solid line is a histogram of dot density. C) The shoulder on the peak is composed of events that occurred primarily in the last half of the sample run (dot density is higher near the top of the chart) suggesting a change in the electrophoresis system with time. D) Restricting the data to the first 30 seconds of the run results in a histogram that shows a single peak primarily without the shoulder that was an artifact of changes in the electrophoresis system.

Example 2. Using predetermined electrophoretic velocity ranges to automatically detect one of two particles in a sample.

An intrinsically fluorescent protein complex, PBXL-3, and a 1.1 kb nucleic acid were used to predetermine characteristic electrophoretic velocity ranges. The nucleic acid was labeled with Alexa Fluor® 647 following the protocol of the ULYSIS® nucleic acid labeling kit (Molecular Probes, Inc., Eugene, Oregon). The samples were subjected to electrophoresis,

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and data was analyzed according to the scheme described above, except that analytical filters for brightness and peak width were applied after cross-correlation. The protein complex and nucleic acid were analyzed independently and the characteristic ranges for the peak height, peak width and elapsed time were used to determine windows where each particle was expected to occur (Table 1).

Table 1

	Window 1 (for PBXL3)	Window 2 (for 1.1 kb nucleic acid)
Peak	>150	<150
Width	>3	<3
Elapsed Time	350-500 ms	250-300 ms

Using these characteristics, four samples were analyzed and the number of cross-correlated events that occurred in each window was determined (Table 2). These predetermined windows can be used to analyze samples whose content is unknown. If events occur within both windows, the sample consists of a mixture of particles. If events occur in only one window, only one type of particle is present.

Table 2

	Window 1	Window 2
PBXL3	59	2
Nucleic acid	3	238
Mixture of PBXL3 and nucleic acid	45	191
Buffer blank	0	0

Example 3. Detection and discrimination of particles in a mixture moving at uniform rates using cross-correlation analysis and filtering.

An intrinsically fluorescent protein complex, PBXL-3, emits at a high intensity relative to a nucleic acid, linearized pUC19 labeled with Alexa Fluor® 647. The pUC19 DNA was labeled with Alexa Fluor® 647 following the protocol of the ULYSIS® nucleic acid labeling kit (Molecular Probes, Inc., Eugene, Oregon). Phosphate Buffered Saline (PBS) (10 mM sodium phosphate, 150 mM NaCl, pH 7.2) was supplemented with 0.01% casein hydrolysate (Sigma-Aldrich Corp., St. Louis, Missouri) and used to make dilution series (2.5, 5, 7.5, 10 and 20 fM) of protein alone, nucleic acid alone or mixtures of both. Samples were moved through the analyzer by pumping at 1 µL/min for 4 min.

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Data was analyzed as described above. The detected signals were filtered to select those that were greater than four standard deviations above the average background. Figure 4A shows plots of cross-correlated filtered signals for the protein complex and nucleic acid alone. The range of elapsed time was restricted to show only the events within the peaks themselves (*see* Fig. 4A) and to emphasize the different characteristic fluorescent intensities of the protein complex and the nucleic acid. A brightness level of 500 photons was chosen to separate a bright window of intensity for the protein complex and a dim window of intensity for the nucleic acid. An analytical filter based on brightness of 15-500 for the nucleic acid and 500-9,000 for the protein complex was applied to the data. The number of events identified by these methods was measured for both the protein complex and nucleic acid at series of concentrations. Standard curves were plotted for the protein and nucleic acid using both brightness windows, and the slopes of the curves were determined.

In three different mixtures, the protein complex and nucleic acid were discriminated based on their intensity. The analytical filter for brightness was applied, and the number of molecules detected in the mixtures of PBXL-3 and pUC19 were used to calculate the concentrations of each component based on the slopes of the standard curves. Comparing the measured concentrations for the protein and nucleic acid to the predicted values demonstrates that the concentration of sample components can be determined by comparing the molecules detected in the sample to a standard curve (Figure 4B).

Example 4. Detection and discrimination of particles in a mixture moving at different rates using optimized cross-correlation analysis and filtering.

IgG and a 1.1 kb PCR product were both labeled with Alexa Fluor® 647 according to the manufacturer's protocols for proteins and nucleic acids respectively (Molecular Probes, Inc., Eugene, Oregon). A mixture of 13 fM IgG and 5 fM nucleic acid was suspended in a buffered sieving solution consisting of 18 mM Tris, 18 mM boric acid, pH 8.6 with 0.2% linear polyacrylamide (LPA, 5,000,000 – 6,000,000 MW), 0.01% sodium dodecyl sulfate and 1 μg/ml each bovine serum albumin, Ficoll®, and polyvinylpyrrolidone. Unbound labels were removed prior to making the mixture and the sample was subjected to electrophoresis at 300 V/cm for one minute to move the molecules through the interrogation volumes of the analyzer.

Data were analyzed as described above, except that a series of analytical filters based on peak width (bins) were applied to the cross-correlated events. Figure 5A shows that with the broadest filter (0-5 bins), peaks for both the protein and nucleic acid are observed. Applying narrower elapsed time filters, selects for the signals of either the protein molecule (Fig. 5B (0-1 bins)) or the nucleic acid molecule (Fig. 3C (1-5 bins)). This

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demonstrates that the analytical filters can be used to confirm the identity of the molecules in a mixture that were separated by their different velocities.

Other Embodiments

The detailed description set-forth above is provided to aid those skilled in the art in practicing the present invention. However, the invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed because these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description which do not depart from the spirit or scope of the present inventive discovery. Such modifications are also intended to fall within the scope of the appended claims.

References Cited

All publications, patents, patent applications and other references cited in this application are incorporated herein by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application or other reference was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. Citation of a reference herein shall not be construed as an admission that such is prior art to the present invention.

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CLAIMS

What is claimed is:

1. A method for enhancing the analysis of particle detection comprising:

measuring a first electromagnetic radiation signal provided by a particle within a first interrogation volume and optionally applying a first analytical filter to the first electromagnetic radiation signal and measuring a second electromagnetic radiation signal emitted by the particle in a second interrogation volume and optionally applying a second analytical filter to the second electromagnetic radiation signal;

comparing by cross-correlation the electromagnetic radiation signal emitted by the particle within the first interrogation volume to the electromagnetic radiation signal emitted by the particle within the second interrogation volume; and

further applying a third analytical filter to the cross-correlation events;

thereby enhancing the analysis of the particle detection.

2. A method according to claim 1, wherein one of or both the first analytical filter and the second analytical filter are applied.

3. A method according to claim 2, wherein both the first analytical filter and the second analytical filter are applied, and wherein the first analytical filter and the second analytical filter are the same analytical filter.

4. A method according to claim 1, wherein the first and second analytical filters are selected from the group consisting of signals that are greater than a predetermined threshold level, signals within a predetermined number of adjacent time segments, and a combination thereof.

5. A method according to claim 1, wherein applying the third analytical filter comprises detecting a particle characteristic selected from the group consisting of emission intensity, burst size, burst duration, fluorescence lifetime, fluorescence polarization, and any combination thereof.

6. A method according to claim 5, wherein the particle characteristic is provided by one of an intrinsic parameter of the particle or an extrinsic parameter of the particle.

7. A method according to claim 6, wherein the extrinsic parameter is provided by marking the particle with at least one label selected from the group consisting of a dye tag, a light-scattering tag, and any combination thereof.

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8. A method according to claim 1, wherein the first analytical filter, the second analytical filter and the third analytical filter are applied before cross-correlating the first electromagnetic radiation signal and second electromagnetic radiation signal.

9. A method according to claim 1, wherein the first and second interrogation volumes are in electromagnetic communication with at least one excitation source selected from the group consisting of a light-emitting diode, a continuous wave laser, and a pulsed laser.

10. A method according to claim 1, wherein the particle is selected from the group consisting of a polypeptide, a polynucleotide, a nanosphere, a microsphere, a dendrimer, a chromosome, a carbohydrate, a virus, a bacterium, a cell, and any combination thereof.

11. A method according to claim 1, wherein the particle is selected from the group consisting of an amino acid, a nucleotide, a lipid, a sugar, a toxin, and any combination thereof.

12. A method according to claim 1, wherein the particle is selected from the group consisting of an aggregate, a complex, an organelle, a micelle, and any combination thereof.

13. A method according to claim 1, further comprising moving a target particle through the first interrogation volume and through the second interrogation volume by a force selected from the group consisting of electro-kinetic force, pressure difference, osmotic difference, ionic difference, gravity, surface tension, centrifugal force, a magnetic field, an optical field, and any combination thereof.

14. A method according to claim 13, wherein the target particle is one of a population of different particles.

15. A method according to claim 14, wherein the target particle is moved through the first interrogation volume and through the second interrogation volume with the population of different particles at a uniform velocity by a force selected from the group consisting of positive pressure, negative pressure, gravity, surface tension, inertial force, centrifugal force, and any combination thereof.

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16. A method according to claim 14, wherein the target particle is moved through the first interrogation volume and through the second interrogation volume with the population of different particles at a different velocity by a force selected from the group consisting of electro-kinetic force, centrifugal force, a magnetic force, an optical force, and any combination thereof.

17. A method according to claim 16, wherein the target particle mobility is determined by an intrinsic parameter of the particle or an extrinsic parameter of the particle.

18. A method according to claim 17, wherein the extrinsic parameter of the target particle is provided by a label selected from the group consisting of a charge tag, a mass tag, a charge/mass tag, a magnetic tag, an optical tag, and any combination thereof.

19. A method according to claim 1, wherein the emitted electromagnetic radiation signal is selected from the group consisting of stimulated emission, fluorescence, elastic light scattering, inelastic light scattering, and any combination thereof.

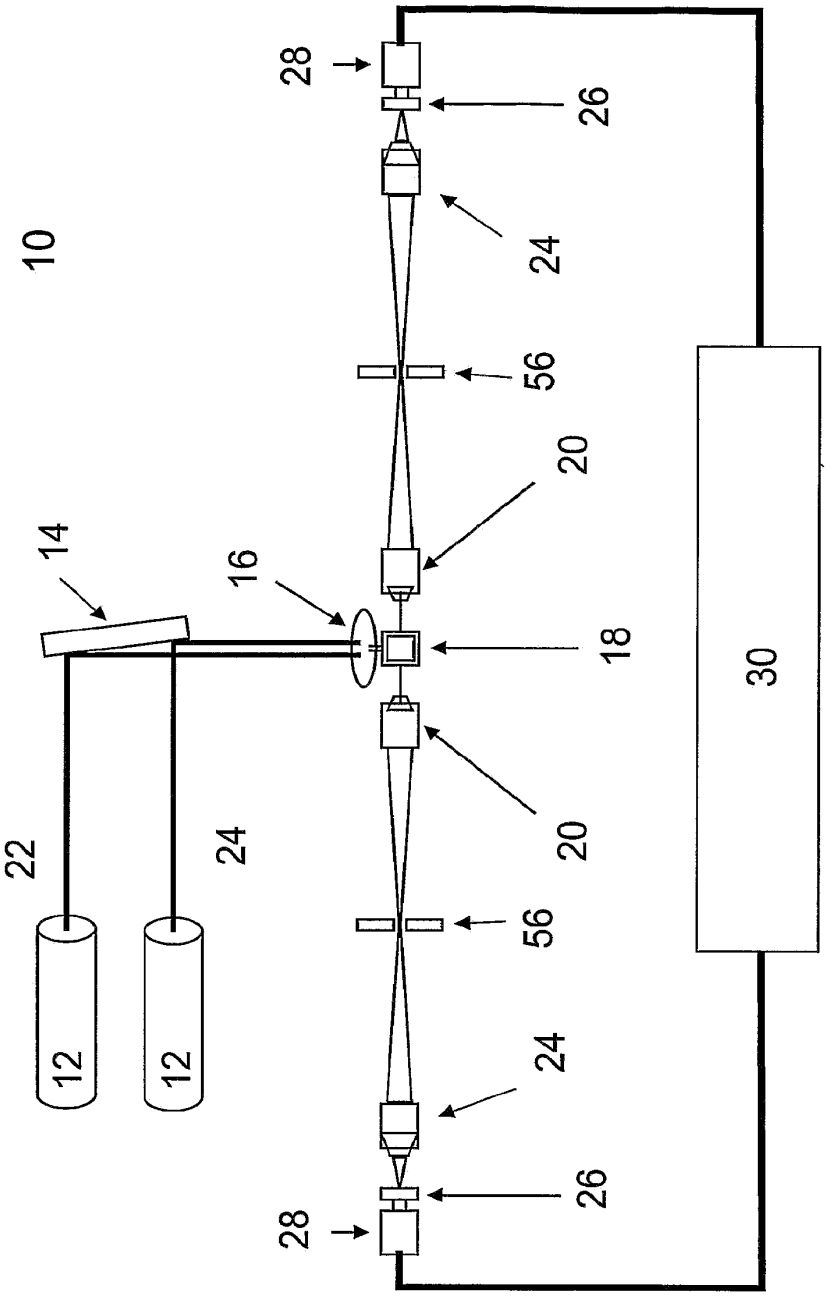
20. A method according to claim 1, wherein the emitted electromagnetic radiation signal passes through an optical band pass filter within an image plane of a detector.

21. A method according to claim 20, wherein the optical band pass filter enables differential detection of emission spectra.

22. A method according to claim 1, wherein the analysis comprises multiple passes through the processes of applying analytical filters and comparing the electromagnetic radiation signal emitted by the particle within the first interrogation volume to the electromagnetic radiation signal emitted by the particle within the second interrogation volume.

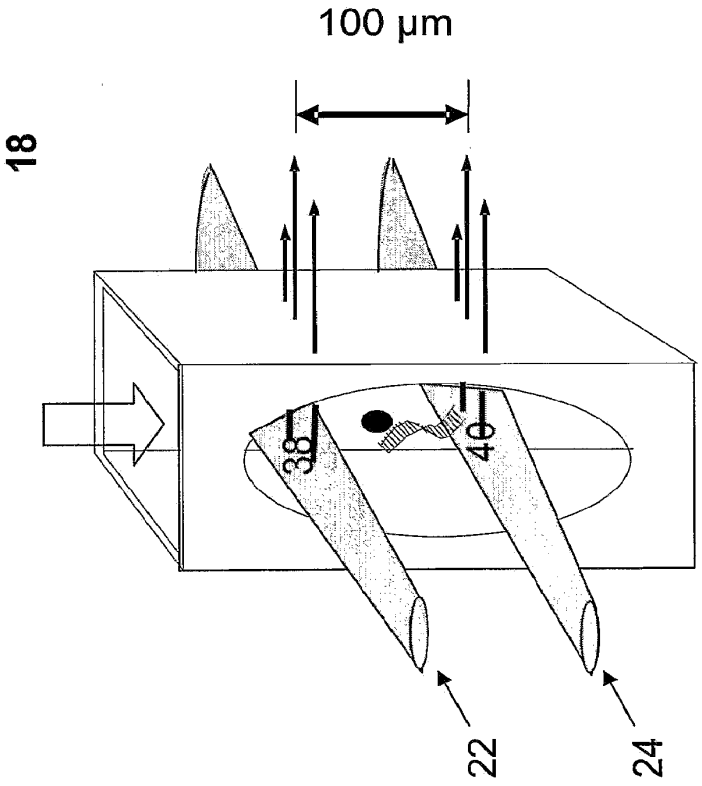
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Figure 1/12



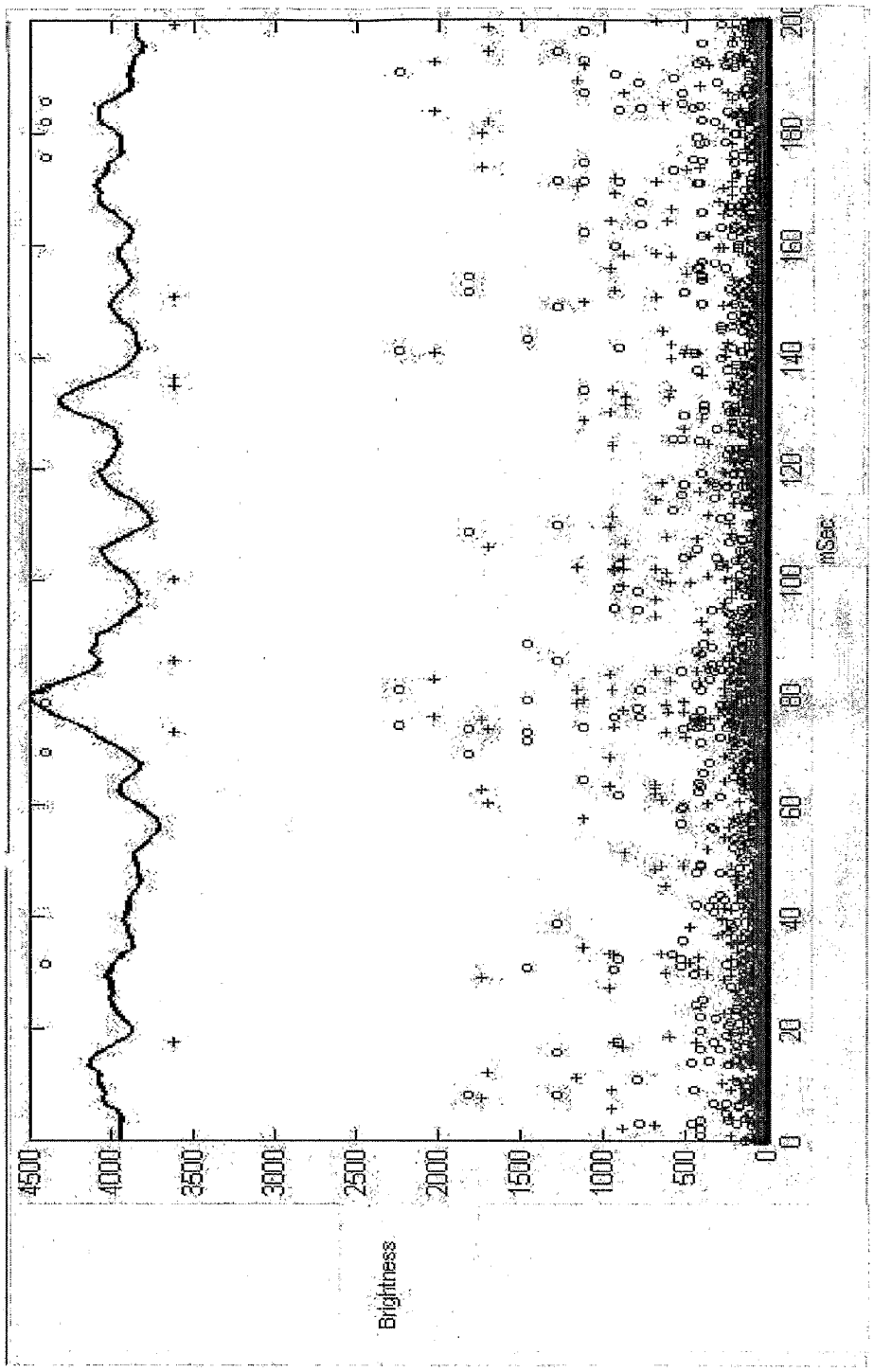
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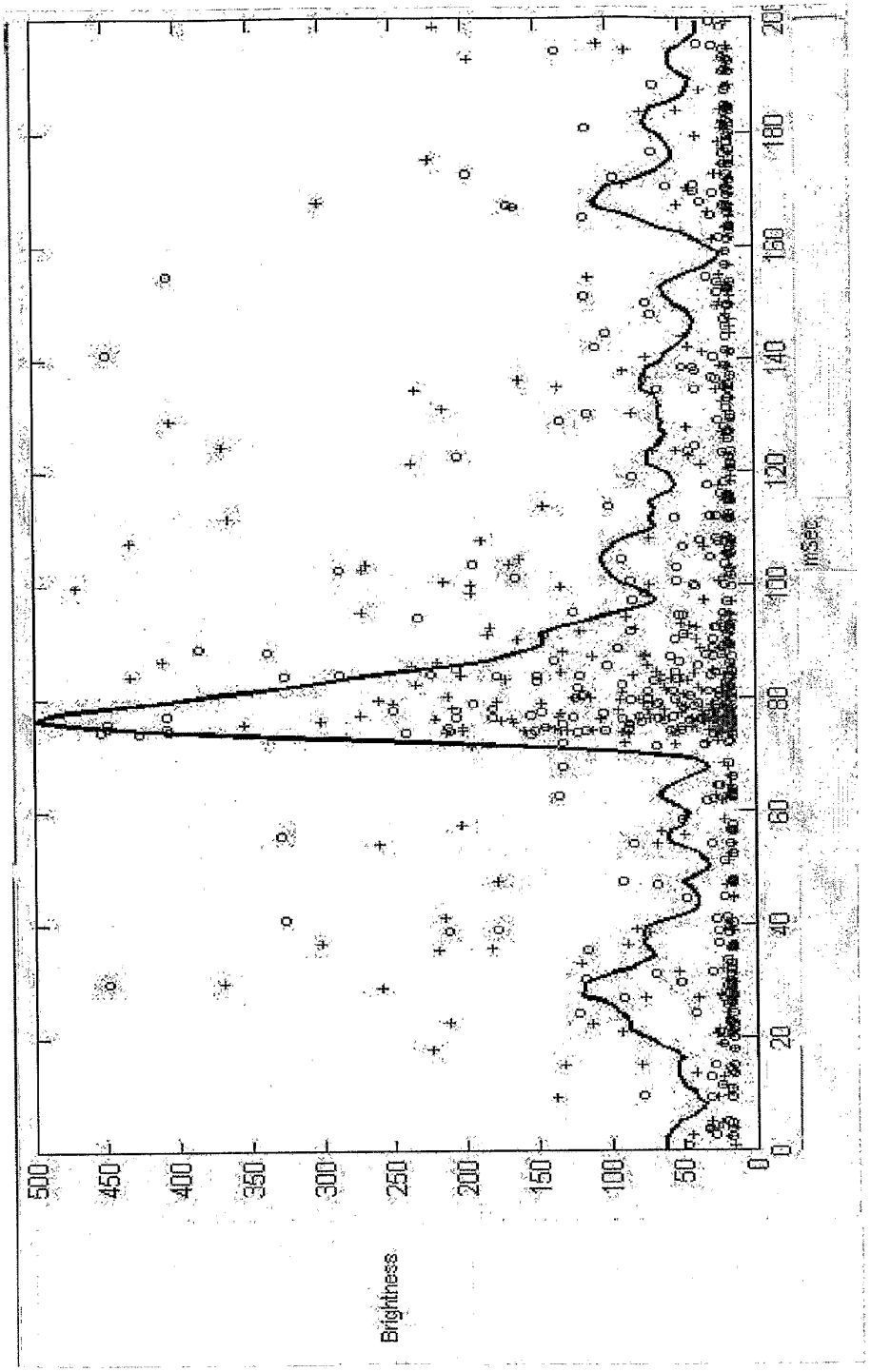
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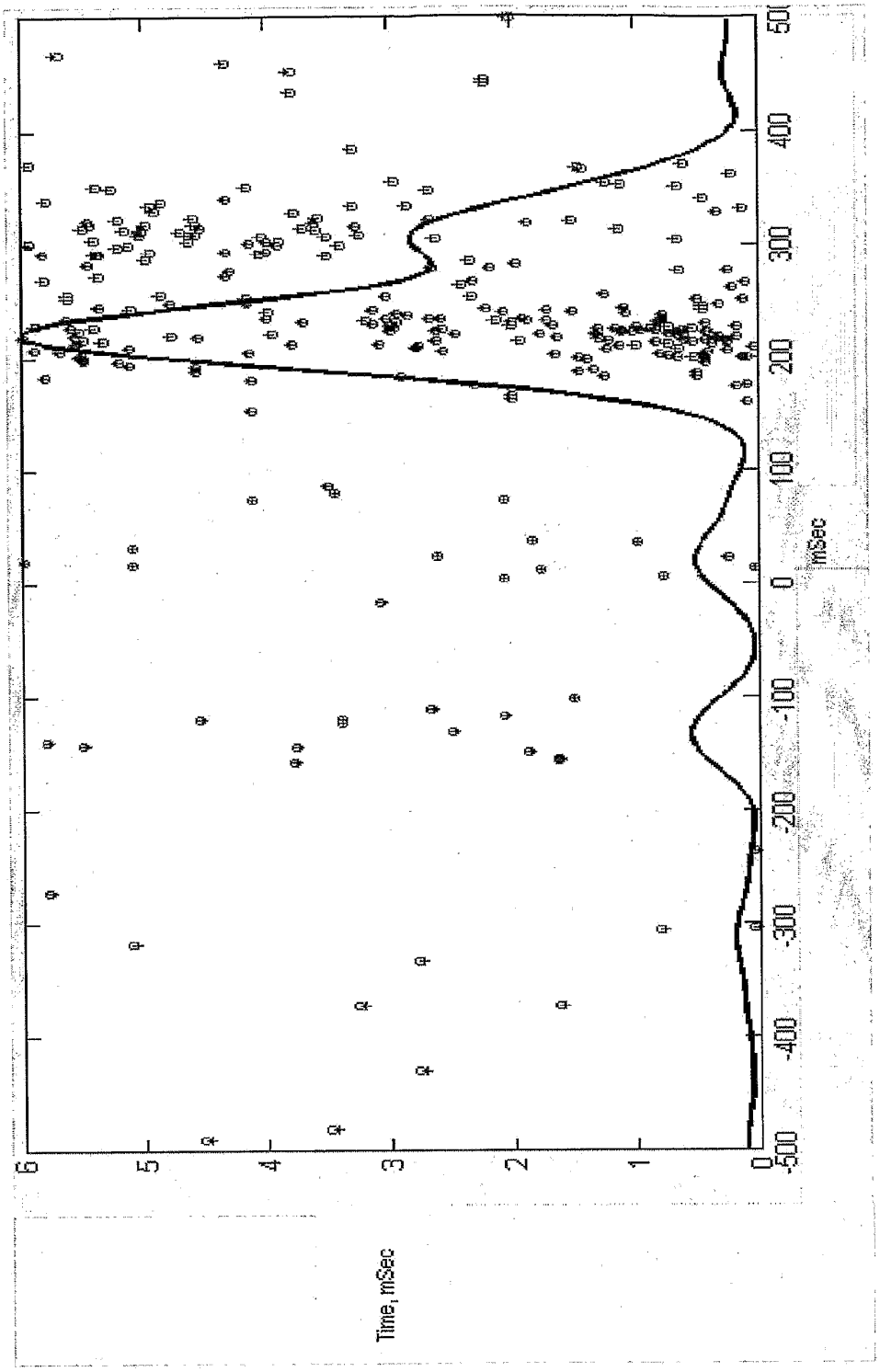
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Figure 4/12



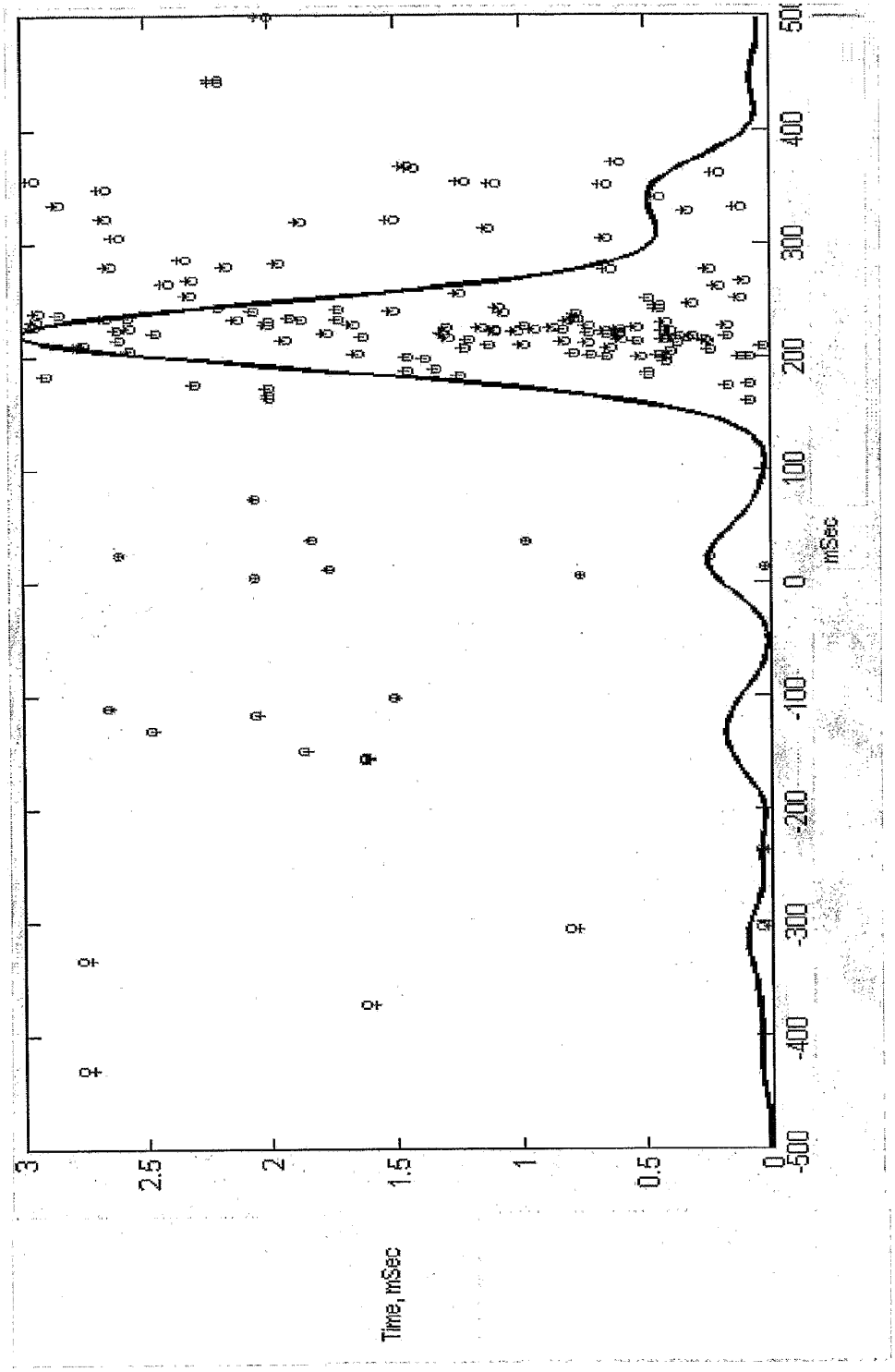
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Figure 5/12



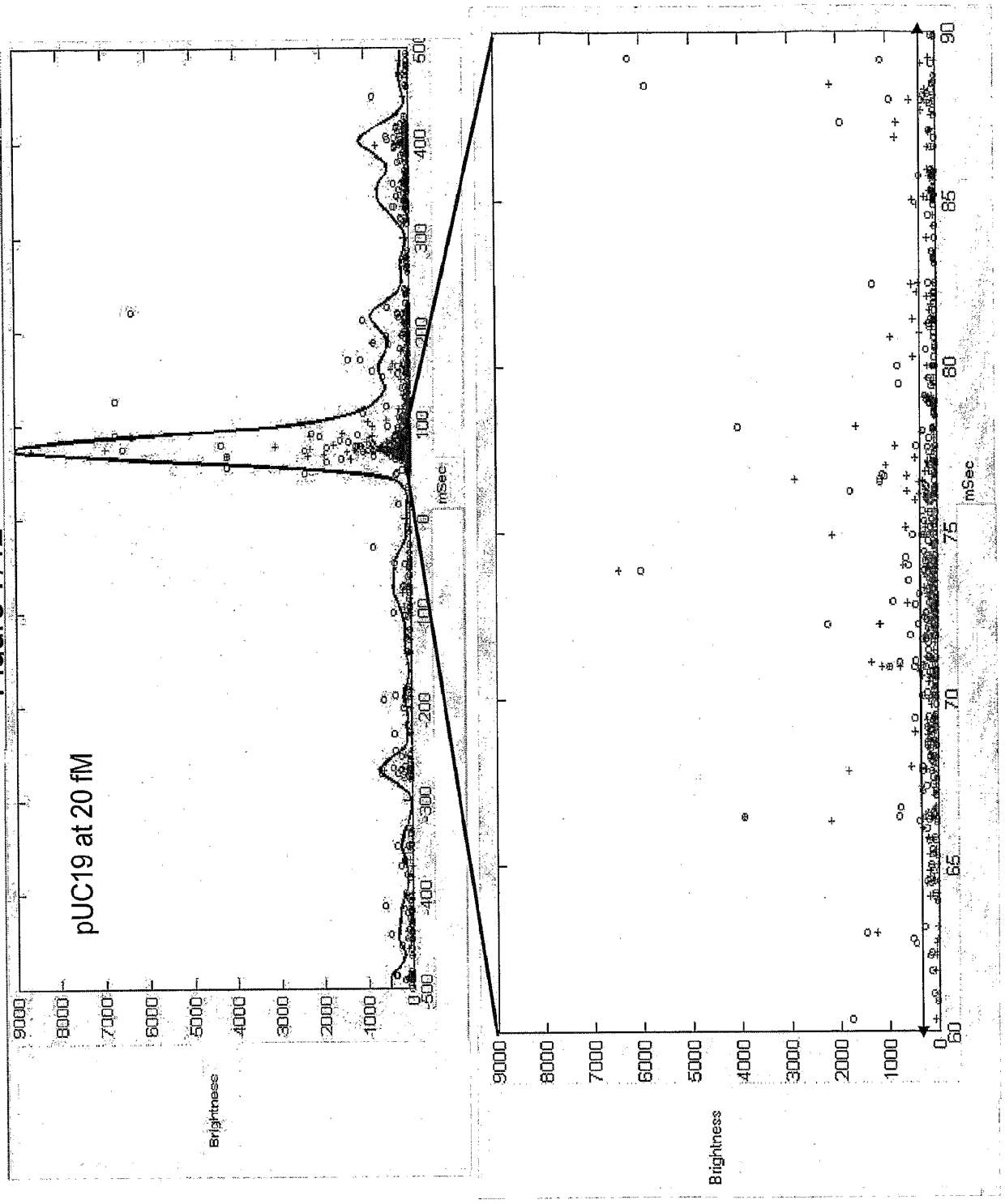
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Figure 6/12



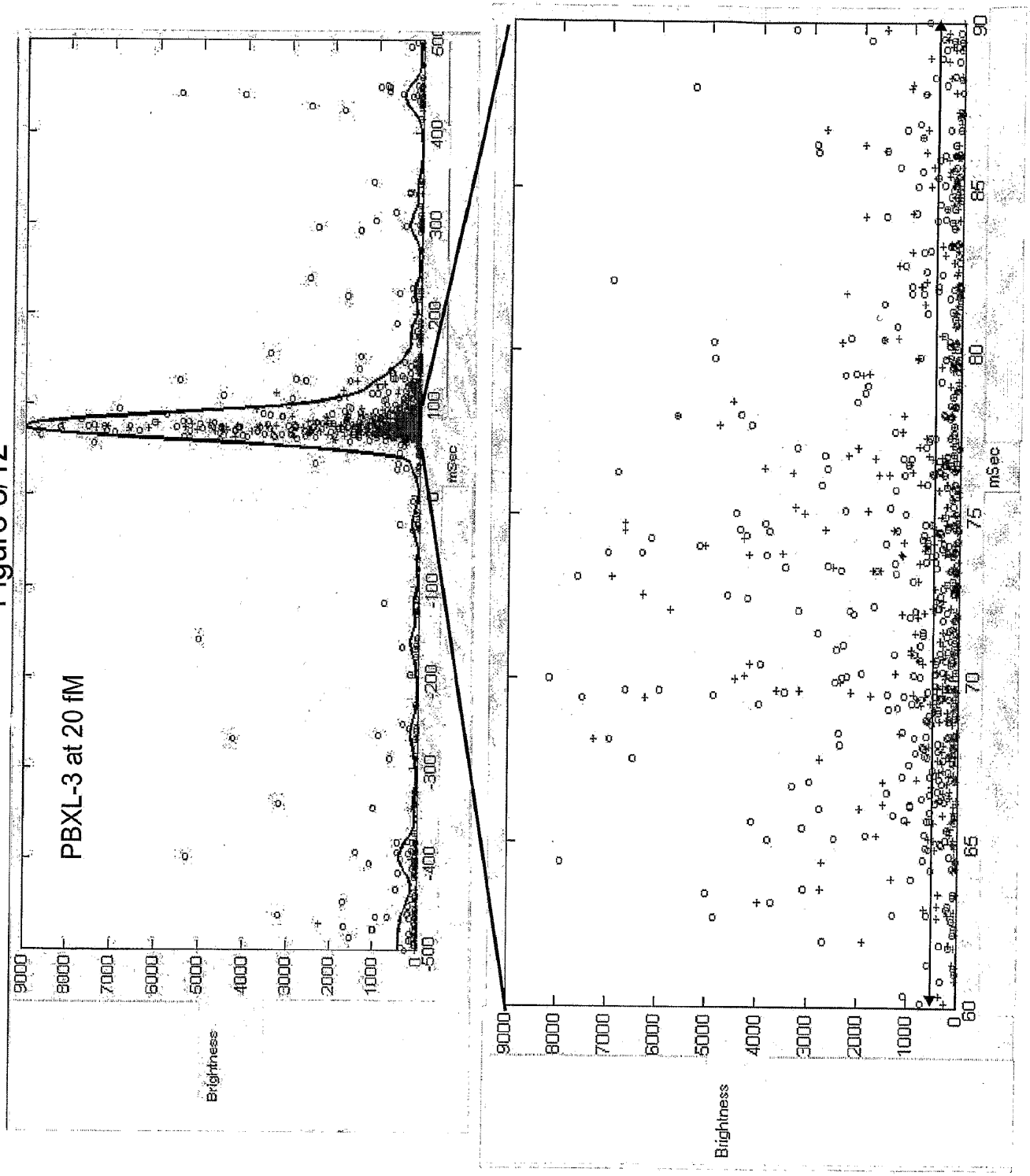
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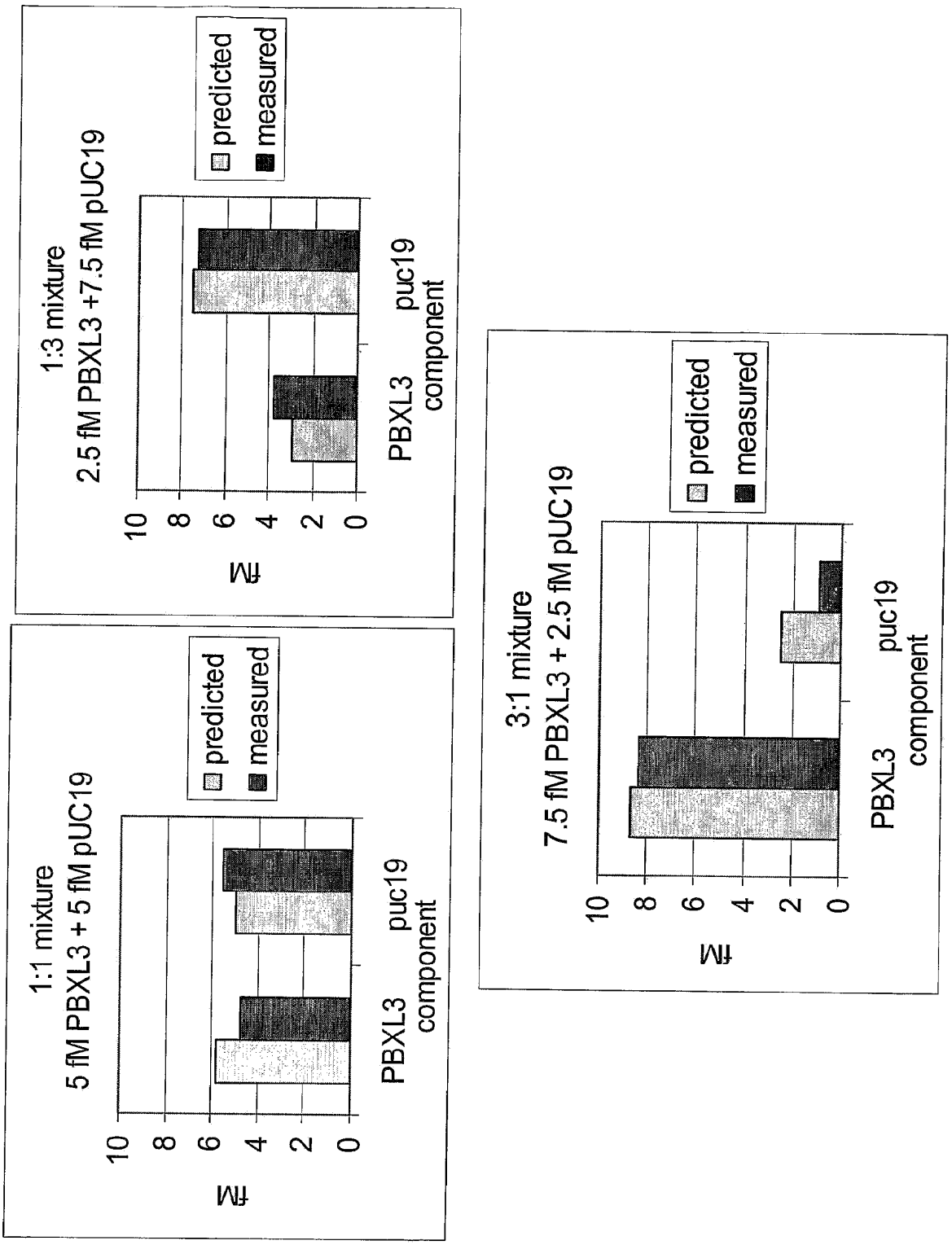
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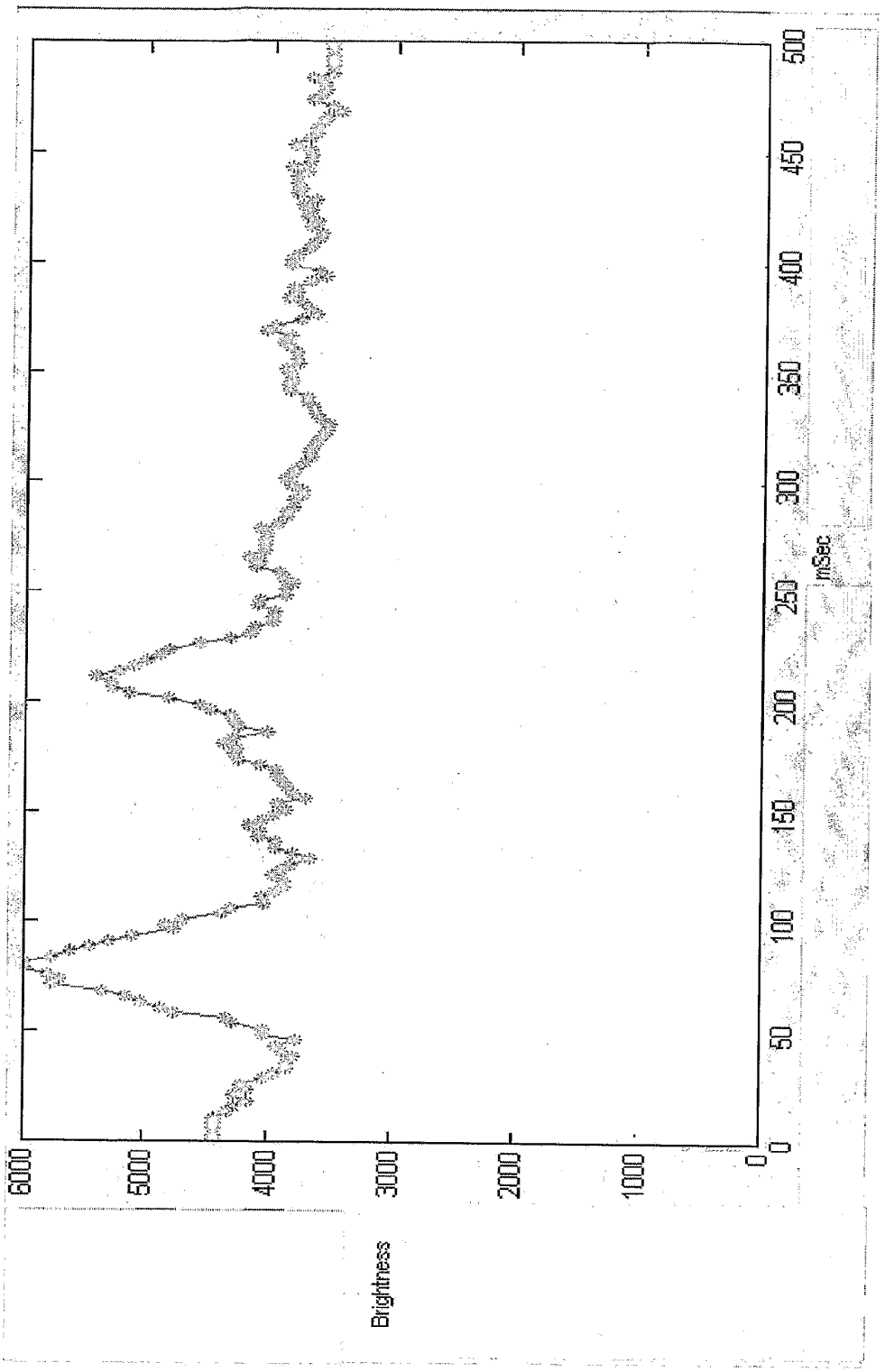
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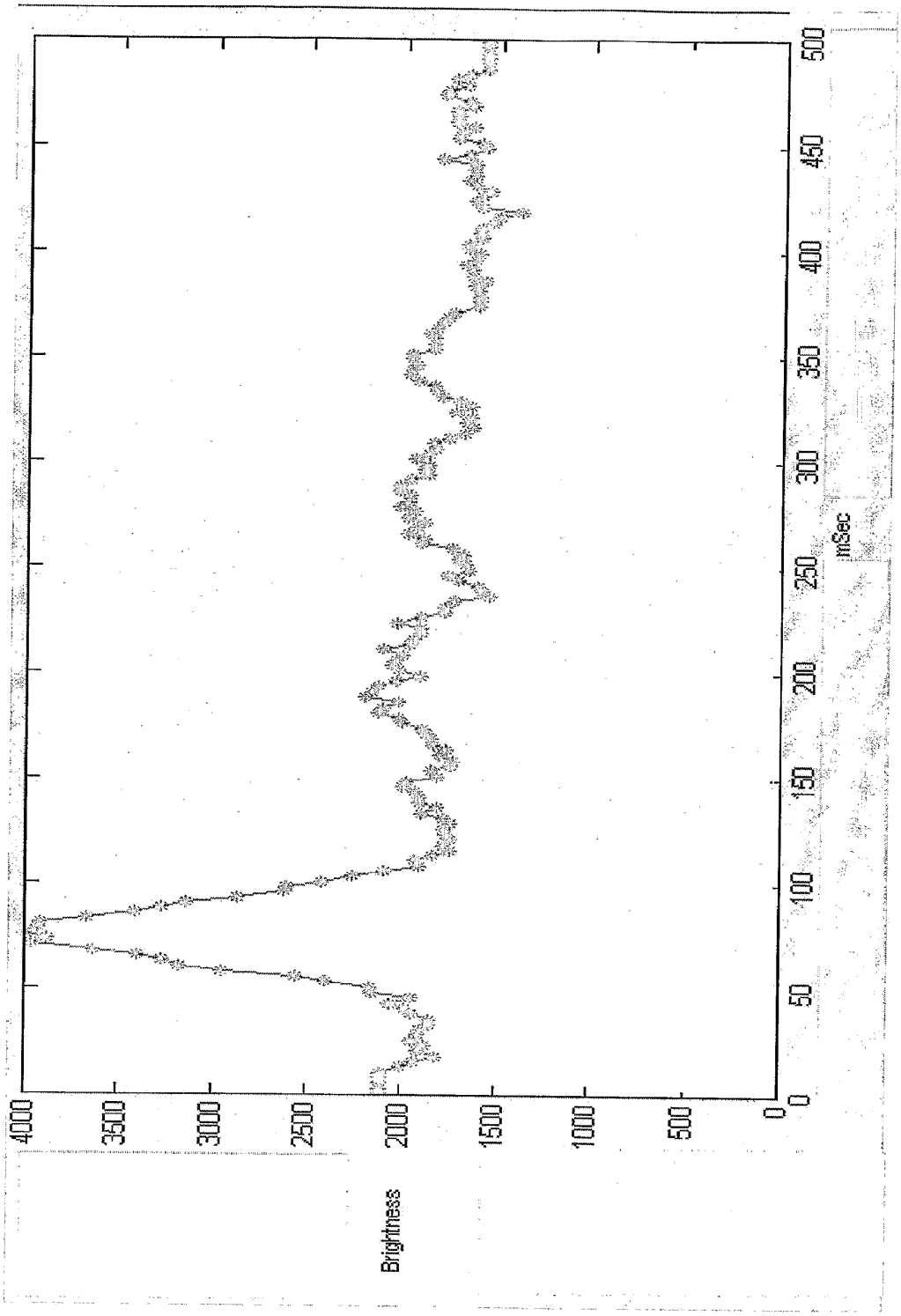
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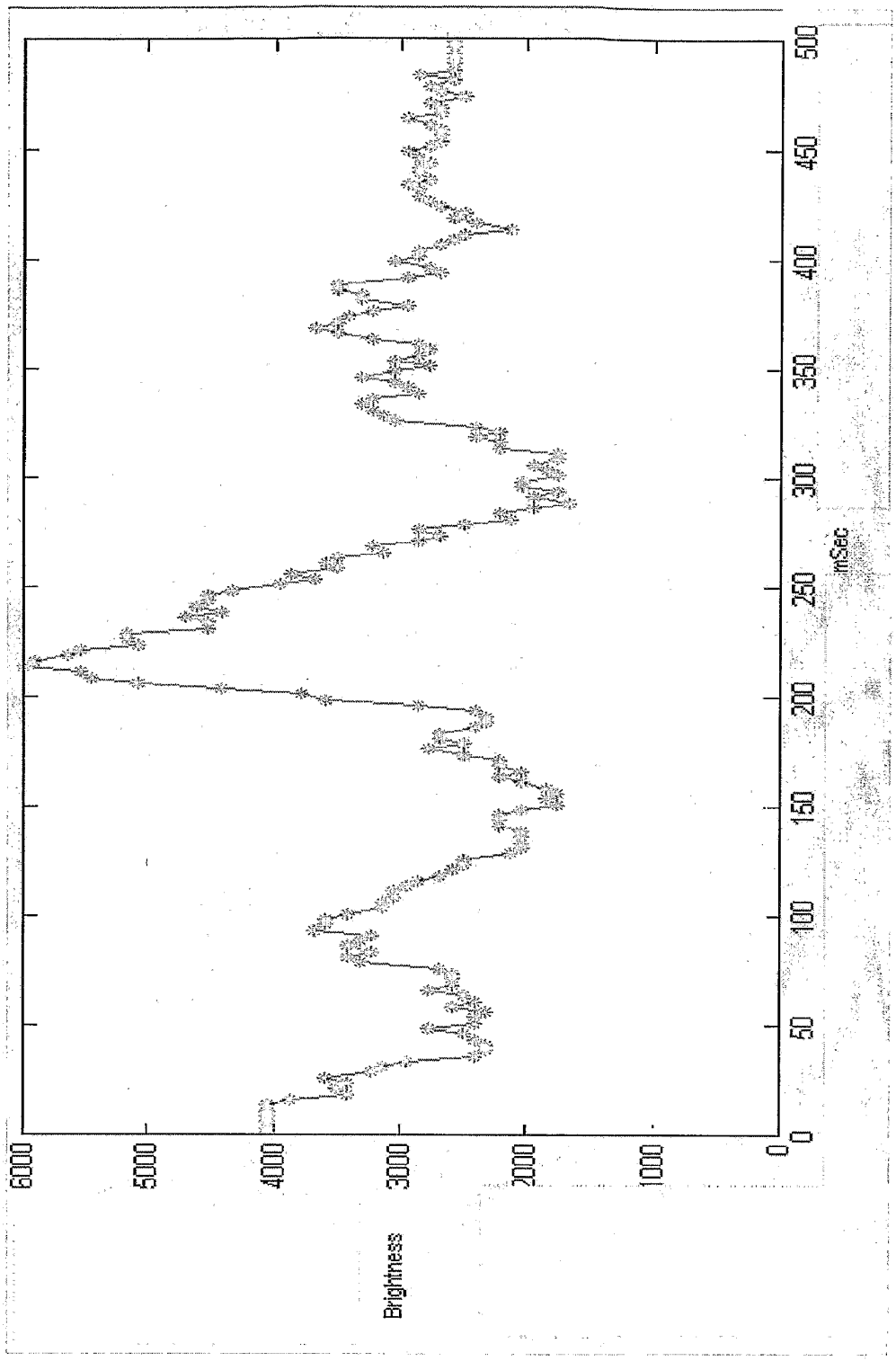
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(54) Title: METHOD FOR ISOLATION OF INDEPENDENT, PARALLEL CHEMICAL MICRO-REACTIONS USING A POROUS FILTER

(57) Abstract: The present invention relates to methods and apparatus for conducting densely packed, independent chemical reactions in parallel in a substantially two-dimensional array. Accordingly, this invention also focuses on the use of this array for applications such as DNA sequencing, most preferably pyrosequencing, and DNA amplification.

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METHOD FOR ISOLATION OF INDEPENDENT, PARALLEL CHEMICAL MICRO-REACTIONS USING A POROUS FILTER

RELATED APPLICATIONS

This application claims the benefit of U.S. Application Serial No. 60/526,160 filed December 1, 2003, which is hereby incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The invention describes methods and apparati for conducting densely packed, independent chemical reactions in parallel in a membrane reactor array with mobile solid supports disposed thereon.

BACKGROUND OF THE INVENTION

High throughput chemical synthesis and analysis are rapidly growing segments of technology for many areas of human endeavor, especially in the fields of material science, combinatorial chemistry, pharmaceuticals (*e.g.*, drug synthesis, testing), and biotechnology (*e.g.*, DNA sequencing, genotyping).

Increasing throughput in any such process requires either that individual steps of the process be performed more quickly, with emphasis placed on accelerating rate-limiting steps, or that larger numbers of independent steps be performed in parallel. One approach for conducting chemical reactions in a high-throughput manner includes performing larger numbers of independent steps in parallel, specifically conducting simultaneous, independent reactions with a multi-reactor system.

A common format for conducting parallel reactions at high throughput comprises two-dimensional (2-D) arrays of individual reactor vessels, such as the 96-well or 384-well microtiter plates widely used in molecular biology, cell biology, and other areas. Individual reagents, solvents, catalysts, and the like are added sequentially and/or in parallel to the appropriate wells in these arrays, and multiple reactions subsequently proceed in parallel. Individual wells may be further isolated from adjacent wells and/or from the environment by sealing means (*e.g.*, a tight-fitting cover or adherent plastic sheet) or they may remain open. The base of the wells in such microtiter plates may or may not be provided with filters of various pore sizes.

Further increasing the number of microvessels or microreactors incorporated in such 2-D arrays has been a focus of much research. This has been and is being accomplished by miniaturization. For instance, the numbers of wells that can be molded into plastic microtiter plates has steadily increased in recent years -- from 96, to 384, and to 1536. Efforts to further increase the density of wells are ongoing (*e.g.* Matsuda and Chung, 1994; Michael *et al.*, 1998; Taylor and Walt, 1998).

Attempts to make arrays of microwells and microvessels for use as microreactors has also been a focal point for development in the areas of microelectromechanical and micromachined systems, applying and leveraging some of the microfabrication techniques originally developed for the microelectronics industry (see Matsuda and Chung, 1994; Rai-Choudhury, 1997; Madou, 1997; Cherukuri *et al.*, 1999; Kane *et al.* 1999; Anderson *et al.*, 2000; Dannoux *et al.*, 2000; Deng *et al.*, 2000; Zhu *et al.*, 2000; Ehrfeld *et al.*, 2000).

Yet another widely applied approach for conducting miniaturized and independent reactions in parallel involves spatially localizing or immobilizing at least some of the participants in a chemical reaction on a surface. This creates large 2-D arrays of immobilized reagents. Reagents immobilized in such a manner include chemical reactants, catalysts, other reaction auxiliaries, and adsorbent molecules capable of selectively binding to complementary molecules. For purposes of this patent specification, the selective binding of one molecule to another – whether reversible or irreversible – will be referred to as a reaction process, and molecules capable of binding in such a manner will be referred to as reactants. Immobilization may be arranged to take place on any number of substrates, including planar surfaces and/or high surface area and sometimes porous support media such as beads or gels. Microarray techniques involving immobilization on planar surfaces have been commercialized for the hybridization of oligonucleotides (*e.g.* by Affymetrix, Inc.) and for target drugs (*e.g.* by Graffinity, AB).

A major obstacle to creating microscopic, discrete centers for localized reactions is that restricting unique reactants and products to a single, desired reaction center is frequently difficult. There are two aspects to this problem. The first is that "unique" reagents – *i.e.*, reactants and other reaction auxiliaries that are meant to differ from one reaction center to the next – must be dispensed or otherwise deployed to particular reaction centers and not to their nearby neighbors. Such "unique" reagents are to be distinguished from "common" reagents like solvents, which frequently are meant to be brought into substantial contact with all the reaction centers simultaneously and in parallel. The second aspect of this problem has to do with restricting reaction products to the vicinity of the reaction center where they were

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created – *i.e.*, preventing them from traveling to other reaction centers with attendant loss of reaction fidelity.

To solve the first problem, reaction centers can consists of discrete microwells with the microvessel walls (and cover, if provided) designed to prevent fluid contact with adjacent microwells. However, delivery of reagents to individual microwells can be difficult, particularly if the wells are especially small. For example, a reactor measuring 100 μm X 100 μm X 100 μm has a volume of only 1 nanoliter. This can be considered a relatively large reactor volume in many types of applications. Even so, reagent addition in this case requires that sub-nanoliter volumes be dispensed with a spatial resolution and precision of at least ±50 μm. Furthermore, addition of reagents to multiple wells must be made to take place in parallel, since sequential addition of reagents to at most a few reactors at a time would be prohibitively slow. Schemes for parallel addition of reagents with such fine precision exist, but they entail some added complexity and cost.

On the other hand, the reaction centers can be brought into contact with a common fluid, *e.g.*, such that microwells all open out onto a common volume of fluid at some point during the reaction or subsequent processing steps. However, this can cause the reaction products (and excess and/or unconverted reactants) originating in one reaction microwell or vessel to travel and contaminate adjacent reaction microwells. Such cross-contamination of reaction centers can occur (i) via bulk convection of solution containing reactants and products from the vicinity of one well to another, (ii) by diffusion (especially over reasonably short distances) of reactant and/or product species, or (iii) by both processes occurring simultaneously.

In certain cases, the individual chemical compounds that are produced at the discrete reaction centers are themselves the desired objective of the process (*e.g.*, as is the case in combinatorial chemistry). For such compounds, any reactant and/or product cross-contamination that may occur will reduce the yield and ultimate chemical purity of this "library" of discrete products. In other cases, the reaction process is conducted with the objective of obtaining information of some type, *e.g.*, information as to the sequence or composition of DNA, RNA, or protein molecules. For these reactions, the integrity, fidelity, and signal-to-noise ratio of that information may be compromised by chemical "cross-talk" between adjacent or even distant microwells.

The issue of contamination of a reaction center or well by chemical products being generated at nearby reaction centers or microwells becomes even more problematic when

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reaction sites are arrayed on a 2-D surface (or wells are arranged in an essentially two-dimensional microtiter plate) over which fluid flows. In such situations, compounds produced at a surface reaction site or within a well undergo diffusive transport up and away from the surface (or out of the reaction wells), where they are subsequently swept downstream by convective transport of fluid that is passing through a flow channel in fluid communication with the top surface of the array.

SUMMARY OF THE INVENTION

A novel technique for densely packing microreactors in a substantially 2-D arrangement is described here. This technique provides not only dense, two-dimensional packing of reaction sites, microvessels, and reaction wells, but also provides for efficient delivery of reagents and removal of products by convective flow rather than by diffusion alone. This latter feature permits much more rapid delivery of reagents and other reaction auxiliaries. In addition, it permits faster and more complete removal of reaction products and by-products than has heretofore been possible using methods and apparatus described in the prior art.

One embodiment of the invention is directed to a membrane reactor array comprising a porous supporting membrane layer attached to a planar mesh array. The planar mesh comprises a plurality of pores with reagent-carrying beads of an appropriate size disposed in the pores. As an example, an appropriate size is one whereby the beads are retained in the pores of the mesh. The mesh array is permeable to an aqueous fluid, such as a fluid or reagent used in sequencing but the mesh array is not permeable to the reagent-carrying beads. In a preferred embodiment, the planar mesh array is weaved from individual fibers with a spacing of less than about 100 μm center to center. In another preferred embodiment, the weaving may be made from two sets of parallel fibers that intersect at right angles. In other words, the weaving may be similar to the strings on a tennis racket at a microscopic scale.

Another embodiment of the invention is directed to a membrane reactor array comprising a planar mesh array with a plurality of beads disposed within the pores of the planar mesh array. Each pore has an opening of less than about 40 μm. The pores are sufficiently small such that the planar mesh array is impermeable to the beads. That is, for a planar mesh array with a pore size of 40 μm, each bead should be somewhat greater than 40 μm in diameter. In a preferred embodiment, the beads are 2 – 3 μm larger than the pore size length. This relationship between bead size and pore size also ensures that only one bead is immobilized to a pore.

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Another embodiment of the invention is directed to a method of identifying a base at a target position in one or more sample nucleic acid, preferably DNA, sequences. In one embodiment of the method, a sample DNA sequence and an extension primer, which hybridizes to the sample DNA immediately adjacent to the target position is provided. The DNA sample and extension primer is subjected to a polymerase reaction in the presence of a deoxynucleotide or dideoxynucleotide so that the deoxynucleotide or dideoxynucleotide will only become incorporated and release pyrophosphate (PP_i) if it is complementary to the base in the target position. Any release of PP_i is detected enzymatically, such as, for example, by detecting a light emission generated by an enzyme in response to the presence of PP_i. It should be noted that the light emission may be generated directly or through a chemical pathway involving additional chemical steps or amplification steps. In the method, different deoxynucleotides or dideoxynucleotides are added successively to the sample-primer mixture and subjected to the polymerase reaction to indicate which deoxynucleotide or dideoxynucleotide is incorporated. Further, the sample DNA is immobilized on a bead within a planar membrane reactor array. In a preferred embodiment, the sequencing reaction is a pyrophosphate sequencing reaction. In another preferred embodiment, the sequencing reagents, including the deoxynucleotides or dideoxynucleotides, are contacted to the nucleic acid by a flow of reagent that is normal (orthogonal) to the plane of the planar membrane reactor array. An advantage of any of the methods of this disclosure where the reagent flow is normal to the plane of the membrane reactor array is reduction or elimination of cross contamination. Because the flow is normal to the plane of the beads, each fluid stream will only contact one bead or one species of DNA before it is disposed into a waste container. The chance of cross contamination is reduced significantly.

Another embodiment of the invention is directed to a method of loading a membrane reactor array with nucleic acid. In the method, a planar mesh array that is substantially permeable to a fluid but substantially impermeable to a population of microreactor beads is provided. A fluid comprising a suspension of said population of microreactor beads is introduced onto the surface of the membrane reactor array. The microreactor beads may be linked to a sample nucleic acid or it may be unlinked. Then the beads are settled onto the pores of the membrane reactor array, preferably using a pump or negative pressure or suction. Settling may be performed, for example by allowing the beads to slowly settle out of solution under gravity. Another method of settling may involve centrifugation. In a preferred method, the fluid is drawn through the planar membrane reactor array. Since the beads are

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bigger than the pores of the planar reactor array, they are trapped (loaded) in the planar reactor array as the fluid is drawn through.

Another embodiment of the invention is directed to a microimaging system for imaging a light emission (*e.g.*, from a pyrophosphate sequencing reaction) from a membrane reactor array. The system comprises two lens groups. The first lens group is the front lens group which is positioned closer to the light source to be detected to collect the light emitted. The second lens group is the rear lens group that is positioned closer to the light detector such as a CCD detection device to image the light onto the detector. In a preferred embodiment, the lens group comprises 50 mm lens with an aperture larger than or equal to 2.8 (*e.g.*, 2.0, 1.8, 1.4, 1.0, etc.). It should be noted that the larger apertures are expressed by a smaller aperture value so that, for example, an aperture of 1 is larger than an aperture of 2.

Another embodiment of the invention is directed to a sequencing cartridge. The cartridge comprises a flow chamber for enclosing an above described membrane reactor array. A membrane supporting structure inside the flow chamber separates the flow chamber into two subchambers. The first subchamber contains the membrane reactor array and also contains an inlet and a first outlet for controlling a fluid flow tangential to the membrane reactor array. The first subchamber also contains a window, covered with a transparent material such as glass or crystal, to allow the optical examination of the membrane reactor array. The second subchamber without the membrane reactor array contains a second outlet allowing fluid to flow normally (orthogonally) from the inlet, through the membrane reactor array, and out through the second outlet. In this manner, both the tangential and normal flow of reagent through the membrane reactor array may be regulated.

Another embodiment of the invention is directed to a method of amplifying a sample nucleic acid on a bead and then loading the bead on a membrane reactor array. In this method, one or more nucleic acid templates to be amplified are individually attached to separate beads to form a population of nucleic acid template-carrying beads. Then the template-carrying beads are suspended in an amplification reaction solution containing reagents necessary to perform nucleic acid amplification. Then, an emulsion is formed to encapsulate the plurality of said template-carrying beads with PCR reaction solution to form a plurality of microreactors (see, *e.g.*, U.S. Application Serial No. 60/476,504, filed June 6, 2003, which is hereby incorporated by reference in its entirety). In a subsequent step one or more nucleic acid templates in fluidic isolation from each other are amplified to form multiple copies of nucleic acid templates. The amplified nucleic acid templates, still in

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fluidic isolation, are attached to the beads. Then the beads are loaded into the membrane reactor array.

Another embodiment of the invention is directed to a method of producing a membrane reactor array by providing one or more nucleic acid templates to be amplified wherein a plurality of nucleic acid templates are individually attached to separate beads to form a population of nucleic acid template-carrying beads. Then the beads are loaded onto the membrane reactor array. After loading, the template-carrying beads are contacted to an amplification reaction solution containing reagents necessary to perform nucleic acid amplification. Then, the nucleic acid template is amplified in fluidic isolation from each other to form amplified nucleic acid. Fluidic isolation may be achieved, for example, by removing most fluids from the membrane reactor array and allowing amplification on the fluids that is still in contact with the bead. Furthermore, oil may be added to the membrane reactor array to prevent evaporation during amplification. Any oil may be removed by organic solvents (such as hexane).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 represents an integral or physical composite of a microchannel array and a porous membrane barrier forming a membrane reactor array. The flow of fluid through the membrane reactor array carries reaction participants along with the fluid.

FIG. 2 shows a schematic of one version of the convective flow sequencing apparatus described herein.

FIG. 3 shows a membrane reactor array comprising a nylon mesh membrane useful for trapping reagent-carrying beads and one embodiment of the reagent-carrying beads. Shown here are sepharose beads).

FIG. 4 shows the size of the sepharose beads relative to the membrane pores. In panel A, the beads are shown to be swollen in liquid. In contrast, panel B shows how the beads are shrunken when dry.

FIG. 5 represents a membrane holder with a circular optical window; a flow chamber with an inlet port and a first outlet port; a nylon membrane with sepharose beads; a fine pore nylon membrane; a membrane support structure with 1.02 mm holes, 1.35 pitch; and a funnel-shaped collector with a second outlet port.

FIG. 6 shows a membrane support structure with a 50 mm capillary plate with 10 μm holes and a 12 μm pitch for supporting the membrane reactor array.

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FIG. 7 shows a schematic of a pyrophosphate-based sequencing method with photon detection.

FIG. 8 shows an automated convective sequencing apparatus.

FIG. 9 shows a sequencing pyrogram indicating the results of sequence analysis.

FIG. 10 shows a sequencing graph. Panel A shows the results for the negative control (no template added), where no sequence was detected. Panel B shows the results for the template, where the correct sequence was detected.

FIG. 11 shows a coupling of amine-primer and amine-biotin to NHS activated sepharose beads.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this patent application, the membrane reactor array is a general term that describes both the confined membrane reactor array (CMRA) and the unconfined membrane reactor array (UMRA) as described by U.S. Application Serial No. 10/191,438 filed July 8, 2002, the entire contents of which are incorporated herein by reference. Methods and apparati are described here for providing a dense array of discrete reaction sites, microreactor vessels, and/or microwells in a substantially two-dimensional configuration (see Fig. 2) and for charging such microreactors with reaction participants by effecting a convective flow of fluid normal to the plane of and through the array of reaction sites or microvessels. The convective flow or delivery of reactants includes both the delivery of sequencing reactants (*e.g.*, dNTPs) towards the reaction site, and convective removal of excess reactants away from the site. Fluid flow sweeps the sequencing reaction products (pyrophosphate (PP_i), ATP) through the reaction region in a normal direction thus countering back-diffusion and resultant contamination.

Reaction participants that may be charged, concentrated, and contained within said reaction sites or microreactor vessels by methods of the present invention include high-molecular-weight reactants, catalysts, and other reagents and reaction auxiliaries. In the context of oligonucleotide sequencing and DNA/RNA analysis, such high-molecular-weight reactants include, for example, oligonucleotides, longer DNA/RNA fragments, and constructs thereof. These reactants may be free and unattached (if their molecular weight is sufficient to permit them to be contained by the method of the present invention), or they may be covalently bound to or otherwise associated with, *e.g.*, high-molecular-weight polymers, high-surface-area beads or gels, or other supports known in the art. Examples of reaction catalysts that may be similarly delivered to and localized within said reaction sites or

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microvessels include enzymes, which may or may not be associated with or bound to solid-phase supports such as porous or non-porous beads. As another example, enzymes such as polymerase may be attached to a support (*e.g.*, beads) as a reagent. In addition, additional polymerase may be delivered to the beads during a reaction to replenish, or supplement the bound polymerase. In this example, a reagent (the polymerase) is both free and unattached and covalently bound or associated with the support. Any reagent of this invention may be both free and bound as described in the above example.

The present invention also includes a means for efficiently supplying relatively lower-molecular-weight reagents and reactants to said discrete reaction sites or microreactor vessels. Also included are means for efficiently removing unconverted reactants and reaction products from said reaction sites or microvessels. More particularly, efficient reagent delivery and product removal are accomplished in the present invention by arranging for at least some convective flow of solution to take place in a direction normal to the plane of the substantially two-dimensional array of reaction sites or microreactor vessels. This flow can lead past or through the discrete sites or microvessels, respectively, where chemical reaction takes place. In this instance, reactants and products will not necessarily be retained or concentrated at the reaction sites or within the reaction microvessels or microwells; indeed, it may be desired that certain reaction products be rapidly swept away from and/or out of said reaction sites or microvessels.

This invention also minimizes the amount of contamination among neighboring reaction sites or "blow-by" which typically occurs in diffusive sequencing. In diffusive sequencing, reaction products from an upstream site have multiple chances to contaminate downstream sites. This contamination is a cumulative effect that may worsen if there are a large number of DNA fragments and multiplets in the upstream reaction sites. In the present invention, the possibility of blow-by has been minimized such that any possible contamination is not cumulative. Specifically, a fluid sheath is formed over each reaction site such that flow downwards relative to the flow laterally is sufficient to prevent blow by or contamination of neighboring reaction sites. Also, each of the beads is washed independently by downward flow of wash solution so that the washing of each reaction site (and any bead disposed therein) is independent of washing of neighboring reaction sites during the washing step.

In addition to including means for providing a controlled convective flux of fluid normal to and across the substantially planar array of reaction sites or microreactors, the present invention also includes permselective, porous filter means capable of discriminating between large (*i.e.*, high-molecular-weight) and small (*i.e.*, low-molecular-weight) reaction

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participants. This filter means is capable of selectively capturing or retaining certain reaction participants while permitting others to be flushed through and/or out the bottom of the microreactor array. By proper selection of the porous filter and the judicious choice of convective flux rates, considerable control over the location, concentration, and fate of reaction participants can be realized.

Membrane Reactor Arrays

In a preferred embodiment, the apparatus of the present invention consists of an array of microreactor elements comprised of at least two functional elements that may take various physical or structural forms. These include: (i) a planar array layer comprised of an array of microchannels or microwells and on average no more than one reagent-carrying bead or support disposed therein (it is recognized that there may be infrequent times where more than one bead is disposed in a microvessel but this is not preferred), and (ii) a porous high flow resistance membrane comprising, *e.g.*, a porous film or membrane in the form of a sheet or thin layer. These two elements are arranged next to and in close proximity or contact with one another, with the plane of the microchannel/microvessel element parallel to the plane of the porous filter element. In other words, the planar array layer and the porous filter element may be in contact with each other to form one sheet with two layers.

As referred to herein, the side of this composite structure containing the microchannel or microvessel array will be referred to hereinafter as the "top", while the side defined by the porous filter will be referred to as the "bottom" of the structure. In a preferred embodiment, contact between the planar array and the porous filter may be tight, in which case a fluid cannot travel from the bottom side of one microchannel into another microchannel without entering and exiting the porous filter element. In another embodiment, the contact may also be loose, in which case some fluid may travel from one microchannel to another on the bottom of the planar array layer without passage through the porous filter element. In practice, the flow of fluids in a direction normal to the plane of the membrane reactor array would prevent significant cross contamination between microchannels even if the contacts were loose.

The membrane reactor array will be discussed in more detail following the discussions of each of its component elements.

Planar Array Layer

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The planar array layer comprises individual pores (also called microchannel or microvessel) elements. Each pore consists of a single microchannel. The planar array layer comprises a plurality of pores, with the longitudinal axes of said pores being arranged in a substantially parallel manner, and with the downstream ends of said channels being in functional contact with a porous filter element (described below). The aspect ratio of the microchannels (*i.e.*, their height- or length-to-diameter ratio) may be small or large, and their cross-section may take any of a number of shapes (*e.g.*, circular, rectangular, hexagonal, etc.). As discussed further below, it is not at all essential that the microchannel walls be continuous or regular. Highly porous, "spongy" matrices with interconnecting pores communicating between adjacent channels will also serve functionally as microchannel elements, despite the fact that they will not necessarily contain discrete or functionally confined or isolated microchannels *per se*. In a preferred embodiment, the porous array layer comprises a nylon membrane.

The fundamental requirement is that the effective pore size of the porous array layer be comparable to or smaller than the diameter of the particles that one desires to retain.

In one embodiment, the porous array layer typically contains more than 10,000 pores, preferably more than 50,000 pores, 100,000 pores, or 250,00 pores, and in one preferred embodiment more preferably between about 100,000 and 1,000,000 pores, and in another preferred embodiment, preferably between about 250,000 and 750,000 pores. The porous array layer is typically constructed to have pores with a center-to-center spacing between about 5 to 200 μm, preferably between about 10 to 150 μm, even more preferably between about 25 to 100 μm, and most preferably between about 50 to 78 μm. In five preferred embodiments, the center to center (c-t-c) spacing is about 58 μm, 64 μm, 68 μm, 70 μm, or 100 μm, respectively.

In one embodiment, we contemplate that each reaction chamber in the porous array layer has a pore size length in at least one dimension of between about 5 μm and 200 μm, preferably between about 10 μm and 150 μm, more preferably between about 15 μm and 100 μm, most preferably between about 20 μm and 35 μm. In one embodiment, the reaction chamber can be square and can have the above cited dimensions (or can be rectangular with those dimensions along one linear dimension of the rectangle). For substantially square pores, the average size can include, *e.g.*, about 15 to 100 μm in length, or preferably, about 20 to 35 μm in length. In four preferred embodiments, the reaction chamber is square with pore size lengths of about 25 μm, 28 μm, 30 μm, or 31 μm, respectively.

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In four preferred embodiments, the porous array layer is selected from a nylon membrane with: (1) a center to center (c-t-c) spacing of about 64 μm and a 31 μm pore size length; (2) a center to center (c-t-c) spacing of about 58 μm and a 25 μm pore size length; (3) a center to center (c-t-c) spacing of about 70 μm and a 30 μm pore size length; and (4) a center to center (c-t-c) spacing of about 68 μm and a 28 μm pore size length.

It is advantageous to deposit or settle a particular reactant molecule (*e.g.*, an oligonucleotide or construct thereof) at discrete sites on the surface of a membrane reactor array (*e.g.*, for pyrosequencing). This may be accomplished by immobilizing said reactants on particulate or colloidal supports (*e.g.*, beads, particles), suspending the supports in a fluid, and then depositing or settling these onto the membrane reactor array surface by drawing the fluid through the membrane reactor array. One method for depositing a bead is to place a fluid suspension of beads on a membrane reactor array and allowing gravity to deposit the beads into the individual pores – a process that can be accelerated by vibration or centrifugation.

In a preferred embodiment, the planar array is substantially permeable to aqueous solutions but is substantially impermeable to the beads. This is possible, for example, if the pores are smaller than the beads so that beads cannot flow through. In another preferred embodiment, the beads reduce the size of the pores but do not eliminate the opening in the pores. In one embodiment, the beads are spherical while the pores are square (see Examples). The deposition of a round bead in a square pore would still allow a flow of fluid through the pore. In another embodiment, the beads and the pores have irregular shapes that deviate, slightly or grossly, from a perfect sphere and a perfect circle. The deposition of an imperfect spherical bead onto an imperfect circular pore would not completely block the pore. For example, in a preferred embodiment, the planar array layer is a woven mesh of polymer (*e.g.*, nylon or nitrocellulose) fibers. The fibers are woven at right angles to each other to form square pores.

Contemplated for use with the invention are commercially available materials. Preferred materials for the planar array layer of invention include precision woven open mesh fabrics, especially monofilament open mesh fabrics, such as those available from Sefar, Inc. (Ruschlikon, Switzerland). Non-limiting examples of such fabrics include Sefar Nitex (PA 6.6), *e.g.*, Cat. # 03-25/14, 03-28/17, 03-30/18, 03-30/20, and 03-35/16. Other preferred materials for the planar array layer include woven nylon net filters such as those available from Millipore (Bedford, MA), including, but not limited to, Cat. # NY41 025 00, NY41 047 00, NY41 090 00, and NY41 000 10.

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Porous High Flow Resistance Membrane

The porous high flow resistance filter element provides significant flow restriction in the membrane reactor array. This flow restriction is useful in achieving uniform flow of reagents through the membrane reactor array.

Membrane reactor arrays without a porous high flow resistance membrane element may suffer from a non-uniform flow of reagent. In the absence of a porous high flow resistance membrane, a pore with a bead would permit a flow that is reduced when compared to a pore without a bead. It follows that a mixture of open pores and bead loaded pores in a membrane reactor array would have uneven flow. In a biochemical reaction, an uneven flow may cause some pores to receive reagents in a non-uniform fashion. Non-uniform delivery of reagents may lead, at least, to a delay in performing reactions because a longer flow is necessary to deliver reagents to all the pores. More significantly, non-uniform delivery may cause errors in interpreting results. For example, some pores may receive more reagents than others and the excess or lack of reagents may change the results of a biochemical reaction. Spatially uneven flow through the porous array layer may also result in the lateral diffusion of reaction products from a reactive pore (*i.e.*, one containing a bead) to a neighboring empty pore, which can lead to cross-contamination, or bleed.

The problem with uneven (non-uniform) flow can be reduced to an insignificant level by the use of a porous high flow resistance membrane element. The porous high flow resistance membrane may be configured in any number of ways to provide satisfactory flow resistance in conjunction with the planar array layer. The porous high flow resistance membrane may comprise pores that are less than one tenth (1/10) or less than one hundredth (1/100) the size of the pores in the planar array layer. The porous high flow resistance membrane, because of its small pores, will have a flow restriction that is about 10-fold or more, preferable about 100-fold or more, than that of the planar array layer. Because the porous high flow resistance membrane provides most of the flow restriction in a membrane reactor array, the pores of the planar array layer, regardless of whether it contains a bead, would provide only a small portion of the flow restriction.

In one embodiment, we contemplate that the porous high flow resistance membrane has an average pore size of between about 0.01 μm and 10 μm, preferably between about 0.01 μm and 5 μm, more preferably between about 0.01 μm and 0.5 μm and even more preferably between about 0.1 μm and 1 μm, or less than 0.1 μm. This is particularly the case when a symmetric membrane is used. In one embodiment, the pore size is about 0.2 μm and in

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another embodiment, the pore size is about 0.02 μm. Preferably, the high flow resistance membrane has a pore diameter that is less than 10%, or less than 1%, of the pore diameter of the planar array layer.

If the membrane porosity is asymmetric (*i.e.*, an anisotropic membrane) then different pores sizes may also be used.

In another embodiment, membranes with altered surface chemistries may be used. For example, the porous high flow resistance membrane may restrict flow of aqueous materials by being composed of a hydrophobic material. In a preferred embodiment of a hydrophobic porous high flow resistance membrane, we contemplate using a 10 - 40 μm PTFE membrane. In another preferred embodiment, the membrane has a pore size of less than or about 20 μm. Additionally, membranes with altered surface chemistries may be used (*e.g.*, hydrophobic membranes).

Commercially available materials are contemplated for use with the invention. Preferred materials for the high-flow resistance membrane of the invention include nylon membrane filters such as those available from Millipore, including, but not limited to, Cat. # GNWP 025 00 and GNWP 047 00. Other preferred materials for the high-flow resistance include membrane ceramic filters such as those available from Refractron Technologies Corp. (Newark, NY), including, but not limited to, alumina or silicon carbide filter plates with 15-30 μm pores and 40-50% porosity (volume %).

Optional Porous Structural Support Layer

In addition to a planar layer and a porous high flow resistance membrane, the membrane reactor array can also employ a porous structural support layer; that is, more porous than the other two layers and that is, in one embodiment, placed against and/or attached to the high flow resistance membrane. This porous support layer can be used to provide mechanical support to membrane reactor array. See, *e.g.*, Fig. 5 where an example of this is shown.

The porous support layer may be made from any material such as metals, glass, polymers, silicon, ceramics with holes formed during manufacture (*e.g.*, sintering, drilled by laser, cracking, etching, and the like). It is noted that while a nonreactive material is generally preferred for the porous support layer, a nonreactive material is not necessary as long as the flow of reagents from the planar array layer to the porous support layer is sufficiently fast to prevent back diffusion of any molecules from the porous support layer to the planar array layer. In one preferred embodiment, we contemplate that the porous support layer comprises a metal mesh.

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In various embodiments, commercially available materials can be used for the support layer. Preferred materials include stainless steel microfiltration meshes, such as Spectra/Mesh® from Spectrum Laboratories (Rancho Dominguez, CA), including, but not limited to, Cat. # 145827, 145936, 145826, and 145935.

Membrane Reactor Array Configuration

It should be appreciated that the membrane reactor arrayed can be constructed in a number of different configurations. For example, the porous high flow resistance membrane may be positioned on the top or the bottom of the planar array layer. In addition, two porous high flow resistance membranes may be utilized, with the planar array layer between them. In addition, the porous structural support layer may be positioned in several different configurations, *e.g.*, on the top, bottom or in the middle of the membrane reactor array. As will be appreciated, the porous structural support layer is optional and may not be required when the membrane reactor array is configured with sufficient inherent support (*e.g.*, when the membrane reactor array is provided with additional support by being affixed to a circumferential support, much like a drum head).

In many cases it will be appropriate to consider the entire array assembly (*i.e.*, the combination of microchannel/microvessel element plus porous filter element) as a single substantially two-dimensional structure comprised of either an integral or a physical composite, as described further below. The membrane reactor arrays of the present invention will be seen to possess some of the general structural features and functional attributes of commercially available microtiter filter plates of the sort commonly used in biology laboratories, wherein porous filter disks are molded or otherwise incorporated into the bottoms of plastic wells in 96-well plates. However, the membrane reactor array is differentiated from these by the unparalleled high density of discrete reaction sites that it provides, by its unique construction, and by the novel and uniquely powerful way in which it can be operated to perform high-throughput chemistries – for example, of the sorts applied to the amplification and/or analysis of DNA.

The composite microreactor/filter structure – *i.e.*, the membrane reactor array of the present invention -- can take several physical forms; as alluded to above, two such forms are represented by physical composites and integral composites, respectively. The two functional elements of the structure include the planar array layer and the porous high flow resistance membrane. These elements may be provided as separate parts or components that are merely laid side-by-side, pressed together, or otherwise attached in the manner of a

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sandwich or laminate. This structural embodiment will be referred to hereinafter as a "physical composite". Additional porous supports (*e.g.*, fine wire mesh or very coarse filters) and/or spacing layers may also be provided where warranted to provide mechanical support. Plastic mesh, wire screening, molded or machined spacers, or similar structures may be provided atop the membrane reactor array to help provide spatial separation between tangential flow of fluid across the top of the membrane reactor array and the upper surface of the membrane reactor array. Similar structures may be provided beneath the membrane reactor array to provide a pathway for egress of fluid that has permeated across the membrane reactor array.

In contrast to the operation of many prior-art microreactor arrays, wherein diffusion of reactants into and products back out of an array of microvessels occurs solely by diffusion, the operation of the membrane reactor arrays of the present invention employs a modest convective flux through the membrane reactor array. In particular, a small pressure difference is applied from the top to the bottom surface of the membrane reactor array sufficient to establish a controlled convective flux of fluid through the structure in a direction normal to the substantially planar surface of the structure. Fluid is thus made to flow first through the microchannel element and then subsequently across the porous filter element. This convective flow enables the rapid delivery to the site of reaction of low-molecular-weight reactants and the efficient and complete removal of low-molecular-weight reaction products from the site of their production. Particularly important is the fact that the convective flow serves to impede or substantially prevent the back-diffusion of reaction products out of the upstream ends of the microchannels, where otherwise they would be capable of contaminating adjacent or even distant microreactor vessels.

In certain reaction systems of interest (*e.g.*, DNA analysis by pyrosequencing, as discussed in more detail below), it may be necessary to avoid covalently immobilizing certain macromolecular reagents altogether. The DNA polymerase used in pyrosequencing is a case in point. It is believed that DNA polymerase should retain at least a certain degree of mobility if it is to function optimally. As a consequence, this particular enzyme must normally be treated as a consumable reagent in pyrosequencing, since it is not desirable to covalently immobilize it and reuse it in subsequent pyrosequencing steps. The polymerase may be in a native form or "tagged" with a moiety that adheres to the beads, such as biotin. For the polymerase, we contemplate: a) placing polymerase in contact with template loaded beads; b) flowing polymerase over the array; and c) both (*i.e.*, employing (a) and (b) together). However, the present invention provides means for localizing this macromolecular

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reagent within the microchannels or microvessels of a membrane reactor array without having to covalently immobilize it.

Applications of the Membrane Reactor Array

Many different types of reactions can be performed in a membrane reactor array. In one embodiment, each cavity or reaction chamber of the array contains reagents for analyzing a nucleic acid or protein. Not all reaction chambers are required to include a nucleic acid or protein target. Typically those reaction chambers that contain a nucleic acid contain only a single species of nucleic acid (*i.e.*, a single sequence that is of interest). There may be a single copy of this species of nucleic acid in any particular reaction chamber, or they may be multiple copies. It is generally preferred that a reaction chamber contain at least 1,000,000 copies of the species of nucleic acid sequence of interest, preferably at least between 2,000,000 and 20,000,000 copies, and most preferably between about 5,000,000 to 15,000,000 copies of the species of nucleic acid sequence of interest. In one embodiment the nucleic acid species is amplified to provide the desired number of copies using polymerase chain reaction ("PCR") (preferred), rolling circle amplification ("RCA"), ligase chain reaction, other isothermal amplification, or other conventional means of nucleic acid amplification. In one embodiment, the nucleic acid is single stranded. In other embodiments the single stranded DNA is a concatamer with each copy covalently linked end to end.

The nucleic acid may be immobilized in the reaction chamber, either by attachment to the chamber itself or preferably by attachment to a mobile solid support (*e.g.*, a bead) that is delivered to the chamber. A bioactive agent could be delivered to the array, by dispersing over the array a plurality of mobile solid supports, each mobile solid support having at least one reagent immobilized thereon, wherein the reagent is suitable for use in a nucleic acid sequencing reaction.

The array can also include a population of mobile solid supports disposed in the reaction chambers, each mobile solid support having one or more bioactive agents (such as a nucleic acid or a sequencing enzyme) attached thereto. The diameter of each mobile solid support can vary, we prefer the diameter of the mobile solid support to be such that only one bead is trapped within a single pore in the sequencing membrane. Not every reaction chamber in the sequencing membrane need contain a mobile solid support. There are numerous contemplated embodiments; in one embodiment, at least 5% to 20% of the reaction chambers can have a mobile solid support having at least one reagent immobilized thereon; a second embodiment has about 20% to 60% of the reaction chambers can have a mobile solid support

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having at least one reagent immobilized thereon; and a third embodiment has about 50% to 100% of the reaction chambers with a mobile solid support having at least one reagent immobilized thereon. Preferably, the percentage of pores loaded with beads is about 5%, 10%, or 25%.

The mobile solid support typically has at least one reagent immobilized thereon. For the embodiments relating to pyrosequencing reactions or more generally to ATP detection, the reagent may be a polypeptide with sulfurylase or luciferase activity, or both. Alternatively, enzymes such as hypoxanthine phosphoribosyltransferase, xanthine oxidase, uricase or peroxidase could be utilized (*e.g.*, Jansson and Jansson (2002), incorporated herein by reference). The mobile solid supports can be used in methods for dispersing over the array a plurality of mobile solid supports having one or more nucleic sequences or proteins or enzymes immobilized thereon.

In another aspect, the invention involves an apparatus for simultaneously monitoring the array of reaction chambers for light generation, indicating that a reaction is taking place at a particular site. In this embodiment, the reaction chambers are sensors, adapted to contain analytes and an enzymatic or fluorescent means for generating light in the reaction chambers. In this embodiment of the invention, the sensor is suitable for use in a biochemical or cell-based assay. The apparatus also includes an optically sensitive device arranged so that in use the light from a particular reaction chamber would impinge upon a particular predetermined region of the optically sensitive device, as well as means for determining the light level impinging upon each of the predetermined regions and means to record the variation of the light level with time for each of the reaction chamber.

In one specific embodiment, the instrument includes a light detection means having a light capture means and a second fiber optic bundle for transmitting light to the light detecting means. We contemplate one light capture means to be a CCD camera. The second fiber optic bundle is typically in optical contact with the array, such that light generated in an individual reaction chamber is captured by a separate fiber or groups of separate fibers of the second fiber optic bundle for transmission to the light capture means.

The membrane reactor array can be utilized to achieve highly parallel sequencing without electrophorectic separation of DNA fragments and associated sample preparation. The membrane reactor array can also be used for other uses, *e.g.*, combinatorial chemistry. For detection purposes, an array of photodetectors is utilized for monitoring light producing reactions within the membrane reactor array. In a preferred embodiment, the array of photodetectors is a CCD camera. Another method of detection of discrete reactions within

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the membrane reactor array is to monitor changes in light absorption as an indicator of a chemical reaction in a membrane reactor array using an array of photodetectors.

Sequencing of DNA via Pyrophosphate Detection

The methods and apparatuses described are generally useful for any application in which the identification of any particular nucleic acid sequence is desired. For example, the methods allow for identification of single nucleotide polymorphisms (SNPs), haplotypes involving multiple SNPs or other polymorphisms on a single chromosome, and transcript profiling. Other uses include sequencing of artificial DNA constructs to confirm or elicit their primary sequence, or to identify specific mutant clones from random mutagenesis screens, as well as to obtain the sequence of cDNA from single cells, whole tissues or organisms from any developmental stage or environmental circumstance in order to determine the gene expression profile from that specimen. In addition, the methods allow for the sequencing of PCR products and/or cloned DNA fragments of any size isolated from any source. The DNA may be genomic DNA or cDNA and may be derived from viral, bacterial, fungal, mammalian, or human (preferably) sources.

Sequencing of DNA by pyrophosphate detection ("pyrophosphate sequencing") is described in various patents (Hyman, 1990, US Patent Number 4,971,903; Nyren *et al.*, US Patents 6,210,891 and 6,258,568 and PCT Patent application WO98/13523; Hagerlid *et al.*, 1999, WO99/66313; Rothberg, US Patent 6,274,320, WO01/20039) and publications (Hyman, 1988; Nyrén *et al.*, 1993; Ronaghi *et al.*, 1998, Jensen, 2002; Schuller, 2002). The contents of the foregoing patents, patent applications and publications cited here are incorporated herein by reference in their entireties. Pyrophosphate sequencing is a technique in which a complementary sequence is polymerized using an unknown sequence (the sequence to be determined) as the template. This is, thus, a type of sequencing technique known as "sequencing by synthesis". Each time a new nucleotide is polymerized onto the growing complementary strand, a pyrophosphate (PP_i) molecule is released. This release of pyrophosphate is then detected. Iterative addition of the four nucleotides (dATP, dCTP, dGTP, dTTP) or of analogs thereof (*e.g.*, α-thio-dATP), accompanied by monitoring of the time and extent of pyrophosphate release, permits identification of the nucleotide that is incorporated into the growing complementary strand. A schematic of pyrophosphate based sequencing is shown in Fig. 7.

Pyrophosphate can be detected via a coupled reaction in which pyrophosphate is used to generate ATP from adenosine 5'-phosphosulfate (APS) through the action of the enzyme

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ATP sulfurylase. The ATP is then detected photometrically via light released by the enzyme luciferase, for which ATP is a substrate. (It may be noted that dATP is added as one of the four nucleotides for sequencing by synthesis and that luciferase can use dATP as a substrate. To prevent light emission on addition of dATP for sequencing, a dATP analog such as α-thio-dATP is substituted for dATP as the nucleotide for sequencing. The α-thio-dATP molecule is incorporated into the growing DNA strand, but it is not a substrate for luciferase.)

Pyrophosphate sequencing can be performed in a membrane reactor array in several different ways. One such protocol follows:

(1) capture DNA whose sequence is to be determined (preferably, many copies of a single sequence) onto beads and load beads onto the membrane reactor array;

(2) for negative control a separate set of beads are prepared as in step (1) with the exception that no DNA is captured. These beads are used along with the DNA loaded beads and serves as a negative control. The negative control is useful, at least, for determining background signal levels; unless specified otherwise, all subsequent steps are performed in parallel using DNA loaded beads and negative control beads;

(3) load: (a) luciferase and sulfurylase onto beads and dispose the loaded beads onto the membrane reactor array (loading beads using any suitable methods of attaching proteins to surfaces known in the art); and (b) DNA polymerase (*e.g.* Klenow fragment) onto the membrane reactor array (can be done concurrently with step (a) above); and

(4) flow a mixture of dXTP, APS (a substrate for sulfurylase), and luciferin (a substrate for luciferase) through the membrane reactor array, cycling through the four nucleotides (dCTP, dGTP, dTTP, α-thio-dATP, or any suitable dATP analog) one at a time. It will be noted that these are all low-molecular weight molecules, so they will pass through the membrane reactor array without said molecules undergoing appreciable concentration polarization.

The upstream-to-downstream flow of fluid into and through the membrane reactor array thus causes:

(a) addition of the appropriate dXTP (dATP, dTTP, dGTP or dCTP) by the polymerase and attendant production of PP_i in the region of the DNA being sequenced (with APS and luciferin flowing through);

(b) production of ATP from APS and PP_i when the latter are brought into contact with the sulfurylase enzyme (with luciferin flowing through); and

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(c) production of light from ATP and luciferin in the vicinity of the luciferase enzyme.

Light production is then monitored by a photodetector – discussed in more detail below. For example, a CCD camera, optically coupled by a lens or other means to the membrane reactor array, is capable of monitoring light production simultaneously from many microchannels or discrete reaction sites. CCD cameras are available with millions of pixels, or photodetectors, arranged in a 2-D array. Light originating from one reaction chamber or discrete reaction site in or on a membrane reactor array can be made to strike one or a few pixels on the CCD. Thus, if each reaction chamber or reaction site is arranged to contain and conduct an independent sequencing reaction, each reaction can be monitored by one (or at most a few) CCD elements or photodetectors. By using a CCD camera or other imaging means comprising millions of pixels, the progress of millions of independent sequencing reactions is simultaneously monitored.

Further, each reaction chamber or reaction site can be made to hold the amplification products from only a single strand of DNA, and if different reaction chambers (or bead disposed therein) hold the amplification products of different strands of DNA, then the simultaneous sequencing of millions of different strands of DNA is possible. The distribution of DNA to be sequenced can be accomplished in many ways, two of which follow:

(a) The amplification products of a single oligonucleotide strand are attached to a bead, and beads from many independent amplification reactions are combined and placed onto a membrane reactor array; or

(b) Many different strands of DNA are added in dilute concentration and applied to the membrane reactor array (each strand of DNA separately attached to a different bead) such that many reaction chambers or discrete reaction sites contain only a single strand of DNA. The DNA is then amplified within or upon the membrane reactor array through one series of reactions, and then it is directly sequenced via addition of the reagents described above. One such technique (polymerase chain reaction or PCR) for amplification of DNA within the pores of a membrane reactor array is described below (see Example 2).

Delivery of the DNA to be sequenced and the enzymes and substrates necessary for pyrophosphate-based sequencing can be accomplished in a number of ways.

In a preferred embodiment, one or more reagents are delivered to the membrane reactor array immobilized or attached to a population of mobile solid supports, *e.g.*, a bead or microsphere. The bead or microsphere need not be spherical; irregular shaped beads may be used. The beads are typically constructed from numerous substances, *e.g.*, plastic, glass or

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ceramic, and cross-linked agarose gel. The bead of the invention may comprise various chemistries, such as, for example, methylstyrene, polystyrene, acrylic polymer, latex, paramagnetic, thoria sol, carbon graphite and titanium dioxide. The construction or chemistry of the bead can be chosen to facilitate the attachment of the desired reagent. In a preferred embodiment, the beads are magnetic or paramagnetic.

Bead sizes depend on the pore size length and width of the reaction chamber. In a preferred embodiment, the diameter of each mobile solid support is chosen so that the mobile solid support cannot pass through the pores (in the membrane reactor array). It should be noted that in other embodiments, the beads may be smaller than the pores – the porous high flow resistance membrane layer can stop the bead from flowing through the membrane reactor array. In a preferred embodiment beads are sized so that only one bead can fit within a single reaction chamber and where the spatial separation between two adjacent reaction chambers has a linear dimension of between about 5 μm and 200 μm, preferably between 10 μm and 150 μm, more preferably between about 25 μm and 100 μm, such as between about 50 μm and 75 μm, and most preferably between about 20 μm and 35 μm. In a specific embodiment, the bead diameter may be 31 μm and the pore diameter may be 33 μm. Even though the 31 μm bead may flow through the 33 μm pore, the porous high flow resistance membrane layer prevents the bead from flowing through.

In some embodiments, one reagent immobilized to the mobile solid support can be a polypeptide with sulfurylase activity, a polypeptide with luciferase activity, or both on the same or different beads, or a chimeric polypeptide having both sulfurylase and luciferase activity. In one embodiment, it can be an ATP sulfurylase and luciferase fusion protein (see, *e.g.*, U.S. Patent Application Serial No. 10/122,706, filed April 11, 2002, and U.S. Patent Application Serial No 10/154,515, filed May 23, 2002; which are incorporated herein by reference in their entirety). Other sulfurylase and/or luciferase that may be used include those described in US patents 5,583,024; 5,674,713, 5,700,673, and in international application WO 00/24878; all incorporated herein in their entirety. Ultra-Glow luciferase (available from Promega) is also suitable. In a preferred embodiment, both luciferase and sulfurylase are immobilized on the same bead. Since the product of the sulfurylase reaction is consumed by luciferase, proximity between these two enzymes may be achieved by covalently linking the two enzymes in the form of a fusion protein. Alternatively, a fusion protein combining functional polymerase, sulfurylase and luciferase activity may be used. In other embodiments, the reagent immobilized to the mobile solid support can be the nucleic

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acid whose sequence is to be determined or analyzed. A DNA or RNA polymerase can be incubated with beads that have nucleic acids attached thereto.

Generally, a membrane reactor array device having normal cross-flow exhibits high levels of wash efficiency. In some cases, proper reactor array membranes and high flow resistance membranes are not available, and the chamber cannot be washed efficiently within a reasonably short period of time. This can have a significant impact on the accuracy of pyrosequencing. In such situations, apyrase may be applied to degrade the leftover nucleotides after each nucleotide delivery. The use of apyrase is typically at concentrations of 1 U/l to 100 U/l, preferably 4 U/l to 40 U/l, most preferably 8 U/l to 20 U/l. In some cases, high fidelity but low processivity polymerase (*e.g.*, Klenow) may have to be used, and in these cases, having polymerase present in the flow may be desirable. Preferably, the flow rate of the membrane reactor array device is about 0.15 ml/minute/cm^2 to 4 ml/minute/cm^2, or about 0.1 ml/minute/cm^2 to 5 ml/minute/cm^2.

Bead Attachment Chemistry

In some embodiment, the bioactive agents (*e.g.*, nucleic acids) are synthesized, and then covalently attached to the beads. As is appreciated by someone skilled in the art, this will be done depending on the composition of the bioactive agents and the beads. The functionalization of solid support surfaces such as certain polymers with chemically reactive groups such as thiols, amines, carboxyls, etc., is generally known in the art. Accordingly, "blank" beads may be used that have surface chemistries that facilitate the attachment of the desired functionality by the user. Additional examples of these surface chemistries for blank beads include, but are not limited to, amino groups including aliphatic and aromatic amines, carboxylic acids, aldehydes, amides, chloromethyl groups, hydrazide, hydroxyl groups, sulfonates and sulfates.

These functional groups can be used to add any number of different candidate agents to the beads, generally using known chemistries. For example, candidate agents containing carbohydrates may be attached to an amino-functionalized support; the aldehyde of the carbohydrate is made using standard techniques, and then the aldehyde is reacted with an amino group on the surface. In an alternative embodiment, a sulfhydryl linker may be used. There are a number of sulfhydryl reactive linkers known in the art such as SPDP, maleimides, α-haloacetyls, and pyridyl disulfides (see for example the 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated here by reference) which can be used to attach cysteine containing proteinaceous agents to the support.

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Alternatively, an amino group on the candidate agent may be used for attachment to an amino group on the surface. For example, a large number of stable bifunctional groups are well known in the art, including homobifunctional and heterobifunctional linkers (see Pierce Catalog and Handbook, pages 155-200).

In an additional embodiment, carboxyl groups (either from the surface or from the candidate agent) may be derivatized using well known linkers (see Pierce catalog). For example, carbodiimides may be used to activate carboxyl groups for attack by good nucleophiles such as amines (see Torchilin *et al.*, Critical *Rev. Therapeutic Drug Carrier Systems*, 7(4):275-308 (1991)). Proteinaceous candidate agents may also be attached using other techniques known in the art, for example for the attachment of antibodies to polymers; see Slinkin *et al.*, *Bioconj. Chem.* 2:342-348 (1991); Torchilin *et al.*, *supra*; Trubetskoy *et al.*, *Bioconj. Chem.* 3:323-327 (1992); King *et al.*, *Cancer Res.* 54:6176-6185 (1994); and Wilbur *et al.*, *Bioconjugate Chem.* 5:220-235 (1994). It should be understood that the candidate agents may be attached in a variety of ways, including those listed above. Preferably, the manner of attachment does not significantly alter the functionality of the candidate agent; that is, the candidate agent should be attached in such a flexible manner as to allow its interaction with a target.

Specific techniques for immobilizing enzymes on beads are known in the prior art. In one case, NH_2 surface chemistry beads are used. Surface activation is achieved with a 2.5% glutaraldehyde in phosphate buffered saline (10 mM) providing a pH of 6.9 (138 mM NaCl, 2.7 mM KCl). This mixture is stirred on a stir bed for approximately 2 hours at room temperature. The beads are then rinsed with ultrapure water plus 0.01% Tween 20 (surfactant), 0.02%, and rinsed again with a pH 7.7 PBS plus 0.01% Tween 20. Finally, the enzyme is added to the solution, preferably after being prefiltered using a 0.45 μm Amicon™ micropure filter.

One method of preparing beads is as follows:

1. Esterification of carboxyl derivative of sepharose beads is achieved with N-hydroxysuccinimide (NHS) and this leads to the formation of activated esters that react rapidly with primer containing amino-groups to give stable amide bonds.

2. Beads to be used for this purpose are supplied (Amersham) in 100% isopropanol to preserve the activity prior to coupling.

3. 25 μl of 1 mM amine-labeled HEG primer are dissolved in coupling buffer (200 mM NaHCO3, 0.5 M NaCl, pH 8.3).

4. Beads were activated by adding 1 ml of ice cold 1 mM HCl.

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5. Beads were washed two times with ice cold coupling buffer.

6. Amine labeled primers and amine labeled biotin, in a ratio of 1:9 respectively) is added to the beads and incubated for 15 to 30 minutes at room temperature with rotation (to allow coupling to happen). Amine-labeled biotin is added so that after coupling the emulsion PCR, the streptavidin is added to be coupled to the biotin. Then the biotinylated sulfurylase and luciferase (454 Life Sciences) will be coupled to the streptavidin.

7. Then the beads were washed one time with coupling buffer.

8. The beads were washed two times with Acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4).

9. The beads were washed three times with coupling buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3).

10. The beads were incubated with 500 µl of blocking buffer for one hour with rotation at room temperature to allow for deactivation or blocking of any leftover active groups.

11. The beads were washed with (a) coupling buffer and then with (b) acetate buffer. This wash ((a) then (b)) was repeated three times.

12. The beads were washed two times in 1x annealing buffer. The annealing buffer also serves as the storage buffer.

This procedure is illustrated in Fig. 11.

In a particularly preferred embodiment, the beads and bioactive agents are linked using a biotin/streptavidin linkage, well known to those skilled in the art.

Apparatus for Detecting a Reaction in the Membrane Reactor Array:

The invention provides an apparatus for simultaneously monitoring an array of reaction chambers for light indicating that a reaction is taking place at a particular site. The reaction event, *e.g.*, photons generated by luciferase, may be detected and quantified using a variety of detection apparati, *e.g.*, a photomultiplier tube, a CCD, CMOS, absorbance photometer, a luminometer, charge injection device (CID), or other solid state detector, as well as the apparati described herein. In a preferred embodiment, the quantitation of the emitted photons is accomplished by the use of a CCD camera fitted with a fused fiber optic bundle. In another preferred embodiment, the quantitation of the emitted photons is accomplished by the use of a CCD camera fitted with a microchannel plate intensifier. A back-thinned CCD can be used to increase sensitivity. CCD detectors are described in, *e.g.*, Bronks, *et al.*, 1995. *Anal. Chem.* 65: 2750-2757. The CCD sensitivity may be enhanced by the known method of chilling the CCD during exposure.

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An exemplary CCD system is a Spectral Instruments, Inc. (Tucson, AZ) Series 600 4-port camera with a Lockheed-Martin LM485 CCD chip and a 1-1 fiber optic connector (bundle) with 6-8 μm individual fiber diameters. This system has 4096 x 4096, or greater than 16 million pixels and has a quantum efficiency ranging from 10% to > 40%. Thus, depending on wavelength, as much as 40% of the photons imaged onto the CCD sensor are converted to detectable electrons.

The invention also provides a microimaging system for imaging a light emission (*e.g.*, from a pyrophosphate sequencing reaction) from a membrane reactor array. The system comprises two lens groups. The first lens group is the front lens group which is positioned closer to the light source to be detected to collect the light emitted. The second lens group is the rear lens group that is positioned closer to the light detector such as a CCD detection device to image the light onto the detector. In one aspect of the invention, the front lens group and rear lens group are identical.

In a preferred embodiment, the lens group comprises 50 mm lens with an aperture larger than 2.8 (*e.g.*, 2.0, 1.8, 1.4, 1.0, etc.). Preferably, a lens group has a focal length of at least 30 mm, at least 50 mm, or at least 70 mm. In specific aspects, a lens group has an aperture brighter than or equal to 4.0, or brighter than or equal to 2.8. In other aspects, a lens group has a numerical aperture larger than 0.1, 0.2, or 0.3. It should be noted that the larger apertures are expressed by a smaller aperture value so that, for example, an aperture of 1 is larger than an aperture of 2. An exemplary imaging system is shown in Fig. 2.

The data from the optical detection device can be analyzed instantaneously or stored electronically (*e.g.*, by computers, hard drives, optical drives, solid state memories) for subsequent analysis as is known to those of skill in the art.

In other embodiments, a fluorescent moiety can be used as a label and the detection of a reaction event can be carried out using a confocal scanning microscope to scan the surface of an array with a laser or other techniques such as scanning near-field optical microscopy (SNOM) which are capable of smaller optical resolution, thereby allowing the use of "more dense" arrays. For example, using SNOM, individual polynucleotides may be distinguished when separated by a distance of less than 100 nm, *e.g.*, 10 nm x 10 nm. Additionally, scanning tunneling microscopy (Binning et al., *Helvetica Physica Acta*, 55:726-735, 1982) and atomic force microscopy (Hanswa *et al.*, *Annu Rev Biophys Biomol Struct*, 23:115-139, 1994) can be used.

Additional material may be found in U.S. Application Serial No. 10/191,438 filed July 8, 2002, U.S. Application Serial No. 60/476,592 filed June 6, 2003, U.S. Application Serial No.

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60/476,602, filed June 6, 2003, U.S. Application Serial No. 60/476,313 filed June 6, 2003, U.S. Application Serial No. 60/476,504 filed June 6, 2003, and U.S. Patent 6,274,320. All patent applications and patents, listed in this disclosure, are hereby incorporated by reference in their entirety.

Many variations and alternative embodiments of the present invention as applied to DNA sequencing and other applications will be readily apparent and are considered to be within the scope of the present invention.

EXAMPLES

Example 1: Sequencing UATF9 DNA Template On Convective Rig

Preparing the Beads

Streptavidin-sepharose beads were size-selected by filtering to obtain diameter between 30-36 μm. The primers and target DNA included: MMP7A sequencing primer (5'-ccatctgttc cctccctgtc-3'; SEQ ID NO:6); target DNA, termed UATF9 (3'-ATGCCGCAAA AACGCAAAAC GCAAACGCAA CGCATACCTC TCCGCGTAGG CGCTCGTTGG TCCAGCAGAG GCGGCCGCCC TTGCGCGAGC AGAATGGCGG TAGGGGGTCT AGCTGCGTCT CGTCCGGGG-5'; SEQ ID NO:7); biotinylated primer and PCR reverse primer, termed Bio-Heg-MMP1B (5'-5Bio//iSp18//iSp18//iSp18/cca tct gtt gcg tgc gtg ct-3'; SEQ ID NO:8); and PCR forward primer, termed MMP1A (5'-cgtttcccct gtgtgccttg-3'; SEQ ID NO:9). For the PCR reverse primer, "5Bio" indicates biotin and "iSp18" indicates Spacer 18.

The biotinylated PCR products were immobilized onto Streptavidin-Sepharose beads. Immobilized PCR product was incubated in 0.10 M NaOH for 10 min, and the supernatant was removed to obtain single-stranded DNA. The beads containing single-stranded DNA were washed 3 times with 100 μl of 1X Annealing Buffer, pH 7.5 (30 mM Tris-HCl, 3 mM magnesium acetate, from Fisher). The beads were pelleted by centrifugation for 1 min at a maximum speed of 13,000 rpm. Supernatant was removed and the beads were suspended in 25 μl of 1X Annealing Buffer. Five microliter of 100 pmol sequencing primer was added to mixture. The beads were incubated at 65°C for 5 min and cooled to room temperature. The beads were washed 3 times with 100 μl of 1X Annealing Buffer and resuspended in final volume of 100 μl.

Loading The Beads Into The Membrane and Assembly into the Loading Jig

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The beads were resuspended at a concentration of about 3,500 beads per microliter in 1X Annealing Buffer. Following this, 25 µl of the suspension was added to 200 µl of 1X Assay Buffer, pH 7.8 (25 mM tricine (Fisher), 5 mM magnesium acetate (Fisher), 1 mM dithiothreitol, 0.4 mg/ml polyvinylpyrrolidone, 0.01% Tween 20, 1 mg/ml BSA, all from Sigma (St. Louis, MO)). In the loading jig, a 0.2 µm nylon membrane was placed below the 30 µm pore nylon membrane (Sefar). The loading jig was then attached to a peristaltic pump having a flow rate of 1 ml per min. Next, 200 µl of 1X Assay Buffer was used to wash the membrane. After washing, and while the membrane was partially dry, 200 µl of the bead suspension was added to the membrane. Negative pressure was applied to the membrane along with 500 µl of 1X Assay Buffer to force the beads onto the pores of the membrane.

After the application of beads, the membrane was washed with 500 µl of 1X Assay Buffer. The membrane was disassembled and placed in 1X Assay Buffer (*e.g.*, 20 ml) in a test tube (*e.g.*, Falcon) for storage. The following solutions were mixed in a container: 500 µl of biotinylated ATP Sulfurylase enzyme at 1 mg/ml (454 Life Sciences); 500 µl of biotinylated Luciferase at 3 mg/ml (454 Life Sciences); 500 µl of 1X Assay Buffer. The bead-loaded nylon membrane was placed in the enzyme mixture. The mixture was rotated with the nylon membrane for 20 min at room temperature at a speed of one rotation per two seconds. The membrane was then placed in 20 ml of 1X Assay Buffer and swirled for 2 minutes. This wash step was repeated once. A solution of 970 µl 1X Assay Buffer with 30 µl of Bst Polymerase enzyme (New England Bio Labs) was prepared. The membrane was immersed in this solution, and the solution/membrane was rotated for 25 min at room temperature at a speed of one rotation per two seconds. After the rotations, the membrane was washed two times in 20 ml of 1X Assay buffer.

Preparing the membrane for sequencing on convective rig:

A 0.2 µm nylon membrane was immersed in 20 ml of 1X Assay Buffer and placed above the wire mesh on the sequencing Jig holder. A 30 µm membrane containing the DNA beads was placed on top of the 0.2 µm nylon membrane. A sequence Jig cover with an optical glass window (13 mm) was placed on top of the membranes. The Jig cover was tightly attached to the lower part of the membrane holder. The cover and holder were threaded and tightening was performed by screwing the two parts together. The sequencing Jig was placed on the z-translation stage below the CCD camera. The acquisition time on the camera was set to 7 sec and the read out time was set to 0.25 sec. The inlet of the membrane holder was connected to the outlet of the pump, which was connected to the Valco valve

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(Valco Instruments, Houston, TX). The lower part of the membrane holder was connected to the second peristaltic pump. The outlet of the sequencing chamber was connected to the waste.

The substrate (25 mM tricine, 5 mM magnesium acetate, both from Fisher; 1 mM dithiothreitol, 0.4 mg/ml polyvinylpyrrolidone, 0.01% Tween 20, all from Sigma; 300 μM D-Luciferin, from Regis; 2.5 μM adenosine-5'-O-phosphosulfate, from Axxora, Inc.) was flowed for 2 min to prime the flow chamber and expel any air bubbles. This allowed the DNA on the nylon membrane to be equilibrated with substrate. The reagents were flowed through the chamber in the following order: 1) dCTP; 2) substrate; 3) Nuke Sp-dATP-α-thio; 4) substrate; 5) dGTP; 6) substrate; 7) dTTP; and 8) substrate. This cycle was repeated 20 times. The flow rates for the lateral and vertical flow were controlled as follows. The nucleotide flow (for steps (1), (3), (5), and (7), above) was at 2 ml/min lateral for 21 sec and then 0.5 ml/min for 7 sec. The vertical flow was 0.5 ml/min for the same time. Total time was 28 sec. The substrate flow (for steps (2), (4), (6), and (8), above) was at 2 ml/min lateral flow and 1 ml/min vertical flow 1 ml/min for 77 sec. The results of the sequencing reaction are shown in Fig. 9.

Example 2: PCR on nylon membrane containing beads and sequencing using a Pyrosequencer: The sequencing step was to confirm the fidelity of the amplified template.

The primers and probe included:

Name	Sequence	SEQ ID NO:
Adeno P1 forward	5' caa tta acc ctc act aaa gg 3'	1
Adeno P2 reverse	5' gta ata cga ctc act ata ggg 3'	2
tf2	3'cgatcaagcgtacgcacgtggttgttaaagctttttgaaagttaatc tcctggttcaccgtctgctcgtatgcggttaccaggtcggcggccgcc acgtgtgcgcgcgcgggactaatcccggttcgcgcgtcgg 5'	3
Biotinylated probe Adeno P1	5'/Bio//iSp18//iSp18/iSp18/caa tta acc ctc act aaa gg 3'	4

The sepharose beads were treated as in Example 1, with a concentration of 3,500 beads per microliter. Next, 90 μl of sepharose beads were washed by resuspension in 200 μl of 1X PCR buffer and this was followed by centrifugation for a total of three washes. After the final wash, 200 μl of 1X PCR buffer was placed on top of the beads pelleted by

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centrifugation. Then, 6 µl of 100 pmol/µl biotinylated P1 probe was added to the top of the beads/PCR buffer. The beads were resuspended and the tube containing the beads was placed on a rotator for 45 min at room temperature. Following the rotation, the beads were washed three times with 200 µl 1X PCR buffer. A 15 µl aliquot of bead suspension was placed in a microcentrifuge tube and briefly centrifuged for 30 s at 13.2k rpm to let the beads settle down. This sample was marked "Sample A". Another sample, "Sample B" was prepared the same way.

The aqueous layer above the beads in Sample A (negative control) was removed and replaced with 50 µl of PCR mix (37.7 µl H_2O, 5 µl 10X PCR buffer, 1 µl dNTPs (10 mM each), 0.4 µl of 100 pmol/µl P1 forward primer, 0.4 µl of 100 pmol/µl P2 reverse primer, 5 µl Betaine (5 M), and 0.5 µl of Taq polymerase (5 U/µl). Nylon membranes were cut into 2 mm circles using a die cutter. The circles were pre-wetted by immersion in 1X PCR buffer. One 2 mm circle of nylon membrane was immersed in Sample A such that the beads were attached to the membrane; filling most of the pores. Sample A and the nylon membrane were placed on a rotator for 3 hours at 4°C to allow the beads to take up the components of the PCR mixture. The nylon membrane was removed and fully immersed in a tube containing about 20 µl of mineral oil.

The aqueous layer above the beads in Sample B was replaced with 50 µl PCR mix (35.45 µl H_2O, 5 µl 10X PCR buffer, 1 µl dNTPs (10 mM each), 0.4 µl of 100 pmol/µl P1 forward primer, 0.4 µl of 100 pmol/µl P2 reverse primer, 5 µl Betaine (5 M), 0.5 µl Taq polymerase (5 U/µl) and 2.25 µl of 1.67 attomol/µl tf2 adeno fragment. One attomole is defined as 1×10^{-18} moles. The estimated concentration of DNA per bead prior to amplification was 10 copies per bead. (Note that where each bead was to contain a distinct template sequence, then a maximum of one unique sequence per bead was preferred). One 2 mm circle of nylon membrane was immersed in Sample B such that the beads were attached to the membrane; filling most of the pores. Sample B and the nylon membrane were placed on a rotator for 3 hours at 4°C to allow the beads to take up the components of the PCR mixture. The nylon membrane was removed and fully immersed in a tube containing about 20 µl of mineral oil.

The tube containing the nylon membrane with Sample A and the tube containing the nylon membrane with Sample B were placed in a thermocycler with the following reaction conditions. Step 1: 96 C for 2 min; Step 2: 96 C for 1 min; Step 3: 58 C for 1 min; Step 4: 72 C for 1 min, go to step 2, 29 times; Step5: 72 C for 10 min; Step 6: 14 C overnight or

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until the reaction was terminated. After the PCR reaction, the PCR tubes with the nylon membranes were removed from the thermocycler. The membranes were removed and placed into separate tubes containing 1 ml chloroform and 200 µl of 1X Annealing Buffer. The tubes were shaken several times. The membranes were transferred to individual tubes containing 1 ml of chloroform and the tubes were rotated several times. The membranes were then transferred to individual tubes with 200 µl of 1X Annealing Buffer. The tubes were then rotated several times. This procedure was repeated an additional two times with 200 µl of Annealing Buffer.

To denature DNA on the beads, the membranes were transferred to individual tubes with 50 µl of 1X Annealing Buffer. The tubes were heated to 90 C for 2 min in a PCR thermocycler. Next, the membranes were transferred to individual tubes containing 50 µl of 1X Annealing Buffer on ice. The membranes were washed two times with 100 µl of 1X Annealing Buffer. The membranes were then incubated with a solution of 5 µl of 100 pmol of P2 primer mixed with 20 µl of 1X Annealing Buffer. The tubes were placed in a thermocycler and heated to 65°C. Next, the tubes were slowly cooled to room temperature to allow the P2 sequencing primer to anneal to the DNA template. Following annealing, the membranes were washed two times with 100 µl of 1X Annealing Buffer.

To confirm the fidelity of the amplified DNA fragment, the reaction product was sequenced on the beads using a pyrophosphate sequencer (PSQ). Methods of pyrophosphate sequencing are generally described, *e.g.*, in U.S. Patent Nos. 6,274,320, 6258,568 and 6,210,891, incorporated *in toto* herein by reference. Briefly, the membranes were soaked for 30 sec in 50 µl of a mixture of ATP sulfurylase and luciferase enzymes (454 life Sciences). Then, the membrane was placed into a well of the PSQ. The nucleotides were flowed into the PSQ plates in the order of G, A, C, and then T. This was repeated five times. For the sample with no DNA (the negative control), no sequence was detected (Fig. 10A). For the amplified tf2 fragment on the beads on the membrane, the proper sequence was detected (Fig. 10B). Thus, detectable sequence was obtained starting from a small number of DNA template fragments (10 copies per bead), using the amplification and sequencing reactions described herein.

Example 3: Methods for Pyrosequencing

Any DNA may be sequenced using the procedure described herein. Briefly, beads are filtered to obtain a diameter of 25-30 µm and resuspended at a concentration of 3,500

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beads/μl, as described above. Next, 14 μl of the bead solution is placed into a tube for each sample to be sequenced. The beads are pelleted at 13,000 rpm. The supernatant is replaced with 500 μl of a mixture of the three enzymes (6 μl of sulfurylase at 1 mg/ml, 6 μl of luciferase at 3 mg/ml, and 60 μl of Bst polymerase at 50 U/μl) and 428 μl of 1X Assay Buffer containing 1 mg/ml BSA. The tube is placed in a rotator for 1 hr at room temperature, at about one turn every 2 sec. Then, the beads are pelleted by centrifugation at 2,000 rpm for 2.5 min. The beads are washed once with 200 μl of 1X Assay Buffer without BSA. Then the beads are loaded onto a membrane with 30 μm pore for pyrophosphate sequencing.

Example 4: Bead Loading Methods

A membrane (*e.g.*, nitrocellulose membrane circle, as described herein) is dipped into bead solution such as 1X Assay Buffer. The membrane is agitated to trap beads in the membrane pores. The membrane/bead mixture is submerged in bead solution. This is stirred or vortexed to trap the beads in the membrane pores. The membrane is used as a bead filter in a sieve, and the bead solution is drained through the membrane using gravity or centrifugal force. On an open-loading Jig and a centered membrane, the bead solution is introduced from the top and drained through the bottom by a pump. Mixing can be used in the cavity of the Jig to ensure uniform bead distribution on the membrane. The loading Jig can include multiple cavities for bead deposits onto different areas of the membrane for different samples or tests. This method can be combined with the loading Jig method as described herein. The beads are loaded using a wicking effect. The membrane is placed on top of other highly hydrophilic membrane or tissue, or other wicking material, and the bead solution is applied to the membrane. Using a pump, the bead solution is flowed across the membrane in an enclosed chamber. The chamber can be placed in any orientation, and the beads can be introduced by various means such as a syringe, pipette, duct, tubing, and the like. The arrayed sample delivery devices (manual or automated) can be used to deposit beads onto discrete regions or patches on the membrane.

Example 5: Automated Convective Sequencing Protocol

Wash buffers, sample DNA, bead solution, enzyme solutions, and sequencing reagents (substrate, PP_i, and nucleotides) are prepared according to the layout of the automated sequencing system (Figure 8). The sequencing and resistance membranes are incubated in 1X Assay Buffer (AB) containing 1% bovine serum albumin (BSA) for at least

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15 min, preferably 30 min, to prevent PP_i drop during a long sequencing run. The sequence chamber system is assembled with the membranes and connected to pumps and reagents. The beads are loaded with Pump 2 at a flow rate of 1-2 ml/min depending on the chamber size. The chamber is washed with a wash buffer (1X AB with 1% BSA). Non-binding beads are removed by running Pump 2 while switching to Pump 1. A proper flow rate is set so that it removes loose beads without disrupting the membrane.

A sulfurylase and luciferase mixture is loaded with both Pump 1 and Pump 2 running. This is incubated for 15 min with mixing by the reciprocal movement by Pump 2. The chamber is washed with a wash buffer for polymerase with both pumps running for 5 min. A Bst polymerase (New England Biolabs, Beverly, MA) solution is loaded and incubated for 30 min with mixing. The chamber is washed with substrate for 5 min with both pumps running. Alternatively, the Bst polymerase can be mixed with sulfurylase and luciferase for combined infusion with all three enzymes. A 0.1 μM PP_i solution is run for signal calibration with a flow rate of 1.5 ml/min for both pumps for 21 sec. After this, Pump 1 is stopped and Pump 2 is continued for another 14 sec. The substrate is washed for 115 sec with Pump 1 at 1 ml/min and Pump 2 at 2 ml/min. The nucleotides are added in order (*e.g.*, C, A, G, T) for a predetermined number of cycles with the same pump procedure as used for PP_i, except that a 65 sec substrate wash is used after each nucleotide is added. At the end of the nucleotide runs, PP_i is run again to check if enzyme activity changes with time. The data is analyzed using DNA sequencing software.

Additional methods that may be used with the invention can be found in U.S. Application Serial No. 10/191,438 filed July 8, 2002, U.S. Application Serial No. 60/476,592 filed June 6, 2003, U.S. Application Serial No. 60/476,602, filed June 6, 2003, U.S. Application Serial No. 60/476,313 filed June 6, 2003, U.S. Application Serial No. 60/476,504 filed June 6, 2003, and U.S. Patent No. 6,274,320, which are hereby incorporated by reference herein in their entirety.

References

Anderson JR, Chiu DT, Jackman RJ, Cherniavskaya O, McDonald JC, Wu H, Whitesides SH, and Whitesides GM. 2000. Fabrication of topologically complex three-dimensional microfluidic systems in PDMS by rapid prototyping. Anal. Chem. 72:3158-64.

Asoh, H, Nishio, K, Nakao, M. Tamamura, T and Masuda, H. 2001. Conditions for fabrication of ideally ordered anodic porous alumina using pretextured aluminum. J. Electrochem. Soc. 148:B152-B156.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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Cherukuri SC, Demers RR, Fan ZHH, Levine AW, McBride SE, Zanzucchi PJ. 1999. Method and system for inhibiting cross-contamination in fluids of combinatorial chemistry device. US Patent Number 5,980,704.

Dannoux T, Pujol G, and Root D. 2000. High-density test plate and process of making. US Patent number US6030829.

Deng T, Wu H, Brittain ST, and Whitesides GM. 2000. Prototyping of masks, masters, and stamps/molds for soft lithography using an office printer and photographic reduction. *Anal. Chem.* 72:3176-80.

Ehrfeld, W, Hessel, V, and Löwe, H. 2000. *Microreactors: New Technology for Modern Chemistry.* New York: Wiley-VCH.

Eykamp, W. 1995. Microfiltration and ultrafiltration, pp. 1-43 in *Membrane Separation Technology: Principles and Applications*, RD Noble and SA Stern, eds. Amsterdam: Elsevier Science BV.

Furneaux, RC, Rigby, WR, and Davidson, AP. 1989. The formation of controlled porosity membranes from anodically oxidized aluminum. *Nature.* 337:147-9.

Hyman ED. 1988. A new method for sequencing DNA. *Anal. Biochem.* 174:423-36.

Jansson V and Jansson K. 2002. Enzymatic chemiluminescence assay for inorganic pyrophosphate. *Analytical Biochemistry.* 304: 135-137.

Jensen N. 2002. Flow-through processing on a microchip for DNA pyrosequencing. *Natl. Nanofabrication* Users *Network.* 66-67.

Kane RS, Takayama S, Ostuni E, Ingber DE, and Whitesides GM. 1999. Patterning proteins and cells using soft lithography. *Biomaterials.* 20:2363-76.

Kulkarni, SS, Funk, EW, and Li, NN. 1992. Ultrafiltration, pp. 391-453 in *Membrane Handbook*, WSW Ho and KK Sirkar, eds. New York: Van Nostrand Reinhold.

Mardilovich, PP, Govyadinov, AN, Mukhurov, NI, Rzhevskii, AM, and Paterson, R. New and modified anodic alumina membranes. Part I: Treatment of anodic alumina membranes. Part II: Comparison of solubility of amorphous (normal) and polycrystalline anodic alumina membranes. *J. Memb. Sci.* 98:131-155.

Martin CR. 1994. Nanomaterials – A membrane-based synthetic approach. *Science.* 266:1961-1966.

Matsuda T and Chung DJ. 1994. Microfabricated surface designs for cell culture and diagnosis. *ASAIO J.* 1:M594-7.

Michael KL, Taylor LC, Schultz SL, and Walt DR. 1998. Randomly ordered addressable high-density optical sensor arrays. *Anal. Chem.* 70:1242-8.

Fujirebio Diagnostics, Inc. v. Quanterix Corp.
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3840

Nyrén P, Pettersson B, and Uhlén M. 1993. Solid phase DNA minisequencing by an enzymatic luminometric inorganic pyrophosphate detection assay. *Anal. Biochem.* 208:171-5.

Rai-Choudhury P. 1997. *Handbook of Microlithography, Micromachining and Microfabrication. Vol. 1: Microlithography* and *Vol. 2: Micromachining and Microfabrication.* SPIE Press.

Ronaghi M, Uhlén M, and Nyrén P. 1998. A sequencing method based on real-time pyrophosphate. *Science.* 281:363-5.

Schuller J. 2002. Microfluidics for DNA pyrosequencing. *Natl. Nanofabrication Users Network.* 76-77.

Taylor LC and Walt DR. 2000. Application of high-density optical microwell arrays in a live-cell biosensing system. *Anal. Biochem.* 278:132-42

Zhu H, Klemic J F, Chang S, Bertone P, Casamayor A, Klemic K G, Smith D, Gerstein M, Reed M A, and & Snyder M. 2000. Analysis of yeast protein kinases using protein chips. Nature Genet. 26:283–89.

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We Claim:

1. A membrane reactor array comprising:

a planar array layer comprising a plurality of pores with reagent-carrying beads disposed in a plurality of said pores, with a maximum of one bead per pore, wherein the array is substantially permeable to aqueous solutions but is substantially impermeable to said beads; and

a porous high flow resistance membrane layer, in contact with the planar array layer, that is permeable to nucleic acids and proteins to provide flow resistance of greater than 10 fold that of the flow resistance of the aqueous solutions through a bead-containing pore in said planar array.

2. The membrane reactor array of claim 1 wherein said planar array layer is formed to provide a spacing of pores of less than 100 μm center to center.

3. The membrane reactor array of claim 1 wherein said planar array layer is formed to provide a spacing of pores of about 50 to 78 μm center to center.

4. The membrane reactor array of claim 1 wherein said planar array layer comprises pores with a pore size length in one dimension of about 15 μm to 100 μm in diameter.

5. The membrane reactor array of claim 1 wherein said planar array layer comprises pores of a pore size length in one dimension of about 20 to 35 μm in diameter.

6. The membrane reactor array of claim 1 wherein said planar array layer comprises substantially square pores with an average size of about 15 to 100 μm.

7. The membrane reactor array of claim 1 wherein said planar array layer comprises substantially square pores with an average size of about 20 to 35 μm.

8. The membrane reactor array of claim 1 wherein said planar array layer comprises substantially square pores with an average size of about, 25 μm, 28 μm, 30 μm or 31 μm.

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9. The membrane reactor array of claim 1 wherein said planar array layer comprises pores that are rectangular.

10. The membrane reactor array of claim 1 wherein said planar array layer comprises at least 10,000 pores.

11. The membrane reactor array of claim 1 wherein said planar array layer comprises at least 50,000 pores.

12. The membrane reactor array of claim 1 wherein said planar array layer comprises at least 100,000 pores.

13. The membrane reactor array of claim 1 wherein said planar array layer comprises at least 250,000 pores.

14. The membrane reactor array of claim 1 wherein said high flow resistance membrane has a pore size between 0.01 μm and 10 μm.

15. The membrane reactor array of claim 1 wherein said high flow resistance membrane has a pore size between 0.01 μm and 0.5 μm.

16. The membrane reactor array of claim 1 wherein said high flow resistance membrane has a pore size between less than 0.1 μm and 1 μm.

17. The membrane reactor array of claim 1 wherein said high flow resistance membrane is a hydrophobic membrane with a pore size less than 20 μm.

18. The membrane reactor array of claim 1 wherein said high flow resistance membrane has a flow resistance to a liquid that is 100 fold greater than that of the planar array layer.

19. The membrane reactor array of claim 1 wherein said high flow resistance membrane has a pore diameter that is less than 10% of the pore diameter of the planar array layer.

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20. The membrane reactor array of claim 1 wherein said high flow resistance membrane has a pore diameter that is less than 1% of the pore diameter of the planar array layer.

21. The membrane reactor array of claim 1 wherein said planar array layer is a woven mesh of a plurality of fibers with pores formed between said fibers.

22. The membrane reactor array of claim 1 wherein said planar array layer is a planar surface comprising pores.

23. The membrane reactor array of claim 1, additionally comprising a porous structural support layer which has less resistance to fluid flow than the planar array or the porous high flow resistance membrane layer.

24. The membrane reactor array of claim 23, wherein said porous structural support layer is made from a material selected from the group consisting of metal, ceramic, and porous silicon.

25. The membrane reactor array of claim 1, further comprising a pyrophosphate degrading enzyme immobilized on one said layer or on said bead.

26. A cartridge comprising a membrane reactor array of claim 1 wherein said membrane reactor array bisects said cartridge into a first and a second chamber, and wherein said cartridge comprise a first opening in said first chamber and a second opening in said second chamber to allow a fluid to flow through said first opening, through said membrane reactor array, and through said second opening.

27. The cartridge of claim 26, further comprising a third opening in said first chamber to allow a fluid to flow through said first opening laterally over said membrane reactor array and through said third opening.

28. A membrane reactor array, comprising

(a) a planar array layer comprising a plurality of pores with reagent-carrying beads disposed in a plurality of said pores such that a maximum of one bead is disposed within any one pore, wherein said beads restrict but do not completely block the flow of fluid

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through said pores, and wherein the array is substantially permeable to aqueous solutions but is substantially impermeable to said beads, and wherein said planar array is made from a fiber material of substantially uniform cross-section weaved to form a mesh layer comprising an array of pores of uniform size, and wherein each pore is an opening of less than 40 μm; and

(b) a porous high flow resistance membrane layer, in contact with the planar array layer, that is permeable to nucleic acids and proteins to provide flow resistance of greater than 10 fold that of the flow resistance of aqueous solutes through a bead containing pore in said planar array.

29. The membrane reactor array of claim 28 wherein said planar array layer comprises two sets of parallel fibers woven together such that the two sets of parallel fibers are substantially at right angle to each other, wherein each individual fiber of each set has a center to center spacing of less than 100 μm, and wherein the fibers form an array of pores.

30. The membrane reactor array of claim 28 wherein said planar array layer comprises pores with a pore size length in one dimension of about 20 μm to 35 μm and a spacing of pores of about 50 to 78 μm center to center.

31. The membrane reactor array of claim 28 wherein the percentage of pores in the mesh layer that are loaded with beads is selected from the group consisting of 5%, 10%, and 25%.

32. The membrane reactor array of claim 28 wherein at least one sequencing enzyme is immobilized on said mesh layer or said bead.

33. The membrane reactor array of claim 32 wherein the sequencing enzyme is selected from the group consisting of ATP sulfurylase, luciferase, polymerase, hypoxanthine phosphoribosyltransferase, xanthine oxidase, uricase, and peroxidase.

34. The membrane reactor array of claim 28 wherein said array is substantially impermeable to a bead with a diameter of more than 40 μm.

35. The membrane reactor array of claim 28 wherein said plurality of beads are attached to at least one nucleic acid molecule.

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36. The membrane reactor array of claim 35 wherein each said bead is attached to a single nucleic acid molecule.

37. The membrane reactor array of claim 35 wherein each said bead is attached to a single species of nucleic acid molecule.

38. The membrane reactor array of claim 32, further comprising a pyrophosphate degrading enzyme immobilized on one said layer or on said bead.

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

providing a sample DNA sequence and an extension primer that hybridizes to the sample DNA immediately adjacent to the target position and subjecting the sample DNA and extension primer to a polymerase reaction in the presence of a deoxynucleotide or dideoxynucleotide,

wherein said sample DNA sequence is immobilized on a bead within a membrane reactor array of claim 1; and

whereby the deoxynucleotide or dideoxynucleotide will only become incorporated and release pyrophosphate (PP_i) if it is complementary to the base in the target position, and any release of PP_i can be detected enzymatically;

adding different deoxynucleotides or dideoxynucleotides successively to the sample-primer mixture; and

determining which deoxynucleotide or dideoxynucleotide is incorporated, thereby identifying a base that is complementary to the base at the target position.

40. The method of claim 39 wherein said different deoxynucleotides or dideoxynucleotides are added to said DNA sequence by a fluid flow that is normal to a plane of the planar membrane reactor array.

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41. The membrane reactor array of claim 40 wherein said fluid flow has a flow rate of 0.15 ml/minute/cm^2 to 4 ml/minute/cm^2.

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

arranging a multiplicity of DNA sequences, each immobilized on a bead, in array format on a membrane reactor array of claim 1;

providing to each sample an extension primer, which hybridizes to the sample DNA immediately adjacent to the target position and subjecting the sample DNA and extension primer to a polymerase reaction in the presence of a deoxynucleotide or dideoxynucleotide, whereby the deoxynucleotide or dideoxynucleotide will only become incorporated and release pyrophosphate (PP_i) if it is complementary to the base in the target position and detecting any release of PP_i enzymatically, and whereby a nucleotide-degrading enzyme is included during the polymerase reaction such that unincorporated nucleotides are degraded;

adding different deoxynucleotides or dideoxynucleotides successively to the same sample-primer mixture in a fluid flow that is normal to the plane of the planar mesh;

determining which deoxynucleotide or dideoxynucleotide is incorporated, thereby identifying a base that is complementary to the base at the target position.

43. The method of claim 42 wherein said nucleotide-degrading enzyme is apyrase.

44. A method of loading a membrane reactor array comprising the steps of:

(a) providing a membrane reactor array of claim 1 which is substantially permeable to a fluid but substantially impermeable to a population of microreactor beads;

(b) depositing said fluid comprising a suspension of said population of microreactor beads on the surface of the membrane reactor array; and

(c) settling said microreactor beads onto the pores at no more than one bead per pore.

45. The method of claim 44 wherein said settling comprises drawing said fluid through said membrane reactor array whereby said microreactor beads are trapped in said membrane reactor array.

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46. The method of claim 44 wherein said microreactor beads have a diameter of between 20 to 35 μm.

47. A microimaging lens system for imaging a light emission from a membrane reactor array of claim 1 comprising:

(a) a front lens group for collecting a light emission from a sequencing reaction; and

(b) a rear lens group for imaging said light emission in a spatially ordered manner onto an optical detector.

48. The microimaging lens system of claim 47 wherein at least one said lens group has a focal length of at least 30 mm focal length.

49. The microimaging lens system of claim 47 wherein at least one said lens group has a focal length of at least 50 mm focal length.

50. The microimaging lens system of claim 47 wherein at least one said lens group has a focal length of at least 70 mm focal length.

51. The microimaging lens system of claim 47 wherein at least one said lens group has an aperture brighter than or equal to 4.0.

52. The microimaging lens system of claim 47 wherein at least one said lens group has an aperture brighter than or equal to 2.8.

53. The microimaging lens system of claim 47 wherein at least one said lens group has a numeric aperture larger than or equal to 0.1.

54. The microimaging lens system of claim 47 wherein at least one said lens group has a numeric aperture larger than or equal to 0.2.

55. The microimaging lens system of claim 47 wherein at least one said lens group has a numeric aperture larger than or equal to 0.3.

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56. The microimaging lens system of claim 47 wherein said front lens group and rear lens group are identical.

57. The microimaging lens system of claim 47, further comprising a solid state optical detector.

58. The microimaging lens system of claim 57 wherein the solid state optical detector is a CCD array.

59. The microimaging lens system of claim 47 further comprising a structure to block light not originating from the membrane reactor array from reaching the optical detector.

60. The microimaging lens system of claim 59 wherein said structure is an opaque tube comprising a first end which forms a light tight fit to one end of the microimaging lens and comprising a second end which forms a light tight fit the optical detector.

61. The microimaging lens system of claim 59 wherein said structure is an opaque tube comprising a first end which forms a light tight fit to one end of the microimaging lens and comprising a second end which forms a light tight fit to a membrane adaptor array.

62. A sequencing cartridge comprising:

(a) a flow chamber enclosing a membrane reactor array of claim 1 with an inlet port and an outlet port,

(b) an optical window for the optical examination of said membrane reactor array;

(c) an inlet port for delivering sequencing reagents;

(d) a first outlet port for removal of sequencing reagents; and

(e) a second outlet port for removal of effluents.

63. The sequencing cartridge of claim 62 wherein the optical window is circular.

64. The sequencing cartridge of claim 62 wherein the flow chamber is substantially funnel shaped with the wide section of the funnel proximal to the membrane support structure and the narrow section of the funnel at the second outlet port.

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65. The sequencing cartridge of claim 62 wherein said support structure comprises a porous solid surface.

66. The sequencing cartridge of claim 62 wherein said inlet is in fluid communication with a pump for controlling a flow of fluids through said inlet.

67. The sequencing cartridge of claim 62 wherein said first outlet port is in fluid communication with a first pump for controlling a flow of fluids through said first outlet port.

68. The sequencing cartridge of claim 62 wherein said second outlet port is in fluid communication with a second pump for controlling a flow of fluids through said second outlet port.

69. The sequencing cartridge of claim 62 wherein said inlet and said first and said second outlet is each in fluid communication with a different pump for controlling a flow of fluids so that a flow of fluids normal to the plane of the membrane reactor array and a flow of fluid tangential to the plane of the membrane reactor array can be controlled simultaneously.

70. A method of producing a membrane reactor array of nucleic acids comprising the steps of:

(a) providing one or more nucleic acid templates to be amplified wherein a plurality of nucleic acid templates are individually attached to separate beads to form a population of nucleic acid template-carrying beads;

(b) suspending said nucleic acid template-carrying beads in an amplification reaction solution containing reagents necessary to perform nucleic acid amplification;

(c) forming an emulsion wherein a plurality of said template-carrying beads and PCR reaction solution are individually isolated in the emulsion to form a plurality of microreactors;

(d) amplifying the one or more nucleic acid templates in fluidic isolation from each other to form amplified nucleic acid;

(e) attaching the amplified nucleic acid to said beads; and

(f) loading said beads onto a membrane reactor array of claim 1.

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71. A method of producing a membrane reactor array of nucleic acids comprising the steps of:

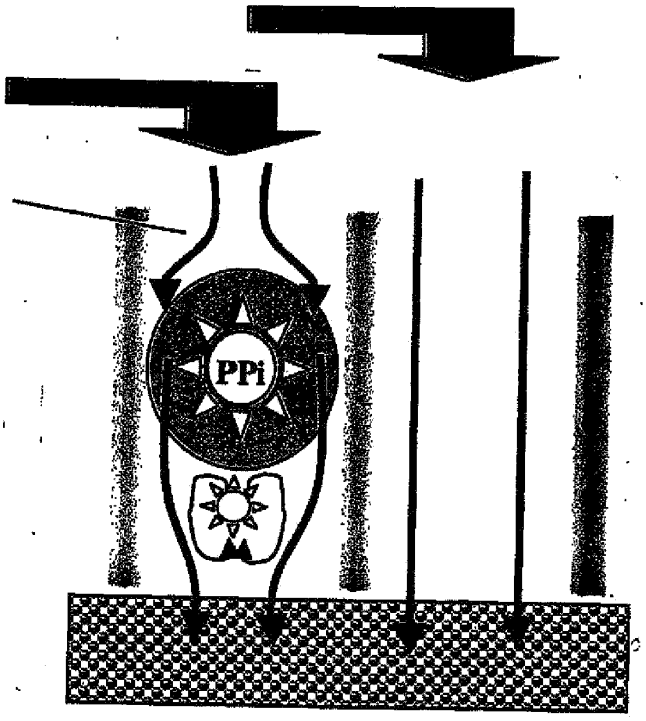
(a) providing one or more nucleic acid templates to be amplified wherein a plurality of nucleic acid templates are individually attached to separate beads to form a population of nucleic acid template-carrying beads;

(b) loading said beads onto a membrane reactor array of claim 1;

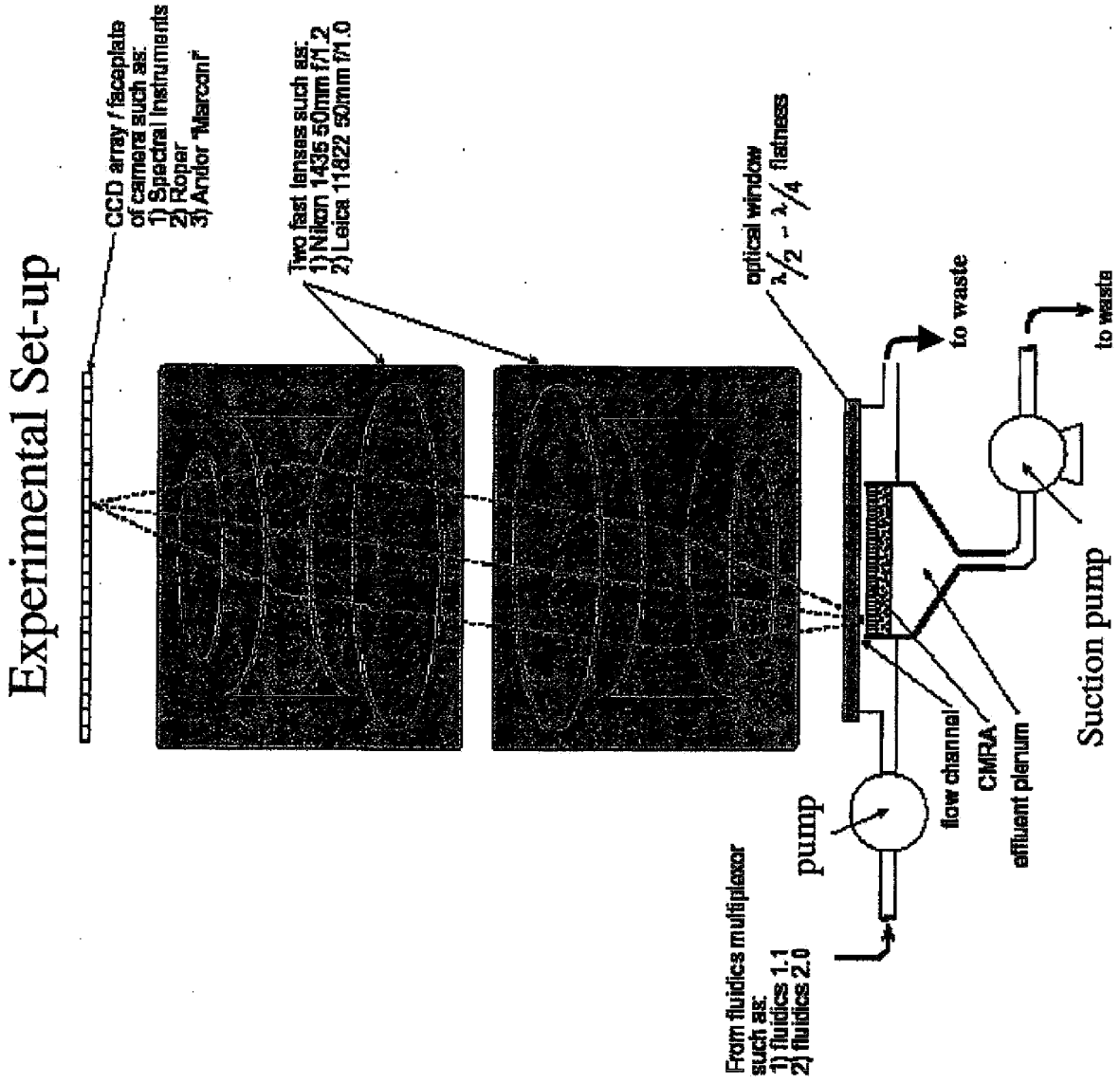
(c) contacting said nucleic acid template-carrying beads to an amplification reaction solution containing reagents necessary to perform nucleic acid amplification; and

(d) amplifying the one or more nucleic acid templates in fluidic isolation from each other to form amplified nucleic acids.

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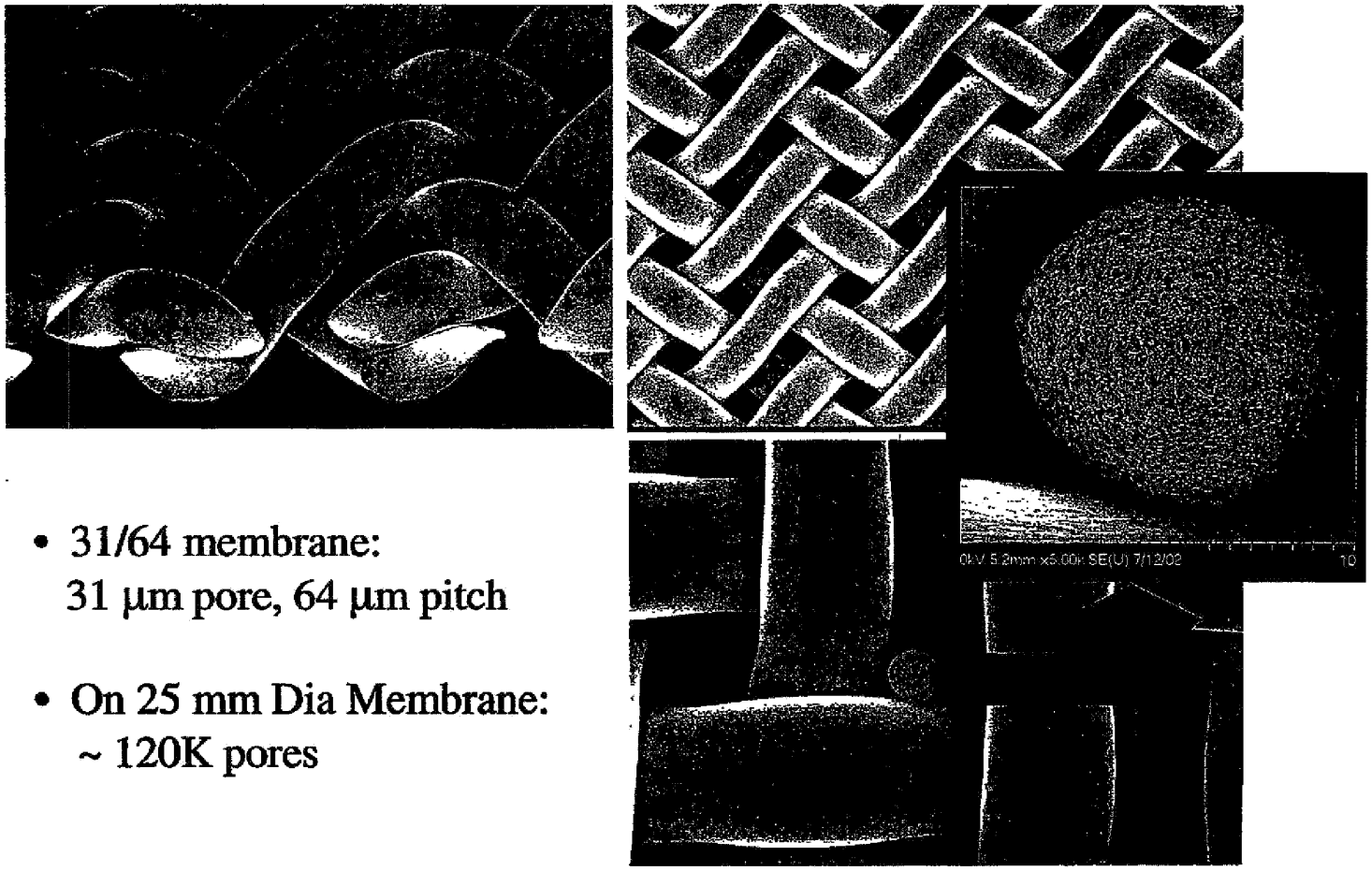


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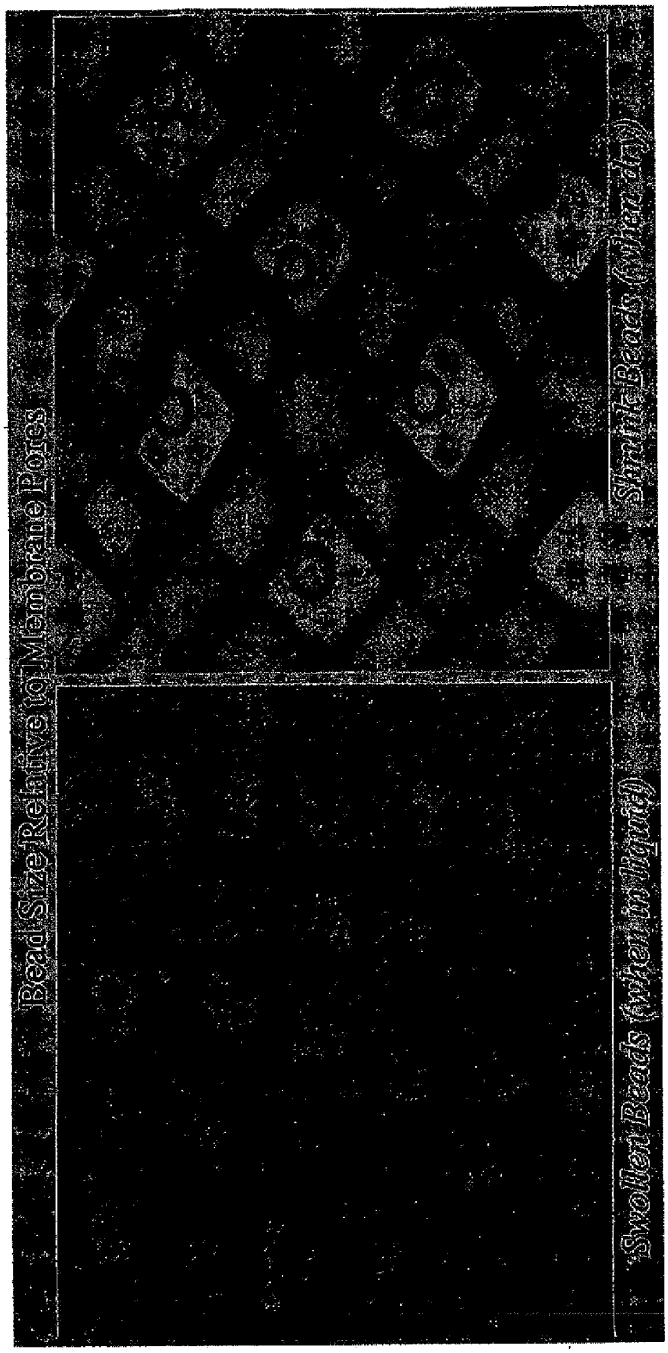
Nylon Mesh Membrane & Sepharose Beads



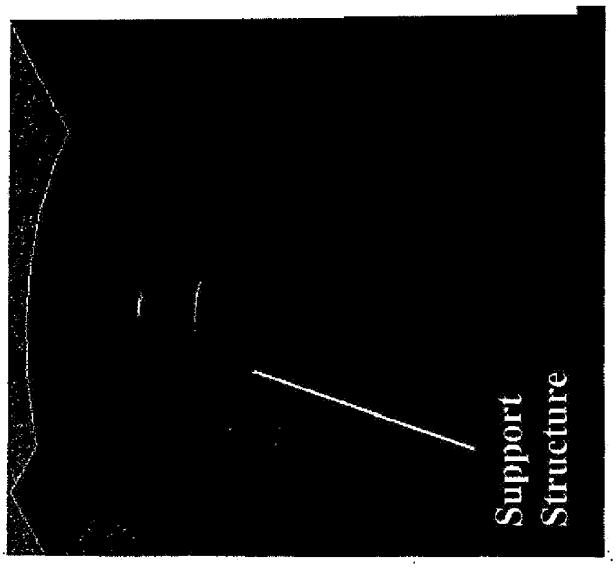
- 31/64 membrane:
  31 μm pore, 64 μm pitch
- On 25 mm Dia Membrane:
  ~ 120K pores

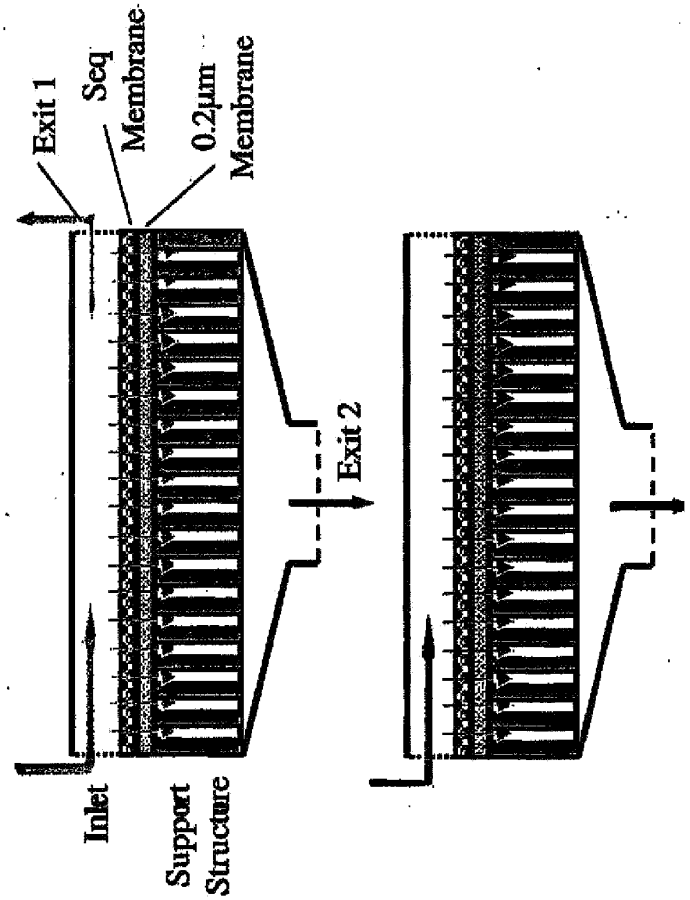
- Commercially available and ready to use (no Micromachining)
- Cheap (~ 10 cents per 25 mm membrane)
- Bio-compatible

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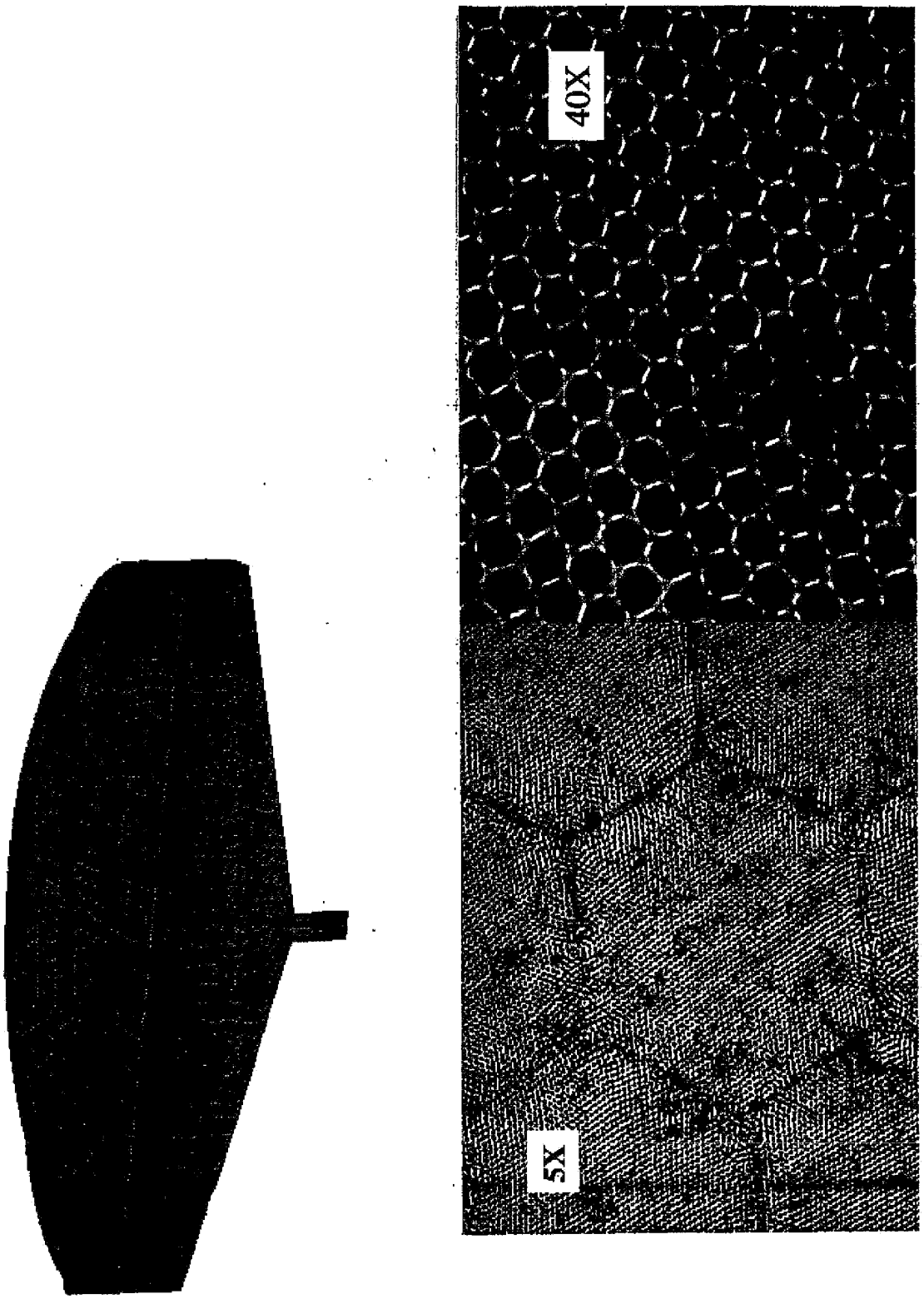


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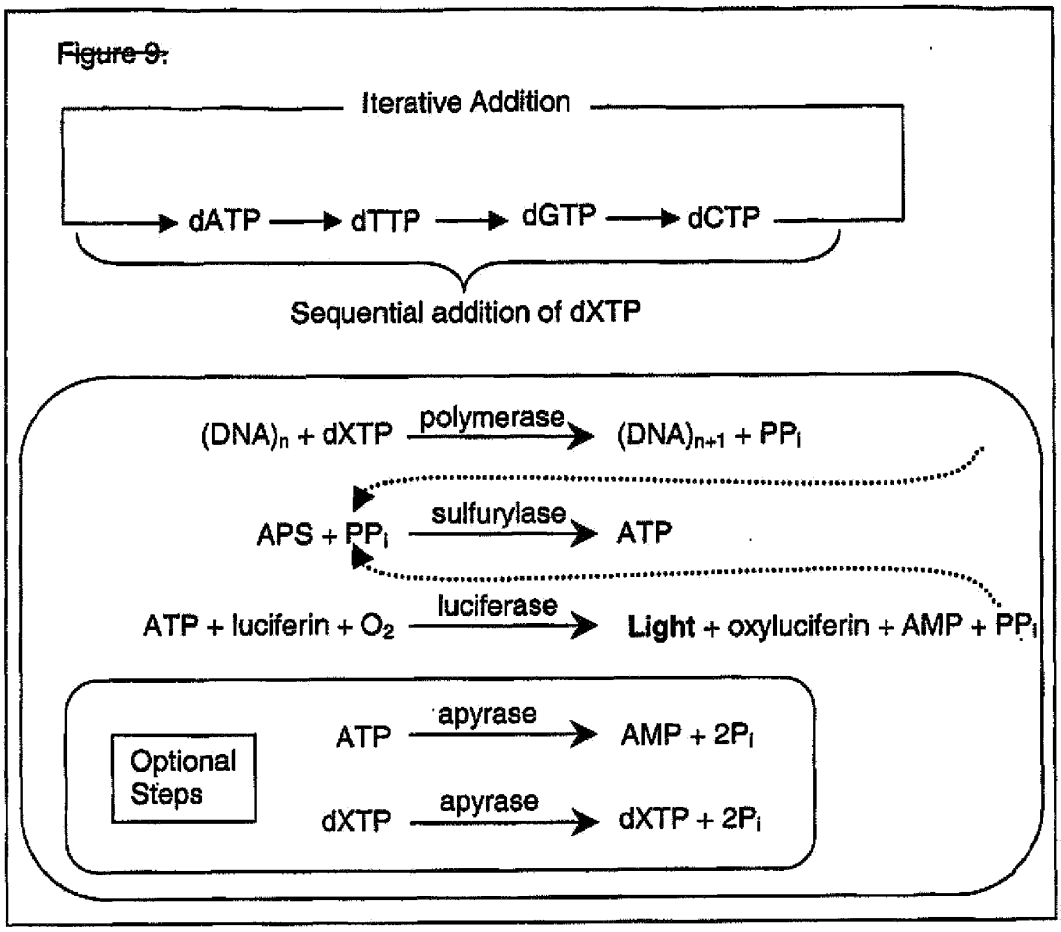




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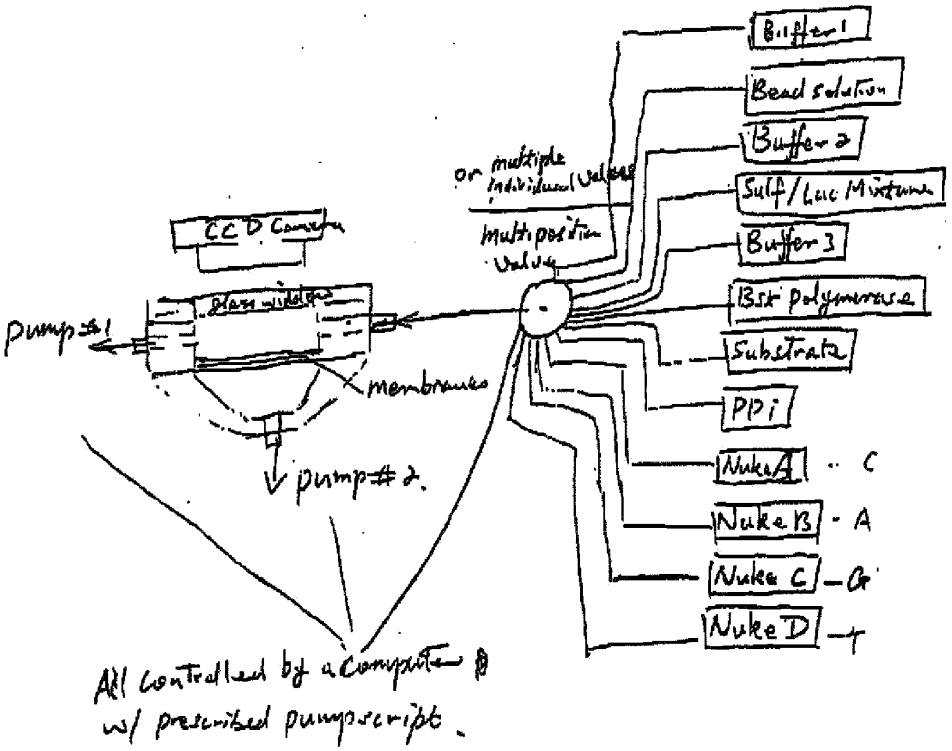


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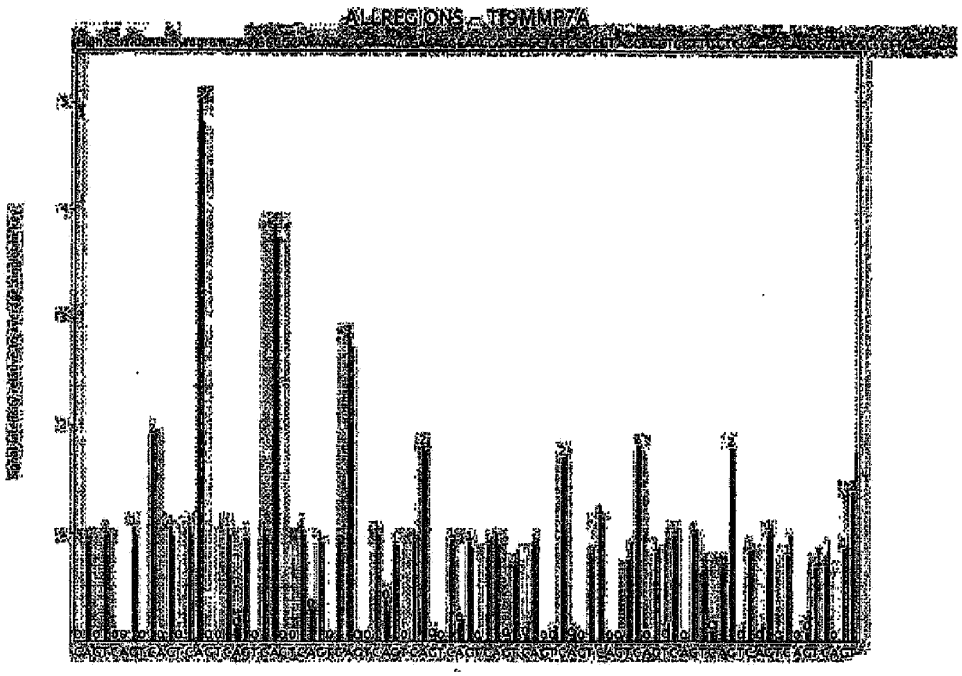
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Fig. 8 Automated Convective sequencing system



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Figure 9



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Figure 10A

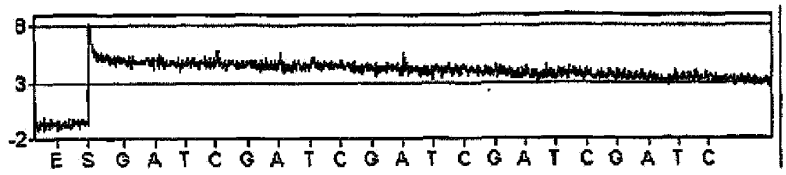
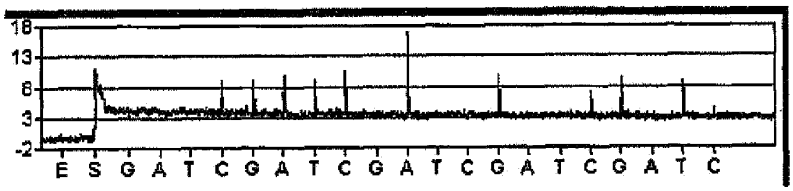
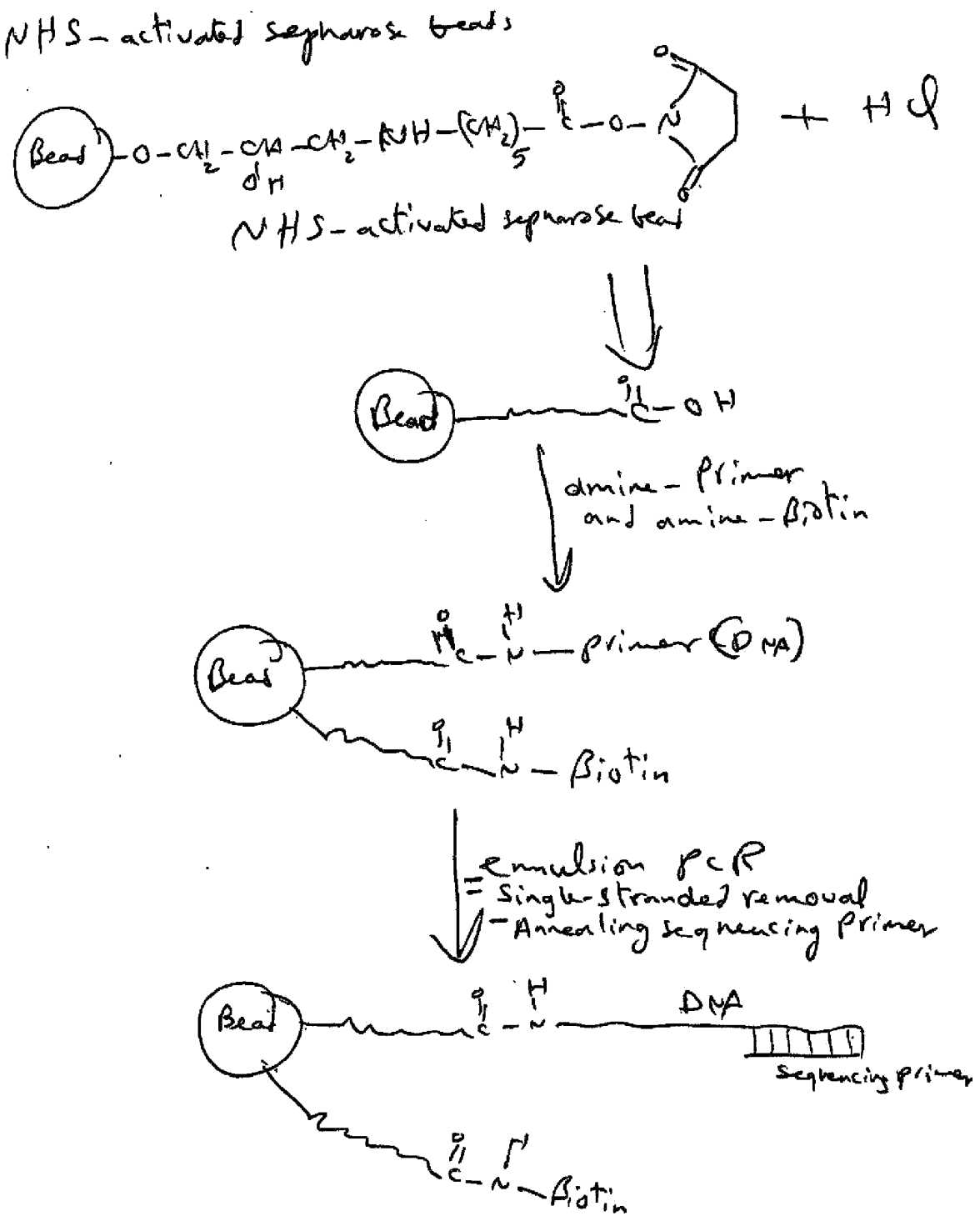


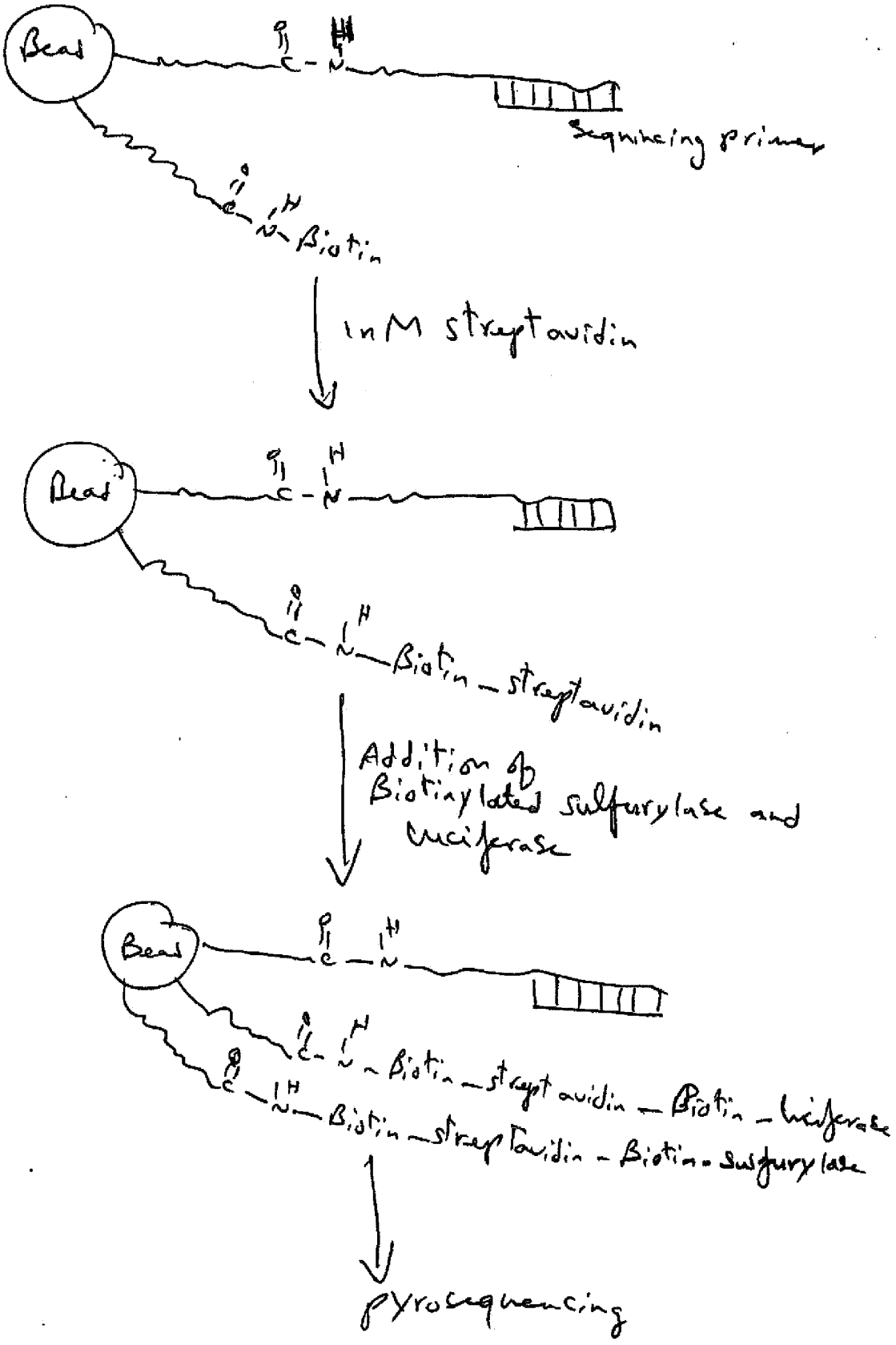
Figure 10B



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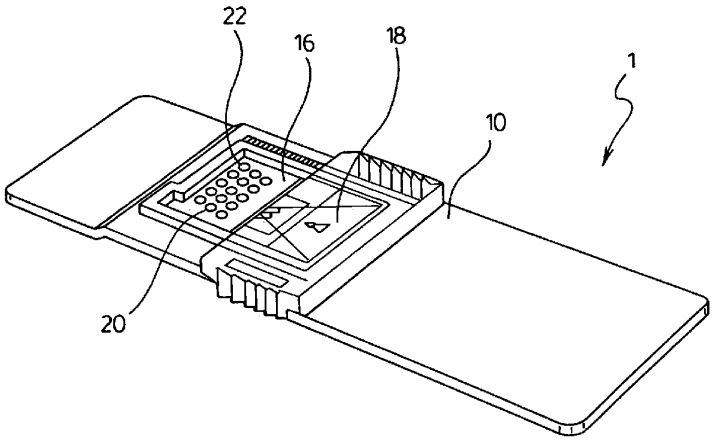
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(54) Title: METHOD TO MEASURE DYNAMIC INTERNAL CALIBRATION TRUE DOSE RESPONSE CURVES



(57) Abstract: A method of determining an amount of analyte in a sample solution is provided. The method involves the use of an assay device that has a substantially planar assay surface. The surface has a plurality of calibration dots a test dot printed thereon. The calibration dots contain pre-determined quantities of the analyte while the test dot includes a capture antibody for binding to the analyte. The analyte is mixed into a solution having a sample antibody for the analyte, where the antibody is labeled with a detectable marker. The sample solution is introduced into the loading portion of the assay device for delivery to said reading portion. The next step is to measure the intensity of detectable marker in the calibration dots. With the data obtained, one then prepares a calibration curve correlating the amount of analyte in said calibration dots to said intensity of detectable marker. The intensity of detectable marker in the test dot can be measures and the amount of anayte present in said test dot calculated by comparing the intensity of detectable marker to the amount of analyte corresponding to the intensity in said calibration curve.

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Method to measure dynamic internal calibration true dose response curves

Field of the Invention

The invention relates to assay devices and methods for constructing assay devices for detecting the presence of an analyte in a biological sample and the quantity of same.

Background of the Invention

Quality standards for immunoassays have traditionally been driven by external calibration reference standards. Current methods of analysis for typical immunodiagnostic assays provide diagnostic test results based on generally accepted external standard reference measurements. A number of known discrepancies have become apparent to be quantitation errors induced when assays are carried out, leading to variations in test results. For example, the concept of assay sensitivity attempts to characterize sensitivity by classic statistical analysis based on repeated measurement of low concentration samples to confirm that the sample result is not statistically different from zero. As the standard error incurred is inversely proportional to the square root of the number of actual measurements, this method does not actually measure the inherent assay sensitivity. Further refinement has led to some improvements. Known in the art as analytical sensitivity, the zero standard is measured several times and the limit of sensitivity becomes a concentration equating to 2–3 standard deviations (SD) from the mean (M). However, the precision for this theoretical determination may be incorrect by an order of magnitude. The concomitant fitting of any derived external calibration curve(s) does not create a true value dynamic dose response curve that can lead to considerable error in the actual sensitivity.

To further measure the accuracy of such analytical measurement, accuracy is used to define how close the average measured value is to the true value. The difference in measurement is known as the bias or degree of accuracy. Bias may vary over the range of the assay. It is known in the art that methods for measuring this true value need to be developed.

The repeatability of an assay or the estimated error in an analytical assay is known in the art as the percentage coefficient of variation (%CV). Automated assay analysis machines can be affected by variations in sample concentration, temperature, heat and edge effects, incomplete suspension of particles and solid phase precipitation. Precision effects also result from fraction separation and counting errors. In optical systems error is due to effects of turbidity, presence of fluorophores, deterioration of lamps and detectors and the deterioration, over time, of reagents. These factors generally lead to significant decreases in signal to noise ratio. Mechanical manipulation errors can result from poor pipetting and instrument stand-by periods.

As a direct result, the assessment for precision of any analytical method requires the measurement of resulting variability at known and relevant concentrations by using defined or standard control solutions to create baseline calibration standards. Accurate determination of such calibrators is based on measurement of known concentrations in dilution series at predetermined intervals, which are then interpolated. Commercially available, as well as in-house prepared reference solutions or reference standards are available, but are often calibrated with standard or pooled matrices, which may vary considerably from actual patient test samples. Part of the solution to overcome these errors is to plot the precision against a wide range of concentrations to obtain a precision profile, or calibration, of the assay.

Cross reactivity, assay specificity, bias causing interference, alterations in antigen, antibody, binding sites, low dose (competitive assay) and high dose (sandwich assay) hook effects, heterophilic antibody interference, endogenous interfering auto-antibodies, complement, rheumatoid factor, interference in solid phase antibody binding, endogenous signal generating substances, enzyme inhibitors, catalysts and co-factors have also been shown to express confounding activity in assays, including cross reactivity, matrix effects and carry over of sample in automated immunoassay instruments and samplers.

For diagnostic applications, the quality control samples may not reflect actual clinical concentrations in the patient, may not reflect the spectrum of present analytes and interfere with the sample matrix to no longer reflect the content of the patient

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samples. The quality control samples may measure performance at discrepant intervals of concentration which may not reflect clinical decision points.

There is therefore a need for an immunoassay that can be reliably calibrated.

Summary of the Invention

The invention is directed to a method for internal dynamic calibration of an assay device for determining the concentration of an analyte in a sample where the assay device has an assay surface. A plurality of calibration dots containing pre-determined quantities of the analyte are printed on the assay surface. A test dot containing a reagent for binding said analyte is also printed on the assay surface. The analyte is labeled with a detectable marker complex prior to introduction onto the assay device. The analyte - detectable marker complex binds to the reagent in the test dot. The amount of antigen in the sample is proportional to the intensity of detectable marker in the test dot. The calibration dots contain differing pre-determined quantities of the analyte. Any unlabeled detectable marker will bind to the calibration dots. An internally calibrated calibration curve is thus prepared. The intensity of detectable marker in the test dot can be compared to the calibration curve to obtain an absolute value.

The method provides a quantitative analysis that is carried out rapidly using a single assay device with the ability to contain a known minimum volume of test fluid and also to have the ability for flowing fluid through the device in order to meet a known concentration of analyte as a function of analyte concentration per tested volume. Both the calibration dots and test dots are printed within a single assay device which then needs only the application of a single, premixed solution containing the analyte and an excess of detecting reagent which is preferably an antibody.

The invention further includes a method for obtaining dynamic true dose response curves by printing both calibrator and test samples onto a common test platform device. The test platform has a minimum of one test dot. Each test dot has multiple corresponding calibration dots. The signal obtained from the total number of comparative concentration dynamic calibration dots, at indexed X / Y co-ordinates is integrated to form the dynamic internal calibration true dose response curve. In a

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similar process, the unknown test dot label response reading is also integrated over all obtained readings. The use of multiple test arrays or matrices in platform format predicates a confidence limit approaching one hundred percent in having obtained the correct test result. The common test platform is exposed to the same test fluid and because the calibrator and test samples are exposed simultaneously to the same test fluid, accurate measurement of the concentration of analyte present in the test sample is determined by the resulting true dose response calibration curve. The invention provides the surprising result that the various errors, incurred using known state of the art methods, are not reflected when a test sample is processed with the disclosed method and device.

According to one aspect of the invention, there is provided a method of determining an amount of analyte in a sample solution comprising the following steps:

- providing an assay device having an assay surface having a plurality of calibration dots printed thereon and a test dot printed thereon, the calibration dots containing pre-determined quantities of the analyte, the test dot including a reagent for binding to said analyte;
- providing a solution having a reagent for binding to the analyte, said reagent being labeled with a detectable marker;
- introducing the analyte into said solution to form a sample solution;
- introducing said sample solution onto said assay device;
- measuring an intensity of detectable marker in said calibration dots;
- preparing a calibration curve correlating the amount of analyte in said calibration dots to said intensity of detectable marker;
- measuring an intensity of detectable marker in said test dot; and
- calculating an amount of analyte present in said test dot by comparing the intensity of detectable marker to the amount of analyte corresponding to said intensity in said calibration curve.

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Brief Description of the Drawings

Figure 1 is a top view of an assay device of the present invention for carrying out a fixed array test;

Figure 2 is a plot of showing a verification that single and aggregate immuno complexes are quantifiable;

Figure 3 is a plot showing that antigen concentration in the test sample does not impact fluorescence intensity in the calibration spots;

Figure 4 is an illustration of a PicoTip array printing, spot size and array matrices;

Figure 5 is a plot showing a correlation of analyte concentration with fluorescence using dynamic true dose response measurement; and

Figure 6 is top view of an assay device to test for the presence of respective antibody response to micro-organisms having been present in human plasma.

Detailed Description of the Invention

The present method is for calibrating an assay device. A preferred assay device is shown in Figure 1. The assay device has an assay surface 10 that preferably includes a loading area 18 and a reading area 16. The reading area 16 has printed thereon at least one and preferably at least two test dots 20. More preferably, a plurality of dots for detecting the presence of the analyte are printed on the reading area 16. The test dots 20 include a reagent that specifically binds to the protein analyte. Preferably, the reagent is bound antibodies that specifically bind to the analyte. Other reagents known in the art to bind a specific analyte can also be used. For the balance of the present discussion the reagent will be referred to as an antibody.

The bound antibodies are preferably spaced apart to make each bound antibody available for binding to the test antigen free of stearic hindrance from adjacent antigen complexes. Preferably, a non-reactive protein separates the bound antibodies in the test dots.

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The reading area 16 has calibration dots 22 printed thereon. The calibration dots include a pre-determined amount of said analyte for reacting with un-reacted reagent in a vessel, conjugated with a detectable marker. The calibration dots allow the intensity of the label to be correlated to the amount of the antigen present. The intensity of label in the test dots can then be used to derive the quantity of antigen present.

The calibration dots have a concentration of the analyte that corresponds to a dynamic range of the analyte. Dynamic range is the concentration of analyte normally found in the patient. The quantitation needs to be within this lowest and highest concentration and relate to the clinically relevant concentrations.

Many of the problems associated with current methods typical for immunoassays derive from the assay calibration being determined by introducing external standard reference samples for calibration. The present method provides more accurate results by not using these standard external calibration samples.

In developing a platform device for measuring the quantity of a respective analyte, instead of using external standards to generate a calibration or base line, both calibration dots as well as test dots at unknown concentration are printed onto the same test platform. The calibration test dots are printed at known concentrations of analyte. The test dots are printed, also at predetermined X-Y locations, containing only capture reagent which is preferably an antibody specific for the analyte under investigation. The test sample is then conjugated with an excess of marker reagent, which is preferably an antibody, and analyte. The marker antibody has previously been conjugated with a respective fluorescent label, emitting at a suitable wavelength (e.g. 650 nanometers). The marker antibody/antigen complex as well as the free, remaining marker antibody is then flowed over the test platform using laminar flow. A person skilled in the art will appreciate however that other assay devices that do not rely on laminar flow may also be employed. For example, an assay device in the form of a vessel where the antibody/antigen complex as well as the remaining free marker antibody move through the device by diffusion limited kinetics may also be employed for the purposes of the present invention.

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In this fashion, marker antibody/unknown concentration of antigen complexes are bound by the capture antibody test dots, whereas free marker antibodies bind to the pre-printed antigen dots at known concentrations. Both the test dots of unknown concentration to be measured and the calibration dots of known concentrations encompassing the dynamic range of the analyte are exposed to the same test fluid sample at the same time. The laminar flow effectively places the analyte components within proximity of the respective binding sites to promote optimal adhesion kinetics for the respective association constants.

The test platform is examined in a reader for determination of the respective concentrations of fluorescent label attached to the dots on the platform when activated by suitable wavelength irradiation. The calibration dots, preferably originally printed at up to ten different concentrations of analyte, result in producing a dynamic internal true dose response curve providing very accurate calibration reference for the assay. The intensity reading obtained from the test dots of unknown concentration is compared to this calibration line. The unknown test concentrations accurately and efficiently interpolate into the dynamic internal true dose response calibration obtained from the known calibration spots.

Each assay device tested provides similarly accurate and reproducible results confirming that the present method for on-platform dynamic calibration provides an accurate, enhanced and sensitive determination of analyte in both quantitative as well as qualitative assays for diagnostic use and detection of analytes in clinical use for humans and animals. This immediate and significant benefit demonstrates that these assays, when processed using the described method (Dynamic Internal Calibration™), do not reflect the errors described in association with current other methods for running these assays while using externally derived calibration standards.

The ability to calibrate and test simultaneously also enhances the confidence level in assuring that the obtained measurements are true. This methodology of the present invention, allows for several different analyte tests to be run on the same assay device at the same time. Several sets of test dots or test arrays for testing for different analytes as well as multiple sets of corresponding calibration dots or calibration arrays for the different analytes can be run on the same assay device at the same time. In

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order to reach a better than 99% confidence level for a test, the test needs to be run a minimum of three times. Multiple panels of different tests may also be used and run, all at the same time, on a single test platform. The surprising results of the present invention prove that errors in the tests are eliminated, reproducibility is enhanced, the dynamic range for any test is easily extended to cover a required analyte concentration range, sensitivity and coefficient of variance is dramatically improved. The combination of these advantages over prior state of the art also results in considerable saving in time to test results. It is an important advantage that the format of the present invention is device independent and may be applied by those skilled in the art.

The assay device and method as described, effectively represent a novel, quantitative and fast method for the accurate determination of analyte and or marker concentrations, typically for diagnostic clinical markers associated with disease processes. The immediate benefit of rapid, accurate measurement of marker concentration allows for rapid dynamic detection of marker concentration as an indicator of a disease process such as increasing, steady or decreasing concentration, as well as rapid quantitative monitoring of drug efficacy in modifying gene expression for the production of specific marker proteins to indicate drug efficacy.

Examples

Example 1: Quantitative Fluorescent Immuno-Assay.

The sandwich immunoassay matrix incorporates a capture antibody that is specific for the antigen of interest and a fluorescence conjugated secondary antibody for detection of analytes and immune complexes. Human chorionic gonadotropin (HCG), a marker of pregnancy in humans, was used as antigen for testing of this platform assay. Currently, the dynamic analytical range for this test is between 1 to 150 fmol/uL (280-37,600mIU/mL) with an assay volume of 5μL. Comparison between the calculated HCG concentration using the device compared with known HCG concentrations has excellent agreement between values (Figure 2, $y=1.0717x + 9.9313$) and high correlation between mean values for each concentration tested ($r=0.9786$). Figure 2 is a graph that

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shows confirmation that single and aggregate immuno complexes provide quantifiable fluorescence when bound to the device platform.

Example 2: Measurement of antigen dot fluorescence at various sample antigen concentrations.

The assay principle is based on quantitative, non-competitive, heterogeneous immunoassays. First generation devices were printed with a series of antigen dots at decreasing concentrations for standard curve auto-calibration; followed by capture antibody dots. Test sample containing the antigen was processed with lyophilized detecting antibody already conjugated to the respective indicator or dye. The test sample reacted with label for 2 minutes and was then dispensed into the chip assay device to be inserted into the reader. The fluorescent intensity of each dot was measured. The reader software compares the fluorescence of the capture antibody dots having unknown antigen concentration with those of the true dose response standard curve having known concentrations and calculates the concentration of the test antigen. Figure 3 is a graph showing data to confirm that antigen concentration in the test sample does not affect the fluorescence intensities of antigen in the calibration dots.

Example 3: Advanced Array Printing on the Assay Device platform.

Each of four 10 x 10 matrices containing 100 test dots as shown in Figure 4, used 2.6 mm x 2.6 mm of platform area. The center-to-center separation from dot to dot was 260 μm. The number of dispensed droplets per spot increments from 1 to 4 droplet(s) per dot per array matrix. Total chip assay device reading area was 8000 μm x 10000 μm. This printing pattern allowed 1200 dots to be printed on the platform reading area. With a minimum of 5 dots per test, 3 for calibration and 2 test dots, each platform supports 240 tests. This technology advantage allows for fmol/ml antigen detection sensitivity and an increasing number of multiplex test arrays for optimal confidence in diagnostic results by being able to multiplex required test matrices to optimize the calibration curves. Figure 4 provides an illustration of advanced PicoTip Array printing technology on the chip assay device platform.

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Example 4. Correlation of human Para Thyroid Hormone Concentration with Fluorescent Intensity calibrated using True Dose Response Arrays measurement.

As shown in Figure 5, the concentrations of human parathyroid hormone was measured to determine the dynamic internal true dose response calibration curve. The tests confirm that the true dose response was accurately plotted over the dynamic range as tested..

Example 6: Layout of multiplex format when testing human plasma for 12 different antibodies in response to having been exposed to 12 micro-organisms.

As shown in Figure 6, the test arrays are printed as triplicate arrays at four concentrations for each test, whereas the calibration arrays, in this case for human IgG immunoglobulin, are printed at 6 concentrations in triplicate. An array of 4 dots x 5 dots is used to measure the response of fluorescent label (Dy647) in a binary coding format to confirm the identity of the assay platform when inserted into a reading device.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the embodiments of the invention described above. Such equivalents are intended to be encompassed in the scope of the following claims.

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WHAT IS CLAIMED IS:

1. A method of determining an amount of an analyte in a sample comprising the following steps:

- providing an assay device having a surface, said surface having a plurality of calibration dots printed thereon and a test dot printed thereon, the calibration dots including pre-determined quantities of the analyte, the test dot including a reagent for binding to said analyte;
- providing a solution having a sample reagent specific for the analyte, said sample reagent being labeled with a detectable marker;
- introducing the sample into said solution to form a sample solution;
- introducing said sample solution onto said assay device;
- measuring an intensity of detectable marker in said calibration dots;
- preparing a calibration curve correlating the amount of analyte in said calibration dots to said intensity of detectable marker;
- measuring an intensity of detectable marker in said test dot; and
- calculating an amount of analyte present in said test dot by comparing the intensity of detectable marker to the amount of analyte corresponding to said intensity in said calibration curve.

2. A method according to claim 1 wherein the reagent is a capture antibody and the sample reagent is an antibody that binds specifically to said analyte.

3. A method according to claim 1 or claim 2 wherein the assay device has a loading portion for receiving the sample solution and a reading portion, the reading portion having the plurality of calibration dots printed thereon and the test dot printed thereon.

4. A method according to one of claims 1-3 wherein the calibration dots are printed in a volume ranging from 1 picoliter to 1 microliter.

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5. A method according to one of claims 1-3 wherein the volume ranges from 1 picoliter to 4 picoliters.

6. A method according to one of claims 1-5 wherein the calibration dots are printed in a volume ranging from 1 picometers to 12 millimeters.

7. A method according to one of claims 1-6 wherein the calibration dots are printed in a volume ranging from 25 micrometers to 300 micrometers.

8. A method according to one of claims 1-7 wherein the calibration dots are printed in arrays at predetermined X-Y co-ordinates.

9. A method according to one of claims 1-8 wherein the calibration arrays are printed at pre-determined concentrations.

10. A method according to claim 1 wherein the calibration dots are printed at concentrations to encompass a dynamic range of test substrate to be found according to the test sample.

11. A method according to claim 1 wherein the calibration arrays are printed at concentrations to encompass a dynamic range of test substrate to be found according to the test sample.

12. A method according to claim 1 wherein the surface of the assay device is substantially planar.

13. A method according to claim 1 wherein the calibration dots have a concentration of the analyte that corresponds to a dynamic range of the analyte.

14. A method according to claim 1 wherein the calibration dots are arranged in at least three arrays for carrying out three or more replicates of a test.

15. A method according to claim 1 wherein the test dots are arranged in at least three arrays for carrying out three or more replicates of the same test.

16. An assay device according to claim 14 or claim 15 wherein the test may be the same or different.

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17. A method according to claim 1 wherein a single analyte in a single test is measured on the assay device.

18. A method according to claim 1 wherein a single analyte in a plurality of arrays for same test is measured on the assay device.

19. A method according to claim 1 wherein different analytes are measured contemporaneously on the assay device.

20. A method and assay device according to claim 1 wherein a plurality of different analytes in a plurality of different tests are measured contemporaneously on the assay device.

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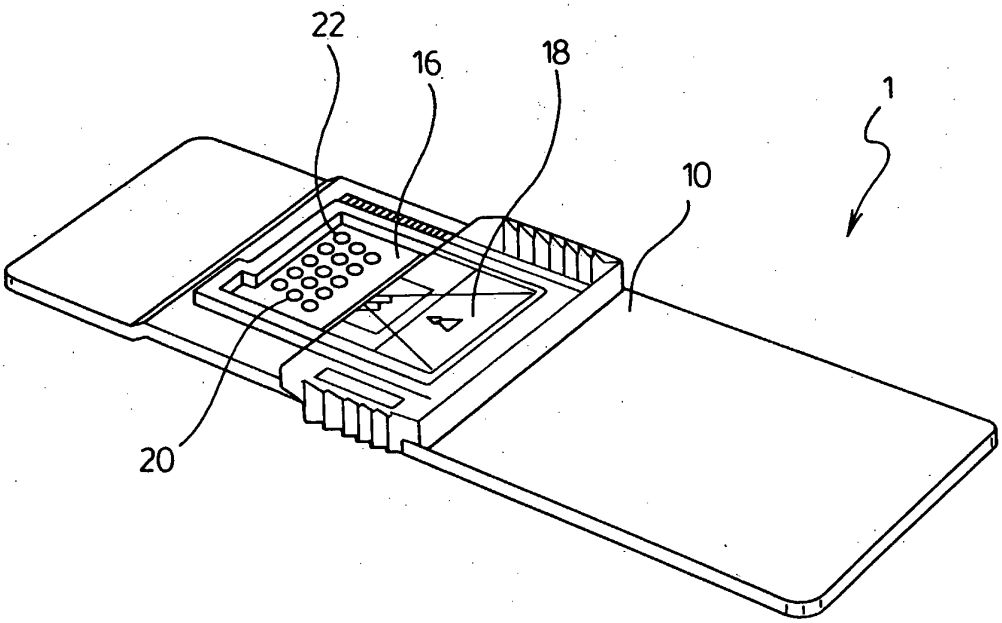


FIG.1.

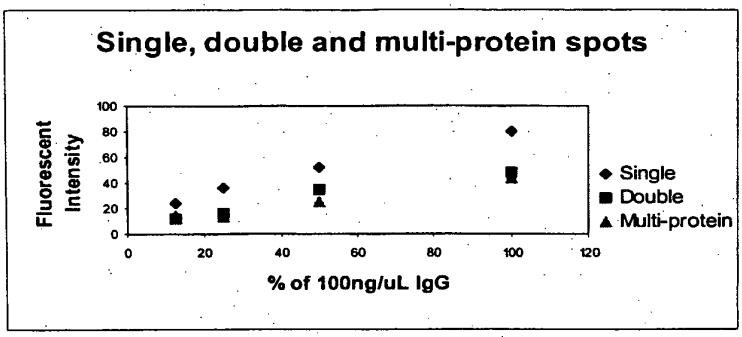

Single, double and multi-protein spots
Fluorescent Intensity
100
80
60
40
20
0
0
20
40
60
80
100
120
% of 100ng/uL IgG
Single
Double
Multi-protein

Figure 2

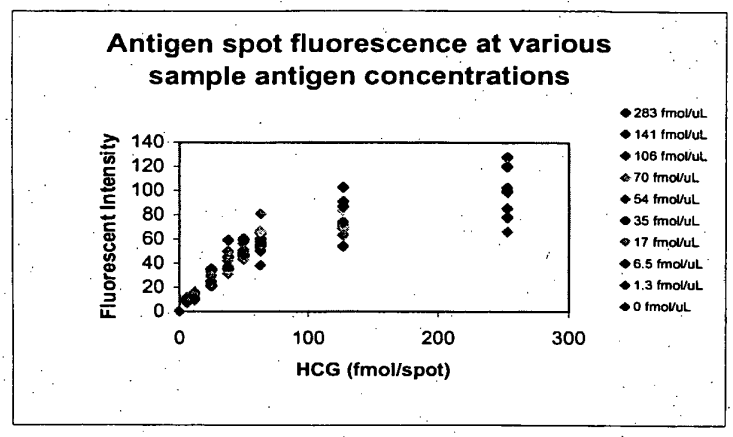


Figure 3

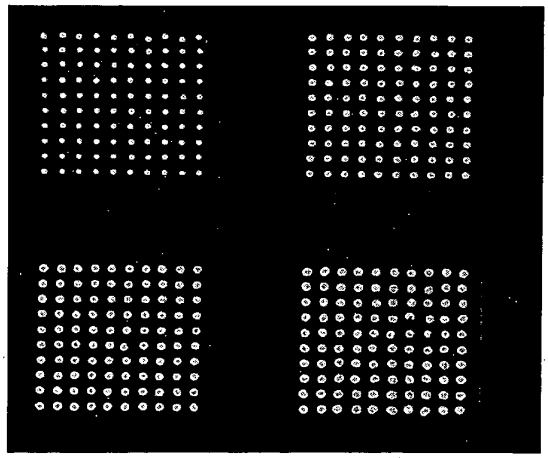


Figure 4

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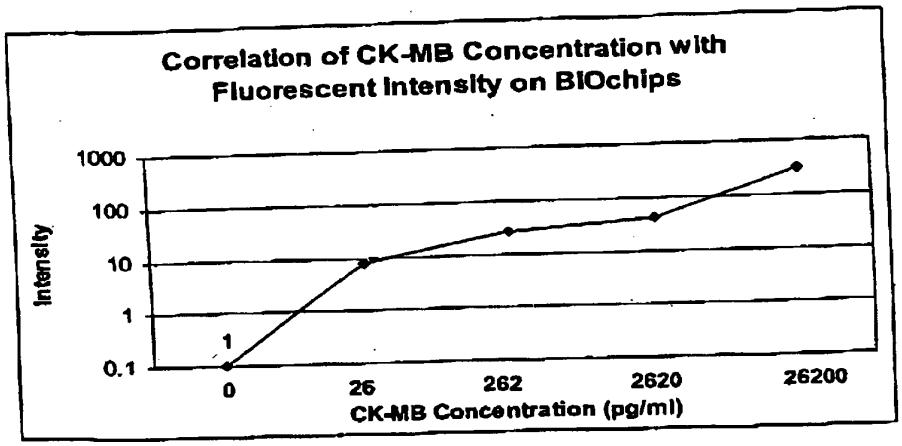


Figure 5

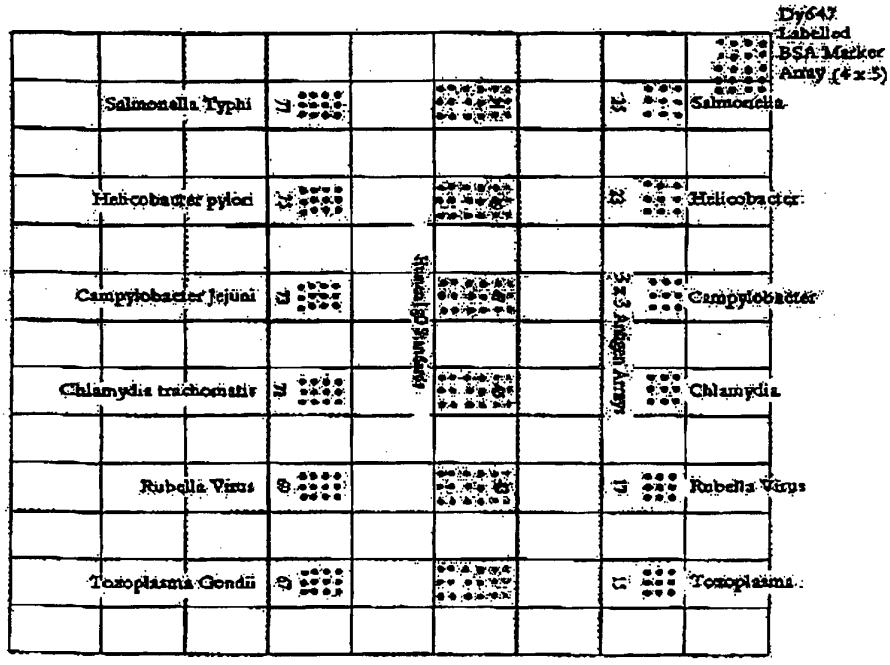


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001147

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7): G01N 33/543, G01N 33/53, G01N 37/00

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)
G01N 33 and G01N 37

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MSN Recherche (internet) and Google (internet)

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Delphion (Derwent), Pubmed and CPD (Canadian Patent Database)

Assay*, calibration*, control*, test*, dot*, spot*, multi-spot, print*, pin-printed, bind*, link*, calibration curve, courbe de calibration (French), dosage (French).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO03025573 A1 (Pharmacia Diagnostics AB) 27 March 2003 (27-03-2003) Refer to the whole document	1-20
A	US5200312 (Oprandy) 6 April 1993 (06-04-1993) Refer to col. 3, lines 4-23	1-20
A	US6265176 (Lin et al.) 24 July 2001 (24-07-2001) Refer to the whole document	1-20
A	WO05031355 A1 (Quidel Corp) 7 April 2005 (07-04-2005) Refer to the whole document	1-20

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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

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Date of the actual completion of the international search	Date of mailing of the international search report
20 October 2005 (20-10-2005)	15 November 2005 (15-11-2005)
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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.
A	WO03034026 (Biocept inc.) 24 April 2003 (24-04-2003) Refer to the whole document	1-20

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001147

Patent Document	Publication Date	Patent Family Member(s)	Publication Date
US5200312	06-04-1993	None	
WO03025573	27-03-2003	CA2460072 A1	27-03-2003
		EP1436621 A1	14-07-2004
		JP2005503556 T	03-02-2005
		SE0103072 D	17-09-2001
		US2003073121A1	17-04-2003
US6265176	24-07-2001	None	
WO05031355	07-04-2005	US20050130120A1	16-06-2005
WO03034026	24-04-2003	EP1444500 A2	11-08-2004
		JP2005506530T	03-03-2005
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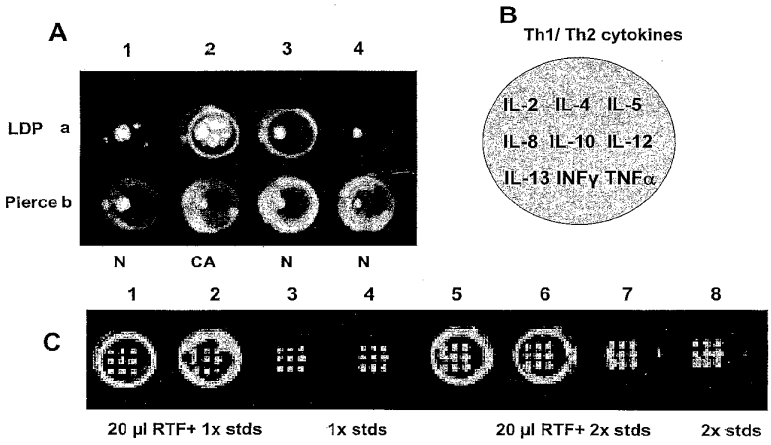
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(54) Title: STATIONARY PHASE ANTIBODY ARRAYS FOR TRACE PROTEIN ANALYSIS



(57) Abstract: The present invention relates to the identification of trace proteins and biomarkers, e.g., TH-1/TH-2, cytokines, MMPs and angiogenic modulators in tear fluids. The present invention provides an antibody-based stationary phase array assay for simultaneously identifying, detecting and characterizing the distribution of a wide range of bioactive trace proteins in a tear fluid sample. A method for simultaneously identifying trace proteins in a biological fluid sample using a highly sensitive antibody-array assay is provided. The present invention also provides methods and kits for treating, preventing, and diagnosing ocular diseases, disorders or pathological conditions.

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STATIONARY PHASE ANTIBODY ARRAYS FOR TRACE PROTEIN ANALYSIS

FIELD OF THE INVENTION

The present invention relates to the identification of trace proteins and biomarkers, e.g., chemokines, cytokines, MMPs and angiogenic modulators, in tear fluids. In particular, the present invention relates to an antibody-based stationary phase array assay for detecting and characterizing the distribution of a wide range of bioactive trace proteins in a tear fluid sample. The invention also relates to a method of differential screening/analysis of the trace proteins in a tear fluid sample. The present invention further relates to methods and kits for diagnosing ocular diseases, disorders or pathological conditions.

BACKGROUND OF THE INVENTION

The pre-ocular tear layer is a complex entity that contains dozens of low abundance proteins (LAPs) including many entities that are bioactive even in trace amounts. Many studies have been carried out monitoring the concentration and distribution of specific targeted LAPs in tears as a function of various parameters. This has aided in a better understanding of homeostatic and pathological processes common to the underling ocular tissue. Particular attention has been focused on those cytokines, chemokines, growth factors, angiogenic modulators and associated molecules that are known to modulate wound healing, apoptosis, cell cycling and migration.

Protein components in a biological fluid sample are involved in key physiological functions in a human individual. Kratoconjunctivitis sicca (KCS) or dry eye syndrome encompasses a diverse spectrum of diseases, which in total affect a large proportion of the population. The classification, diagnosis, characterization, pathophysiology processes and therapeutic approaches to the management of KCS have been extensively reported. One major subgroup of dry eye syndromes arises from processes attributed in part to a non Sjögren's syndrome (SS) lacrimal secretory deficiency. To identify biomarkers related to KCS progression and prognosis as well as

to understand the underlying pathophysiological processes of KCS, studies have been carried out in attempt to correlate the concentration and distribution of specific proteins in the pre-ocular tear layer with the clinical symptoms of KCS. Besides the well-known general decrease in the concentrations of the constitutive lacrimal gland proteins, lysozyme, lactoferrin and certain other proteins have been identified in tear fluid which are either up-regulated or down-regulated in KCS. It has been suggested that aqueous deficient triggers an inflammatory cascade through decreased fluid turnover and increased osmolarity. The bifurcated characteristics of the tear (i.e., increased tear osmolarity and decreased in fluid turnover) may induce an epithelium related up-regulation of the production and the accumulation in the pre-ocular tear film of IL-1b and TNF-alpha. It has been hypothesized that such up-regulation results in the activation of proMMP-9 through the up-regulation of MMP-3 and thereby ultimately results in KCS.

The above hypothesis appears to be supported by certain studies of human tear fluid and animal model. However, in conducting such studies and developing methods for diagnosing and treating KCS, it has remained significantly difficult to obtain human proteomics data on tear composition due to the limited tear sample size. Until recently, virtually all studies in this field have relied either upon ELISA assays or, when appropriate, upon zymographic analysis that is carried out on either individual or, if necessary, pooled tear samples. Sample size constraints have restricted analysis in most instances to the study one or at most a few targeted proteins for a given tear sample and limited the availability of matched samples to serve as controls.

Antibody (protein) array technology offers an alternative approach for obtaining proteomic data. To this end, several laboratories have employed the enhanced multiplex analyses in the form of moving phase immuno-bead arrays coupled with flow cytometry to assay tear samples for as many as 18 low abundance proteins (LAP) in individual tear samples. Although very sensitive, such technology requires significant initial capital investment in the form of equipment and trained personnel. Moreover, at present, only a limited number of arrays have been validated for use with open eye tear

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fluid. Extending the range of the assay to other trace proteins and validating the methodology for use with closed eye tear fluid may be problematic. In addition, tear fluid contains "blocking factor(s)" that have been shown to interfere with the binding and the efficacy of ELISA-type assays. Although the antibody-based membrane array (MA) system has been employed to carry out differential analysis of the distribution of trace proteins in tissue extracts, tissue culture filtrates, serum and urine samples, one major disadvantage of this system is the lack of sensitivity relative to ELISA. For example, many trace proteins have yet to be identified or characterized in tear fluids by the presently available protein or antibody assay arrays.

Despite much progress, serious problems remain. One of the long-standing problems when using ELISA or multiplex assay has been the widely divergent values for the baseline levels of concentrations of many cytokines, chemokines and growth factors in the normal basal (open) eye tear fluid. It has not been uncommon to find reports of the mean concentrations for given cytokines or growth factors in the open eye tear fluid that vary by a factor of one hundred fold or more. These significant variations have made it extremely difficult to integrate the data from different studies and laboratories.

Angiogenin (ANG), which was originally identified as a tumor-derived protein, is a normal blood constituent. It was found to induce vascular growth. ANG was also reported to bind to the membrane of different cell lines and to have the ability to intervene in cellular signal transduction. Abnormality in granulocyte function can cause a subject to become susceptible to infections. ANG, including recombinant ANG, was found to inhibit the degranulation of polymorphonuclear leukocytes (PMNL), even at minute concentrations in the nanomolar (10^{-12}) range. Although ANGs have also been implicated in tumor-associated angiogenesis, their normal physiologic function only started to be revealed recently. ANGs have been identified to be microbicidal proteins involved in innate immunity. A previously uncharacterized angiogenin, Ang4, which is produced by mouse Paneth cells, was found secreted into the gut lumen and has bactericidal activity against intestinal microbes. Furthermore, mouse Ang1 and human angiogenin, circulating proteins induced during inflammation,

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exhibit microbicidal activity against systemic bacterial and fungal pathogens, suggesting that ANGs contribute to systemic responses to infection. These results establish angiogenins as a family of endogenous antimicrobial proteins.

The pre-ocular tear film plays a critical major role in host defense mechanisms and homeostatic processes that differ markedly in open and closed eye environments. Studies have shown that on eye closure, inducible lacrimal flow largely ceases with ongoing flow consisting of a much slower constitutive secretion consisting primarily of secretory IgA (SIgA). This is accompanied by the recruitment of massive numbers of polymorphonuclear (PMN) cells and the induction of a sub-clinical state of inflammation. Various leukochemotactic factors including IL-8 and 12 rHETRE have been identified to accumulate in closed eye tear fluid (CTF), which drive the recruitment of PMN cells. These appear to be derived at least in part from the ocular surface tissue and may be induced by hypoxia driven up-regulation.

The present invention provides an MA system coupled with an ultra-sensitive substrate system and optimized signal-to-noise ratio that can achieve unexpectedly stable high sensitivity, e.g., one-hundred fold increased sensitivity, relative to the sensitivity obtained by the presently commercially available MA system. The present invention thus provides a facile and less expensive antibody-based MA system with high sensitivity and methods for analyzing trace protein components in a tear fluid sample.

SUMMARY OF THE INVENTION

The present invention recognizes the successful adaptation of microwell plate and membrane antibody array technology for tear protein analysis and utilization of this technology to analyze the distribution of low abundance proteins (LAPs) in tear fluids from subjects in normal and pathological conditions (e.g., allergic or KCS basal tear fluids). The present invention is also based on the surprising discovery that ANG exists in virtually all tear samples and exhibits high levels of signal intensity in the assay array employed by the present invention.

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It is an object of the present invention to simultaneously identify and analyze in one assay the trace proteins in a tear sample with sufficient sensitivity and specificity in a cost effective and rapid means.

Particularly, the present invention provides arrays to improve the sensitivity of the ELISA assays by optimizing the conditions of each assay. The present invention also discloses vast improvements of the signal-to-noise ratio by excluding from the arrays any antibody pairs that gave rise to even low levels of cross-talk between capture and probe antibodies, which allows to increase the sensitivity of assay for many LAPs (including cytokines) at least one hundred fold and thereby providing arrays suitable for the analysis of clinically obtainable size samples. The present invention also contemplates adapting a commercially available antibody array system, e.g., a 96 microwell plate formatted antibody array system, for obtaining quantitative data.

One aspect of the present invention is directed to the identification of LAPs or trace proteins in biological fluids, preferably, tear fluids. Examples of LAPs or trace proteins include, but are not limited to, matrix metalloprotease (MMP, e.g., MMPs-1, 2, 3, 8, 9, 10, 13), angiogenin (ANG, e.g., ANG-2), hematopoietic growth factor (HGF), basic fibroblast growth factor (FGFb), thromopoietin (TPO), vascular endothelial growth factor (VEGF), keratocyte growth factor (KGF), HB- epidermal growth factor (HB-EGF) and plate derived growth factor-BB (PDGF-BB), interleukins (ILs, e.g., ILs-2, 4, 5, 8, 10, 12 and 13), interferons (IFNs, e.g., IFNγ), tumour necrosis factor (TNF) (e.g., TNFα), and tissue inhibitor of metalloprotease (TIMP) (e.g., TIMPs-1 and 2). Accordingly, a method for detecting the presence and distribution of LAPs in a tear fluid sample from a subject is provided by the present invention. In a particular aspect, the method of the present invention is a qualitative method.

Another aspect of the present invention is directed to an antibody-based stationary phase array system comprising an array matrix of dot grids on a stationary phase/support/surface, preferably a membrane, bounded or attached by at least one antibody that is capable of binding with a specific protein species, secondary or

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detection antibodies, and an ultra-sensitive substrate that is recognized by an enzyme linked to the secondary antibodies.

Still another aspect of the present invention is directed to a method for simultaneously identifying LAPs or trace proteins in a fluid sample, preferably, a biological fluid sample, more preferably, a tear sample, comprising the steps of obtaining the sample, incubating an antibody-based stationary phase array with a blocking buffer, incubating the sample with the array, incubating the array with detection/secondary antibodies, incubating the array with an ultra-sensitive substrate that is reacted with an enzyme lined to the detection antibodies. In a particular aspect, the method can also be described to comprise the steps of: a) obtaining the sample, b) incubating the sample with an antibody-based stationary phase array comprising capture antibodies on the array, c) incubating the array from Step b with secondary antibodies, d) incubating the array from Step c with an ultra-sensitive substrate that is reacted with an enzyme linked to said secondary antibodies thereby providing a detectable signal of the binding between a capture antibody and a LAP, e) detecting the signals and analyzing data. In a particular aspect of the present invention, the method can further comprise the steps of optimizing conditions for increasing or maximizing signal-to-noise ratio, e.g., incubating an antibody-based stationary phase array with a blocking buffer prior to incubation of the sample and the array. In another particular aspect, the present invention is directed to a method for identifying trace proteins in a fluid sample, preferably, a biological fluid sample, more preferably, a tear sample, comprising obtaining the sample and assaying the sample by the antibody-based stationary phase array system of the present invention. In another particular aspect, the analysis is carried out using more than one array, e.g., three off the shelf antibody array kits, with the individual samples assayed by sequential transfer from one array to another.

Yet another aspect of the present invention is directed to a method for differential screening/analysis of trace proteins in biological fluid samples, preferably, tear samples, that are obtained from different physiological conditions or stages or status, comprising the steps of a) obtaining the samples, and b) identifying and comparing/analyzing the trace proteins in each sample.

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A further aspect of the present invention is directed to a method for diagnosing pathological conditions, particularly, an ocular disease or pathological condition, of a subject, preferably, a human, comprising the steps of a) obtaining a biological fluid sample, preferably, a tear sample, b) identifying the protein distribution or level, e.g., MMP or ANG level, in the sample by the method of the present invention or by the antibody-based stationary phase array system of the present invention, and c) detecting and analyzing the changes of the trace protein distribution or level in the sample relative to that of a normal sample or to a database comprising known trace protein distribution/level patterns under normal or pathological conditions. In a particular aspect, the present invention provides a method for diagnosing an ocular infection and/or inflammations, e.g., microbial infections, including but not limited to infections related to eyes caused by bacteria, fungi or virues, or infections/inflammations caused by trauma or contact lense, or risk of susceptibility to such infections/inflammations in a subject, by detecting a varied ANG level beyond a normal range in a biological fluid sample, preferably, a tear fluid sample, from the subject.

A still further aspect of the present invention is directed to treatment of ocular infections and/or inflammation in a subject, comprising a) detecting or diagnosing an ocular microbial infection in the subject, e.g., by a detecting pathological level of ANG in a biological fluid sample, preferably, a tear fluid sample, from the subject, and b) administering ANG and/or other anti-microbial agents.

A further aspect of the present invention is directed to prevention of ocular infections and/or inflammation in a subject, comprising a) detecting or diagnosing level of ANG in a biological fluid sample, preferably, a tear fluid sample, from the subject, and b) if the ANG level is lower than its normal range, administering ANG and/or other anti-microbial agents.

One aspect of the present invention is directed to a kit for diagnosing ocular pathological conditions comprising an instruction manual, an antibody-based membrane array, a reaction-well tray, blocking and washing buffer solutions, detection antibodies, e.g., biotinylated secondary antibodies, at least one indicator that detects a

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specific binding of trace proteins in a test sample to the capture antibody or antibodies carried by the array, e.g., streptavidin-linked peroxidase (SPO) and a luminol-amplifier based substrate system.

Another aspect of the present invention is directed to a kit containing a composition in the form of eye drops for anti-ocular microbial infection, comprising angiogenin, preferably, recombinant angiogenin, and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts Th1/Th2 well plate array assayed for OTF samples from three normal (N) individuals and an individual with active chronic rhino-conjunctivitis (CA) with one set of lanes using the LDP (top row) and the other set of lanes using the manufacturer's protocol (bottom row). Duplicate OTF samples (5 µl each) were assayed from three normals (lanes 1, 3 and 4) and from one donor with active chronic rhino-conjunctivitis (lanes 2). Arrow points to minor amount of IL-4 detected in one well using the LDP. Figure 1B depicts array format, left to right - top row within the circle contains antibodies for IL-4, IL-5 and IL-10, middle row-antibodies for IL-8, IL-10 and IL-12, bottom row antibodies for IL-13, INFγ and TNFα. Figure C shows duplicates containing two sets of dilutions of recombinant protein standards both neat and spiked in RTF as assayed using the TH-1/Th-2 array with the LDP. Lanes 1 and 2 contain recombinant cytokine standards (IL-2 200 pg/ml, IL-4 400 pg/ml, IL-8 200 pg/ml, IL-12 200 pg/ml, IL-13 2000 pg/ml, INF gamma 200 pg/ml, and TNF alpha 800 pg/ml each) spiked in 20 µl RTF; lanes 3 and 4 contain neat standards (IL-2 200 pg/ml, IL-4 400 pg/ml, IL-8 200 pg/ml, IL-12 200 pg/ml, IL-13 2000 pg/ml, INF gamma 200 pg/ml, and TNF alpha 800 pg/ml each); lanes 5 and 6 duplicates contain 2x recombinant cytokine standard spiked in 20 µl RTF; wells 7 and 8 contain 2x standards (IL-2 400 pg/ml, IL-4 800 pg/ml, IL-8 400 pg/ml, IL-12 400 pg/ml, IL-13 4000 pg/ml, INF gamma 400 pg/ml, and TNF alpha 1600 pg/ml each) neat. Note that despite the modified protocol residual non-specific deposition still occurs at the edge of the wells that contain RTF. Blank control well plate (not shown) was devoid of significant luminescence.

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Figure 2 depicts recovery of a cocktail of recombinant cytokine spiked standards depicted in Figure 1 C assayed using the Th1/Th2 array with the LDP.

Figure 3A and 3B illustrates the approximate concentration ranges of inflammatory and anti-inflammatory Th1/Th2 cytokines in RTF, OTF and CTF samples obtained from two normals (N) and an individual with active chronic rhino-conjunctivitis (CA) as assayed using the well plate array with the LDP.

Figures 4A-4E depict custom membrane array specific for 16 cytokines assayed for pooled OTF and CTF (30 μl) samples from two normals (N) and the corresponding control membrane. Figure 4F depicts the array format used in the arrays of Figures 4A-4E. Note that in this and the other arrays the capture antibodies for several cytokines were spotted in duplicates at two or more concentrations to systematically evaluate the optimal conditions for obtaining maximal sensitivity and specificity for each assay while maintaining optimum signal to noise ratio. The number listed in the parenthesis of each of the locations on the array represents the relative concentrations of capture antibodies compared to a starting level. The effects of variation in the concentration of capture antibodies can be readily seen upon examining the signals for or IL-8 can be readily seen in this and the following two figures. Note that the positive control, which consists of a biotinylated protein, was spotted on this and the array in Figure 5 at one hundredth the concentration that is normally found on the array in Figure 6. This was done to avoid the signal blooming and the resultant obscuring of the adjacent weaker signals. Also note under some conditions of ultra-sensitive assay and detection the negative control as depicted in this array gives rise to a false positive signal. This possibility necessitates the use of a control membrane which in this case was incubated with an equivalent volume of a <1 kDa ultrafiltrate of RTF.

Figures 5B-5D depict OTF, CTF (7.5 μl) samples obtained from the same chronically allergic individual as depicted in Figure 1A and a membrane control (incubated with an equivalent volume of the < 1 kDa ultra filtrate of RTF) assayed with a different array format as shown in Figure 5A. Note that the array configuration, the concentrations of capture antibody and the conditions of this array development differs from those utilized in Figure 4. Under these conditions there is increased noise in the

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control membrane but a reduction in non-specific reactivity with the negative controls. These differences have no direct bearing upon the significance of these findings but are important in the array design.

Figures 6A-6C depict OTF from an individual with seasonal acerbated chronic conjunctivitis and a normal individual along with a control membrane assayed with a third array fromat. Note that the array configuration as shown in Figure 6D, the concentrations of capture antibody and the conditions of this array differs from those utilized in Figures 4 and 5 with the concentration of the positive control not diluted resulting in blooming and a partial obscuring of adjacent signals.

Figure 7A depicts microwell plate angiogenic array assaying normal OTF samples and protein standards using the LDP. Wellplate 1: 5µl; wellplate 2: 2.5µl; and wellplate 3:1µl of normal (N) OTF (arrow points to ANG-2); Wellplates 4, 5, 6 contain serial dilutions of recombinant protein standards. Figure 7B depicts array format: top row- TIMP-1, ANG-2 and PDGF, middle row-TPO (can be blank see text and Figure 7), KGF, HGF, bottom row- FGFb, VEGF and HB-EGF. Figure 7C depicts wellplates containing (in duplicate) two dilutions of recombinant angiogenic protein standards neat and spiked in 20 µl RTF as assayed using the LDP. Wellplates 1 and 2: duplicates with angiogenic standards (TIMP-1 5000 pg/ml, Ang-2 5000 pg/ml, PDGF-bb 1,000 pg/ml, TPO 3300 pg/ml, KGF 1000 pg/ml, HGF 1600 pg/ml, HGF 1600 pg/ml, FGFbasic 2800 pg/ml, VEGF 2000 pg/ml, and HB-EGF 625 pg/ml each) in the presence of 20 µl RTF; wellplates 3 and 4: duplicates of neat angiogenic standards; wellplates 5 and 6: duplicates containing 2x angiogenic standards in 20 µl RTF: wellplates 7 and 8: neat 2x angiogenic standards. Figure 7D depicts an assay of 5µl OTF from normal and chronic allergic individuals (in duplicate). C: allergic samples; N: normal samples. Note that while high levels of VEGF and HGF were common to this set of samples, these growth factors were absent in many normal and pathological samples. In contrast, positive signals for FGFb and Hb-EGF were exclusive to all but one of the chronic allergic OTF samples.

Figure 8 shows percent recovery of a cocktail of recombinant angiogenic standards assayed using an array without a TPO assay employing the LDP. Samples in

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duplicate consist of two concentrations of recombinant standards with and without 20 µls of a pooled RTF sample.

Figure 9 depicts wellplates of angiogenic modulators assaying composite OTF and CTF samples (volumes as indicated) from three normal (N) donors and a donor with active chronic rhino-conjunctivitis (CA) using the LDP (Note that this array contains an assay for TPO). Donors a, b and c are normal (N) individuals (shown in Figures 9A-9C) while donor d has active chronic rhino-conjunctivitis (CA) (shown in Figure 9D). Figure 9 illustrates the range of distribution of the varying angiogenic factors in OTF and CTF samples. Note that the CTF sample from subject b was atypical compared to that from other normal individuals in that FGFb and Hb-EGF were detected. Tears for this individual are also assayed in figure 4 showing high levels of cytokines in CTF.

Figure 10A depicts wellplates of MMPs assayed with a series of dilutions of RTF and CTF samples obtained from an individual with active chronic rhino-conjunctivitis (CA). Volumes of samples are as indicated. Figure 10B depicts array format within the circle; top row-MMP-1, MMP-2, MMP-3; middle row-MMP-8, MMP-9, MMP-10; bottom row-MMP-13, TIMP-1, TIMP-2. Figure 10C depicts serial dilution of MMP standards neat and with added RTF assayed using the MMP array. Top row- 1 through 4 contain serial dilutions of MMP standards. Bottom row: control, MMP standards with and without added RTF. Figure 10D shows 5 µl of representative OTF samples from pathological and normal individuals assayed for MMPs illustrating the range of distribution of MMPs and TIMPs in OTF. Wellplates 1-3 contain OTF samples (5µl) from three chronic allergic (CA) individuals. Wells 4-6 contain OTF (5 µl) from three normal individuals (N). Note that arrow points to an artifact.

Figure 11 illustrates recovery of a cocktail of recombinant TIMP and MMP protein standards at two concentrations neat and spiked in 20 µls of a pooled RTF sample assayed using the Pierce MMP array. Note that similar curves were obtained with recombinant protein standards spiked in different sources of RTF.

Figure 12 depicts segments from four membrane arrays developed with two sets (20 µl) of OTF (recovered one month apart) after recovered from the affected

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and follow eyes induction of an acute unilateral allergic conjunctival reaction with samples. Samples were probed on the same array shown in Figure 4. Array segment shows the dot ELISA arrays for IL-8 using two sets of antibody dilutions--1:2 (arrow head) and 1:5 for IL-8. Note that the difference is particularly pronounced in the 1:2 dot ELISA assays.

DETAILED DESCRIPTION OF THE INVENTION

The present invention recognizes the successful adaptation of microwell plate and antibody array, particularly, membrane antibody array, techniques for tear protein analysis and utilization of this technology to analyze the distribution of low abundance proteins (LAPs) or trace proteins in tear fluids from subjects in normal and pathological conditions (e.g., allergic or KCS basal tear fluids). The present invention also recognizes that ANG exists in virtually all tear samples and exhibits high levels of signal intensity in the assay array employed by the present invention. The present invention discloses methods of using stationary phase forms of array analysis carry out quantitative and qualitative analysis of clinically obtainable size tear samples.

Particularly the present invention recognizes the marked difference in the pattern of distribution of various angiogenic modulators and MMPs in the normal and pathological tear samples. FGFb and Hb-EGF which are virtually absent or barely detectable in virtually all of the normal tear samples are very prominent entities in many of the dry eye tear samples, reaching concentrations which at times approaches the ng/μl range. That these growth factors are absent, or found at most in trace levels, in normal tear fluid confirms the findings of other reports. The finding of a very marked increase in Hb-EGF in the pathological tear samples is surprising since that the level of EGF was reported lower in tear fluid from individuals with both SS and non-SS aqueous deficiency dry eye syndromes. The result of the present example illustrates that Hb-EGF and EGF two closely related growth factors exhibit an inverse pattern of regulation. All of these growth factors are known to be synthesized by corneal epithelium, keratocytes, endothelium, and the lacrimal gland. Much less is known about the conjunctiva. Hb-EGF is known to secreted and bound to the corneal epithelium and other epithelial where it is found complexed with glycoproteins on the

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cell membrane through its heparin-binding domain. Several MMPs and other proteases are known to clip the glycoprotein releasing free HP-EGF from the cell membrane. This maybe the source of the marked increase in HB-EGF in the pathological tear samples. HB-EGF in turn is known to bind to the EGFr. Thus, chronic allergic reactions are associated with an exponential increase in the concentrations of FGFb and Hb-EGF in tear fluid. HB-EGF can be derived from HB-EGF normally bound to the epithelial cell membrane. This can be cleaved by various ADAM-like proteases including MMP-3. Released Hb-EGF can modulate apoptosis, cell migration and turnover through binding to the EGF receptor. Without intending to be limited in a particular mechanism, it is believed that FGFb can up regulate wound healing through stimulation of keratocytes.

The present invention recognizes that using both microwell plate and the membrane array techniques, OTF recovered from almost all individuals with active chronic allergic ocular surface diseases contain detectable and what is likely to be exponentially higher levels of many of the probed cytokines. According to the present invention, those cytokines that are particularly elevated include IL-2, 4, 5, 12 and INFγ. The marked increase in the levels of IL-2, 4 and INFγ confirms the premise of a strong Th2 component to chronic ocular surface allergic diseases, e.g., vernal conjunctivitis, atopic keratoconjunctivitis and giant papillary conjunctivitis. Elevated species, however, also include Th1 cytokines and encompass a mix of both activators and suppressors of inflammation. Without intending to be limited by any particular theory, it is believed that once established these pathologies represent complex cascades of events involving a multitude of pathways and the ocular surfaces has the adaptive nature to maintain homeostatic processes.

According to the present invention, the tear cytokine profiles are indicative of a chronic rather than an acute allergic reaction. Other than a modest increase in the level of IL-8 (and possibly IL-6) the cytokine profile of OTF remained largely unchanged after induction of an acute allergic reaction.

According to the present invention, the concentrations of many assayed LAPs in the pathological samples are elevated in the closed eye environment relative to

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the open eye environment. Without intending to be limited by any particular theory, it is believed that prolonged eye closure is associated with a marked decrease in the rate of inducible lacrimal secretion, a marked decrease in the rate of tear turnover, the induction of a sub-clinical inflammation, the recruitment and activation of PMN cells and the ensuing accumulation in CTF of a wide range of ocular surface tissue and PMN cell secretion products. Thus, the present invention contemplates detecting high levels of inflammatory mediators in the pathological CTF. The present invention also contemplates that the CTF can serve as an ideal vehicle for the recovery and detection of LAPs that are biomarkers of other ocular surface diseases because these biomarkers are much harder to identify diluted by turnover in the open eye environment.

The present invention further recognizes that the presence of antigenic species is consistent with elevated levels of f MMPs 1, 2, 3, 8, 9 and 10 in the pathological compared to normal tears especially in CTF.

One embodiment of the present invention is directed to the identification of LAPs, e.g., TH-1/TH-2 cytokines, Hb-EGF, FGF basic (FGFb), angiogenic modulators (such as ANG) and MMP, in tear fluids and the anti-microbial and an anti-inflammatory property of ANG in tear fluids. Accordingly, a method for detecting the presence of LAP, e.g., MMP or ANG, in a tear fluid sample from a subject is provided by the present invention. Without intending to be limited in a particular mechanism, it is believed that ANG exists in normal bodily fluids, e.g., blood and tears, and can function as an anti-microbial infection agent and an anti-inflammatory agent.

An object of the present invention is to simultaneously identify and analyze LAPs or trace proteins in a fluid sample, preferably, a biological fluid sample, more preferably, a tear sample, with sufficient sensitivity and specificity by a simple, cost-effective and rapid means.

According to the present invention, the stable sensitivity of an antibody-based stationary phase/support/surface array, preferably, an antibody-based membrane array (MA), can be increased, e.g., up to several-hundred fold, by modifying the array. In accordance with the present invention, a commercially available or custom-made array can be modified to maximize the sensitivity of detection, preferably, by

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employing an ultra-sensitive substrate, preferably, a luminol based substrate system. According to the present invention, maximizing the signal-to-noise ratio can further stabilize the high sensitivity.

Accordingly, another embodiment of the present invention is directed to an antibody-based stationary phase array system comprising an ultra-sensitive substrate, preferably, a luminol based substrate system, e.g., SuperSignal™ West Femto (Pierce). The array system of the present invention also includes, but is not limited to, an array matrix of dot grid on a stationary phase/surport/surface, preferably a membrane, e.g., a Hybond nylon membrane, bounded or attached by at least one antibody, i.e., capture antibody, that is capable of binding with a specific protein species, secondary or detection antibodies that are, or can be, labeled or linked by enzymes, e.g., horseradish peroxidase (HRP), which reacts with the ultra-sensitive substrate thereby providing a detectable or visible indicator/signal of the binding between a capture antibody and a protein. For example, the detection antibodies can be HRP-conjugated secondary antibodies or biotinylated secondary antibodies that bind to HRP-conjugated streptavidin.

By a "LAP" or "trace protein" is meant a protein of minute and barely detectable amount or concentration or of very small quantity, e.g., less than 10 μg/ml in concentration. By "biological fluid sample" is meant a physical sample in liquid, fluid or mucus form that is obtained or derived from a biological source, e.g., a cell, cell culture, tissue, tissue extract, an animal or a human, which includes, but is not limited to, blood, serum, plasma, saliva, urine, tears and other bodily fluids. By "test sample" is meant a sample containing the biological fluid to be tested that is prepared for and/or carried by an antibody array.

By "negative control array/membrane" is meant an array obtained according to the array procedure in which the test sample is replaced by an appropriate mock buffer which contains substantially the same variables as the test sample except without the biological fluid to be tested. By "positive control array/membrane" is an array obtained according to the array procedure in which the test sample is replaced by

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an appropriate mock buffer which contains at least one, preferably, all the proteins specifically recognized by the immobilized/capture antibodies on the array.

By "differential screening" or "differential analysis" is meant a means to differentiate different trace protein distributions in test samples that are obtained, particularly from the same or similar sources, under varied physiological conditions or stages or status, e.g., from healthy and diseased subjects or at different stages of protein expression.

By "enhancing agent" is meant a chemical that can increase or improve the quality or level of detectability of a signal.

The array used for the present invention can be a standard array (e.g., RayBio™ Human Cytokine Array V (RayBiotech Inc. Norcross, GA)) or a variant of this array in which the concentration of the positive controls on the array is reduced, preferably, by a factor of 10.

According to the present invention, the array matrix can comprise dot grids on a membrane with at least one unique capture antibody, preferably, at least one of each of a positive control, a negative control and a sample buffer control (see Figure 6). The capture antibody or antibodies are specific for the trace protein or proteins that are contemplated to be detected. In accordance with the present invention, an array of reduced size and number of dot matrix is preferred. An example of such array is shown in Figure 5, of which the overall matrix dimensions are reduced and the complexity of the array greatly reduced to form a 4 x 4 dot matrix on 12x8 mm Hybond Nylon membrane. Without intending to be limited to a particular mechanism, it is believed that background noise in the small custom array is minimal. A particularly preferred array system of the present invention is designed to allow the simultaneous screening of two or more samples for the relative distribution of more than 120 growth factors, chemokines, cytokines, angiogenic modulators and other trace proteins using a dot sandwich ELISA assay protocol.

Still another embodiment of the present invention is directed to a method for simultaneously identifying trace proteins in a fluid sample, e.g., a conditioned media sample, preferably, a biological fluid sample, more preferably, a tear sample,

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comprising the steps of obtaining the sample, incubating an antibody-based stationary phase array with a blocking buffer, incubating the sample with the array, incubating the array with detection/secondary antibodies, incubating the array with an ultra-sensitive substrate that is reacted with an enzyme lined to the detection antibodies.

By "ultra-sensitive substrate" is meant a substrate, particularly, a substance acted upon by an enzyme, that can be detected, at a level of femtogram (10^{-15}) or attogram (10^{-18}) by any chemical/biological/biochemical detection means available in the art.

According to the present invention, the standard MA kits are far too insensitive to be employed for tear analysis when they are used as directed by the manufacturer. According to the present invention, the standard assay protocol is therefore modified with the dual objectives of increasing the assay sensitivities and increasing the signal-to-noise ratio. The present invention surprisingly recognizes that the sensitivity of an MA can be increased, e.g., by several hundred fold, by substituting the supplied substrate with an ultra-sensitive substrate, preferably, luminol based substrate system, e.g., SuperSignal™ West Femto (Pierce). In a particular embodiment, the method can also be described to comprise the steps of: a) obtaining the sample, b) incubating the sample with an antibody-based stationary phase array, c) incubating the array from Step b with secondary antibodies, d) incubating the array from Step c with an ultra-sensitive substrate that is reacted with an enzyme linked to said secondary antibodies, e) detecting the signals and analyzing data.

In accordance with the method of the present invention, an antibody-based stationary phase array, preferably, a membrane array (MA), is incubated, preferably with constant mixing at an appropriate temperature, with a sufficient amount of a blocking solution of appropriate pH for a sufficient period of time. For example, a standard size membrane array, e.g., RayBio™ Human Cytokine Array V, can be incubated in 2 ml of 5 % blocking grade non-fat milk (Bio-Rad) in phosphate buffered saline (PBS) at pH 7.4 for 2 hours at room temperature or overnight at 4^0C. The blocking solution is then discarded and the array/membrane is incubated with sufficient volume of a biological fluid sample, for a sufficient period of time, e.g., 2 hours. For

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example, a standard array can be incubated with a biological fluid sample, e.g., tears, of volumes ranging from about 20 to about 200 µl brought up to a final volume of 1ml with an appropriate blocking buffer. At least one parallel negative control array/membrane is incubated, preferably, with PBS diluted in an equivalent manner with the blocking buffer. The membrane is then washed for a sufficient time, e.g., 4 times of five minutes each with 2 ml aliquots of laboratory prepared PBS and 0.05% Tween 20 or with the manufacturer's supplied washing buffer. This is followed by a second series of additional similar washes in the same or similar type of buffer except without detergent (e.g., Tween 20). The membrane is then incubated with secondary/detection antibodies, preferably, biotinylated secondary antibodies, in a sufficient concentration and amount, e.g., the supplied cocktail of biotinylated secondary antibodies diluted to one-half of the recommended concentration in 1 ml of biotin-free casein colloidal buffer (RDI, Flanders, NJ), for a sufficient period of time, preferably, 2 hours at room temperature or overnight at 4^0C. The solution is then discarded and the washing sequence (without detergent) repeated. The array is then incubated with a sufficient amount of a blocking solution for a sufficient period of time, e.g., 2 ml of the supplied APO diluted 1:20,000 (one half the normal concentration) in the casein blocking solution for 30 minutes. The membrane is subjected to the washing sequence (without detergent) as described above.According to the present invention, for comparative analysis, matched sets of samples and control arrays are developed and imaged in tandem. For maximal sensitivity, each of the membranes is incubated with sufficient amount of the ultra-sensitive substrate, preferably, 1ml of a freshly prepared solution of SuperSignal^R West Femto (Pierce), for a short period of time, preferably, 1 minute. The membranes are then imaged by any of the well-established methods, e.g., the membranes can be stained, sandwiched between sheets of Saran Wrap and imaged using a hand luminometer (Analytical Luminescence Laboratory, San Diego, CA) equipped with Fuji FB-3000B film. Preferably, imaging is initiated within 5 minutes of addition of the substrate, with the film serially exposed for varying lengths of time, e.g., ranging from 10 seconds up to more than 30 minutes. In accordance with the method of the present invention, imaging continues to generate multiple images as the signal

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decays. As the signal decays, the difference between the samples and negative control array/membrane often becomes more pronounced.

In another particular embodiment, the present invention is directed to a method for identifying trace proteins in a fluid sample, preferably, a biological fluid sample, more preferably, a tear sample, comprising obtaining the sample and assaying the sample by the antibody-based stationary phase array system of the present invention.

In still another particular embodiment of the present invention, the method can further comprise steps of optimizing conditions for increasing or maximizing signal-to-noise ratio.

According to the present invention, it is further recognized that to improve assay sensitivity for trace proteins, the blocking process can be altered and the concentration of the biotinylated secondary antibodies optimized. In addition, membranes can be pre-treated with a sensitivity-enhancing agent, e.g., Millennium Enhancer™ used as directed by the manufacturer (BioChain Institute Inc. Hayward, CA).

Without intending to be limited by a particular theory, it is believed that use of an ultra-sensitive substrate system may result in background luminescence on the capture antibodies on the negative control array. This is believed to be attributed to trace levels of non-specific binding of the biotinylated secondary antibodies to the vast majority of capture antibodies as detected on the negative-control array. Therefore, according to the present invention, optimizing conditions for increasing or maximizing signal-to-noise ratio can further stabilize the high sensitivity of an assay achieved by the present invention.

According to the present invention, increasing the sample size, e.g., to several hundred µl, can optimize or increase the signal-to-noise ratio. For example, a sample size of 500 µl to 800 µl can optimize or increase signal-to-noise ratio.

According to the present invention, further increasing or maximazing the signal-to-noise ratio can achieve stable sensitivity in the method of the present invention. Thus, the signal-to-noise ratio can be improved by the partial removal from

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both the array and the biotinylated antibodies of cross-reacting species. For example, this can be accomplished by incubating casein-blocked membranes with the supplied cocktail of biotinylated secondary antibodies in blocking solutions. After two hours of incubation, the residual biotinylated secondary antibody solution is harvested and set aside for later use. The membranes are washed several times in PBS and then incubated for one half hour in 2 ml of 1mM avidin (Sigma, St. Louis, Mo) in blocking solution. This procedure serves to cap any bound biotinylated secondary antibodies (as well as the biotinylated positive controls) with avidin thereby making these species non-reactive. After washing the membrane in buffer several times to remove the residual unbound avidin, the membranes are ready for use. This process not only reduces non-specific background but also results in a decreased signal from the positive controls.

According to the present invention, a further improvement in the signal-to-noise ratio and visualization of cryptic positive entities can be accomplished by re-probing the arrays. For example, in a particular protocol, after completing SPO imaging, the arrays are incubated overnight in TRIS buffered saline (TBS) containing 0.05 % Tween-20. This is followed by a series of 5-minute washes with TBS. The membranes are then incubated for 2 hours at room temperature with a 1:20,000 dilution of streptavidin-linked alkaline phosphatase (SAP) (Tropix Bedford, MA) in TRIS-buffered saline containing blotting-grade skim milk (BioRad). The membranes are subsequently washed five times with TRIS-buffered saline with Tween-20 followed by a second series of washes in buffer without detergent. The membranes are then re-imaged using CDP star^{TM} (Tropix) with the signal detected on film.

Yet another embodiment of the present invention is directed to a method for differential screening/analysis trace proteins in biological fluid samples, preferably, tear samples, that are obtained, particularly from the same or similar sources, under different physiological conditions or stages or status, comprising the steps of a) obtaining the samples, and b) identifying and comparing/analyzing the trace proteins in each sample.

Taking advantage of the methods provided by the present invention, which identify trace proteins in the amount as low as femtogram (10^{-15}) or attogram (10^{-}

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^{18}) level, differential screening/analysis can be achieved or performed by rapidly identifying the differentially expressed or distributed proteins, particularly trace proteins in biological fluid samples from different stage or conditions, e.g., open tear fluid (OTF) and close tear fluid (CTF).

A further embodiment of the present invention is directed to a method for diagnosing pathological conditions, particularly, an ocular disease or pathological condition, of a subject, preferably, a human, comprising the steps of a) obtaining a biological fluid sample, preferably, a tear sample, b) identifying the protein distribution or level, e.g., ANG level, in the sample by the method of the present invention or by the antibody-based stationary phase array system of the present invention, and c) detecting and analyzing the changes of the trace protein distribution or level in the sample relative to that of a normal sample or a sample obtained by substantially the same or similar manner as the test sample from a normal subject, or to a database comprising known trace protein distribution/level patterns under normal or pathological conditions. According to the present invention, a normal or typical ANG level in a tear fluid sample is about 0.1 ng/ml to about 1 ng/ml, most typically, about 0.7 ng/ml.

In a particular embodiment, the present invention provides a method for diagnosing an ocular inflammation and infection, e.g., ocular infections and/or inflammation caused by bacteria, fungi or viruses or other factors, e.g., trauma or contact lenses, or the risk of susceptibility to such infections and/or inflammation in a subject, by detecting a varied ANG level beyond a normal range in a biological fluid sample, preferably, tear fluid sample, from the subject.

According to the present invention, an ocular microbial infection includes, but is not limited to, bacterial conjunctivitis, herpes simplex infection, bacterial keratitis (corneal ulcer), chlamydial and gonococcal conjunctivitis, viral conjunctivitis (pharyngoconjunctival fever and epidemic keratoconjunctivitis). Without intending to be limited by a particular theory, it is believed that the ocular ANG level, particularly the ANG level in a tear sample of a subject, increases when the subject has an ocular infection and/or inflammation. It is also believed that if the ocular ANG level, particularly the ANG level in a tear sample of a subject, is below a normal range,

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e.g., about 0.7 ng/ml, the subject is at risk of having, or is susceptible to ocular infections/ , particularly, microbial infections.

By a "normal subject" is meant a healthy subject without any detectable pathological condition by all the available medical means or a subject without a detectable particular pathological condition by all the available medical means. The contemplated pathological conditions include, but are not limited to, cancers/tumors, infections and inflammations, arterial occlusive diseases, acute myeloid leukaemia, and myelodisplastic syndromes. The contemplated ocular pathological conditions include, but are not limited to, bacterial conjunctivitis, herpes simplex infection, bacterial keratitis (corneal ulcer), chlamydial and gonococcal conjunctivitis, viral conjunctivitis (pharyngoconjunctival fever and epidemic keratoconjunctivitis).

A still further embodiment of the present invention is directed to treating ocular infections and/or inflammation in a subject, comprising a) detecting or diagnosing an ocular microbial infection in the subject, e.g., by detecting pathological level of ANG in a biological fluid sample, preferably, a tear fluid sample, from the subject, and b) administering ANG and/or other anti-microbial agents.

According to the present invention, ANG can be employed alone or in combination with one or more of other anti-microbial agents, including but not limited to, antibiotics (natural substances produced by microorganisms), synthetic antibiotics, chemotherapeutic agents (chemically synthesized), semisynthetic antibiotics (hybrid substances, which are a molecular version produced by the microbe and subsequently modified by the chemist to achieve desired properties). ANG or ANG in combination with other anti-microbial agents can be administered in oral, intravenous, or eye drop routes, or in the form of an aerosol spray.

A further embodiment of the present invention is directed to the prevention of ocular infections and/or inflammation, or having a risk of susceptible to ocular microbial infections, in a subject, comprising a) detecting or diagnosing level of ANG in a biological fluid sample, preferably, tear fluid sample, from the subject, and b) if ANG level is lower than its normal range, administering ANG and/or other anti-microbial agents.

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One embodiment of the present invention is directed to a kit for diagnosing ocular pathological conditions comprising an instruction manual, an antibody-based membrane array, a reaction-well tray, blocking and washing buffer solutions, detection antibodies, e.g., biotinylated secondary antibodies, at least one indicator that detects a specific binding of trace proteins in a test sample to the capture antibody or antibodies carried by the array, e.g., streptavidin-linked peroxidase (SPO) and a luminol-amplifier based substrate system.

Another embodiment of the present invention is directed to kit containing a composition in the form of eye drops for anti- ocular microbial infection, comprising angiogenin, preferably, recombinant angiogenin, and a pharmaceutically acceptable carrier.

The present invention is illustrated by the following Examples which are not to be considered limiting the scope of the present invention in any way.

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Example 1

Tear collection and subject selection

Tear samples were collected with informed consent according to the guidelines established by the Association for Research in Vision and Ophthalmology and the appropriate institutional review board.

Three types of tear samples were obtained from a subgroup of donors. These consisted of a reflex-type tear fluid (RTF) sample, a slowly collected basal or open eye-type tear fluid (OTF) sample and a small tear sample recovered immediately upon awaking after overnight sleep (closed eye tear fluid, CTF).

Sets of samples were recovered on an irregular basis, over a several month to a several year period, from 9 individuals who ranged in age (on onset of this study) from twenty to fifty-nine. Seven of these individuals consistently exhibited no symptoms of ocular allergies or ocular surface tissue disease while two of these individuals experienced chronic ocular allergies with acute episodes occurring during allergy seasons (beginning of spring and fall). One of these donors was diagnosed with rhino-conjunctivitis and had a clinical history that included inflamed nasal membranes and nasal polyps that required surgical intervention. The other donor had been diagnosed with seasonally enhanced atopic keratoconjunctivitis (AKC). Both individuals had undergone RAST testing with results indicating a hypersensitivity to common pollen allergens. Both donors exhibited ocular symptoms including stinging and a burning sensation that were greatly enhanced during spring and late summer, correlating with high pollen counts. Samples were divided from these two donors into those samples that were obtained during seasonal activation and those samples obtained during clinically quiescent periods.

These samples were self-collected using techniques that the present inventor has detailed elsewhere (Sack et al. *Invest Ophthalmol Vis Sci.* 1992; 33:626-640). Briefly, RTF was collected following nasal stimulation (employing a cotton swab), with 50 µl glass microcapillaries at a rapid rate of tear flow with the total sample volume at times exceeding 1 ml. 5 µl OTF samples were collected slowly over several minutes using a calibrated 5 µl capillary with similar size tear samples recovered

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immediately upon eye opening after overnight sleep (CTF). Samples when available were pooled from individual donors providing sufficient volume to allow multiple analyses of the same samples.

7-10 µl sized OTF samples were also obtained in the spring from 25 normal individuals (professional school students) who had no history of recent ocular surface disease with similar samples collected, one or more times, from 7 individuals who self-reported experiencing a seasonal activation of chronic allergic conjunctivitis (these individuals to varying degrees reported ongoing year round symptoms of burning, itchy eyes with the intensity of these symptoms greatly increasing during the pollen seasons).

In addition to these samples, sets of OTF samples were obtained from a single atopic male upon laboratory provocation of an acute unilateral allergic conjunctivitis. This was accomplished by exposure of the lid to a known specific allergen (mouse nest dander). OTF samples were collected and pooled from both the fellow and the provoked eye from time zero and approximately at 15 minute intervals continuing up to two hours after initiation of the allergic reaction. This process was repeated on two other occasions separated by approximately one month apart with the acute reaction provoked in the same eye. Each set of samples were independently assayed.

Lastly, large RTF samples were recovered from several normal donors with the majority of this fluid pooled to serve as a common stock fluid. Some of this stock was used to prepare a protein-free tear ultra filtrate by passage of the sample through a 1 Kda cut off micro-centrifugal ultra-filter. This ultra-filtrate was used in the preparation of some of the controls in this study. A portion of the pooled RTF fluid was separated using preparative high-pressure liquid chromatography with the resultant molecular weight fractions concentrated by ultra-filtration. Lastly, some of the pooled RTF was used as a matrix for the preparation of spiked RTF samples that were employed in the calibration of the microwell plate assays.

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All samples were kept on ice, centrifuged (11,000 rpm, 30 minutes, 40°C) and the supernatants from a given donor, when appropriate, pooled and stored at –78°C until analyzed.

Saliva and nasal secretion samples

Nasal secretion samples were collected by capillary tube as a by-product of induction of nasal instigation of reflex tearing. Crude sputum samples were also recovered with the samples centrifuged (11,000 rpm, 30 minutes, 40°C) before storage.

Microwell plate array assays

Assays were carried out using a laboratory designed protocol (LDP) employing the contents of three commercially available antibody array kits (Pierce SearchLight™ Human Angiogenic Array, Pierce SearchLight™ matrix metalloprotease array and Pierce SearchLight™ TH1/TH2 cytokine array (Pierce Rockford, IL).

The SearchLight™ TH-1/TH-2 array is designed to simultaneously measure Interleukin (IL)s, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) using volumes up to 40 µl of biological fluids. The sensitivities of each of the assays is reported to be 0.2, 0.4, 0.2, 0.4, 0.2, 0.4, 7.8, 0.2 and 1.56 pg/ml, respectively. The Pierce SearchLight™ MMP array is designed to simultaneously measure matrix metalloprotease (MMP) MMP1, MMP-2, MMP-3, MMP-8, MMP-9, MMP- 10, MMP-13 and TIMP-1 and 2. While the sensitivities of each of the assays is reported to be 24, 7.8, 9.7, 24, 20, 2.4, 9.7, 8 and 1.2 pg/ml, respectively, no information was available from the manufacturer as to the molecular nature of the antigens that are used for calibration or the relative sensitivity of the assays for the pro, active and complexed forms of the MMPs. The Pierce SearchLight™ angiogenic array (as currently manufactured) is designed to allow the simultaneously assay of angiopoietin-2 (ANG-2), vascular endothelial growth factor (VEGF), heparin binding epithelial growth factor (HB-EGF)), basic fibroblast growth factor (FGFb), platelet-derived growth factor-BB (PDGF-BB), hepatocyte growth factor (HGF), keratocyte growth factor (KGF), and tissue inhibitor of metalloprotease-1

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(TIMP-1) using volumes of up to 40 µl of biological fluids with the sensitivities of each of the assays reported to be 93, 12, 4, 17, 6, 10, 6, and 31 pg/ml, respectively. In the initial portion of this study an earlier version of this array was used that contained an additional dot ELISA assay for thrombopoietin-2 (TPO). Data from both versions of this array are provided with the particular version of the array identified.

All assays are based upon a classical dot sandwich ELISA protocol employing biotin-streptavidin-HRP amplification and chemiluminescence for detection. Samples were separately assayed using all three arrays. In addition as a proof of principal, pilot analysis was also carried out by successive transfer of a single set of samples and pooled mixed standards from the MMP array to the cytokine array and lastly to the angiogenic array.

Preliminary tear analysis was carried out employing the array kits as directed by the manufacturer using the instructions accompanying each of the kits (see the manufacturer's web site). This methodology has been extensively validated for use with serum, urine and tissue culture media. However, when used by both this laboratory and Pierce in house as directed for tear fluid assay, two types of matrix affects detailed below were evident that profoundly impacted the ability to obtain meaningful data. Based upon extensive studies the following laboratory designed protocol (LDP) was developed that greatly reduces the impact of both effects for most but not all of the ELISA assays.

To carry out analysis, a pre-blocking step was added to the kit protocols. This consisted of pre-incubation of the wells with 50 µl of MEGA BLOCK 3™ (a proprietary synthetic blocking agent (Cel Associates, Inc. Pearland, TX)) in buffer for one hour at room temperature. (This greatly decreased the extent and incidence of the aggregation of a highly sticky tear factor(s) onto the well surfaces which subsequently binds biotinylated secondary antibodies resulting in non specific reactivity). The tear samples (2-40 µl) along with a serial dilution of the supplied recombinant protein standards were then reconstituted in the same buffered blocking agent rather than the supplied sample buffer. 50 µl volumes of each of the samples were added in duplicate (whenever possible) to separate individual wells (this reduced the capacity of tear fluid

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to block the binding of targeted proteins to the well-bound capture antibodies). To carry out an assay, the samples were added to each well and incubated at room temperature with agitation for 60 minutes. In most instances the residual tear fluid and the standard protein fluids were decanted and discarded. In some instances the residual fluids in the wells were quantitatively harvested and stored for further use in sequential array analysis. The wells were washed six times as directed in the kits using the reconstituted supplied wash buffer. 50 µl of the supplied cocktail of the diluted biotinylated secondary antibodies was added to each well and the plate was incubated for 30 minutes at room temperature with agitation. The residual solutions were discarded and the washing sequence repeated. The wells were incubated for 30 minutes with 50 µl of the supplied streptavidin-peroxidase linked reporter enzyme with agitation, and the wells sequentially washed as directed.

Either the supplied substrate or 50 µl of freshly prepared solution of ChemiGlowTM™ (Alpha Innotech, San Leandro, CA) was added to each well and the wells checked for the presence of interfering bubbles. The later substrate was preferred since it had a much longer half-life. The plates were visualized using a Chemdoc XRS image station (Biorad) equipped with a deep cooled CCD camera. Imaging was carried out without binning for periods ranging from 1 minute to 10 minutes with the supplied substrate or as long as 1 hour with ChemiGlow with the background noise subtracted. The images of each of the wells in the plate were visually examined for artifactual background chemiluminescence. Most often this occurred primarily around the well edges. When affected, nearby dot ELISA assays were either excluded from analysis or whenever possible, processed with Quantity one-4.5.0™ (Biorad proprietary software) and the background chemiluminescence estimated and subtracted. Using appropriate wells with standards and spiked tear samples the same software package was used to construct two sets of standard curves: one based upon the data obtained from analysis of serial dilutions of neat recombinant protein standards and the other based upon the data obtained using the same standards spiked in pooled RTF.

To determine the effect of individual variations of the source and volume of tear fluid on the reliability and recovery of proteins in each of the dot ELISA assays,

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varying volumes of tear samples from different individuals were spiked with recombinant protein standards and the results of each of the ELISA assays were compared to those obtained using neat recombinant proteins standards in the absence of tear fluid. Analysis was also carried out using HPLC molecular weight fractions obtained from a pooled RTF sample.

Whenever possible tear samples were assayed in duplicate at two dilutions. Since limited volumes were available from many of the pathological samples that had to be shared for use with several microwell plate assays, these samples were often subject to a single point assay. To further conserve samples in some instances, the same samples were sequentially transferred from one array to another. This provided data suitable to obtain approximate rather than quantitative data.

Partial characterization of tear interfering factors

Reflex tear fluid was pre-absorbed onto a variety of affinity beads with the objective of eliminating from the tear fluid interfering factors which prevented assay using the standard protocols, which is followed by centrifugation using routine protocols described by the bead manufacturers. These beads included plastic affinity beads, agarose beads linked antibodies to the heavy chain of IgA, laboratory linked anbodies to lactoferrin, lysozyme and lysine linked, and wheat germ lectin and jacalin linked agarose.

Pooled RTF samples were separated by molecular sieve high pressure liquid chromatography (HPLC) under isocratic conditions in acidic phosphate buffer on a TSK-3000 column as we have detailed elsewhere(33). Major eluting protein fractions as determined by UV absorption at 254 nm were concentrated by centrifugal utrafiltration using appropriate cut off filters and the concentrates used for array analysis.

Membrane array assays

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A series of arrays were custom manufactured courtesy of RayBiotech, Inc.(Norcross, GA). Several of the prototype arrays were garnered in this study to serve as a proof of principal.

In the design of these experimental arrays the concentrations of the positive controls and nature and concentrations of the each of the capture antibodies were varied to contain a minimum of duplicate dots of capture antibodies specific for granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, INFγ, monocyte chemotatic protein (MCP-1) and TNF-α. In various arrays many of the specific capture antibodies were spotted in two or more sets of dilutions. This was carried out in order to determine the optimum conditions of capture and detection for each protein and is not directly germane to this study. Dot ELISA assays were carried out using a sandwich ELISA assay employing a biotin-streptavidin amplification step and an ultra-sensitive chemiluminescent substrate for detection. Various parameters of the assay were also varied with the objective of maximizing the sensitivity of detection of individual assays while decreasing background and nonspecific interactions. To determine the sensitivity of the dot ELISA assays, arrays were calibrated using a serial dilution of recombinant protein standards (provided by Ray Biotech Inc) as well as standards from other sources. The experimental arrays that are depicted in the study allowed the detection of many of the probed protein standards well into the sub-picogram/ml range and in many instances exhibited sensitivities over one hundred fold greater sensitivity than that obtainable using standard arrays and ECL technology. The enhanced sensitivity of detection resulted in a blooming of the signal for the positive controls. To avoid this problem, in many of the arrays the concentration of positive controls was reduced 1/100 fold that of the concentration employed on current commercial products. Many array and assay parameters were modified in this broad study only a few specific arrays were chosen for purposes of illustration of specific phenomena. Given this variability a general protocol is described as follows, which is by no means to limit the scope of the present invention in any way.

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The arrays were incubated in 2 ml of 5 % biotin-free casein colloidal buffer (RDI, Flanders, NJ) in phosphate buffered saline (PBS) at pH 7.4. This and all subsequent incubations were carried out with constant rocking at room temperature. After two hours, the blocking solution was discarded and the membranes were incubated with the tear samples (5 to 30 µl) that had been brought up to a final volume of 1ml in the presence of the above blocking buffer. Each array was assayed with a parallel negative control array incubated either with PBS or in some instances with a <1 Kda ultrafiltrate from RTF diluted in an equivalent manner with the blocking buffer. In some instances, arrays were run in parallel with recombinant protein standards spiked in RTF.

After two hours of incubation with tear or other samples, the membranes were washed three times for five minutes each with 2 ml of laboratory prepared PBS and 0.05% Tween 20 followed by a second series of three washes for five minutes each in PBS buffer without detergent. The membranes were then incubated with the supplied cocktail of biotinylated-secondary antibodies. This was diluted to one-half of the recommended concentration in 1 ml of biotin-free casein colloidal buffer. After incubation for 2 hours at room temperature, the solution was discarded and the washing sequence repeated. The membranes were then incubated with 2 ml of the supplied horse radish peroxidase (HRP) conjugated streptavidin diluted 1:20,000 (one half the normal concentrations) in the casein blocking solution. After one half-hour, the residual fluid was discarded and the membranes subjected to a final washing sequence.

For comparative analysis, matched sets of samples and control arrays were developed and imaged in tandem. For maximal sensitivity, each of the membranes was incubated with 1ml of a freshly prepared solution of ECL Advance Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ) for 1 minute. The membranes were stained, sandwiched between sheets of Saran Wrap and imaged using a hand luminometer (Analytical Luminescence Laboratory, San Diego, CA) equipped with Fuji FB-3000B film. Imaging was initiated after 1 minute of addition of the substrate, with the film serially exposed for varying lengths of time ranging from 10 seconds up to more than one half hour. As the signal decayed, the difference between

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the samples and negative control membrane often became more pronounced. Imaging was also accomplished in a Biorad ChemiDoc XRS image station equipped with an enhanced sensitivity -45^{0}C cooled-backed 12–bit CCD with a dynamic range >3.

Results

Microwell plate array assays- tear matrix effects and the development of a LDP

When tear samples were assayed using the Pierce SearchLight^{TM} array kits as configured without any modification, two tear specific matrix effects were evident that profoundly limited the capacity to obtain meaningful data. These consisted of a high level of non-specific background chemiluminescence (Figure 1A- row b) and the partial to complete obstruction of the capacity to detect recombinant protein standards spiked into tear fluid (not shown). The latter process also resulted in the presence in tear fluid of cryptic non-reactive pools of targeted proteins (Figure 1A-rows a and b as an example).

Non-specific background chemiluminescence was most evident when assaying larger sized samples (RTF and OTF) using the more sensitive cytokine array. This artifact can be attributed to the presence in tear fluid of a highly sticky factor(s) or complex that exhibits a predilection for the microwell plastic. This substance preferentially aggregates around the well edges and subsequently binds the biotinylated secondary antibodies resulting in non-specific background chemiluminescence. A similar phenomenon could be observed on the assay of sputum but not nasal secretions.

Passage of RTF through a molecular sieve HPLC column resulted in an eluent largely devoid of aggregating activity when the eluted fractions assayed either separately or re-combined (not shown). This would suggest that the interfering factor(s) either represents a dissociable complex or that it is retained on the column.

Attempts to reduce or eliminate these artifacts by selectively removing the interfering factors from tear fluid prior to assay by the pre-incubation of tear fluid with surface active plastic beads, or by precipitation in the presence of commercially prepared lectin (jacalin, wheat germ agglutinin), protein L, IgA and IgG specific-beads and laboratory prepared lysozyme and lactoferrin specific beads all proved

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unsuccessful. Pre-incubation of the microwell plates, however, with various blocking agents including I-block ™ (Tropix Bedford, MA), Casein (Bio-Rad), Bovine serum albumin and fish gelatin and inclusion of these blocking substances in the sample incubation buffer to varying degrees, reduced the impact of both matrix effects. Best results were obtained incorporating MEGA-BLOC 3 ™ (a proprietary pre-buffered synthetic blocking agent) in a LDP protocol (see above). This allowed for the assay of up to 30 µl of OTF, while greatly reducing the risk and extent of non-specific background chemiluminesence and allowing for the detection of previously cryptic pools of targeted proteins (Compare Figure 1A top and bottom rows).

Significantly, irrespective of the choice of blocking agents or the conditions of assay, neither matrix effects could be totally eliminated. Low levels of non-specific aggregation still occurred occasionally while the per cent recovery of spiked proteins proved quite variable (Figures 3, 7, 8, 10 and 11). In most instances, however, the residual non-specific chemiluminescence was restricted to the well edge. Since this often is geographically separate from the dot ELISA assays, by carefully avoiding the integration of the luminescence of the well edges it often proved possible to salvage the data from individual affected wells. In those rare instances where non-specific deposition led to a relatively uniform background, this could be subtracted (using the Bio-Rad proprietary software) thereby allowing the approximation of the concentration of the probed proteins. For the above reasons analysis requires the visual inspection of each of the wells in the plate prior to computerized densitometry analysis. Quantitative estimations were based upon standard curves constructed from assay of recombinant protein standards in the presence and absence of added RTF with the per cent recovery of protein standards spiked in RTF used for correction (see Figures 3, 7 and 10).

Since the extent of interference with the recovery of spiked protein standards was found to vary with the size and nature (RTF, OTF and CTF) of the tear sample, the results in this study can at best be viewed as semi-quantitative rather than quantitative in nature.

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Microwell plate assay of cytokines

Assay of spiked tear samples with the TH-1/TH-2 array and the laboratory designed protocol (LDP) allowed for the recovery of ~60-80 % of each of the recombinant cytokine standards (Figure 1B and table II) and revealed the cytokine profiles of the OTF samples from the normal and chronic allergic populations to be decidedly different.

The normal OTF profile invariably exhibited a moderate to strong signal for IL-8, which was occasionally accompanied by much lower and more variable signals for IL-4, TNFα or other cytokines (Figures. 1 A-row a and figure 3). In contrast, the OTF samples from all but one of the pathological population (6 of 7 individuals) exhibited extremely intense signals for Il-2, Il-4, IL-8, IL-10, Il-12 and TNFα. These signals were far more intense and the differential was far greater when contrasting the CTF rather than OTF samples (see Fig. 3). In longitudinally collected pathological OTF samples, the level and distribution of cytokines was found to parallel the changes in clinical symptomology with the cytokine profile approaching that of normal tear fluid during the quiescent clinical periods.

In contrast to OTF collected during chronic allergic condition, OTF recovered subsequent to the induction of an acute monocular atopic reaction proved strikingly similar to that of the control eye exhibiting only a marginal increase in the level of IL-8 and possibly IL-6 (Fig. 11).

Membrane array analysis of cytokines

A number of experimental membrane arrays were manufactured courtesy of RayBiotech with the objectives of minimizing the extent of cross-reactivity between capture and probe antibodies, and optimizing the conditions of each assay and increasing the signal-to-noise ratio. Under optimum conditions the sensitivity of the individual dot ELISA assays was increased in some instances over one e hundred fold or more over that obtained with conventional ECL assays thereby allowing the visualization of many cytokines in concentrations well down into the femtogram range (Figure 5). This allowed for the differential assay and the determination of the relative

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distribution of all 16 of probed cytokines and inflammatory mediators on the arrays using small pooled OTF (30-15µl) samples as well as individual (~5-8 µl) CTF samples obtained from the normal and pathological populations (Figures 4-6). Note that these arrays contained capture antibodies for many probed proteins in two or more dilutions. This was done in order to assess the effect of variation in the concentration of capture antibody upon the visualization of the targeted protein and non-specific cross reactivity as seen in the negative control membrane. The latter factor highlights the critical importance of running a parallel control membrane when using this ultra sensitive assay protocol. The specific results parallel and dramatically expand upon the findings of the microwell plate assays revealing markedly elevated levels of virtually all 16 of the probed proteins in the pathological samples. As anticipated the intensity and difference in signals proved far more extreme when contrasting CTF to OTF (Figures 4-6).

Angiogenic array assays

SearchLight angiogenic array assay of spiked RTF samples with the LDP revealed very pronounced differences in the levels of recovery of specific protein standards (Figures 7 row A and Figure 8). Virtually all of the OTF samples exhibited very intense signals for TIMP-1. Within the sample size selected for assay (10-20 µl) the signal for TIMP-1 often exceeded the linear range of the assay. A minority of both the normal as well as pathological OTF also exhibited strong signals for VEGF and less frequent and less intense signals for HGF (Figure 8). OTF samples from all but one individual with chronic allergic conjunctivitis exhibited strong to very intense signals for HGFb and HB-EGF, two growth factors that were virtually absent in all of the OTF samples from the normal population (Figure 6 D). Moreover, longitudinal analysis revealed that the presence and levels of HGFb and HB-EGF in OTF paralleled the clinical course of disease waxing and waning of the clinical symptoms. In contrast, neither of the growth factors could be detected in OTF obtained after instigation of a unilateral acute allergic reaction.

Comparative analysis of OTF and CTF samples from the normal and the chronic allergic populations revealed far more intense signals in the CTF compared to

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OTF samples with the CTF sample from one asymptomatic donor also exhibiting signals for EGFb and HB EGF (Figure 8).

MMP microwell plate array assays

Comparative assays of TIMPs and MMP species using the Pierce array with the LDP reveals a low level of recovery for many of the MMP (MMP-1, 8, 9 10) standards when spiked in RTF samples (Figures 9 and 10). It is believed that RTF can contain high levels of various entities (i.e. inhibitors or receptors) that can complex or transform these spiked protein standards converting them into antigenically non-reactive entities.

Differential analysis of OTF and CTF obtained from the normal and the pathological populations, however, revealed striking differences in the patterns of distribution of detectable signals. OTF samples from normals exhibited intense signals for TIMPs 1 and 2, but at most only trace signals for MMP-3 and/or MMP-10. In contrast, OTF samples from virtually all of the allergic individuals exhibited intense signals for a variable mix of MMP species including MMP-1, 2, 3, 8, 9 and 10 (Figure 9). As anticipated the array profile for OTF and CTF proved strikingly different with the latter samples exhibiting far more intense signals for both TIMP 1 and 2, as well as strong signals for several MMP species including MMPs 1, 2, 8 and 9 (Figure 9).

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Example 2

Tear Collection

Tear samples were routinely recovered over a several month period from 6 normal male and female subjects, who ranged from twenty-five to fifty-nine years of age. Reflex tear fluid (RTF) was collected following nasal stimulation using a 50µl glass capillary tube at a rapid rate of tear flow. Open eye (basal) tear samples (OTF) were collected slowly over a several minute period using a 5 µl calibrated glass microcapillary tube. Immediately upon eye opening after overnight sleep, similar size closed eye tear samples (CTF) were collected. Samples were transported to the laboratory on ice and centrifuged (11,000 rpm, 30 minutes, 4^0C) with the RTF, OTF and CTF supernatants from each donor separately pooled and stored at -78^0 C until analyzed.

Membrane-Micro array Assays

The majority of the work was carried out either with a standard array (RayBio™ Human Cytokine Array V (RayBiotech Inc. Norcross, GA)) or a variant of this array in which the concentration of the positive controls on the array was reduced by a factor of 10. The array matrix consisted of an 11 by 8 dot grid on a 20x30 mm Hybond membrane with 79 unique capture antibodies, 6 identical positive controls containing a biotinylated protein standard and three negative controls consisting of two dots of Bovine Serum Albumin (BSA) and one dot of the sample buffer. The capture antibodies were specific for Angiogenin (ANG), B-lymphocyte chemoattractant (BLC), Brain-derived neurotrophic factor (BDNF), Chemokine-beta-6 (Eotaxin-2), Chemokine-beta-8-1 (Ck beta 8-1), Serotoxin (Eotaxin), Epidermal growth factor (EGF), Epithelial neutrophil-activating protein 78 (ENA-78), Fibroblast growth factor-4 (FGF-4), Fibroblast growth factor-6 (FGF-6), Fibroblast growth factor-7 (FGF-7), Fibroblast growth factor-9 (FGF-9), Fractalkine (FKN), Fms-like tyrosine kinase-3 ligand (Flt-3 Ligand), Glial-derived Neurotrophic Factor (GDNF), Granulocyte Chemotactic Protein 2 (GCP-2), Granulocyte-colony Stimulating Factor (GCSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), Growth Related Oncogene (GRO), Growth

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Related Oncogene-Alpha (GRO-α), hematopoietic growth factors, hepatocyte growth factor (HGF), I-309 (I-309), IFN-γ Inducible Protein 10 (IP-10), Insulin-like growth factor 1(IGF-1), Insulin-like growth factor binding proteins 1 (IGFBP-1), Insulin-like growth factor binding proteins 2 (IGFBP-2), Insulin-like growth factor binding proteins 3 (IGFBP-3), Insulin-like growth factor binding proteins 4 (IGFBP-4), Interferon gamma (IFN-gamma), Interleukin 1 Alpha (IL-1a), Interleukin 1Beta (IL-1b), Interleukin 2 (IL-2), Interleukin 3 (IL-3), Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 7 (IL-7), Interleukin 8 (IL-8), Interleukin 10 (IL-10), Interleukin 12 (IL-12), Interleukin 13 (IL-13), Interleukin 15 (IL-15), Interleukin 16 (IL-16), Leptin (Leptin), LIGHT (LIGHT), Leukemia inhibitory factor (LIF), Macrophage Inflammatory Protein 1Beta (MIP-1b), Macrophage Inflammatory Protein 1Delta (MIP-1d), Macrophage Inflammatory Protein 3Alpha (MIP-3 a), Macrophage-Colony Stimulating Factor (MCSF), Macrophage-derived Chemokine (MDC), Mesoderm inducing factor (MIF), Monokine induced by gamma interferon (MIG), Monocyte Chemoattractant Protein 1 (MCP-1), Monocyte Chemoattractant Protein 2 (MCP-2), Monocyte Chemoattractant Protein 3 (MCP-3), Monocyte Chemoattractant Protein 4 (MCP-4), Neutrophil Activating Peptide 2 (NAP-2), Neurotrophin-3 (NT-3), Neurotrophin-4 (NT-4), Oncostatin M (OSM), Osteoprotegerin (OPG), Placenta growth Factor (PIGF), Platelet-derived Growth Factor-BB (PDGF-BB), Regulated upon activation, normal T-cell expressed and presumably secreted (RANTES), Pulmonary and Activation-Regulated Chemokine (PARC), Stem Cell Factor (SCF), Stromal cell-derived factor (SDF-1), Thrombopoietin (TPO), Thymus and Activation-Regulated Chemokine (TARC), Tissue inhibitor of metalloproteinases-1 (TIMP-1), Tissue inhibitor of metalloproteinases-2 (TIMP-2), Transforming growth factor beta 1 (TGF-beta 1), Transforming growth factor beta 2 (TGF-beta 2), Transforming growth factor beta 3 (TGF-beta 3), Eotaxin-3, Tumor necrosis factor-alpha (TNF-a), Tumor necrosis factor-beta (TNF-b) and Vascular Endothelial Growth Factor (VEGF).

The second array that was used in this Example was a prototype provided as a gift of the manufacturer (RayBiotech Inc.). The overall matrix dimensions were reduced and the complexity of the array greatly reduced to form a 4 x

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4 dot matrix on 12x8 mm Hybond Nylon membrane. This matrix consisted of three positive controls (at one-tenth the standard concentration), one negative control, and 12 capture antibodies. The capture antibodies were specific for ANG, ENA-78, Eotaxin, FGF-7, IL-8, TIMP-1, VEGF, TNF-a, IGFBP-3, OSM and NT-3 and a previously unprobed protein, Angiopoietin-2 (APO-2). The array composition was selected in part to provide qualitative data to complement quantitative data obtained by micro-well plate formatted array assays data (Pierce SearchLight array ™).

A standard size array was processed in the manufacturer supplied well-plate chambers. A mini-array was developed in smaller chambers of a 16 well tissue culture micro-well-plate thereby allowing a 50% reduction in the volumes of all of the added solutions. The standard size arrays were incubated in 2 ml of 5 % blocking grade non-fat milk (Bio-Rad) in phosphate buffered saline (PBS) pH 7.4. This and all subsequent incubations were carried out with constant rocking at room temperature. After two hours, the blocking solution was discarded and the membranes were incubated with the tear samples (volumes ranging from 20 to 200 µl) brought up to a final volume of 1ml with the blocking buffer. A parallel negative control membrane was incubated with PBS diluted in an equivalent manner with the blocking buffer. After two hours of incubation with tear samples, the membranes were washed 4 times (for five minutes each) with 2 ml aliquots of laboratory prepared PBS and 0.05% Tween 20or with the manufacturer's supplied washing buffer. This was followed by a second series of four additional five minutes washes in buffer without detergent. The membranes were then incubated with the supplied cocktail of biotinylated secondary antibodies that was diluted to one-half of the recommended concentration in 1 ml of biotin-free casein colloidal buffer (RDI, Flanders, NJ). After incubation for 2 hours at room temperature, the solution was discarded and the washing sequence repeated. The membranes were then incubated with 2 ml of the supplied APO diluted 1:20,000 (one half the normal concentration) in the casein blocking solution. After one half-hour the membranes were subjected to the washing sequence.

For comparative analysis, matched sets of samples and control arrays were developed and imaged in tandem. For maximal sensitivity, each of the

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membranes was incubated with 1ml of a freshly prepared solution of SuperSignal^{R} West Femto (Pierce) for 1 minute. The membranes were stained, sandwiched between sheets of Saran Wrap and imaged using a hand luminometer (Analytical Luminescence Laboratory, San Diego, CA) equipped with Fuji FB-3000B film. Imaging was initiated within 5 minutes of addition of the substrate, with the film serially exposed for varying lengths of time ranging from 10 seconds up to more than one half hour. Imaging continued as the signal decayed providing multiple images. As the signal decayed, the difference between the samples and negative control membrane often became more pronounced. Imaging was also accomplished in a Biorad ChemiDoc XRS image station equipped with an enhanced sensitivity -45^{0} C cooled-backed 12–bit CCD with a dynamic range >3. Images were acquired using 3 binning at 3 minute intervals with the image summed over a period of as long 30 minutes. It should be emphasized that while use of an imaging station was not mandatory, this produced data that was linear over a broader dynamic range relative to films.

Evaluation of Data

As the luminescence decayed with time, the distribution and signal-to-noise ratio of the samples and the negative control arrays changed. To carry out analysis, several images of a particular set of assays were photographed, scanned and digitized. These sets of data or the equivalent CCD-acquired data were image-processed to eliminate the background haze, and the pixel intensity x area density of each of the dot ELISA assays was then calculated using Quantity One,^{TM} a proprietary software (Bio-Rad). Whenever possible, the data was normalized against the average density of the five positive controls on a given array, which was arbitrarily set at one. In theory, the presence of a positive signal could be simply ascertained by subtraction of the data on the control membrane. In practice, this was not always possible. In some instances the intensity of background on a given array was not uniform or the intensity of the signals from the positive controls on the top of the membrane differed significantly from those at the bottom of the array. Moreover, this type of analysis was not possible using a standard array where the intensity of luminescence on the positive controls greatly exceeded the linear range of either the film or camera. This type of

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analysis was also not possible using the capped pre-conditioned membranes. In these situations, arrays were visually analyzed and to ensure the reliability of the data, the results were blindly and independently scaled by three observers and compiled.

While the obtained data was non-quantitative in nature, at times it was used to obtain a crude estimate of the likely concentration range of some of the detected proteins. This estimation was based on the relative ratio of the sensitivities of each of the dot ELISA assays as posted on the manufacturer's web site, the known concentration range established for TIMP-1 and for IL-8 in RTF, OTF and CTF fluids and the relative area intensity ratios of the dot ELISA data obtained for the protein in question and that of TIMP-1 and IL-8. The validity of this analysis is predicated on the assumption that the signal for each of the ELISA assays (minus that on the negative control array) is within the linear range of the assay and specific in nature, can be attributed solely to the probed protein, and that the relative sensitivities of the assays as listed on the manufacturer's web site are accurate.

The specificity and lack of cross-reactivity of each of the ELISA assays found in the array has been determined by the manufacturer using a panel of recombinant standard proteins with the array partially validated for use for serum and urine analysis. This relationship does not necessarily hold for other complex biological fluids such as tears. In evaluating the data of the present invention, it is therefore important to recognize that the invention provides the presence of an antigenic reactive species that most likely represents the probed protein.

Western blot analysis

Western blot analysis was used to confirm the presence and to determine the approximate concentration range of ANG in tear fluid. In this instance RTF, OTF and CTF samples and a serial dilution of recombinant ANG were separated on 16 % SDS PAGE mini-gel under reduced conditions and blot transferred onto nitrocellulose following a previously published protocol. Probing was carried out using a goat polyclonal antibody specific for ANG as the primary antibody (antibodies and recombinant ANG purchased from R and D systems Inc. Minneapolis, MN). Detection was carried out using an AP-linked anti-goat IgG (Sigma) and NBT/BCIP (Sigma) as a

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substrate. While it would have been preferred to carry out western blot analysis on several of the other detected tear proteins as well, time and cost constraints made this impossible.

Results

In a typical assay of a 300 µl RTF sample using the large array kit as configured and imaged with the supplied luminol based substrate, only three signals could be detected. These consisted of an intense signal for ANG (a protein not previously known to be present in tear fluid) and far less intense signals for two known tear constituents, TIMP-2 and TIMP-1. Western blot analysis confirmed that ANG is present in RTF in the form of an ~14 kDa species and that it is present in RTF in concentrations in the sub-ng/µl range.

Coupling the array to an ultra-sensitive substrate system and optimizing the assay protocol as contemplated herein greatly enhanced the sensitivity of detection thereby allowing the visualization of positive signals for at least 11 antigenic species in 50-100 µl tear samples obtained from all 6 donors (Table 1) with the intensity of all of these signals markedly higher in the CTF compared to OTF and RTF samples.

Increasing the sample size to several hundred µl of RTF increased the signal-to-noise ratio thereby allowing the tentative identification of numerous additional species with the vast majority present in much higher levels in CTF compared to RTF and OTF fluids.

A further increase in the signal-to-noise ratio was obtained by the partial removal of non-specific interacting species from both the array and the cocktail of biotinylated probe antibodies prior to the assay (as described above) allowing the visualization of upwards of 39 antigenic reactive species in a pooled CTF sample (see Table II). Stripping of the membrane after visualization followed by re-probing with SAP (see above) further improved the signal-to-noise ratio and allowed the visualization of several previously cryptic species. This includes numerous chemokines and leukochemokines (Table III).

In order to explore further the sensitivity limits of this technology, a mini-array was constructed in which the grid geometry was compacted and the array

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complexity greatly reduced to probe for only 12 proteins. Decreasing the complexity of the cocktail of biotinylated antibodies resulted in a profound reduction in the level of non-specific reactivity on the negative control array thereby markedly increasing the signal-to-noise ratio. This permitted the detection of up to 10 of the 12 probed proteins in 20 µl of CTF. Detected species included trace levels of several entities such as VEGF that could not be detected above the noise using a four-fold larger CTF sample with the standard array. Calibrating the array assay with a known mixture of recombinant protein standards suggested a threshold of sensitivity for some of the assays well into the sub-picogram/ml range. Therefore, the approach of the present invention was most successful when using the small custom array where the problem of high background noise was minimal.

Under conditions in this Example, as many as ten of the twelve probed proteins were detected using as little as 15 µl of CTF. This included several proteins (i.e. VEGF) that were not detectable (due to the high background noise level) using larger pooled tear samples and the standard array. However, using larger pooled samples, with the standard array some proteins such as IL 1β that were not detected in normal tear fluid using standard ELISA assays were detected.

The array in this Example detected positive signals for as many as 40 of the 79 probed proteins in tear fluid. These include many proteins that are bioactive in trace amounts, many of which have never been observed in tear fluid. Also, this Example unequivocally demonstrated a profound difference in the relative distribution of many of these entities in tear samples collected under two distinct physiological conditions in a manner consistent with the different physiological functions of the pre-ocular tear film under open and closed eye conditions. Based upon this finding, array analysis of the present invention can be a useful tool in identifying biomarkers and mediators of ocular surface diseases in tear fluid.

Strong positive signals were consistently obtained for 11 of the 79 probed proteins in all types of tear samples from all donors (Table I). Not surprisingly, the vast majority of these signals came from dot ELISA assays that exhibit a low threshold of sensitivity (exception being TIMP-1). Five of these signals came from

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proteins (IL-8, EGF, TIMP-1, TIMP-2 and MCP-1) that have been previously documented to be present in tear fluid in concentration ranges that are consistent with the obtained data (In the case of MCP-1, detection has previously been restricted by ELISA assay solely to CTF).

Significantly, the intensity of the signals that are obtained for these and the vast majority of the other detected species were consistently higher in CTF compared to the RTF and OTF samples. In some instances the magnitude of the difference was exponential in nature. This finding is strikingly different from the pattern of distribution that has been established for the three major inducible lacrimal secretory proteins (lysozyme, lactoferrin and tear specific pre-albumin). The latter proteins have been shown to remain relatively constant in tear fluid irrespective of the mode of sample collection. Since this relationship proved true for EGF as well as for trace signals for HGF, two cytokines that have previously been attributed at least in the open eye condition to a lacrimal gland origin, one must conclude that in the closed eye environment, the inducible lacrimal gland secretion is at best only a minor contributing source for the vast majority of bioactive trace tear proteins.

The other six prominent signals represent proteins that have not been previously reported in tear fluid. These include IP-10, GRO (generic), IGFBP-2, ANG and ENA-78. The most surprising of these findings was the very high levels of signals that were seen for ANG in virtually all tear samples.

Array analysis reveals the accumulation in CTF of several members of the CXC family of chemokines-- the most prominent being IL-8, ENA-78, IP-10, and GRO, as well as two CC macrophage specific chemokines, MCP-1 and MIP-1beta (Table II).

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Example 3

Using the antibody array analysis (AAA) describe in Example 3, the relative distribution of more than 80 growth factors, cytokines, chemokines and angiogenic modulators in openand CTF was characterized. This allowed the identification of a wide range of CXC (some antibacterial) and CC chemokines, including MCP-1, GRO, ENA-78 and NAP-1 that accumulate in CTF. These findings illustrated an extensive epithelial contribution to the closed eye defense mechanism. These factors can also involve in PMN recruitment and enhance the efficiency of SIgA and surfactant D opsonization of entrappedmicroorganism. PMN cell degranulization has been known to result in the accumulation in CTF of toxic reactive products such as MMPs, elastase,cathepsin G. This in turn is balanced by the accumulation of anti-proteases in part derived from the ocular surface tissue.

Many multifunctional antimicrobial agents were identified in tears, many of which accumulate in CTF. These included high concentrations of many small highly basic multifunctional proteins such as angiogenin and SLIPI that are ideally suited to complex with the acidic groups common to the mucosal matrix and interacting with the resident pool of PMN cells.

Angiogenic assays revealed that CTF exhibited net angiogenic activity. Partial purification of the active species illustrated properties consistent with possible new bioactive species, such as IGFBP-2 and neurotrophic growth factors. AAA analysis revealed the accumulation in CTF of high levels of numerous angiogenic modulators. AAA also revealed the presence of high levels of several previously undetected growth factors as well as markedly higher levels of well-known growth factors such as VEFG, EGF and HGF. These findings can demonstrate that the ocular surface and or recruited inflammatory cells rather than the lacrimal gland is the major source of these proteins, many of which were found in bioactive concentrations.

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Example 4

The present example assayed proteins consisted of 9 TH-1/TH-2 cytokines, 8 angiogenic modulators and 9 MMP constituents (one redundant with the angiogenic array), each of which are known to modulate inflammatory and immune processes, epithelial cell migration, angiogenesis, apoptosis and differentiation or wound healing in ocular and other tissues. The results demonstrated the usefulness of this technology for tear protein analysis, which can identify biomarkers of KCS.

Methods

Subjects and tear collection

The tear samples were obtained by two methods. The vast majority of tear samples ranging in size from 3 to 10 μl were slowly collected over a ten to twenty minute period using calibrated glass microcapillary tubes from the lower fornix. A minority of tear samples was collected by a novel procedure based upon the adoption of a protocol pioneered by Phuegfelder.

Tears were collected by placing a sterile wick composed of a contact lens polymer (was available from CIBA) on the surface of the lacrimal lake where the wick functioned much as a sponge. Once the bottom was wetted, the wick was sealed inside of an Eppendorf tube, which was kept on dry ice. On thawing, the strip was placed within a disposable plastic pipette tip which rested in an Eppendorf tube and the tear fluid eluted off the wick by centrifugation.

Samples were collected before any ocular examination from 25 individuals who were free of any history or symptom of ocular allergies or ocular surface tissue diseases. Pathological tears samples were recovered from 23 individuals who had been previously diagnosed with non-SS aqueous deficiency dry eye syndrome. The diagnosis and relative severity of the disease was graded based upon clinical symptoms, tear breakup time, Rose Bengal cornea staining, and Schirmer strip evaluation into three categories consisting of those individuals who exhibited mild, moderate and severe dry eye symptoms and pathologies. 8 to 10 μl of tear fluid was collected from the majority of these individuals. In three instances insufficient tear fluid volumes could be recovered from a given individual to allow analysis. In these

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instances the tear samples were combined from all three individual giving a 12 µl sized sample. After collection the samples were double blind coded transferred to siliconized eppendorf tubes and sent to the laboratory in dry ice and the stored at -78^0C. Prior to analysis all the samples were centrifuged (11,000 rpm, 30 minutes, 4^0C) and the supernatants used for assay.

In addition to the assayed basal type tear samples, large volumes of reflex type tear fluid (RTF) was recovered from several normal individuals using nasal stimulation. Most of this fluid was pooled to form a stock solution. This and the remainder of the individual RTF samples were used to prepare spiked tear samples to calibrate the recovery of recombinant protein standards in the presence of RTF. This proved possible for all of the recombinant proteins except for TIMP1 and 2 and IL-8 which were present in too high concentrations in RTF to allow calibration.

Microwell plate sandwich ELISA array assays

Analysis was carried out using three off the shelf antibody array kits (the Pierce SearchLight™Human Angiogenic Array, the Pierce SearchLight™TH1/TH2 cytokine array and the Pierce MMP array) with the individual tear samples assayed by sequential transfer from one array to another in the order listed.

The initial assays were carried out using the Searchlight ™ TH-1/TH-2 array a kit which is designed to simultaneously measure IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα). The sensitivities of each of the assays are 0.2, 0.4, 0.2, 0.4, 0.2, 0.6, 7.8, 0.2 and 4.7 pg/ml, respectively. After incubation of the tear samples and dilutions of standards ((a combined pooled composites of the recombinant proteins standards from all three of the arrays (see below)) were quantitatively harvested from the respective and transferred to wells on a pre-blocked (see below) Pierce SearchLight™angiogenic array. This array was designed to allow the simultaneously assay eight proteins, e.g., angiopoietin-2 (ANG-2), vascular endothelial growth factor (VEGF), heparin binding epithelial growth factor (EGF-(1, 4)), basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-BB) (HGF), keratocyte growth factor (KGF), transforming growth factor (TGF), and tissue inhibitor metalloprotease-1 (TIMP-1). After incubation, the

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residual tear fluids and combined recombinant standards were once again salvaged and quantitatively transferred to a third array specific for matrix metalloprotease (MMP) MMP1, MMP-2, MMP-3, MMP-8, MMP-9, MMP- 10, MMP-13 and TIMP-1 (redundant assay) and 2.

All of the utilized arrays are designed to be used in a plug and play basis and as such are sold as kits which include all the necessary reagents and standards to carryout analysis of samples of volumes of up to 50 µls using a methodology that is described is detailed in the supplied instruction manuals available on the manufacturer's Web site. During the course of preliminary studies, it was found that when these arrays were used as directed for tear analysis, two specific matrix effects predominated which rendered these assays useless. Briefly, these matrix effects consist of the non-specific deposition of a highly sticky tear protein(s) or complex which exhibited a marked affinity for the well plastic matrix. This substance(s) subsequently bound the secondary biotinylated antibodies resulting in extremely high background reactivity. The second matrix effect consisted of the partial to complete blocking of the capacity of antigens to the intended capture antibodies. This particular matrix effect phenomenon has also been reported. The assay protocols in the present invention were modified on the basis of preliminary studies to greatly reduce the impact of these artifacts on the reliability of almost all of the 23 ELISA assays. The exception being the assay for ANG-2, which cannot at present be validated in the presence of tear fluid. The following protocol allows the semi-quantification of all of the remaining assayed proteins.

To carryout analysis, a pre-incubation step consisting of incubation in 50 µl of MEGA Block3 ™ in buffer (a proprietary synthetic blocking agent) for one hour at room temperature was added to the assay protocol. This served to block reactive binding sites which are common to the well plastic surface and thereby greatly decreased the predilection of a highly sticky tear factor(s) to aggregate around the well matrix. The tear samples (8-10 µl) along with a serial dilutions of a combined cocktail consisting of dilutions of all three of the supplied recombinant standards were reconstituted in MEGA Block3™ and the 50 µl volumes were added to each well. The same set of standards were spiked in 10 µl of pooled stock RTF which were added in

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duplicates to individual set of wells in order to approximate the efficiency of standard recovery in the presence of tear fluid. Preliminary studies were conducted to compare the variability of protein recovery using standards spiked in different volumes of RTF from different individuals. MEGA Block3™ was included as a diluent to all of these samples since it greatly increased the capacity to recovery recombinant protein standards in spiked tear samples. Due to the sample size limitation and the relative lack of sensitivity of some of the assays, most of the pathological tear samples contained sufficient volumes to carryout only a single assay although occasionally sufficient volumes were present to allow the assay in duplicate. In most of the normal tear samples, assays were carried out in duplicate in two dilutions. After incubation for 30 minutes at room temperature, the residual fluid in the samples and the standard wells are quantitatively harvested and transferred along with a 5 µl wash from each well to a pre-incubated angiogenic array and the incubation process repeated. After this incubation the samples were transferred to a third pre-blocked array specific for MMP proteins. To obtain information on the likely degree of loss of protein from the multiple transfer process, several wells on the angiogenic and MMP arrays contained fresh solutions of protein standards.

After 30 minutes incubation at room temperature with agitation, the wells were emptied, washed five times with a supplied washing buffer and replaced with 50 µl of a cocktail of the biotinylated secondary antibodies in blocking buffer. The plate was incubated with agitation at room temperature as directed. The wells were again emptied and the washing sequence was repeated. The wells were then incubated for 30 minutes with 50 µl of a supplied solution of streptavidin-linked peroxidase (SPO) reporter enzyme in blocking buffer. After discarding this fluid, the wells are subjected to a final series of washes.

Within ten minutes of the addition of the supplied luminol-based enhanced sensitive substrate (e.g., Femtogram SuperSignal™ from Pierce), detection was carried out by imaging using a Chemdoc XRS image station (Biorad) equipped with a deep cooled CCD camera. Imaging was carried out without binning, using the supplied substrate for periods of one to ten minutes or over a several hour period with

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background noise subtracted when using ChemiGlow™. The images of each of the wells in the plate were visually examined for artifactual background chemoluminescence before densitometric analysis. In the few instances where no specific deposition occurred this was restricted primarily to the well edges. In these instances the data from affected nearby dot ELISA assays were either excluded from analysis or when possible processed with Quantity one-4.5.0™ (Biorad proprietary software). This allowed for background subtraction. Using the same software, two sets of standard curves were constructed: one based upon the data obtained from analysis of serial dilutions of neat recombinant protein standards and the other based upon the data obtained using spiked pooled RTF. The protein concentrations in the samples were estimated by extrapolating the standardizations curves from the spiked samples. This approach as mentioned was not possible in the cases of TIMP-1, TIMPs -2 and IL-8 where the high base line levels of these proteins in RTF exceeded the linear range of the assay.

RESULTS

The results showed the profound impact of two matrix effects (aggregation and blocking) on the capacity to carryout to ELISA assays for TH-1/TH-2 cytokines using the Pierce array kit as proscribed by the manufacturer.

The result showed a set of two dilutions of the other recombinant proteins standards neat and spiked in a stock RTF matrix using the modified protocol of the present invention as described above.

The results reveal a suppression of both matrix effects and the capacity to recovery approximately 50 to 80 % of each of the recombinant protein standards. The extent of quenching of the signal varies with the source and volume of the spiked tear fluid, making the obtained data semi-quantitative in nature.

Assay of the normal and the dry eye tear obtained samples revealed a very similar pattern of cytokine distributions. Other than increased level of IL-8 in many of the dry eye tear samples, only trace levels of the other probed cytokines could be detected in both the pathological and normal tear samples. The result showed that normal OTF contains high levels of IL-8 and at most trace levels of other measured TH-

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1/TH-2 cytokines (IL-4, TNF). Overnight eye closure is associated with a massive build up of IL-8 and an increase in the concentrations of many other cytokines in tears. Chronic allergic reactions associated with elevated levels of IL-2, 4, 5, 8, 10, INF and TNFa with this increase most pronounced in CTF.

Comparative assays of the recombinant protein standards neat and in spiked RTF reveals widely divergent patterns in the degree to which the addition of RTF quenched the signal of the recombinant proteins. Particularly dramatic was the total loss of signal for ANG-2 and an inverse pattern of ANG-2 detection on sample dilution made the assay for this protein impractical.

Far better results were obtained for the remaining 6-targeted proteins with the percent recovery of TIMP-1 not quantifiable due to the very high levels of this protein in the RTF. Comparative analysis of the normal and dry eye tear samples revealed a marked difference in the concentration and pattern of distribution of many of these entities in a subgroup of the pathological tear samples. The normal tear samples invariably contained exceptionally high levels of TIMP-1 and often-variable levels of VEFG and HFG with other entities only occasional detected in minute quantities. In contrast, the majority of the dry eye obtained tear samples in addition to these entities contained anywhere from low to very high levels of FGFb and Hb-EGF growth factors not detectable in the normal tear fluid.

Assay of the recombinant proteins standards in net and spiked tear samples using the MMP array revealed a partial variable degree of quenching of the protein standards with quenching particularly evident in the case of MMP-9. The percentage of the recovery of TIMPs 1 and 2 could not be ascertained in the spiked sample due to the exceptionally levels of these proteins in the RTF. Comparative analysis of normal and pathological reveals marked different pattern and variable patterns in the distribution of MMPs in the normal and the majority of the pathological tear samples. In many samples keratosicca samples the levels of MMP 2, 3, 8, 9 and 10 were decidedly higher than that in the normal tear samples.

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Discussion

To the best of the inventor's knowledge, this is the first time that microwell plate formatted protein array technology has been employed in tear analysis. The format offers an unparalleled vantage for the simultaneous assay of a small volume of tear fluid for a multitude of LAPs while at the same time allowing for the screening of each of the assays for tear matrix effects which might not be evident using other methods of analysis. As shown above, these matrix effects can profoundly influence the reliability and validity of analysis. Two tear specific matrix effects were evident: the consistent of high non-specific background deposition and the partial to complete blockage of the capacity of the targeted protein to bind the capture antibody. The former effect can be attributed the aggregation of a highly sticky tear factor on the plastic matrix. This effect subsequently complexes with secondary antibody thereby resulting high background reading. Blockage in turn can be attributed to the presence in tear fluid of various blocking factors. The failure to recognize and adequately account for these matrix effects could well be a factor in the hundred fold or more difference that has been reported for the levels of various cytokines, chemokines and growth factors in tear fluids in earlier studies.

The above example of present invention greatly minimized the impact of these effects by altering the assay protocol thereby allowing the assay of most of the probed targeted proteins. Significantly, in the case of ANG-2, assay in tear fluid was not feasible as demonstrated the total loss of the recombinant protein standard in the spiked tear fluid. These findings certainly highlight the necessity to always check the validity and accuracy of any ELISA type assay by carrying out standardization with spiked tear samples.

In other assays, the recovery of recombinant proteins varied anywhere from approximately 50 to 80 % of the neat protein standard. At present it is uncertain to what extent the efficiency of recovery or the prevalence blocking varies from tear sample to sample or with the type of tear sample (open, reflex or closed). This coupled with the fact that the small size of many of the dry eye samples precluded multi assay results in data which at best should viewed as semi-quantitative in nature.

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The present example increased the breath of assay by sequentially transferring the same samples through three arrays. While larger arrays are available which allow the simultaneous dot ELISA assay of as many of 36 proteins per well, increasing the array size has the decided disadvantages. As the array size is increased the signal to noise ratio of individual assays often is impaired due to an enhanced level of cross talk between the capture antibodies and the cocktail of secondary antibodies. Moreover, it becomes increasingly more difficult to design arrays in which all of the probed proteins in a biological sample lie within the linear range of all of the assays. In theory, these problems can be circumvented by passage of the same sample through multiple arrays. In the present invention, the quality of the obtained data could have been further improved by utilizing custom configured and validated arrays which would segregate and compartmentalize the assays of IL-8, TIMPs-1 and 2 into a separate array. These proteins could be assayed with much smaller tear samples (<1 μl) thereby bring data within the linear range of these assays and eliminating the redundancy for the assay of TIMP-1. Budgetary and time constraints, however, precluded this possibility. Irrespective of these limitations, the obtained data serves as a proof of principal and provides a wealth of data on the relative distribution of a wide range of bioactive LAP in normal and dry eye tear fluid. Some of these entities can participate in the pathophysiological processes.

Particularly striking is the marked difference in the pattern of distribution of various angiogenic modulators and MMPs in the normal and pathological tear samples. FGFb and Hb-EGF which are virtually absent or barely detectable in virtually all of the normal tear samples are very prominent entities in many of the dry eye tear samples, reaching concentrations which at times approaches the ng/ μl range. That these growth factors are absent, or found at most in trace levels, in normal tear fluid confirms the findings of previous reports. The finding of a very marked increase in Hb-EGF in the pathological tear samples is surprising since the level of EGF was reported to be lower in tear fluid from individuals with both SS and non-SS aqueous deficiency dry eye syndromes. The result of the present example illustrates that Hb-EGF and EGF two closely related growth factors exhibit an inverse pattern of regulation. All of these

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growth factors, are known to be synthesized by corneal epithelium, keratocytes, endothelium, and the lacrimal gland. Much less is known about the conjunctiva. Hb-EGF is known to secreted and bound to the corneal epithelium and other epithelial where it is found complexed with glycoproteins on the cell membrane through its heparin-binding domain. Several MMPs and other proteases are known to clip the glycoprotein releasing free HP-EGF from the cell membrane. This can be the source of the marked increase in HB-EGF in the pathological tear samples. HB-EGF in turn is known to bind to the EGFr. Chronic allergic reactions are associated with an exponential increase in the concentrations of FGFb and Hb-EGF in tear fluid. It is believed that HB-EGF can be derived from HB-EGF normally bound to the epithelial cell membrane. This could be cleaved by various ADAM-like proteases including MMP-3. Released Hb-EGF could modulate apoptosis, cell migration and turnover through binding to the EGF receptor. FGFb could up regulate wound healing through stimulation of keratocytes.

It is of interest to note that virtually all tear samples exhibited a detectable signal for stromolysin 3 (i.e., MMP- 10) and that the signal is elevated in many of the dry eye tear samples. This was often companied by the detection of stromolysin (i.e., MMP-3) which cannot be detected in the normal tear sample. To the best of inventors' knowledge this is the first time that MMP-10 has been reported in normal tear fluid with the presence of MMP-3. MMP-3 was previously detected only in tears from individuals with active acne rosaeia. Pflugfelder's laboratory has presented data that strongly suggests that MMP-10 participates in the pathophysiological process in acne rosaeia by cleaving and activating proMMP-9. MMP-9 and other disintegrins, such as ADAM-12, that are known to cleave the epithelial bound HB-EGF complex releasing HB-EGF. The recognition of the present invention certainly supports the concept of a functional role for MMPs in the pathophysiology of dry eye syndrome.

The presence of a strong signal for MMP-8 which is specific for neutrophiles in many of the dry eye tear samples supports the premise of the recruitment and activation of PMN cells in this processes. The result supports an earlier report from the inventor's laboratory of elevated levels of neutrophil associated lipocalin

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(NGAL) in tear fluid from individual experiencing dry eye type symptomology. This finding is also compatible with recently reported of SELDI-TOF-MS that two of seven protein biomarkers associated with KCS consist of calgranulin A (a neutrophil specific protein) and the C terminal fragment of alpha1 anti-trypsin. The inventor has previously shown that alpha-1-antitrypsin is one of the major serpins that is present in tear fluid; that its concentration increases markedly in tear fluid during overnight eye closure; that its concentration appears to be upregulated in response to the build up of PMN cell proteases; and that it rapidly reacts in the tear fluid with PMN cell derived elastase and proteinase-3 giving rise to protease-antiprotease complexes and the C-terminal fragment. Taken in total this data strongly suggest an active participation of PMN in at least a sub group of the individuals with KCS symptoms.

Hb-EGF is known to bind to the EGFr and thereby modulate epithelial cell function by regulating apoptosis and cell turnover. Thus one can postulate that that the presence of high levels of Hb-EGF suggestion a shift in epithelial turnover. These pathways are unlikely to be unique to the pathophysiology of dry eye and may instead be representative of a common down stream process common to other surface conditions.

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Table 1: Major antigenic species detected in all RTF samples

Protein	Relative Sensitivity of Assay	Corroborating Data
ANG	10	wb
EGF	1	
ENA-78*	1	-
GRO	1	-
IL-8	1	wpma, wb,
MCP-1	3	
IGFBP-2	10	
IP-10	10	-
TIMP-1	100	wpma, wb,
TIMP-2	1	wb,
MIP-1b	10	-

wb western blot, wpma well plate membrane array assay, * variable;

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Table 2: Proteins detected in closed eye tear fluid (CTF) by MA

Protein	relative sensitivity	high background	intense signal	moderate signal	faint signal	75-100% samples	50-75% samples
ANG	10		x			x	
EGF	1		x			x	
ENA-78	1	x		x		x	
EOTAXIN	1			x		x	
EOTAXIN-2	1			x			x
GCSF	2000				x	x	
GDNF*	100	x			x	x	
GM-CSF	100				x	x	
GRO	1 (gamma)		x			x	
GROa	1000				x	x	
HGF	200				x		x
IFN-γ	100				x	x	
IGFBP-1	1				x	x	
IGFBP-2	10		x			x	
IL-6	1			x		x	
IL-8	1		x			x	
IL-10	10				x	x	
IL-12	1				x		x
IL-15	100				x		x
IL-16	1				x		x
IP-10	10		x			x	
MCP-1	3		x			x	
MCP-2	100	x		x			x
MCSF	1				x		x
MDC	1000				x		x
MIP-1b	10	x		x		x	
NAP-2*	100	x		x		x	
NT-3	20				x		x
NT-4	2				x		x
TGF-b2*	1000	x			x	x	
TIMP-1	100	x	x			x	
TIMP-2	1		x			x	
TNF-a	10				x	x	

* uncertain findings because of high background and/or faint signal

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Table 3: Chemotactic, angiogenic and antibacterial characteristics of CXC-Chemokines present in tears

Protein	Chemotactic properties	Angiogenic properties	Antibacterial properties
IL-8	Np, T, B, Ba, EC	+	-
GRO	Np, Ba, EC	+	-
NAP-2	Np, EC	+	-
ENA-78	Np, EC	+	-
GROα	Np, T, B, Ba, EC	+	-
IP-10	Tac, M, NK, EO	angiostatic	+
MIG	Tac, EO	angiostatic	+

B B-lymphocytes, T T-lymphocytes, Tac activated T-lymphocytes, EC Endothelial cells, Np Neutrophil granulocytes, Ba basophil granulocytes, Eo eosinophil granulocytes, M Monocytes, NK Natural killer cells;

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WHAT IS CLAIMED:

1. A method for simultaneously identifying low abundance proteins (LAPs) in a tear fluid sample comprising the steps of a) obtaining the sample, b) incubating an antibody-based stationary phase array with a blocking buffer, c) incubating the sample from a) with the array from b), d) incubating the array from c) with detection/secondary antibodies, e) incubating the array from d) with an ultra-sensitive substrate that is reacted with an enzyme linked to the detection antibodies thereby providing a detectable signal of the binding between a capture antibody on the array and a LAP, f) detecting the signal.

2. The method of Claim 1, wherein the LAP is selected from the group consisting of matrix metalloprotease (MMP), angiogenin (ANG), HGF, FGFb, TPO, VEGF, KGF, HB-EGF and PDGF-BB, interleukins (ILs), interferons (IFNs) and TNF, and TIMP.

3. A method for diagnosing a pathological condition in a subject comprising the steps of a) obtaining a biological fluid sample from the subject, b) identifying the LAPs in the sample by the method of Claim 1, and c) detecting and analyzing the changes of the LAPs in the sample relative to that of a normal sample or to a database comprising known LAP distribution/level patterns under normal or pathological conditions.

4. The method of Claim 3, wherein the pathological condition is an ocular disease and wherein the sample is a tear sample.

5. The method the Claim 4, wherein the ocular disease is an ocular infection or ocular inflammation.

6. A method for treatment of ocular infections and/or inflammation in a subject, comprising a) detecting or diagnosing an ocular microbial infection in the subject according to Claim 3, and b) administering one or more anti-microbial agents to the subject.

7. A method for prevention of ocular infections and/or inflammation in a subject, comprising a) detecting or diagnosing angiogenin (ANG) level in a tear fluid sample from the subject, b) comparing ANG level of the sample with a normal

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reference range of ANG from tear samples, and c) if the ANG level is lower than its normal range, administering ANG and/or at least one other anti-microbial agent.

8. A method for differential screening/analysis of LAPs in tear samples obtained from different physiological conditions, comprising the steps of a) obtaining the samples, and b) identifying and comparing/analyzing the LAPs in each sample.

9. An antibody-based stationary phase array system comprising an array matrix of dot grids on a stationary phase bounded by at least one antibody that is capable of binding with a specific protein species, at least one secondary or detection antibody, and an ultra-sensitive substrate that is recognized by an enzyme linked to the secondary antibodies.

10. A kit for diagnosing ocular pathological conditions comprising an instruction manual, an antibody-based membrane array, a reaction-well tray, blocking and washing buffer solutions, detection antibodies, at least one indicator that detects a specific binding of trace proteins in a test sample to the capture antibody or antibodies carried by the array.

11. A kit containing a composition in the form of eye drops for anti-ocular microbial infection, comprising recombinant angiogenin and a pharmaceutically acceptable carrier.

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Figure 1

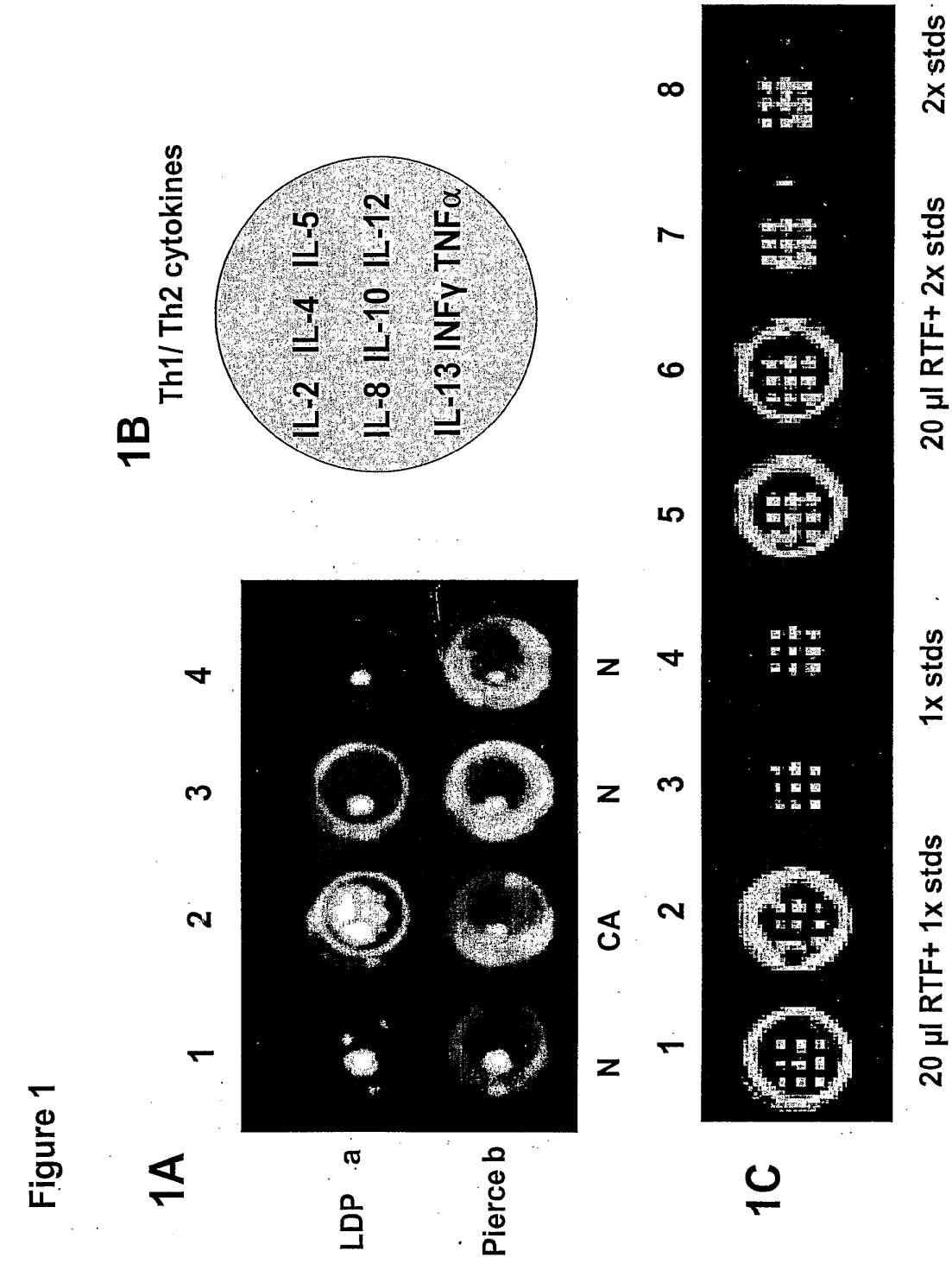
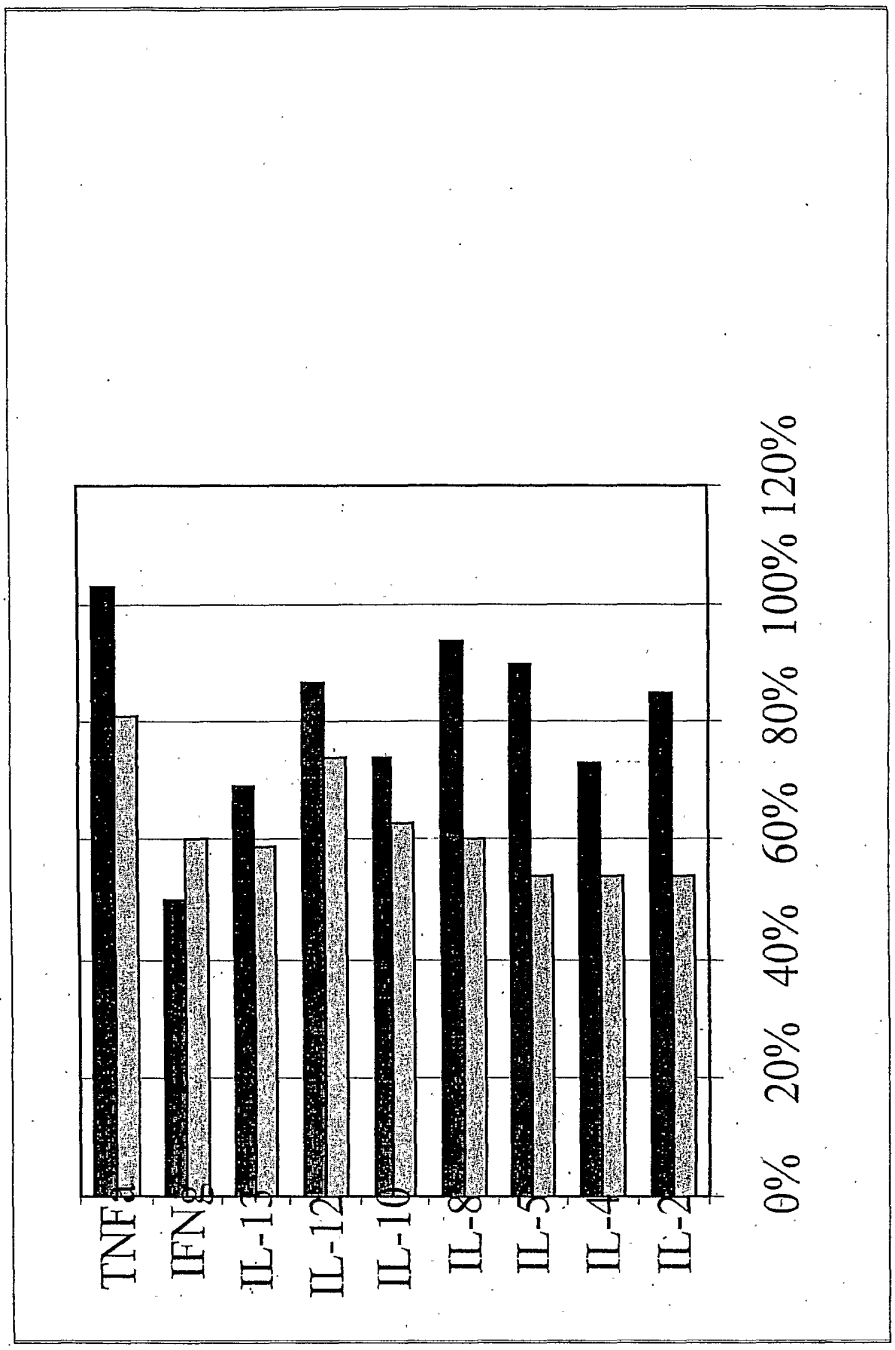

1A
1 2 3 4
LDP a
Pierce b
N CA N N
1B
Th1/ Th2 cytokines
IL-2 IL-4 IL-5
IL-8 IL-10 IL-12
IL-13 INFγ TNFα
1C
1 2 3 4 5 6 7 8
20 µl RTF+ 1x stds
1x stds
20 µl RTF+ 2x stds
2x stds

Figure 2


% Recovery of Cytokine Std at two dilutions in the Presence of RTF
TNF
IFN
IL-13
IL-12
IL-10
IL-8
IL-5
IL-4
IL-2
0% 20% 40% 60% 80% 100% 120%

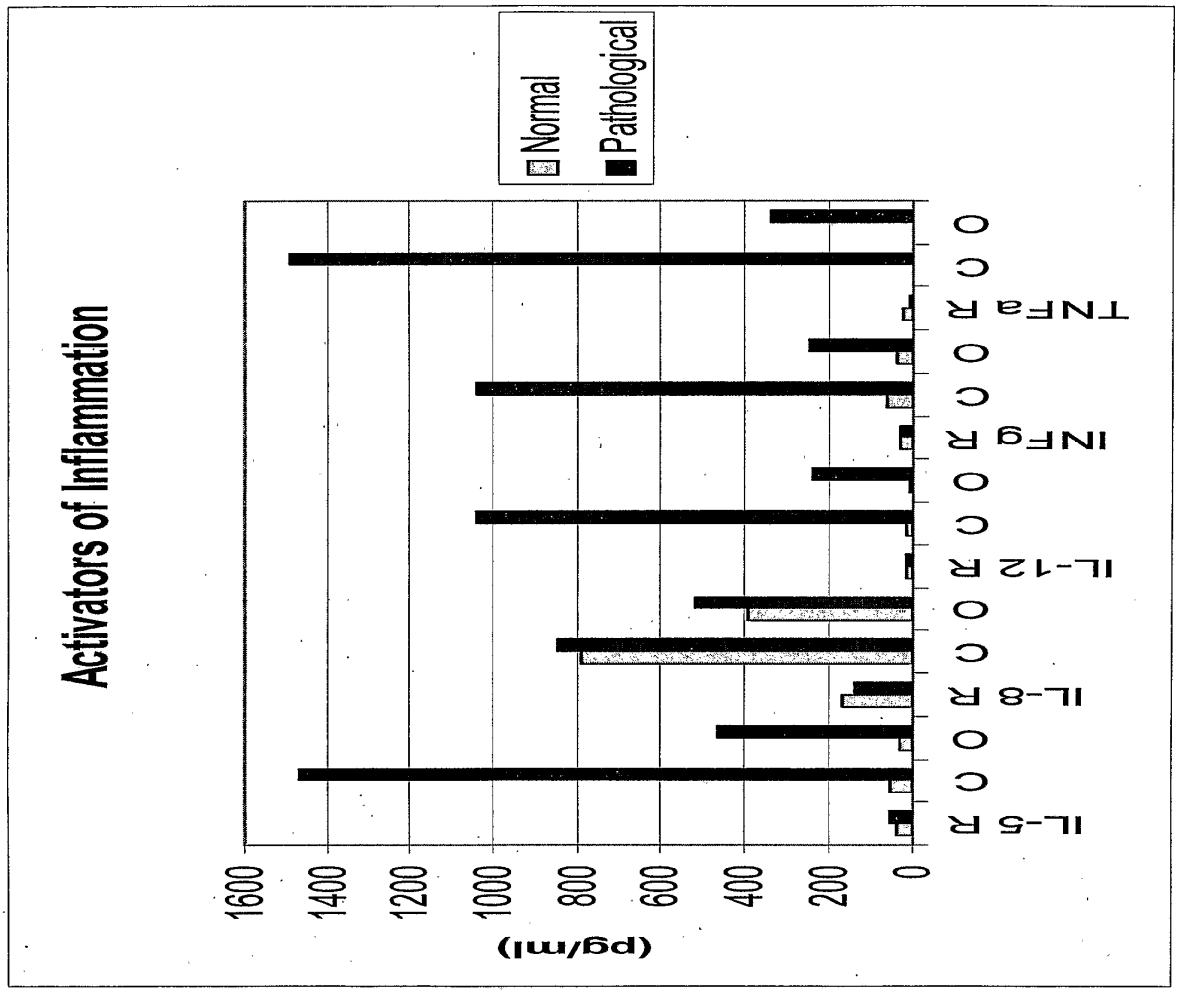

Activators of Inflammation
Normal
Pathological
(pg/ml)
0
200
400
600
800
1000
1200
1400
1600
IL-5 R
IL-8 R
IL-12 R
INFg R
TNFa R

Figure 3A

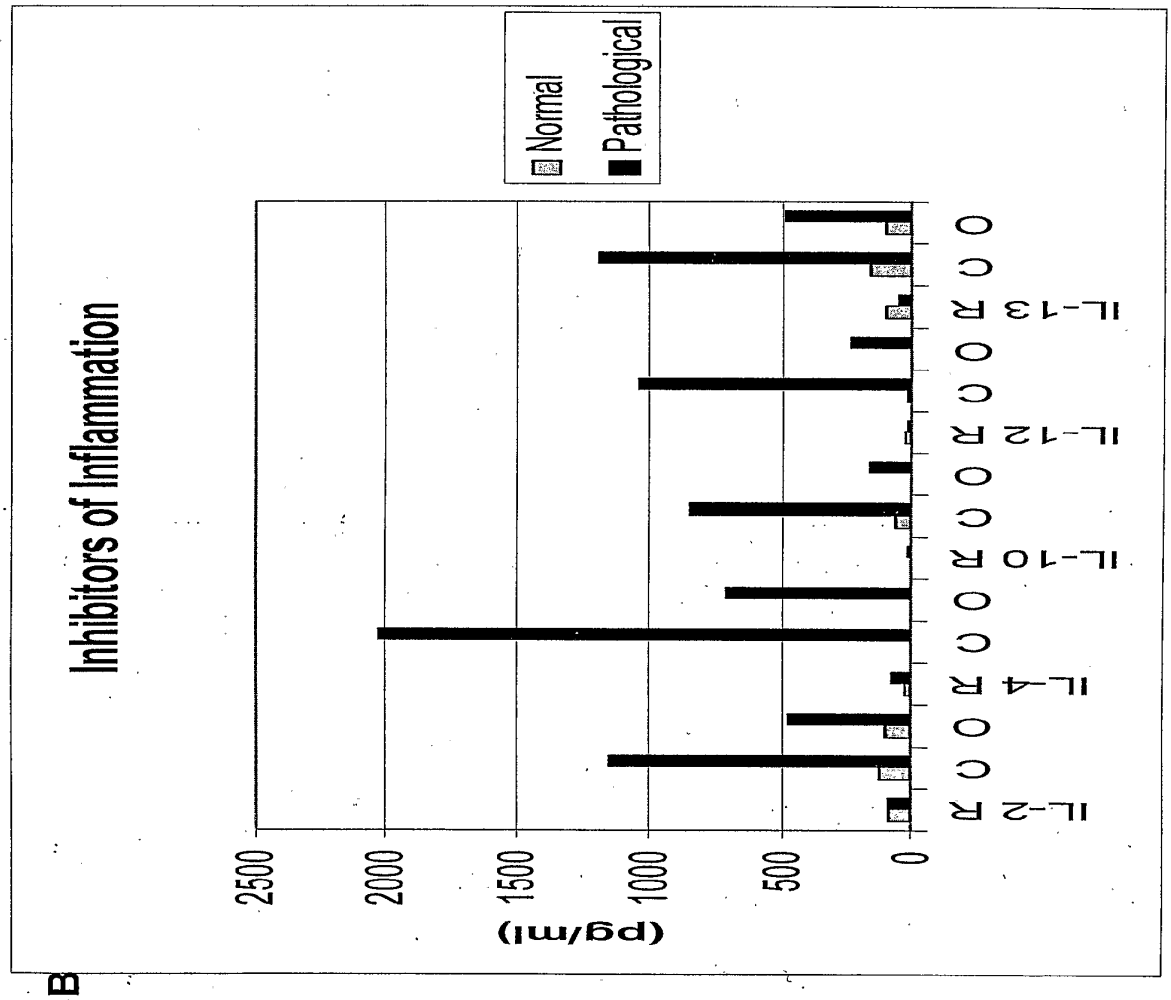
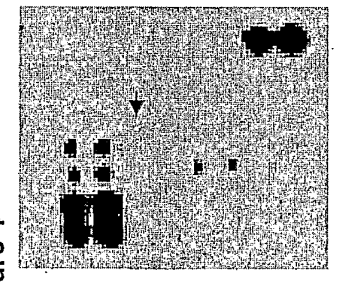

Inhibitors of Inflammation
Normal
Pathological
(pg/ml)
2500
2000
1500
1000
500
0
IL-2 R
IL-4 R
IL-10 R
IL-12 R
IL-13 R

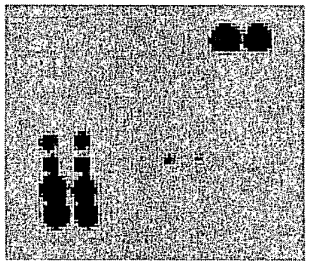
Figure 3B

Figure 4



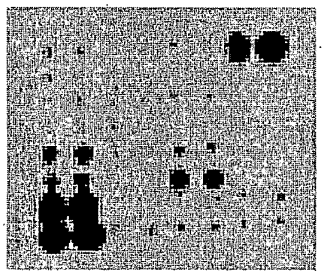
OTF- N1

4A



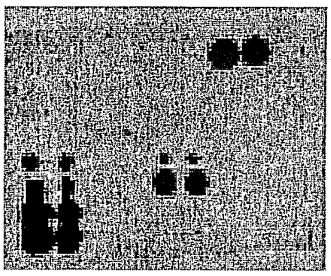
OTF- N 2

4B

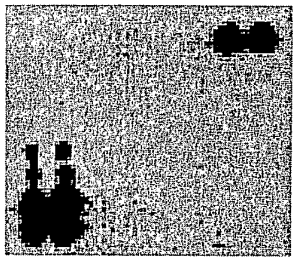


CTF- N1

4C



CTF- N 2



control

4D

POS	POS	NEG	NEG	GMCS F (1/5)	IL-1α (1/1)	IL-1α (1/5)	IL-1β (1/5)
POS	POS	NEG	NEG	GMCS F (1/5)	IL-1α (1/1)	IL-1α (1/5)	IL-1 β (1/5)
IL-2 (1/1)	IL-2 (1/5)	IL-3 (1/1)	IL-3 (1/5)	IL-4 (1/1)	IL-4 (1/5)	IL-5 (1/1)	IL-5 (1/5)
IL-2 (1/1)	IL-2 (1/5)	IL-3 (1/1)	IL-3 (1/5)	IL-4 (1/1)	IL-4 (1/5)	IL-5 (1/1)	IL-5 (1/5)
IL-6 (1/5)	IL-7 (1/5)	IL-8 (1/1)	IL-8 (1/5)	IL-10 (1/5)	IL-12 (1/5)	IL-13 (1/5)	INF-γ (1/5)
IL-6 (1/5)	IL-7 (1/5)	IL-8 (1/1)	IL-8 (1/5)	IL-10 (1/5)	IL-12 (1/5)	IL-13 (1/5)	INF-γ (1/5)
MCP-1 (1/5)	TNF-α (1/5)	BLANK	BLANK	BLANK	BLANK	BLANK	POS
MCP-1 (1/5)	TNF-α (1/5)	BLANK	BLANK	BLANK	BLANK	BLANK	POS

4E

Figure 5

POS	POS	NEG	NEG	GM-CSF (1/5)	IL-1α (1/1)	IL-1α (1/5)	IL-1β (1/5)
POS	POS	NEG	NEG	GM-CSF (1/5)	IL-1α (1/1)	IL-1α (1/5)	IL-1β (1/5)
BLANK	BLANK	IL-2 (1/1)	IL-2 (1/5)	IL-3 (1/1)	IL-3 (1/5)	IL-4 (1/1)	IL-4 (1/5)
BLANK	BLANK	IL-2 (1/1)	IL-2 (1/5)	IL-3 (1/1)	IL-3 (1/5)	IL-4 (1/1)	IL-4 (1/5)
IL-5 (1/1)	IL-5 (1/5)	IL-6 (1/1)	IL-7 (1/5)	IL-8 (1/1)	IL-8 (1/5)	IL-10 (1/5)	IL-12 (1/5)
IL-5 (1/1)	IL-5 (1/5)	IL-6 (1/5)	IL-7 (1/5)	IL-8 (1/1)	IL-8 (1/5)	IL-10 (1/5)	IL-12 (1/5)
IL-13 (1/5)	IFN-λ (1/5)	MCP-1 (1/5)	TNF-α (1/5)	BLANK	BLANK	BLANK	POS
IL-13 (1/5)	IFN-λ (1/5)	MCP-1 (1/5)	TNF-α (1/5)	BLANK	BLANK	BLANK	POS

5A

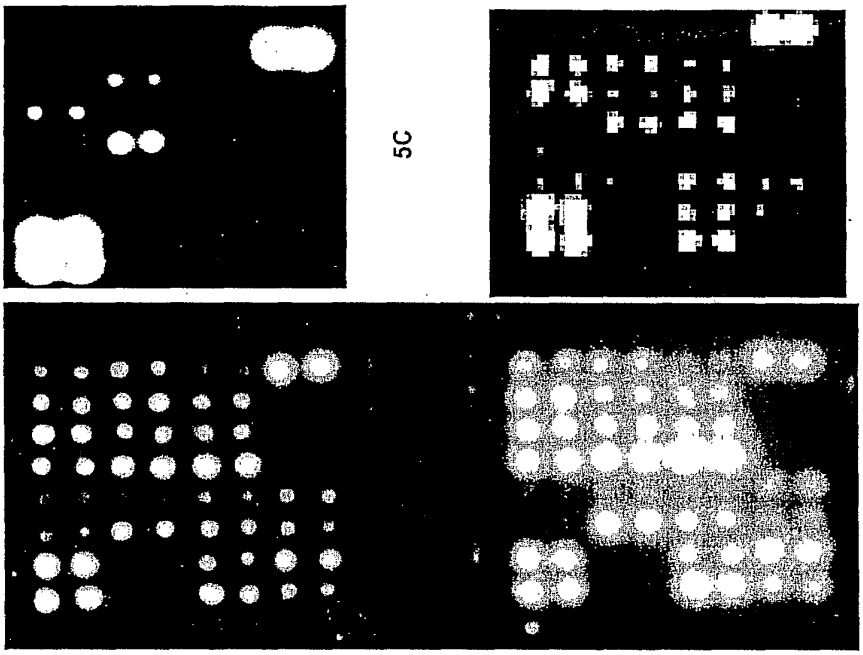
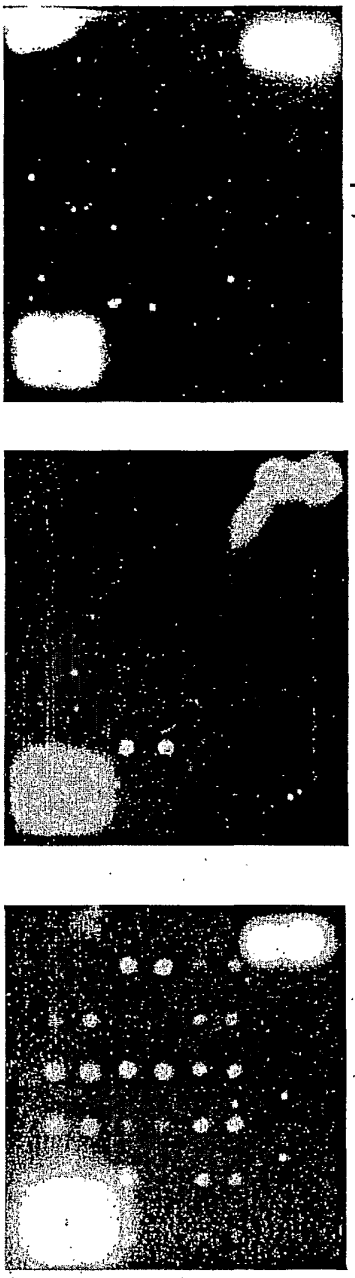


Figure 6



OTF CA 6A

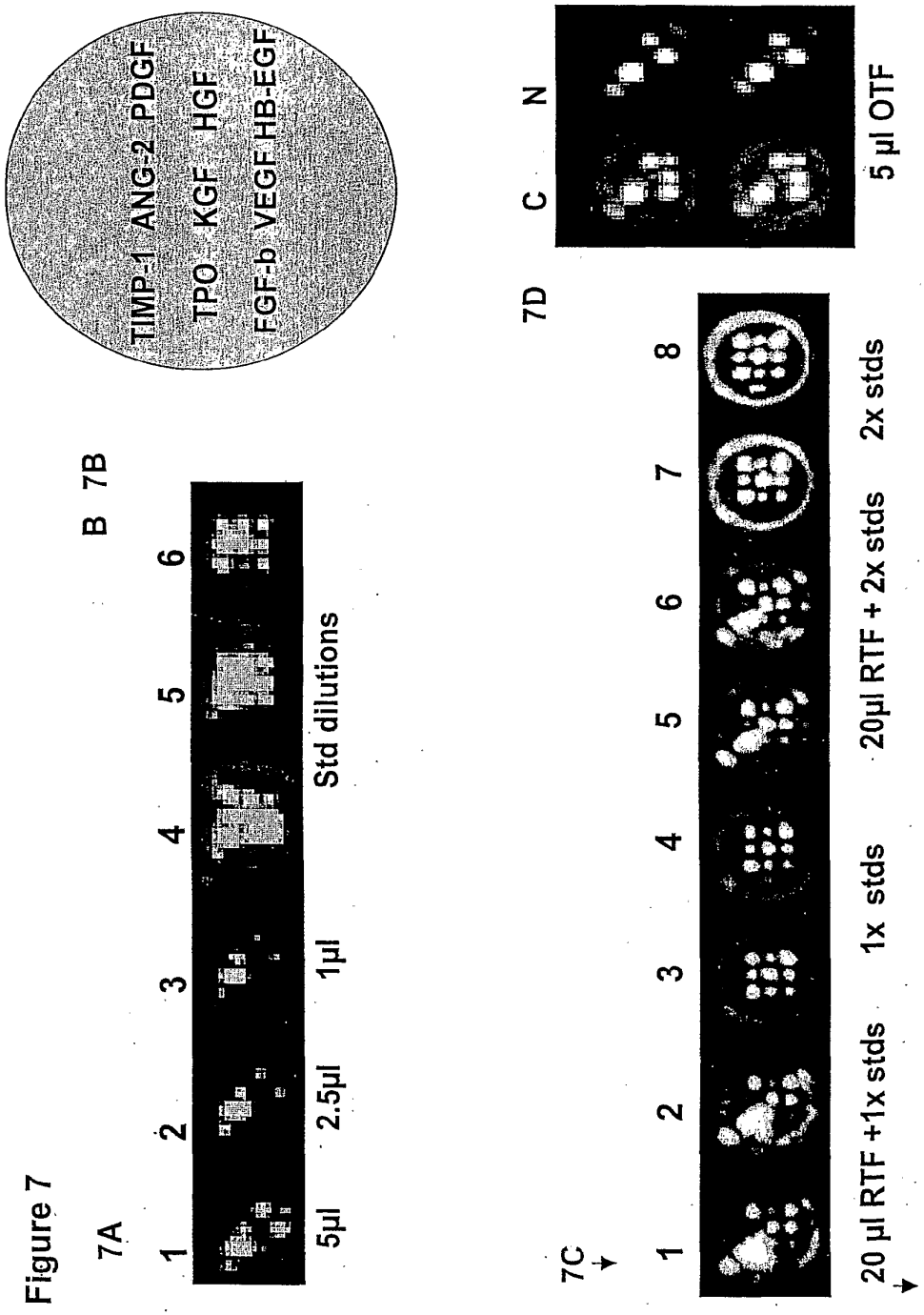
OTF N 6B

control 6C

Pos	Pos	Neg	Neg	GM-CSF (1/5)	GM-CSF (1/20)	IL-1a (1/5)	IL-1a (1/20)	IL-1b (1/5)	IL-1b (1/20)	IL-2 (1/5)	IL-2 (1/20)
Pos	Pos	Neg	Neg	GM-CSF (1/5)	GM-CSF (1/20)	IL-1a (1/5)	IL-1a (1/20)	IL-1b (1/5)	IL-1b (1/20)	IL-2 (1/5)	IL-2 (1/20)
IL-3 (1/5)	IL-3 (1/20)	IL-4 (1/5)	IL-4 (1/20)	IL-5 (1/5)	IL-5 (1/20)	IL-6 0(1/5)	IL-6 (1/20)	IL-7 (1/5)	IL-7 (1/20)	IL-8 (1/5)	IL-8 (1/20)
IL-3 (1/5)	IL-3 (1/20)	IL-4 (1/5)	IL-4 (1/20)	IL-5 (1/5)	IL-5 (1/20)	IL-6 0(1/5)	IL-6 (1/20)	IL-7 (1/5)	IL-7 (1/20)	IL-8 (1/5)	IL-8 (1/20)
IL-10 (1/5)	IL-10 (1/20)	IL-12 (1/5)	IL-12 (1/20)	IL-13 (1/5)	IL-13 (1/20)	IFNγ (1/5)	IFNγ (1/20)	MCP-1 (1/5)	MCP-1 (1/20)	TNF-α (1/5)	TNF-α (1/20)
IL-10 (1/5)	IL-10 (1/20)	IL-12 (1/5)	IL-12 (1/20)	IL-13 (1/5)	IL-13 (1/20)	IFNγ (1/5)	IFNγ (1/20)	MCP-1 (1/5)	MCP-1 (1/20)	TNF-α (1/5)	TNF-α (1/20)
blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	Pos
blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	Pos

6D

Figure 7


7A
1
2
3
4
5
6
5µl
2.5µl
1µl
Std dilutions
B 7B
TIMP-1 ANG-2 PDGF
TPO KGF HGF
FGF-b VEGF HB-EGF
7C
1
2
3
4
5
6
7
8
20 µl RTF +1x stds
1x stds
20µl RTF + 2x stds
2x stds
7D
C
N
5 µl OTF

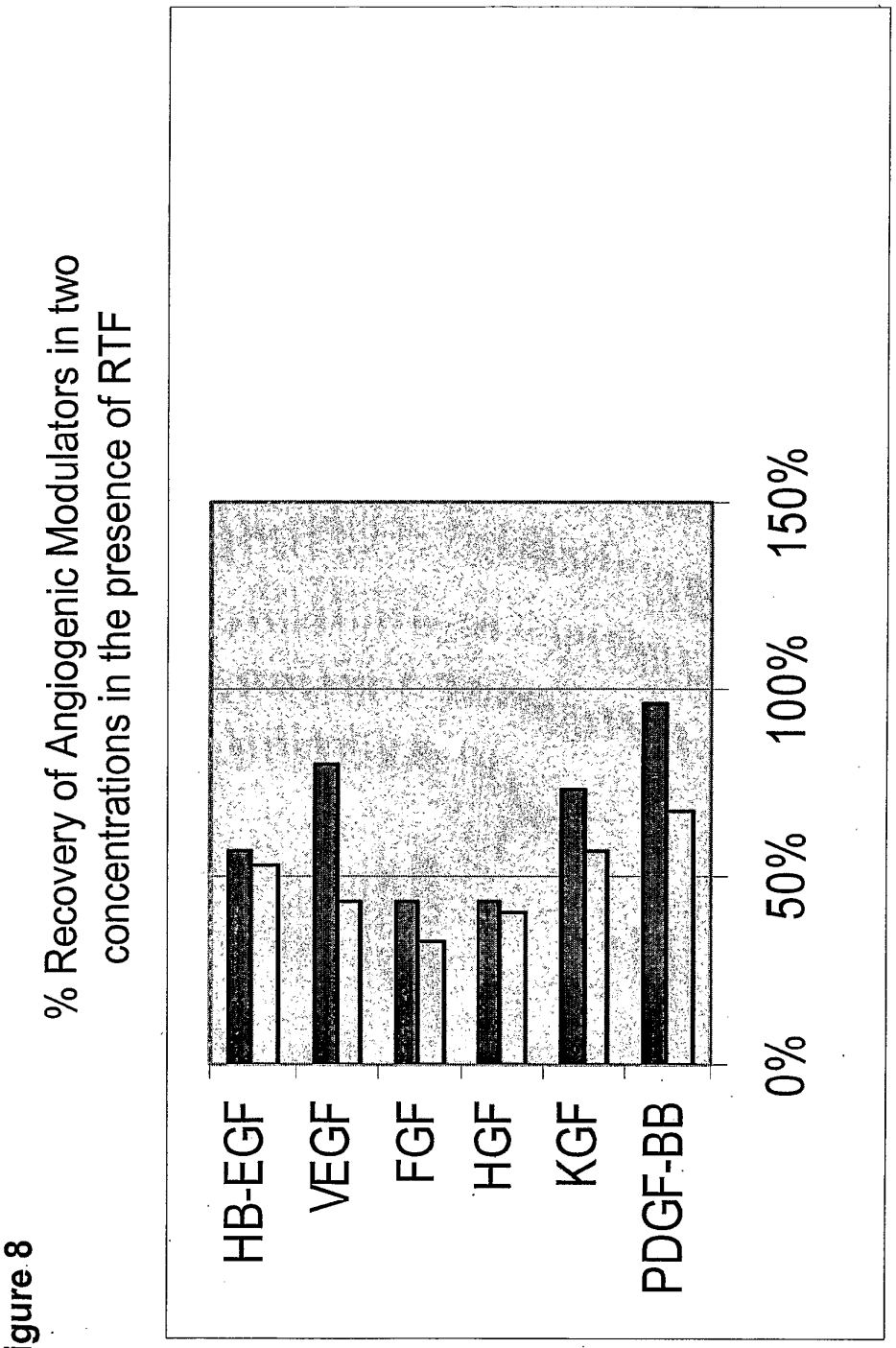
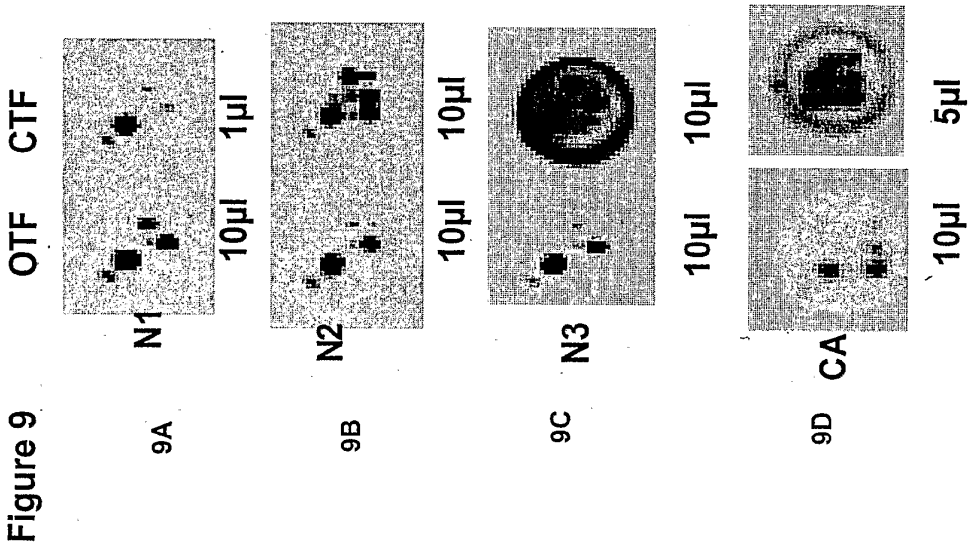

% Recovery of Angiogenic Modulators in two concentrations in the presence of RTF
HB-EGF
VEGF
FGF
HGF
KGF
PDGF-BB
0%
50%
100%
150%

Figure 8


Figure 9
OTF CTF
N1
1µl
10µl
9A
N2
10µl
10µl
9B
N3
10µl
10µl
9C
CA
5µl
10µl
9D

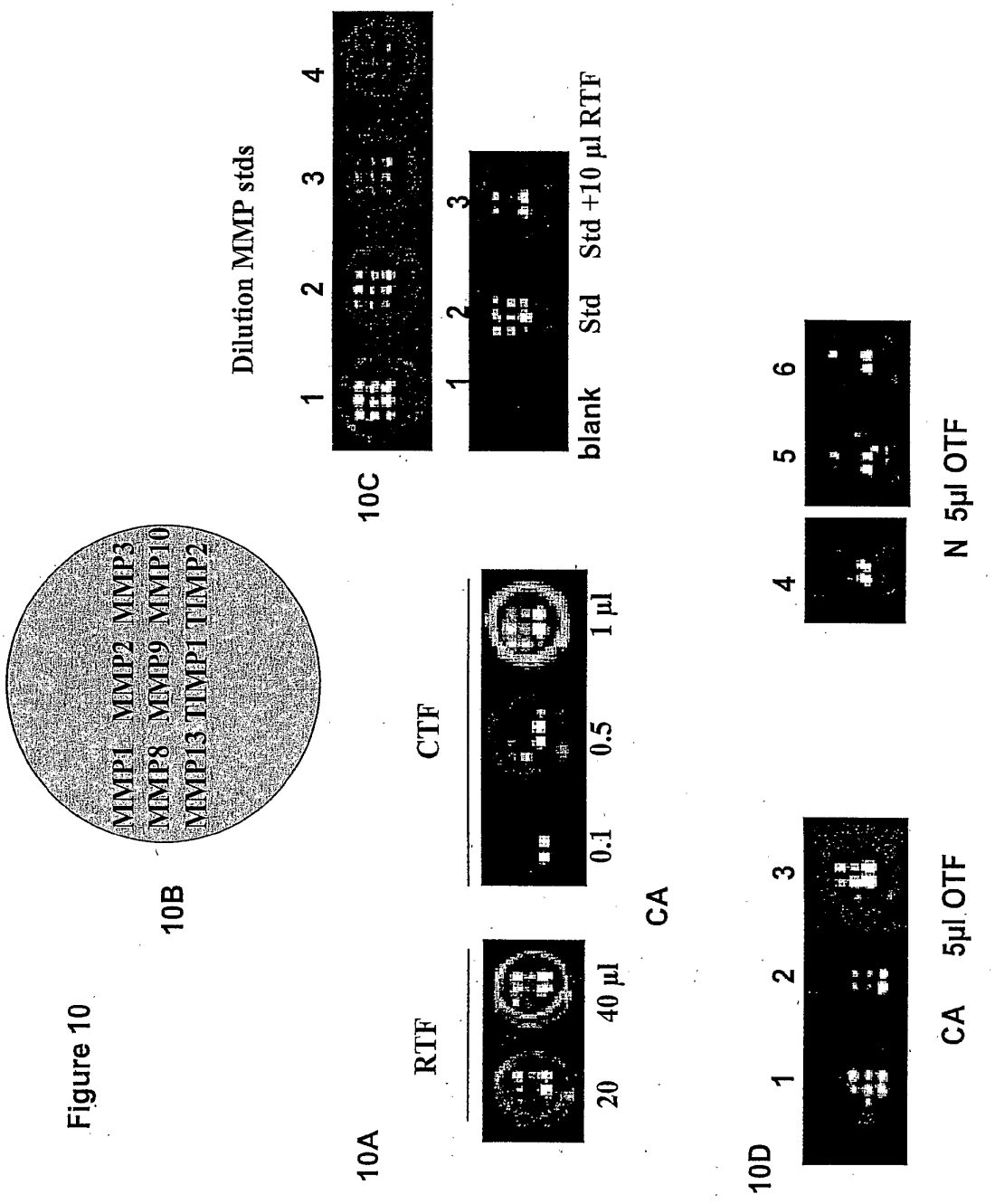
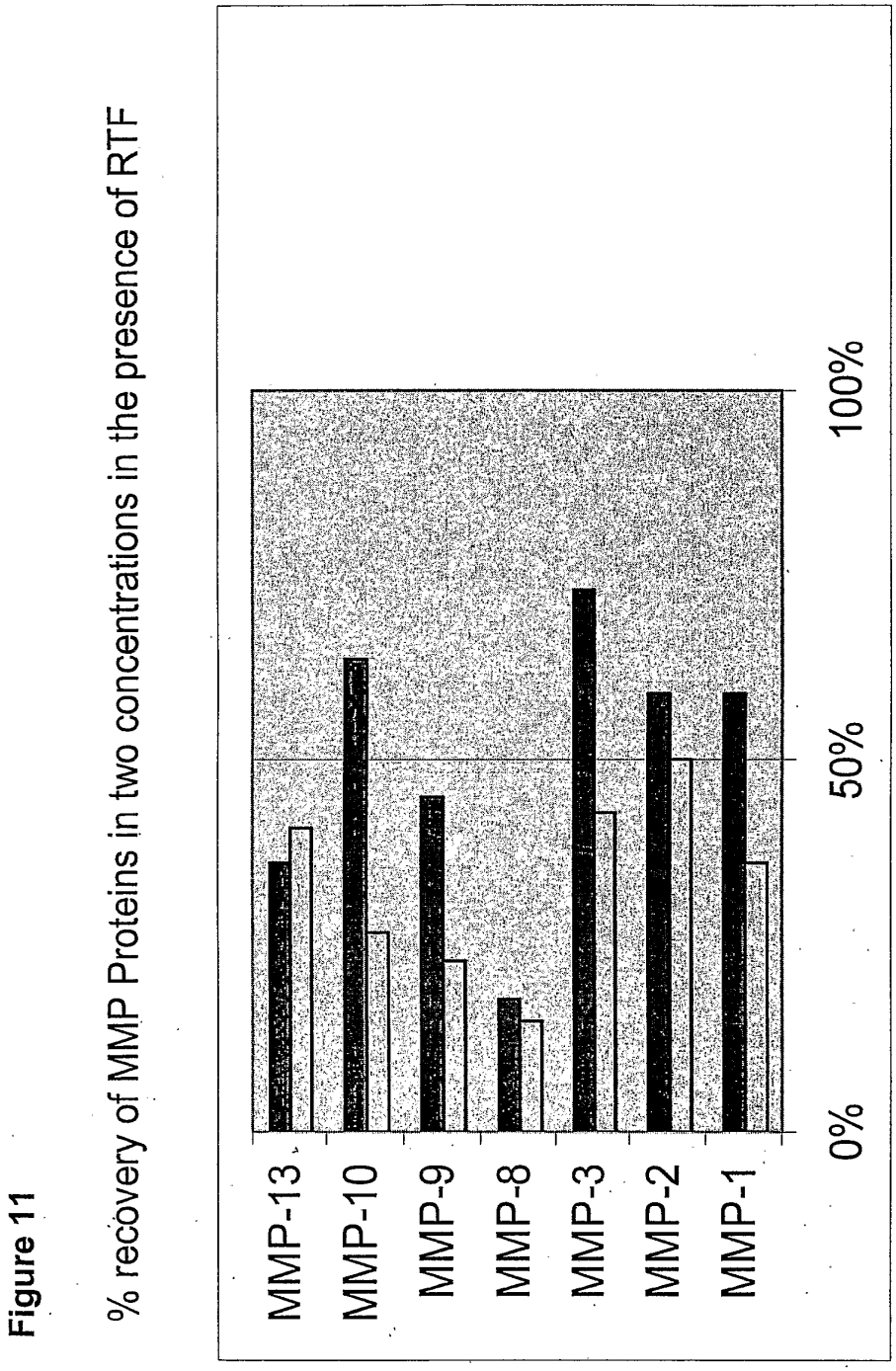

Figure 10
10A
RTF
20 40 µl
CTF
0.1 0.5 1 µl
CA
10B
MMP1 MMP2 MMP3
MMP8 MMP9 MMP10
MMP13 TIMP1 TIMP2
Dilution MMP stds
10C
1 2 3 4
1 2 3
blank Std Std +10 µl RTF
10D
1 2 3
CA 5µl OTF
4 5 6
N 5µl OTF

Figure 11


% recovery of MMP Proteins in two concentrations in the presence of RTF
MMP-13
MMP-10
MMP-9
MMP-8
MMP-3
MMP-2
MMP-1
0%
50%
100%

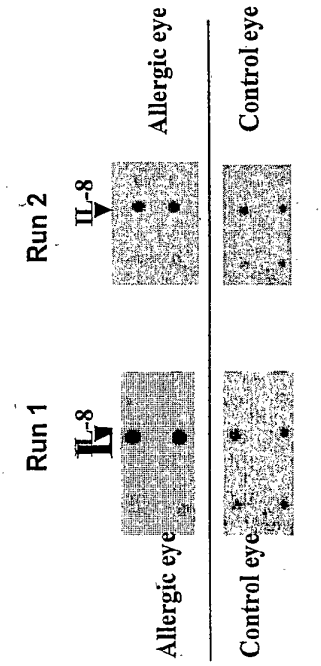

Run 1
IL-8
Allergic eye
Control eye
Run 2
IL-8
Allergic eye
Control eye

Figure 12

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(54) Title: BIO-BARCODE BASED DETECTION OF TARGET ANALYTES

(57) Abstract: The present invention relates to screening methods, compositions, and kits for detecting for the presence or absence of one or more target analytes, e.g. biomolecules, in a sample. In particular, the present invention relates to a method that utilizes reporter oligonucleotides as biochemical barcodes for detecting multiple protein structures or other target analytes in a solution.

BIO-BARCODE BASED DETECTION OF TARGET ANALYTES

FIELD OF THE INVENTION:

The present invention relates to a screening method for detecting for the presence or absence of one or more target analytes, e.g., proteins, nucleic acids, or other compounds in a sample. In particular, the present invention relates to a method that utilizes reporter oligonucleotides as biochemical barcodes for detecting one or more analytes in a solution.

CROSS-REFERENCE:

This application is a continuation-in-part of U.S. patent application 10/877,750, filed June 25, 2004 and International application PCT/US04/020493, filed June 25, 2004, both of which are incorporated by reference in their entirety. This application claims the benefit of provisional application Nos. 60/570,723, filed May 12, 2004; 60/585,294, filed July 1, 2004; and _____ (Atty Docket No. 05-192) filed January 19, 2005. This application also claims the benefit of provisional application Nos. 60/506,708, filed Sept. 25, 2003; 60/482,979, filed June 27, 2003; 60/496,893, filed August 21, 2003; 60/515,243, filed Oct. 28, 2003; 60/530,797, filed Dec. 18, 2003 and is a continuation-in-part of U.S. patent application serial no. 10/108,211, filed March 27, 2002, which in turn claims the benefit of U.S. Provisional application nos. 60/192,699, filed March 28, 2000; and 60/350,560, filed November 13, 2001, which are incorporated by reference in their entirety, and which is a continuation-in-part of U.S. patent application serial no. 09/820,279, filed March 28, 2001. The work reported in this application is funded, in part, by NSF, ARO, AFOSR, DARPA, and NIH grants. The work herein was also supported in part under the National Institutes of Health Pioneer award 1DP1-0D000285-01 and Air Force Office of Scientific Research grant F496-20-01-1-0401 and Edison grant 6144601-05-0001. Accordingly, the U.S. government has certain rights to the invention described in this application.

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BACKGROUND OF THE INVENTION:

The detection of analytes is important for both molecular biology research and medical applications. Diagnostic methods based on fluorescence, mass spectroscopy, gel electrophoresis, laser scanning and electrochemistry are now available for identifying a variety of protein structures.[1-4] Antibody-based reactions are widely used to identify the genetic protein variants of blood cells, diagnose diseases, localize molecular probes in tissue, and purify molecules or effect separation processes.[5] For medical diagnostic applications (e.g. malaria and HIV), antibody tests such as the enzyme-linked immunosorbent assay, Western blotting, and indirect fluorescent antibody tests are extremely useful for identifying single target protein structures. [6,7] Rapid and simultaneous sample screening for the presence of multiple antibodies would be beneficial in both research and clinical applications. However, it is difficult, expensive, and time-consuming to simultaneously detect several protein structures under assay conditions using the aforementioned related protocols.

Polymerase chain reaction (PCR) and other forms of target amplification have enabled rapid advances in the development of powerful tools for detecting and quantifying DNA targets of interest for research, forensic, and clinical applications [26-32]. The development of comparable target amplification methods for proteins could dramatically improve medical diagnostics and the developing field of proteomics [33-36]. Although one cannot yet chemically duplicate protein targets, it is possible to tag such targets with oligonucleotide markers that can be subsequently amplified with PCR and then use DNA detection to identify the target of interest [37-45]. This approach, often referred to as immuno-PCR, allows one to detect proteins with DNA labels in a variety of different formats (Figure 5). To date, all immuno-PCR approaches involve heterogeneous assays, which involve initial immobilization of a target analyte to a surface with subsequent detection using an antibody with a DNA label (for example, see U.S. Patent nos. 5,635,602, and 5,665,539). The DNA label is typically strongly bound to the antibody (either through covalent interactions or strepavidin-biotin binding). Although theses approaches are notable advances in protein detection, they have several drawbacks: 1) limited sensitivity because of a low ratio of DNA identification sequence to detection antibody; 2) slow target binding kinetics due to the heterogeneous nature of the target capture procedure, which increases assay time and decreases assay sensitivity (Step 3 in

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Figure 5); 3) complex conjugation chemistries that are required to chemically link the antibody and DNA-markers (Step 4 in Figure 5); and 4) require a PCR amplification step[45]. Therefore, a sensitive, and rapid method for detecting target analytes in a sample that is amenable to multiplexing and easy to implement is needed.

For DNA detection methods, many assays have been developed using radioactive labels, molecular fluorophores, chemiluminescence schemes, electrochemical tags, and most recently, nanostructure-based labels.[61-70] Although some nanostructure-based methods are approaching PCR in terms of sensitivity, none thus far have achieved the 1-10 copy sensitivity level offered by PCR. A methodology that allows for PCR-like signal amplification without the complexity, expense, and time and labor intensive aspects associated with PCR would provide significant advantages over such PCR-based methods.

SUMMARY OF THE INVENTION:

The present invention relates to methods, probes, compositions, and kits that utilize oligonucleotides as biochemical barcodes for detecting multiple analytes in one solution. The approach takes advantage of recognition elements of specific binding pairs functionalized either directly or indirectly with nanoparticles, and the previous observation that hybridization events that result in the aggregation of gold nanoparticles can significantly alter their physical properties (e.g. optical, electrical, mechanical).[8-12] The general idea is that each recognition element of a specific binding pair can be associated with a different oligonucleotide sequence with discrete and tailorable hybridization and melting properties and a physical signature associated with the nanoparticles. The discrete hybridization and melting properties can be used to decode a series of analytes in a multi-analyte assay by creating a change in a physical signature associated with the nanoparticles or by detection of oligonucleotide sequence(s), through hybridization/dehybridization or melting/annealing events.

The invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

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a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are optionally labeled with a reporter label, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

e) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes; and

f) detecting for the presence of the DNA barcode wherein the presence of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

In addition, the invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the

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specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are fluorescently labeled, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

e) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes; and

f) detecting for the presence of a fluorescent signal from the DNA barcodes wherein the presence of a fluorescent signal is indicative of the presence of a specific target analyte in the sample.

The invention also provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

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b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are fluorescently labeled, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

e) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes;

f) subjecting the isolated washed capture substrate to conditions effective to release the DNA barcodes; and

g) detecting for the presence of the fluorescent signal of the DNA barcodes wherein the presence of a fluorescent signal of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

Furthermore, the invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

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b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes comprising a reporter label, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

e) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes;

f) subjecting the isolated washed capture substrate to conditions effective to release the DNA barcodes; and

g) detecting for the presence of the reporter label of the DNA barcodes wherein the presence of the reporter label of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

The invention also provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

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b) providing at least one type of particle detection probes, the particle detection probes comprising a microparticle or nanoparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are optionally labeled with a reporter label, wherein the specific binding complement bound to the particle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of particle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the particle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

e) optionally isolating and washing the capture substrate to remove unbound particle detection probes;

f) releasing the DNA barcodes from the particle detection probes by a chemical releasing agent; and

g) detecting for the presence of the DNA barcode wherein the presence of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

The invention further provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates that are separable from the sample, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

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b) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

c) optionally separating the capture substrate and any target analytes bound thereto from the sample;

d) providing at least one type of particle detection probes, the particle detection probes comprising a particle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of oligonucleotides that are optionally labeled with a reporter label, wherein the specific binding complement bound to the particle detection probes binds to at least a second binding site of the target analyte;

e) contacting the immobilized target analytes with at least one type of particle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the particle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate, wherein the complex comprises the target analyte and capture substrate and particle detection probe;

f) optionally separating the complex from unbound particle detection probes; and

g) detecting for the presence of the complex wherein the presence of the complex is indicative of the presence of a specific target analyte in the sample.

As discussed herein, the assay methods of the invention are ultrasensitive and can be performed in less than 60 minutes without the need for enzymatic amplification or a scanometric detection scheme. By eliminating both PCR and scanometric method without losing sensitivity, the methods of the invention overcome some of the drawbacks that both of these conventional methods, such as long assay times and assay complexity. Moreover, non-scientists such as nurses, medical doctors, and soldiers should be able to

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learn how to use the methods of the invention after simple training due to its simplicity. The methods of the invention can be easily multiplexed by using different fluorophores for different target proteins and total assay time can be even more decreased by optimizing probe concentrations and reaction conditions. In addition, the methods of the invention can be used to detect any target that can be detected in a sandwich assay, including proteins, DNA, RNA, small molecules, and metal ions. Furthermore, other types of reporter groups can be attached to the barcodes allowing for methods other than fluorescence-detection to identify the barcodes and amplified detection signal. These include, but are limited to, redox-active groups, groups with electrical signatures, radioactive groups, catalytic groups, groups with distinct absorption characteristics, and groups with Raman signatures. A reporter group can be anything with a distinct and measurable chemical or physical signature.

In one embodiment of the invention, a method is provided for detecting for the presence or absence of one or more target analytes, the target analyte having at least two binding sites, in a sample comprising the steps of:

providing a substrate; providing one or more types of particle probes, each type of probe comprising a particle having one or more specific binding complements to a specific target analyte and one or more DNA barcodes bound thereto, wherein the specific binding complement of each type of particle probe is specific for a particular target analyte, and the DNA barcode for each type of particle probe serves as a marker for the particular target analyte;

immobilizing the target analytes onto the substrate;

contacting the immobilized target analytes with one or more types of particle probes under conditions effective to allow for binding between the target analyte and the specific binding complement to the analyte and form a complex in the presence of the target analyte;

washing the substrate to remove unbound particle probes; and

optionally amplifying the DNA barcode; and

detecting for the presence or absence of the DNA barcode wherein the presence or absence of the marker is indicative of the presence or absence of a specific target analyte in the sample.

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In one aspect of this embodiment of the invention, the target analyte is a protein or hapten and its specific binding complement is an antibody comprising a monoclonal or polyclonal antibody.

In another aspect of the invention, DNA barcode is amplified by PCR.

In another aspect of the invention, the particle is labeled with at least two DNA barcodes.

In another aspect of the invention, the substrate is arrayed with one or more types of capture probes for the target analytes.

In another embodiment of the invention, a method is provided for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites, the method comprising:

providing one or more types of capture probes bound to a substrate, each type of capture probe comprising a specific binding complement to a first binding site of a specific target analyte;

providing one or more types of detection probes, each type of detection probe comprising a nanoparticle having oligonucleotides bound thereto, one or more specific binding complements to a second binding site of the specific target analyte, and one or more DNA barcodes that serve as a marker for the particular target analyte, wherein at least a portion of a sequence of the DNA barcodes is hybridized to at least some of the oligonucleotides bound to the nanoparticles

contacting the sample, the capture probe, and the detection probe under conditions effective to allow specific binding interactions between the target analyte and the probes and to form an aggregate complex in the presence of the target analyte;

washing the substrate to remove any unbound detection probes;

detecting for the presence or absence of the DNA barcode in any aggregate complex on the substrate, wherein the detection of the presence or absence of the DNA barcode is indicative of the presence or absence of the target analyte in the sample.

In one aspect of this embodiment of invention, the detection probe comprises (i) one or more specific binding complements to the second binding site of a specific target analyte, (ii) at least one type of oligonucleotides bound to the nanoparticle, and a DNA barcode having a predetermined sequence that is complementary to at least a portion of at

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least one type of oligonucleotides, the DNA barcode bound to each type of detection probe serving as a marker for a specific target analyte;

In another aspect of this embodiment, prior to said detecting step, the method further comprising the steps of:

subjecting the aggregate complex to conditions effective to dehybridize the complex and release the DNA barcodes; and

amplifying the DNA barcode prior to said detecting.

In another aspect of this embodiment, the DNA barcode is amplified by PCR.

In another aspect of this embodiment, the capture probe is bound to a magnetic substrate such as a magnetic particle.

In another aspect of this embodiment, the target analyte is a target nucleic acid having a sequence of at least two portions, the detection probe comprises a nanoparticle having oligonucleotides bound thereto, at least a portion of the oligonucleotides having a sequence that is complementary to the DNA bar code, the specific binding complement of the detection probe comprising a first target recognition oligonucleotide having a sequence that is complementary to a first portion of the target nucleic acid, and the specific binding complement of the capture probes comprises second target recognition oligonucleotide having a sequence that is complementary to at least a second portion of the target nucleic acid.

In another aspect of this embodiment, the target analyte is a target nucleic acid having a sequence of at least two portions, the detection probe comprising a nanoparticle having oligonucleotides bound thereto, the DNA barcode having a sequence that is complementary to at least a portion of the oligonucleotides bound to the detection probe, the specific binding complement comprises a target recognition oligonucleotide having a sequence of at least first and second portions, the first portion is complementary to a first portion of the target nucleic acid and the second portion is complementary to a least a portion of the oligonucleotides bound to the nanoparticles, the specific binding complement of the substrate comprising a target recognition oligonucleotide having at least a portion that is complementary to a second portion of the target nucleic acid.

In another aspect of this embodiment, the detection probe comprises a dendrimer.

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In yet another embodiment of this invention, a method is provided for detecting for the presence or absence of one or more target analytes in a sample, each target analyte having at least two binding sites, the method comprising:

providing one or more types of capture probes, each type of capture probe comprising (i) a magnetic particle; and (ii) a first member of a first specific binding pair attached to the magnetic particle, wherein the first member of the first specific binding pair binds to a first binding site of a specific target analyte;

providing one or more types of detection probe for each target analyte, each type of detection probe comprising (i) a nanoparticle; (ii) a first member of a second specific binding pair attached to the nanoparticle, wherein the first member of the second specific binding pair binds to a second binding site of the target analyte; (iii) at least one type of oligonucleotides bound to the nanoparticle; and (iv) at least one type of DNA barcodes, each type of DNA barcode having a predetermined sequence that is complementary to at least a portion of a specific type of oligonucleotides and serves as a marker for a specific target analyte;

contacting the sample with the capture probe and the detection probe under conditions effective to allow specific binding interactions between the target analyte and the probes and to form an aggregated complex bound to the magnetic particle in the presence of the target analyte;

washing any unbound detection probes from the magnetic particle; and

detecting for the presence or absence of the DNA barcodes in the complex, wherein the detection of the DNA barcode is indicative of the presence of the target analyte.

In one aspect of this embodiment, the method further comprises, prior to said detecting step, the steps of:

isolating the aggregated complex by applying a magnetic field;

subjecting the aggregated complex to conditions effective to dehybridize and release the DNA barcodes from the aggregated complex;

isolating the released DNA barcodes.

In another aspect of this embodiment, the method further comprises amplifying the released DNA barcodes.

In another aspect of this embodiment, the method further comprises:

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providing a substrate having oligonucleotides bound thereto, the oligonucleotides having a sequence complementary to at least a portion of the sequence of the DNA barcode;

providing a nanoparticle comprising oligonucleotides bound thereto, wherein at least portion of the oligonucleotides bound to the nanoparticles have a sequence that is complementary to at least a portion of a DNA barcode; and

contacting the DNA barcodes, the oligonucleotides bound to the substrate, and the nanoparticles under conditions effective to allow for hybridization at least a first portion of the DNA barcodes with a complementary oligonucleotide bound to the substrate and a second portion of the DNA barcodes with some of the oligonucleotides bound to the nanoparticles.

In another aspect of this embodiment, the DNA barcode is amplified by PCR prior to detection.

In another aspect of this embodiment, the method further comprises isolating the aggregated complexes prior to analyzing the aggregated complex.

In another aspect of this embodiment, the aggregated complex is isolated by applying a magnetic field to the aggregated complex.

In another aspect of this embodiment, the nanoparticles are metal nanoparticles such as gold nanoparticles or semiconductor nanoparticles.

In another aspect of this embodiment, the specific binding pair is an antibody and an antigen; a receptor and a ligand; an enzyme and a substrate; a drug and a target molecule; an aptamer and an aptamer target; two strands of at least partially complementary oligonucleotides.

In another aspect of this embodiment, the DNA barcode may be biotinylated, radioactively labeled, or fluorescently labeled.

In another embodiment of the invention, a method is provided for detecting for the presence or absence of one or more target analytes in a sample, the method comprises:

providing at least one or more types of particle complex probes, each type of probe comprising oligonucleotides bound thereto, one or more specific binding complements of a specific target analyte, and one or more DNA barcodes that serves as a marker for the particular target analyte, wherein at least a portion of a sequence of the

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DNA barcodes is hybridized to at least some of the oligonucleotides bound to the nanoparticles;

contacting the sample with the particle complex probes under conditions effective to allow specific binding interactions between the target analytes and the particle complex probes and to form an aggregate complex in the presence of a target analyte; and

observing whether aggregate complex formation occurred.

Another embodiment of the invention provides for a method for detecting the presence or absence of one or more target analytes, each target analyte having at least two binding sites. The method comprises at least one type of capture probe and at least one type of detection probe for each target analyte used. These probes may be generated prior to conducting the actual assay or in situ while conducting the assay. The capture probe comprises a first member of a first specific binding pair, wherein the first member of the first specific binding pair binds to the first binding site of the target analyte, and wherein the first member of the first specific binding pair optionally binds to a substrate. In one preferred embodiment, the substrate comprises a magnetic particle. The detection probe comprises (1) a nanoparticle; (2) a first member of a second specific binding pair attached to the nanoparticle, wherein the first member of the second specific binding pair binds to a second binding site of the target analyte; and (3) at least one type of oligonucleotides bound to the nanoparticle; and (4) at least one type of DNA barcodes, each type of DNA barcode having a predetermined sequence that is complementary to at least a portion of a specific type of oligonucleotides. When employed in a sample containing the target analyte, the first member of a first specific binding pair on the capture probe binds to the first binding site of the target analyte, and the first member of a second specific binding pair on the detection probe binds to the second binding site of the target analyte. Aggregation occurs when capture probes and detection probes are brought together by the target analyte. The aggregates may be isolated and subjected to further melting analysis to identify the particular target analyte where multiple targets are present as discussed above. Alternatively, the aggregates can be dehybridized to release the DNA barcode.

In one aspect of this embodiment, the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

In another aspect of this embodiment, the method further comprises the steps of:

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isolating aggregated complexes; and

analyzing the aggregated complexes to determine the presence of one or more DNA barcodes having different sequences.

In another aspect of this embodiment, the method further comprises the steps of:

isolating the aggregated complex;

subjecting the aggregated complex to conditions effective to dehybridize the aggregated complex and release the DNA barcode;

isolating the DNA barcode; and

detecting for the presence of one or more DNA barcodes having different sequences, wherein each DNA barcode is indicative of the presence of a specific target analyte in the sample.

In another aspect of this embodiment, the method further comprises the steps of:

isolating the aggregated complex;

subjecting the aggregated complex to conditions effective to dehybridize the aggregated complex and release the DNA barcode;

isolating the DNA barcode;

amplifying the isolated DNA barcode; and

detecting for the presence of one or more amplified DNA barcodes having different sequences, wherein each DNA barcode is indicative of the presence of a specific target analyte in the sample.

In another aspect of this embodiment, the target has more than two binding sites and at least two types of particle complex probes are provided, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type of probe having a specific binding complement to a second binding site on the probe. A plurality of particle complex probes may be provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

In another aspect of this embodiment, the detecting step for the presence of one or more DNA barcodes comprises:

providing a substrate having oligonucleotides bound thereto, the oligonucleotides having a sequence complementary to at least a portion of the sequence of the DNA barcode;

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providing a nanoparticle comprising oligonucleotides bound thereto, wherein at least portion of the oligonucleotides bound to the nanoparticles have a sequence that is complementary to at least a portion of a DNA barcode; and

contacting the DNA barcodes, the oligonucleotides bound to the substrate, and the nanoparticles under conditions effective to allow for hybridization at least a first portion of the DNA barcodes with a complementary oligonucleotide bound to the substrate and a second portion of the DNA barcodes with some of the oligonucleotides bound to the nanoparticles; and

observing a detectable change.

In another aspect of this embodiment, the substrate comprises a plurality of types of oligonucleotides attached thereto in an array to allow for the detection of one or more different types of DNA barcodes.

In another aspect of this embodiment, the detectable change is the formation of dark areas on the substrate.

In another aspect of this embodiment, the detectable change is observed with an optical scanner.

In another aspect of this embodiment, the substrate is contacted with a silver stain to produce the detectable change.

In another aspect of this embodiment, the DNA barcodes are contacted with the substrate under conditions effective to allow the DNA barcodes to hybridize with complementary oligonucleotides bound to the substrate and subsequently contacting the DNA barcodes bound to the substrate with the nanoparticles having oligonucleotides bound thereto under conditions effective to allow at least some of the oligonucleotides bound to the nanoparticles to hybridize with a portion of the sequence of the DNA barcodes on the substrate.

In another aspect of this embodiment, the DNA barcodes are contacted with the nanoparticles having oligonucleotides bound thereto under conditions effective to allow the DNA barcodes to hybridize with at least some of the oligonucleotides bound to the nanoparticles; and subsequently contacting the DNA barcodes bound to the nanoparticles with the substrate under conditions effective to allow at least a portion of the sequence of the DNA barcodes bound to the nanoparticles to hybridize with complementary oligonucleotides bound to the substrate.

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In another aspect of this embodiment, the DNA barcode is amplified prior to the contacting step.

In another aspect of this embodiment, at least two types of particle complex probes are provided, a first type of probe having a specific binding complement to a first binding site of the target analyte and a second type of probe having a specific binding complement to a second binding site of the target analyte.

In another embodiment of the invention, particle complex probes are provided. Thus, in one aspect of this embodiment, the particle complex probe comprises a particle having oligonucleotides bound thereto, one or more DNA barcodes, and an oligonucleotide having bound thereto a specific binding complement to a specific target analyte, wherein (i) the DNA barcode has a sequence having at least two portions; (ii) at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode; (iii) the oligonucleotide having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode; and (iv) the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

In another aspect of this embodiment, the particle complex probe comprises a particle having at least two types of oligonucleotides bound thereto, one or more DNA barcodes, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein a first type of oligonucleotides bound to the probe having a sequence that is complementary to at least a portion of the DNA barcode, the second type of oligonucleotide bound to the probe having a sequence that is complementary to at least a portion of the sequence of the oligonucleotide having a specific binding complement.

In another aspect of this embodiment the particle complex probe comprising a particle having oligonucleotides bound thereto, one or more DNA barcodes, and a specific binding complement to a target analyte, wherein at least a portion of the oligonucleotides bound to the particle have a sequence that is complementary to at least a portion of the sequence of the DNA barcode and where the DNA barcode serves as an identifier for a specific target analyte.

In yet another embodiment of the invention, a particle complex probe is provided. Thus in one embodiment of the invention, a particle complex probe is provided which

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comprises a particle having oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a specific target analyte, wherein (i) the DNA barcode has a sequence having at least two portions; (ii) at least some of the oligonucleotides attached to the particle have a sequence that is complementary to a first portion of a DNA barcode; (iii) the oligonucleotide having bound thereto a specific binding complement have a sequence that is complementary to a second portion of a DNA barcode; and (iv) the DNA barcode in each type of particle complex probe has a sequence that is different and that serves as an identifier for a particular target analyte.

In another embodiment of the invention, a particle complex probe is provided which comprises a particle having at least two types of oligonucleotides bound thereto, a DNA barcode, and an oligonucleotide having bound thereto a specific binding complement to a target analyte, wherein a first type of oligonucleotides bound to the probe having a sequence that is complementary to at least a portion of the DNA barcode, the second type of oligonucleotide bound to the probe having a sequence that is complementary to at least a portion of the sequence of the oligonucleotide having a specific binding complement.

In yet another embodiment of the invention, a particle complex probe is provided which comprises a particle having oligonucleotides bound thereto, a DNA barcode, and a specific binding complement to a target analyte, wherein at least a portion of the oligonucleotides bound to the particle have a sequence that is complementary to at least a portion of the sequence of the DNA barcode and where the DNA barcode serves as an identifier for a specific target analyte.

In yet another embodiment of the invention, a detection probe is provided which comprises a nanoparticle; a member of a specific binding pair bound to the nanoparticle; at least one type of oligonucleotide bound to the nanoparticle; and at least one type of DNA barcode each having a predetermined sequence, wherein each type of DNA barcode is hybridized to at least a portion of the at least one type of oligonucleotide.

In another embodiment of the invention, kits are provided which comprise the particle complex probe described above.

These and other embodiments of the invention will become apparent in light of the detailed description below.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a DNA/Au nanoparticle-based protein detection scheme. (A) Preparation of hapten-modified nanoparticle probes. (B) Protein detection using protein binding probes. Notice that there are nine G,C pairs in sequence A and there are only two G,C pairs in sequence B.

Figure 2 illustrates thermal denaturation profiles for Au nanoparticle aggregates linked by DNA and proteins. Extinction at 260 nm was monitored as a function of increasing temperature (1 °C/min, 1 min holding time). Each UV-Vis spectrum was measured under constant stirring to suspend the aggregates. All the aggregates were suspended in 1 ml of 0.3 M PBS prior to performing the melting analyses. A) Two probes with one target antibody present IgE (——), IgG1 (---)); all data have been normalized; (B) Two probes with both target antibodies present. Inset; first derivative of the thermal denaturation curve.

Figure 3 illustrates an array-based protein detection scheme using DNA as a biobarcode for the protein.

Figure 4 illustrates scanometric DNA array detection of the DNA biobarcodes. Left column is for the detection of the biobarcode associated with IgG1 and the right column is for the biobarcode associated with IgE. The capture oligonucleotides are 5' -thiol-modified ataactagaacttga (SEQ ID NO:1)for the IgG1 system and 5' –thiol-modified ttatctattatt (SEQ ID NO:2) for the IgE system. Each spot is approximately 250 um in diameter and read via gray-scale with an Epson Expression 1640XL flatbed scanner (Epson America, Longbeach, California). These assays have been studied and work comparably well over the 20 nM to 700 nM target concentration range.

Figure 5 illustrates a common type of immuno-PCR-based analyte detection scheme.

Figure 6 depicts the use of Barcode PCR (*B*PCR) protocol to detect a target analyte, prostate specific antigen (PSA). Panel A illustrates probe design and preparation. Panel B depicts PSA detection and barcode DNA amplification and identification.

Figure 7 illustrates the control experiment to assess primer-dimer formation, DNA barcode amplification, and the effect of increasing DMSO concentration using 25 thermal cycles. Lanes 1 through 5 are those with DNA barcode present in the PCR reaction

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mixture while there is no DNA barcode in lanes 6 through 10. Note that DMSO is increased from lane 1 to 5 and 6 to 10 (0 to 2 % in 0.5 % increments).

Figure 8 shows gel electrophoresis images and relative band intensity graph of barcode DNA amplified by PCR after PSA detection. Panel A, lanes 1 and 2 are control experiments (lane 1: with background proteins anti-dinitrophenyl and β-galactosidase without PSA, lane 2: with no protein). From lanes 3 to 8, PSA concentrations in the sample (10μl) are 300 aM, 3 fM, 30 fM, 300 fM, 3 pM, and 30 pM, respectively. The standard biobarcode DNA 40-mer for PSA is run in lane 9 to compare with other gel bands after PCR. Panel B, relative gel electrophoresis band intensity graph after *B*PCR. Panel C, low concentration detection of PSA. Concentrations are from 3 aM to 300 fM in 10x dilutions from lane 2 (3 aM) to lane 7 (300 fM). A negative control with only background proteins is shown in lane 1, and the standard biobarcode 40-mer (6 μM biobarcode duplex) is shown in the fist lane (lane C). Panel D, relative gel electrophoresis band intensity after *B*PCR.

Figure 9 illustrates scanometric detection of PSA-specific barcode DNA. PSA concentration (sample volume of 10 ml) was varied from 300 fM to 3 aM and a negative control sample where no PSA was added (control) is shown. For all seven samples, 2 ml of anti-dinitrophenyl (10 pM) and 2 ml of β-galactosidase (10 pM) were added as background proteins. Also shown is PCR-less detection of PSA (30 aM and control) with 30 nm NP probes (inset). Chips were imaged with the Verigene ID system.

Figure 10 illustrates theoretical detection limit of *B*PCR. Left panel, The gel image shows bands after PCR at decreasing starting barcode DNA concentrations. Lane 1: 3×10^9 copies, lane 2: 3×10^8 copies, lane 3: 3×10^7 copies, lane 4: 3×10^6 copies, lane 5: 3×10^5 copies, lane 6: 3×10^4 copies, lane 7: 3×10^3 copies, lane 8: 3×10^2 copies, lane 9: 3×10^1 copies, and lane 10: no barcode DNA. Right panel, relative gel electrophoresis band intensity graph.

Figure 11 illustrates detection of PSA-specific barcode DNA where the PSA is dissolved in a complex goat serum medium. Each panel shows the signal generated by the *B*PCR amplified barcode DNA at various concentrations of analyte (3 pM to 3 aM).

Figure 12 illustrates PCR-less detection of PSA with 30 nm NP probes. Each panel and associated relative intensity value on the bar graph shows the signal generated by direct detection of barcode DNA (*i.e.*, non-*B*PCR amplified) at various concentrations

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(30 aM to 3 pM, and control). Chips were imaged with the Verigene ID system (Nanosphere, Inc., Northbrook, IL).

Figure 13 illustrates the DNA-BCA assay. **A.** Nanoparticle and Magnetic Microparticle Probe Preparation. **B.** Nanoparticle-Based PCR-less DNA Amplification Scheme.

Figure 14 illustrates amplified Anthrax Bar-Code DNA Detection with Verigene ID system. **A.** Anthrax Bar-Code DNA Detection with 20 nm NP Probes. **B.** Anthrax Bar-Code DNA Detection with 30 nm NP Probes.

Figure 15 illustrates intensity graph of the bar-code DNA and NP probe sandwiched spots after silver enhancement for 20 nm and 30 nm NP probes.

Figure 16 illustrates one embodiment of the "universal" nanoparticle probe detection scheme. **A.** The universal probes are synthesized for specificity to one or more target nucleic acid sequences. The target recognition DNA can be used to control specificity of the probe for a target. The probes can be used in assay systems where single or multiple target nucleic acid sequences are present in a given test solution. **B.** The universal probes are used in conjunction with a second type of recognition oligonucleotide bound to a substrate, such as a magnetic microparticle or glass slide. The second type of recognition oligonucleotide, the universal probe, and the test solution thought to contain the target nucleic acid are mixed and reacted under conditions that allow for hybridization and complex formation. The complex is separated from the unreacted universal probes and test solution components, and the reporter oligonucleotides are detected.

Figure 17 illustrates another embodiment of the universal nanoparticle probe with dendrimer probes, amplified dendrimer probes, and dendrimer-nanoparticle hybrid probes. The first type of dendrimer probes comprise a recognition oligonucleotide sequence and a nucleic acid sequence that is complementary to a reporter oligonucleotide, such as a barcode DNA. The recognition oligonucleotide sequence on the first type of dendrimer probe can hybridize to a second type of dendrimer probe. Under hybridization conditions, this generates a dendrimer probe complex (or matrix) that can be extended as desired. The second type of dendrimer probe can bind a plurality of dendrimer probes and functions to amplify the amount of reporter oligonucleotide contained within the entire matrix. Similarly, the amount of recognition oligonucleotide present on the first

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type of dendrimer can be increased or decreased, in order to provide more sites of complexation with the second type of dendrimer, or in order to provide more reporter oligonucleotide. The first or second type of dendrimer probes can be used with other particle probes, such as gold nanoparticle probes, or magnetic particle probes in order to generate a hybrid probe complex (or matrix) system, as required by the particular assay.

Figure 18 illustrates a fluorophore-based bio-barcode amplification assay. Barcode DNA for each polystyrene probe was about $1.1x10^5$ and total assay time was about 50 minutes.

Figure 19 shows fluorescence signal (Alexa-647) for prostate specific antigen (PSA) (10 μL sample). Sensitivity was about 30 aM PSA. The micro-fluorometer cell (50 μL capacity) was used with Alexa-647.

Figure 20 shows a schematic representation of an exemplary assay of the present invention wherein the probe strands on the surface of the gold nanoparticle probes also represent the reporter moieties. Thiolated oligonucleotides doubling as probes for mRNA specific target sequences and reporter moieties were covalently immobilized to the surface of the gold nanoparticle. As shown, the sphere next to the gold nanoparticle surface represents either a fluorophore for fluorescent readout or a "universal" DNA sequence that can be used for scanometric gold nanoparticle labeling.

Figure 21 shows a schematic representation of the detection of mock mRNA target. Probes are as in Figure 20. Fluorescent readout was used to detect DTT-liberated fluorescently-labeled probes specific to the detected mRNA target.

Figure 22 shows a schematic representation of multiplexed detection of numerous nucleic acid targets where they are sorted out on a high density microarray which is subsequently readout using any standard method. Importantly, the sphere can represent a fluorescent readout method or can represent a universal capture sequence that can be probed using a "universal" gold nanoparticle probe in the Scanometric technique.

Figure 23 shows fluorimetric detection of fluorescently-labeled probe strands liberated after the detection of a mock mRNA sequence as shown in Figure 20. The 6 pM fluorescent 4 detection limit is as low as is possible using 13 nm probes liberating approximately 100 fluorescently-tagged reporter moieties per detection event in light of the fluorescent detection limit of approximately 1 nM for Alexa 488.

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DETAILED DESCRIPTION OF THE INVENTION

As used herein, a "type of" nanoparticles, conjugates, particles, latex microspheres, etc. having oligonucleotides attached thereto refers to a plurality of that item having the same type(s) of oligonucleotides attached to them. "Nanoparticles having oligonucleotides attached thereto" or "Nanoparticles having oligonucleotides attached thereto" are also sometimes referred to as "nanoparticle-oligonucleotide conjugates" or, in the case of the detection methods of the invention, "nanoparticle-oligonucleotide probes," "nanoparticle probes," or just "probes."

As used herein, the term "particle" refers to a small piece of matter that can preferably be composed of metals, silica, silicon-oxide, or polystyrene. A "particle" can be any shape, such as spherical or rod-shaped. The term "particle" as used herein specifically encompasses both nanoparticles and microparticles as defined and described hereinbelow.

As used throughout the invention "barcode", "biochemical barcode", "biobarcode", "barcode DNA", "DNA barcode", "reporter barcode", "reporter barcode DNA", etc. are all interchangeable with each other and have the same meaning. The DNA barcode may be a nucleic acid such as deoxynucleic acid or ribonucleic acid. Preferably, the DNA barcode is an oligonucleotide of a predefined sequence. If desired, the DNA barcode may be labeled, for instance, with biotin, a radiolabel, or a fluorescent label.

The term "nanoparticle complex" or "nanoparticle complex probe" refers to a conjugate comprised of nanoparticle-oligonucleotide conjugates, a reporter oligonucleotide, and an oligonucleotide having bound thereto a specific binding complement to a target analyte.

The term "analyte" or "target analyte" refers to the compound or composition to be detected, including drugs, metabolites, pesticides, pollutants, and the like. The analyte can be comprised of a member of a specific binding pair (sbp) and may be a ligand, which is monovalent (monoepitopic) or polyvalent (polyepitopic), preferably antigenic or haptenic, and is a single compound or plurality of compounds, which share at least one common epitopic or determinant site. The analyte can be a part of a cell such as bacteria or a cell bearing a blood group antigen such as A, B, D, etc., or an HLA antigen or a microorganism, e.g., bacterium, fungus, protozoan, or virus. If the analyte is

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monoepitopic, the analyte can be further modified, e.g. chemically, to provide one or more additional binding sites. In practicing this invention, the analyte has at least two binding sites.

The polyvalent ligand analytes will normally be larger organic compounds, often of polymeric nature, such as polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations include components of bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes and the like.

For the most part, the polyepitopic ligand analytes to which the subject invention can be applied will have a molecular weight of at least about 5,000, more usually at least about 10,000. In the polymeric molelecule category, the polymers of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight; among the hormones of interest, the molecular weights will usually range from about 5,000 to 60,000 molecular weight.

A wide variety of proteins may be considered as belonging to the family of proteins having similar structural features, proteins having particular biological functions, proteins related to specific microorganisms, particularly disease causing microorganisms, etc. Such proteins include, for example, immunoglobulins, cytokines, enzymes, hormones, cancer antigens, nutritional markers, tissue specific antigens, etc.

The types of proteins, blood clotting factors, protein hormones, antigenic polysaccharides, microorganisms and other pathogens of interest in the present invention are specifically disclosed in U.S. Pat. No. 4,650,770, the disclosure of which is incorporated by reference herein in its entirety.

The monoepitopic ligand analytes will generally be from about 100 to 2,000 molecular weight, more usually from 125 to 1,000 molecular weight.

The analyte may be a molecule found directly in a sample such as a body fluid from a host. The sample can be examined directly or may be pretreated to render the analyte more readily detectible. Furthermore, the analyte of interest may be determined by detecting an agent probative of the analyte of interest such as a specific binding pair member complementary to the analyte of interest, whose presence will be detected only when the analyte of interest is present in a sample. Thus, the agent probative of the analyte becomes the analyte that is detected in an assay. The body fluid can be, for

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example, urine, blood, plasma, serum, saliva, semen, stool, sputum, cerebral spinal fluid, tears, mucus, and the like.

The term "specific binding pair (sbp) member" refers to one of two different molecules, which specifically binds to and can be defined as complementary with a particular spatial and/or polar organization of the other molecule. The members of the specific binding pair can be referred to as ligand and receptor (antiligand). These will usually be members of an immunological pair such as antigen-antibody, although other specific binding pairs such as biotin-avidin, enzyme-substrate, enzyme-antagonist, enzyme–agonist, drug-target molecule, hormones-hormone receptors, nucleic acid duplexes, IgG-protein A/protein G, polynucleotide pairs such as DNA-DNA, DNA-RNA, protein-DNA, lipid-DNA, lipid-protein, polysaccharide-lipid, protein-polysaccharide, nucleic acid aptamers and associated target ligands (*e.g.*, small organic compounds, nucleic acids, proteins, peptides, viruses, cells, etc.), and the like are not immunological pairs but are included in the invention and the definition of sbp member. A member of a specific binding pair can be the entire molecule, or only a portion of the molecule so long as the member specifically binds to the binding site on the target analyte to form a specific binding pair.

The term "ligand" refers to any organic compound for which a receptor naturally exists or can be prepared. The term ligand also includes ligand analogs, which are modified ligands, usually an organic radical or analyte analog, usually of a molecular weight greater than 100, which can compete with the analogous ligand for a receptor, the modification providing means to join the ligand analog to another molecule. The ligand analog will usually differ from the ligand by more than replacement of a hydrogen with a bond, which links the ligand analog to a hub or label, but need not. The ligand analog can bind to the receptor in a manner similar to the ligand. The analog could be, for example, an antibody directed against the idiotype of an antibody to the ligand.

The term "receptor" or "antiligand" refers to any compound or composition capable of recognizing a particular spatial and polar organization of a molecule, e.g., epitopic or determinant site. Illustrative receptors include naturally occurring receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, nucleic acid aptamers, avidin, protein A, barstar, complement component C1q, and

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the like. Avidin is intended to include egg white avidin and biotin binding proteins from other sources, such as streptavidin.

The term "specific binding" refers to the specific recognition of one of two different molecules for the other compared to substantially less recognition of other molecules. Generally, the molecules have areas on their surfaces or in cavities giving rise to specific recognition between the two molecules. Exemplary of specific binding are antibody-antigen interactions, enzyme-substrate interactions, polynucleotide interactions, and so forth.

The term "non-specific binding" refers to the binding between molecules that is relatively independent of specific surface structures. Non-specific binding may result from several factors including hydrophobic interactions between molecules.

The term "antibody" refers to an immunoglobulin which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of another molecule. The antibody can be monoclonal or polyclonal and can be prepared by techniques that are well known in the art such as immunization of a host and collection of sera (polyclonal) or by preparing continuous hybrid cell lines and collecting the secreted protein (monoclonal), or by cloning and expressing nucleotide sequences or mutagenized versions thereof coding at least for the amino acid sequences required for specific binding of natural antibodies. Antibodies may include a complete immunoglobulin or fragment thereof, which immunoglobulins include the various classes and isotypes, such as IgA, IgD, IgE, IgG1, IgG2a, IgG2b and IgG3, IgM, etc. Fragments thereof may include Fab, Fv and $F(ab')_2$, Fab', and the like. In addition, aggregates, polymers, and conjugates of immunoglobulins or their fragments can be used where appropriate so long as binding affinity for a particular molecule is maintained.

In certain embodiments, the invention provides a novel detection reagent containing a reporter particle of a convenient size that has been derivatized to include on its surface some number of selective binding moieties that specifically bind a pre-selected target chemical species and a large number of copies of a pre-selected reporter moiety. The reporter moiety is covalently attached to the surface of the reporter particle using a standard chemistry.

The preferred detection method utilizing this amplification material is similar to that used in a sandwich immunoassay. In particular, the sample being analyzed is

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exposed to a capture phase capable of selectively and specifically binding to species of interest, the capture phase being immobilized on an insoluble material. Any unbound materials are then separated from the immobilized analyte through standard means. Immobilized analyte is then exposed to the detection reagent of this invention. The detection reagent binds to the immobilized analyte through the selective binding moieties incorporated thereon. The "sandwich" structure thus formed (insoluble substrate - analyte - detection reagent) therefore effectively immobilizes the detection reagent on the insoluble substrate. Unbound detection reagent can be separated from this immobilized structure through standard methods. Amplification can be performed by exposing the immobilized insoluble substrate - analyte - detection reagent sandwich to a means of rupturing the bond between the reporter moiety and the reporter particle, resulting in the release of the reporter moieties into the medium. As the ratio of the numbers of reporter moieties and selective binding moieties initially bound to the reporter particle can be established at greater than one during preparation of the detection reagent, release of the reporter moieties from a particle results in more reporter moieties entering the medium than there are target analyte molecules abound to the insoluble substrate. Detection, and optionally quantitation, of the released reporter moieties can be performed using any method that is appropriate to the chemical nature of the reporter moiety. The significant amplification of the detected signal of the reporter moiety from the detection of individual target analyte molecules results in an extremely sensitive, reliable and adaptable chemical detection assay.

In certain embodiments, a detection reagent of the present invention is composed of a reporter particle of a convenient size that has been derivatized to include on its surface selective binding moieties that specifically bind to the target chemical or target analyte and a plurality of pre-selected reporter moieties. The reporter particle can be any material that is compatible with the sample containing the target analyte and capable of binding both the selective binding moieties and the reporter moieties. Examples of suitable reporter particles include, but are not limited to, metals, silica, silicon-oxide, and polystyrene.

The selective binding compound may be any compound capable of selectively recognizing and binding to the target analyte without interfering with the binding between the target analyte and the capture phase. Example of suitable selective binding

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compounds include, but are not limited to, antibodies, enzymes, proteins, oligonucleotides and inorganic compounds.

The reporter particles are selected for a functional size typically having a diameter in the nanometer to micrometer range. As the ratio of the numbers of reporter moieties and selective binding moieties initially bound to a reporter particle can be established at greater than one during preparation of the detection reagent of the present invention, release of the reporter moieties from a particle will result in more reporter moieties entering the medium than there are analyte molecules bound to the reporter particle. This ratio establishes the amplification of the signal from the detection of a target analyte molecule. For example, the release of the reporter moieties from one reporter particle bearing 1000 copies of the reporter moiety that is bound to one molecule of immobilized analyte will result in 1000 molecules of reporter moiety appearing in the medium for each molecule of analyte in the original sandwich. This results in the chemical signal represented by the target analyte being amplified by a factor of 1000. This amplification can be adjusted during the synthesis of the detection reagent by manipulating parameters such as the surface area of the reporter particle and the ratio between and the packing densities of the selective binding and reporter moieties on the surface of the reporter particle. Thus, the size of the reporter particle dictates the number of reporter moieties that can be released, and the ultimate amplification factor that is obtained with regard to labeled target molecules. For example, 13 nm gold particles can carry with them up to 200 thiolated surface oligomers serving as reporter moieties thereby achieving an amplification factor of 200 reporter moieties to a single target molecule bound by a single reporter particle in the sandwich formed in the detection assay. Larger size (e.g. micron-sized) particles will obviously lead to larger amplification factors.

The reporter moiety is preferably attached to the surface of the reporter particle by a means sufficiently strong enough to prevent significant non-specific release of the reporter moiety during the steps of the detection method but simultaneously susceptible to separation and release of the reporter moiety immediately prior to the detection step. Preferably, the reporter moiety is attached to the surface of the reporter particle through a covalent bond that can be broken when contacted with a specific reagent added to the solid support immediately prior to the detection step. In the embodiment in which a covalent bond is used to anchor the reporter moiety to the reporter particle, the chemical

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used to release the reporter moiety from the reporter particle is a molecule that effectively takes the place of the reporter moiety on the surface of the reporter particle by preferentially binding to, or reacting with, the reporter particle while displacing the reporter moiety.

Examples of the reporter moieties may include, but are not limited to, fluorophores, chromophores, oligonucleotides with or without attached fluorophores or chromophores, proteins including enzymes and porphyrins, lipids, carbohydrates, synthetic polymers and tags such as isotopic or radioactive tags. These chemicals can be linked to the reporter particle through a covalent link between sulfur atoms such as thiols, disulfides, phosphorylthiolates and the like. In this instance, contacting the reporter particle with a chemical that displaces the reporter moiety by preferentially binding a sulfur atom in the covalent link liberates the reporter moiety for subsequent detection. Chemicals that would effectively displace the reporter moiety include any molecule that will preferentially bind to the reporter particle through the thiol link such as other thiol- or disulfide-containing molecules, dithiothreitol (DTT), dithioerythritol (DTE), mercaptoethanol and the like. Similarly, reducing agents such as sodium borohydride will cleave a disulfide linkage releasing the reporter moieties.

In a specific embodiment of the present invention, the reporter moiety and the selective binding compound are the same chemical entity. For example, in a preferred embodiment, the reporter moiety and the selective binding compound are described by a single oligonucleotide that is complementary to the target analyte and can additionally be detected after removal from the reporter particle (for example through a fluorescent tag present on the oligonucleotide).

In the first step of a detection method of the present invention, the sample being analyzed for the presence of the target molecule is exposed to a capture phase such as an antibody, oligonucleotide, lectin or similar material that is capable of selectively and specifically binding to the target specie of interest. The capture phase is immobilized on an insoluble material that is compatible with the assay chemistry and that it can readily be separated from the reaction medium. The immobilized capture phase is constructed such that it specifically binds, captures and immobilizes the analyte of interest, but preferably does not bind any other materials that may be present in the sample. Examples of the insoluble material suitable for use in the methods of the present invention include, but are

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not limited to, wells of a microtiter plate, a microparticle, fibrous or membrane filters, or other such insoluble materials.

The capture phase is preferably selected such that it binds to a different determinant on the analyte than does the selective binding moiety component of the detection reagent. Any unbound materials are then separated from the immobilized target analyte by any suitable means including, for example, decantation, sedimentation, washing, centrifuging or combinations of these processes. The net result of this process is that the analyte of interest is present in a purified and concentrated state on the surface of the insoluble material.

In a subsequent step of a method of the present invention, the immobilized target analyte is exposed to the detection reagent of this invention which contains the reporter particle bound to the reporter moiety and at least one selective binding moiety. The selective binding moiety specifically binds to the target analyte forming a "sandwich" structure including the insoluble substrate bound to the target analyte which is, in turn, bound to the detection reagent. This sandwich structure effectively immobilizes the detection reagent on the insoluble substrate, and any unbound detection reagent can be separated from this immobilized structure by any suitable methods such as decantation, sedimentation, washing, centrifuging or combinations of these processes as noted above.

In another step of a present method the signal from the binding and detection of the target analyte is amplified by exposing the immobilized insoluble substrate - target analyte -detection reagent sandwich to a medium containing a chemical that can liberate the reporter moiety from the reporter particle. The liberated reporter moiety then enters the media surrounding the detection reagent bound to the target analyte as described in detail above.

The media containing the released reporter moiety can be analyzed for the presence of the released reporter moieties using any method that is appropriate to the chemical nature of the reporter moiety. For example, a fluorescently-labeled reporter moiety may be detected and even quantitated by measurement of the fluorescence intensity or fluorescence depolarization of the medium while the presence of a chemiluminescent-labeled reporter can be determined by measuring the luminescence that occurs upon addition of an appropriate trigger reagent. Oligonucleotide-based reporter moieties can further be amplified by the polymerase chain reaction or captured by

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complimentary oligonucleotides immobilized on an insoluble substrate and detected and quantitated using methods commonly used in conjunction with nucleic acid-based microarrays. Numerous other options including electrochemical, impedance, enzymatic and radioactivity detection are also available.

Therefore, in certain embodiments, the invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are optionally labeled with a reporter label, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

e) optionally isolating and washing the capture substrate to remove unbound microparticle detection probes; and

f) detecting for the presence of the DNA barcode wherein the presence of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

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In other embodiments, the invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

a) providing at least one type of magnetic substrates, the magnetic substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are fluorescently labeled, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the magnetic substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the magnetic substrate so as to immobilize the target analytes onto the magnetic substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the magnetic substrate;

e) optionally isolating and washing the magnetic substrate to remove unbound microparticle detection probes; and

f) detecting for the presence of a fluorescent signal from the DNA barcodes wherein the presence of a fluorescent signal is indicative of the presence of a specific target analyte in the sample.

In additional embodiments, the invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

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a) providing at least one type of magnetic substrates, the magnetic substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are fluorescently labeled, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the magnetic substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the magnetic substrate so as to immobilize the target analytes onto the magnetic substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the magnetic substrate;

e) optionally isolating and washing the magnetic substrate to remove unbound microparticle detection probes;

f) subjecting the isolated washed magnetic substrate to conditions effective to release the DNA barcodes; and

g) detecting for the presence of the fluorescent signal of the DNA barcodes wherein the presence of a fluorescent signal of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

In still other embodiments, the invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

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a) providing at least one type of magnetic substrates, the magnetic substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of microparticle detection probes, the microparticle detection probes comprising a microparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes comprising a reporter label, wherein the specific binding complement bound to the microparticle detection probes binds to at least a second binding site of the target analyte;

c) contacting the magnetic substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the magnetic substrate so as to immobilize the target analytes onto the magnetic substrates;

d) contacting the immobilized target analytes with at least one type of microparticle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the microparticle detection probes to the analyte and to form a complex in the presence of the target analyte on the magnetic substrate;

e) optionally isolating and washing the magnetic substrate to remove unbound microparticle detection probes;

f) subjecting the isolated washed magnetic substrate to conditions effective to release the DNA barcodes; and

g) detecting for the presence of the reporter label of the DNA barcodes wherein the presence of the reporter label of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

In further embodiments, the invention provides methods for detecting for the presence of one or more target analytes in a sample, each target analyte having at least two binding sites for specific binding interactions with specific binding complements, in a sample comprising the steps of:

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a) providing at least one type of capture substrates, the capture substrates having bound thereto at least one specific binding complement of the specific target analyte that binds to at least a first binding site of the specific target analyte;

b) providing at least one type of particle detection probes, the particle detection probes comprising a microparticle or nanoparticle having bound thereto (i) at least one specific binding complement to a specific target analyte and (ii) a plurality of DNA barcodes, the DNA barcodes are optionally labeled with a reporter label, wherein the specific binding complement bound to the particle detection probes binds to at least a second binding site of the target analyte;

c) contacting the capture substrates with a sample believed to contain target analytes under conditions effective to allow for binding of the specific target analyte to the specific binding complement bound to the capture substrate so as to immobilize the target analytes onto the capture substrates;

d) contacting the immobilized target analytes with at least one type of particle detection probes under conditions effective to allow for binding between the target analytes and the specific binding complement bound to the particle detection probes to the analyte and to form a complex in the presence of the target analyte on the capture substrate;

e) optionally isolating and washing the capture substrate to remove unbound particle detection probes;

f) releasing the DNA barcodes from the particle detection probes by a chemical releasing agent; and

g) detecting for the presence of the DNA barcode wherein the presence of the DNA barcode is indicative of the presence of a specific target analyte in the sample.

In certain embodiments, the methods of the invention can further comprise the step of washing the capture substrate after the target analytes are immobilized thereon and prior to contacting the capture substrate with particle detection probes.

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A target analyte can be a protein or hapten and its specific binding complement can be an antibody comprising a monoclonal or polyclonal antibody. A target analyte can also be a nucleic acid molecule.

A microparticle can be labeled with a plurality of DNA barcodes.

In other embodiments, the methods of the invention can comprise prior to said detecting step and subsequent to said isolating and washing step, the steps of: subjecting the complex to conditions effective to release the DNA barcodes from the microparticle detection probes; and optionally amplifying the DNA barcode prior to said detecting.

Released DNA barcodes can be immobilized on a substrate prior to said detecting.

DNA barcodes can be directly bound to the microparticles or can be bound to the microparticles through any attachment moiety. The microparticles can further comprise oligonucleotides bound thereto and the DNA barcodes can be hybridized to at least a portion of the oligonucleotides bound to the microparticles.

A microparticle used in a method of the invention can be polymeric (such as polystyrene), glass, metallic, semiconductor, or ceramic.

A specific binding pair can be, for example: an antibody and an antigen; a receptor and a ligand; an enzyme and a competitive inhibitor; a drug and a target molecule; or two strands of at least partially complementary oligonucleotides.

In certain embodiments, a DNA barcode is biotinylated, radioactively labeled, or fluorescently labeled.

A target analyte can have more than two binding sites. In some instantces, in addition to its first binding site, a target analyte can be modified to include a second binding site.

In some embodiments, at least two types of microparticle detection probes are provided in a method of the invention, the first type of probe having a specific binding complement to a first binding site on the target analyte and the second type of probe having a specific binding complement to a second binding site on the probe.

In some instances, a plurality of microparticle detection probes are provided, each type of probe having a specific binding complement to different binding sites on the target analyte.

A specific binding complement and target analyte can be members of a specific binding pair. A "member of a specific binding pair" can comprise nucleic acid,

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oligonucleotide, peptide nucleic acid, polypeptide, antibody, antigen, carbohydrate, protein, peptide, amino acid, hormone, steroid, vitamin, drug, virus, polysaccharides, lipids, lipopolysaccharides, glycoproteins, lipoproteins, nucleoproteins, oligonucleotides, antibodies, immunoglobulins, albumin, hemoglobin, coagulation factors, peptide and protein hormones, non-peptide hormones, interleukins, interferons, cytokines, peptides comprising a tumor-specific epitope, cells, cell-surface molecules, microorganisms, fragments, portions, components or products of microorganisms, small organic molecules, nucleic acids and oligonucleotides, metabolites of or antibodies to any of the above substances. Nucleic acid and oligonucleotide comprise genes, viral RNA and DNA, bacterial DNA, fungal DNA, mammalian DNA, cDNA, mRNA, RNA and DNA fragments, oligonucleotides, synthetic oligonucleotides, modified oligonucleotides, single-stranded and double-stranded nucleic acids, and natural and synthetic nucleic acids.

The following are non-limiting examples of target analyte:specific binding complements. A target analyte can be a nucleic acid and the specific binding complement can be an oligonucleotide. A target analyte can be a protein or hapten and the specific binding complement can be an antibody comprising a monoclonal or polyclonal antibody. A target analyte can be a sequence from a genomic DNA sample and the specific binding complements can be oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the genomic sequence. Genomic DNA can be eukaryotic, bacterial, fungal or viral DNA. A target analyte can be a sequence from episomal DNA sample and the specific binding complements can be oligonucleotides, the oligonucleotides having a sequence that is complementary to at least a portion of the episomal DNA sequence. A specific binding complement and the target analyte can be members of an antibody-ligand pair.

A "capture substrate" can be any insoluble material to which analytes can be immobilized as described above and throughout this disclosure. A "capture substrate" as used herein captures target analytes from a sample, and can facilitate the separation of these captured target analytes (both before and after treatment with the detection probe) from the sample. Such substrates are typically physically large relative to the analyte and are preferably insoluble in the sample. In particular instances, the methods of the

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invention comprise the use of magnetic substrates, as described herein, which can be isolated by subjecting the magnetic substrate to a magnetic field.

Additional isolating steps in methods of the invention can comprise any separation methods known to those of skill in the art. For example, separation methods include, but are not limited to, filtration, sedimentation, flotation, hydrodynamics, or gradient methods. A filtration step can comprise, for example, a membrane that removes sample components that do not comprise DNA barcodes.

The DNA barcodes can be released from the microparticles to which they are attached by a chemical releasing agent that will disrupt binding of the barcode to a surface as described above and elsewhere in this disclosure. Such agents, as described herein, include, but are not limited to, any molecule that will preferentially bind to a microparticle through a thiol link such as other thiol- or disulfide-containing molecules, dithiothreitol (DTT), dithioerythritol (DTE), mercaptoethanol and the like, and reducing agents such as sodium borohydride that will cleave a disulfide linkage thereby releasing DNA barcodes from the microparticles to which they are attached.

DNA barcodes can also be released from microparticles by exposing the barcodes to conditions under which the DNA barcodes will dehybridize from oligonucleotides by which the barcodes were attached to the microparticles.

As described herein, a barcode can be labeled with a detectable reporter group. Suitable reporter groups include, but are not limited to, a fluorophore, a chromophore, a redox-active group, a group with an electrical signature, radioactive group, a catalytic group, or Raman label.

The Raman labels can be any one of a number of molecules with distinctive Raman scattering spectra. Unlike the enzymes used in enzyme immunoassays, these label species can be stable, simple, inexpensive molecules which can be chemically modified as required. The following attributes enhance the effectiveness of the label in this application: (a) A strong absorption band in the vicinity of the laser excitation wavelength (extinction coefficient near 10^4; (b) A functional group which will enable covalent attachment to a specific binding member; (c) Photostability; (d) Sufficient surface and resonance enhancement to allow detection of analyte in the subnanogram range; (e) Minimal interference in the binding interaction between the labeled and unlabeled specific binding members; (f) Minimal exhibition of strong fluorescence emission at the

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excitation-wavelength used; (g) A relatively simple scattering pattern with a few intense peaks; and/or (h) Labels with scattering patterns which do not interfere with each other so several indicator molecules may be analyzed simultaneously.

The following is a listing of some, but not all potential candidates for these Raman-active label: 4-(4-Aminophenylazo)phenylarsonic acid monosodium salt, arsenazo I, basic fuchsin, Chicago sky blue, direct red 81, disperse orange 3, HABA (2-(4-hydroxyphenylazo)-benzoic acid), erythrosin B, trypan blue, ponceau S, ponceau SS, 1,5-difluoro-2,4-dinitrobenzene, cresyl violet and p-dimethylaminoazobenzene. The chosen labels may be covalently attached to the specific binding members of interest or attached or associated with.

An important aspect of the invention is that multiple Raman labels may be bound to the particle to provide a multicoding Raman labels for indexing different particles. Thus, the invention includes a reagent which has multiple Raman dyes and a specific binding substance, such as DNA, RNA, antibody, antigen, small molecule bound to the particle. For particle-based detection probes, the Raman labels or dyes can be attached directly or indirectly to the particle. The Raman label can be modified with a functional group, e.g., a thiol, amine, or phosphine that can bind to the surface of the particle such as a metallic nanoparticle. If desired, the Raman dye can be further functionalized with a molecule such as oligonucleotides (e.g., polyadenosine, polythymidine) for enhanced nanoparticle stability or with a specific binding pair member (such as an oligonucleotide having a sequence that is complementary to at least a portion of a nucleci acid target or a receptor for a particular ligand). Alternatively, the Raman label can be conjugated with a molecule or any linker, e.g., polyA or polyT oligonucleotide, that bears a functional group for binding to the particle. Raman labels can be detected as described, for example, in US Patent Application Serial No. 10/431,341 filed May 7, 2003, which is incorporated by reference.

Examples 1-10 provided herein demonstrate the ability to detect simultaneously and, optionally, to quantitate very low concentrations of a large number of different target analytes in a clinical specimen, an environmental sample, an industrial process stream, or other such medium that potentially contains the target analytes. Examples 11 and 12 demonstrate the ability to more rapidly detect fewer different target analytes at similar to higher concentrations. Specifically, Examples 11 and 12 provide modifications of the

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methods presented and described in Examples 1-10 that allow the methods to be used in less demanding applications while retaining the advantageous sensitivity of the methods. Such modifications also facilitate the use of alternative detection techniques.

One example of a less demanding application requires the simultaneous detection of only a small number, typically between one and ten, of target analytes rather than the tens to hundreds that are within the scope of the methods described in Examples 1-10. In this instance, Examples 11 and 12 conveniently differentiate between and identify target analytes on the basis of the emission wavelengths of fluorescent labels attached to the oligonucleotides of the detection probes rather than by recovering and separating the oligonucleotides prior to detection as practiced in Examples 1-10. Furthermore, the methods described herein permit the detection step to be performed directly upon the ternary sandwich complexes formed between the substrate, target antigen and detection probe, or in solution depending upon the particular end use of the invention. These methods can additionally be extended to emulate the recovery and separation of oligonucleotides as practiced in Examples 1-10, but in a faster and simpler manner.

The methods of the invention require that a target have at least a first and a second feature that differ from each other and that permit the target analyte to be differentiated from all other materials that are present in the medium containing the target analyte. For illustrative purposes, it will be assumed that the target analyte is a protein and that the different features are different epitopes, each of which can be selectively bound by the corresponding antibody. However, any of the types of features and complementary chemical binding moieties, also referred to below as specific binding complements and binding pair members, described herein may be utilized in a similar manner in the practice of these improvements. Where it is desired to detect multiple target analytes that may be present in the medium, one preferred arrangement is for the pair of distinctive features that characterize any one of the target analytes that may be present to be different than the pairs of distinctive features that characterize all of the other target analytes that might be present in the same medium. Alternatively, one distinctive feature may be the same for all of the target analytes that may be present while the second distinctive feature is unique to each type of target analyte. In either case, it is desirable that none of the other materials that may be present in the medium have any of the distinctive features associated with the target analytes.

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