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(54) **Title:** METHOD FOR DETECTING MICROORGANISMS

(57) **Abstract:** As a method for Chlamydia assay, Europium-nanoparticles and a Chlamydia antibody are conjugated using an amide bond to trigger an immune reaction to a Chlamydia antigen. Thus, the conjugate sensitively reacts to the Chlamydia antigen to detect even a small amount of Chlamydia antigen. A time-resolved fluorescence (TRF) apparatus uses TRF to detect a signal generated by Europium. Since the Europium has a relatively long emission frequency, a more sensitive and precise reaction in detecting background interference or fluorescence is obtained than a short wavelength. An object of the present invention is to provide a fluorescence reader with a simple structure which leads to a low-cost design.



METHOD FOR DETECTING MICROORGANISMS

[Background of the Invention]

The present application relates to a method for developing a Rapid Diagnostic Test
5 (RDT) kit through active conjugation of Europium nanoparticles and a highly sensitive
method for detecting Chlamydia antigen (Chlamydia Ag) through Time Resolved
Fluorescence (TRF) image analysis.

Chlamydia may be divided into two species: *Chlamydia trachomatis* and *Chlamydia*
proctitis. *Chlamydia trachomatis* includes trachoma, inclusion conjunctivitis,
10 lymphogranuloma venereum conjunctivitis, nonspecific or non-gonorrheal urethritis, and
proctitis and is an etiologic agent for diseases in human eyes or genital organs.

Generally, *Chlamydia trachomatis* infection spreads throughout the group. It is
estimated that non-gonorrheal urethritis occurs over a million cases every year. Since
Chlamydia spreads widely, a reliable and highly sensitive test is believed to play an important
15 role in testing existence of organisms.

An immunoassay has been used as a test that employs an antibody-antigen immune
reaction. Such an immune test mainly uses ELISA or Western blot as a method of combining
an optical label with the catalysis of enzyme through the measurement of a signal obtained by
labeling a radiation luminescent or fluorescent material on an antibody or an antigen.
20 However, these methods suffer from the disadvantages that an apparatus for complex test
method and analysis is a large-sized apparatus and long time is required for analysis.

An antibody or the like, which is a target material of an immune sensor, exists in
small amount in a biological sample. For this reason, there is a need for highly sensitive

signaling technologies that are much superior in detection limit of a sensor to biosensor technologies. In addition, since an antibody and protein are susceptible to be structurally denaturalized by change in an external environment, an antibody and an antigen are susceptible to be transmuted. Thus, a biometric function is susceptible to be lost.

5 Ultrasensitive immunoassay methods are developed and used in clinical diagnostics to measure extremely low concentrations of specific compounds in highly complex samples. Although the sensitivity, reliability, rapidity, simplicity, and cost of these methods have steadily improved, further improvements are still needed and possible. The interest in new label technologies has especially increased because none of the commonly used direct or
10 enzyme-amplified radioactive, colorimetric, luminescent, or fluorescent reporters fulfills all of the requirements for an ideal label, including specific activity, size, non-toxicity, cost, stability, localization, and detection. Directly detectable labels such as fluorophores suffer from limited sensitivity, and enzyme-amplified or dissociation-enhanced methods lose spatial information.

15 Recently, new detection methods based on high specific-activity particulate labels, such as quantum dots, luminescent inorganic crystals, up-converting phosphors, fluorescent nanoparticles, and plasmon resonant particles, have been introduced to respond to future demands for clinical diagnostics and biological, genomic, and pharmaceutical research. These submicrometer-sized labels are coupled to specific binding reagents such as nucleic
20 acid probes, receptors, lectins, enzymes, and antibodies to detect specific molecules with sensitivities equal to or better than the best conventional labels available. In spite of the large molecular size and obvious steric problems, these particular labels have also been used successfully in solid-phase immunoassays. It has been recognized, however, that the

production, colloidal stability, and nonspecific binding of particle-protein bioconjugates may still require further improvements.

Time-resolved fluorometry and lanthanide labels were introduced for immunoassays 20 years ago. Since then, the dissociation-enhanced lanthanide fluoroimmunoassay 5 (DELFA) technology has been known as one of the most sensitive and reliable immunoassay platforms. The research on intrinsically fluorescent, inert, and stable lanthanide chelate and cryptate labels has led to the development of novel homogeneous and heterogeneous assays that are expected to be introduced into routine clinical diagnostics. Moreover, an advanced dissociation-enhanced technology, based on lanthanide cofluorescence, which amplifies the 10 long-lifetime fluorescence of Europium(III), terbium(III), samarium(III), and dysprosium(III), has been published. A unique feature of lanthanide chelate fluorescence, the absence of self-quenching effects from multiple labeling, makes them ideal and suitable for high-density cluster labels such as dyed latex nanoparticles. The highly fluorescent chelates used in the DELFA technology can also be used in fluorescent lanthanide(III) chelate nanoparticles 15 because the hydrophobic environment inside the latex protects the fluorescent chelates from environmental effects, such as solvent quenching, and stabilizes the kinetically weak complexes. The adaptation of appropriate chelates for all four lanthanides would enable a nanoparticle-based, quadruple-labeling technology with an extremely low detection limit and a direct, surface readout measurement.

20 Detecting low levels of target marker in a sample using classic fluorochrome is sometimes difficult and prone to errors because specific fluorescence signals tend to be low and are usually mixed with nonspecific signals. Furthermore, autofluorescence produced from specimen can cause interference. The fluorescence half-life of complex chelate of

lanthanide elements, e.g., Europium (EU) is as much as six orders of magnitude longer than conventional fluorescent labels. Consequently, the emission from lanthanide chelate can be distinguished from background fluorescence (which has a short decay half-life) by using a time-resolved fluorometer with an appropriate delay, counting, and cycle times. This unique dye confers luminescence with a decay time of >500 μ s, far longer than that of conventional fluorescent probes or autofluorescent samples, typically having decay times of <50 μ s. Thus, time-resolved fluorometry can virtually eliminate autofluorescence.

These Europium luminescent dyes feature long-wavelength emissions (~610 nm) that are well separated from the excitation peak (~365 nm). This unusually large Stokes shift permits the use of filter combinations that effectively isolate the desired luminescence signal (Harma et al., Europium nanoparticles and time-resolved fluorescence of ultrasensitive detection of prostate-specific antigen. 2001;47:561-568).

In the DELFIA system, lanthanide ions are dissociated from the chelating structure into the fluorescence enhancement solution. This additional enhancing step is required to provide an environment that effectively eliminates quenching water and contains energy-absorbing chelating compounds to transfer energy further to lanthanide ions. The lanthanide phosphors can be detected directly without any enhancing steps due to the water-protecting crystal structure. The disadvantage of using the lanthanide phosphors is the lack of light-absorbing groups that effectively transfer the absorbed energy to the lanthanide ions. By combining these properties into a latex particle, fluorescent particles with a very high specific activity could be prepared. They prepared latex particles which contained a thenoyltrifluoroacetone lanthanide chelate complexed with tri-n-octylphosphine oxide (naphthoyltrifluoroacetone in the DELFIA technology complexed with tri-n-octylphosphine

oxide) providing particles without quenching effects but having a light-absorbing group inside the particle. The polymer shell efficiently removes fluorescence-quenching water from the vicinity of the chelate by producing a hydrophobic environment. Extremely sensitive assays can be carried out using such particle labels. These nanosized polymer labels contain 5 30,000-2,000,000 Europium molecules entrapped by β -diketones, which have one of the highest quantum yields of the known lanthanide chelators (Harma et al., Europium nanoparticles and time-resolved fluorescence of ultrasensitive detection of prostate-specific antigen. 2001;47:561-568). This encapsulation has no negative effect on the fluorescence efficiency. For a 100 nm size Europium particle, the fluorescence yield is equivalent to about 10 3,000 molecules of fluorescein. Phycobiliprotein B-PE (perhaps the most fluorescent substance known) has a fluorescence yield equivalent to about 30 fluorescein molecules. Since a 100 nm particle is about 10 times the diameter of phycobiliprotein B-PE and a thousand times greater in volume/mass, these Europium particles are 100 times more fluorescent than B-PE on a molar basis. This particle will give ultrasensitivity for assay 15 because of its great fluorescence, broad Stokes shift and long-lived luminescence.

A rapid diagnostic test (RDT) kit is designed such that the amount of chemical coloring is determined according to the amount of an analysis target in a sample and the chemical coloring is observed by naked eyes. For this reason, the RDT kit encounters a limitation in finding out an objectified value. Accordingly, there is a need to develop an 20 apparatus for overcoming the limitation and enhancing sensitivity and accuracy.

Assays have been developed that employ fluorescent labels to facilitate detection of the analyte. Fluorescence is generally the result of a three-stage process. In the first stage, energy is supplied by an external source, such as an incandescent lamp or a laser, and

absorbed by the fluorescent compound, creating an excited electronic singlet state. In the second stage, the excited state exists for a finite time during which the fluorescent compound undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. During this time, the energy of the excited state is partially
5 dissipated, yielding a relaxed state from which fluorescence emission originates. The third stage is the fluorescence emission stage wherein energy is emitted, returning the fluorescent compound to its ground state. The emitted energy is lower than its excitation energy (light or laser) and thus of a longer wavelength. This shift or difference in energy or wavelength allows the emission energy to be detected and isolated from the excitation energy.

10 In response to some of the problems with conventional fluorescence detection techniques, a technique known as “time-resolved” fluorescence was developed. Time-resolved fluorescence involves exciting the fluorescent label with a short pulse of light, then waiting a certain time (e.g., between approximately 100 to 200 microseconds) after excitation before measuring the remaining long-lived fluorescent signal. In this manner, any short-lived
15 fluorescent background signals and scattered excitation radiation are eliminated. With the device development, the present invention is intended to overcome the limitation of a rapid diagnostic test (RDT) kit.

[Summary of the Invention]

20 According to the present invention, sensitivity is significantly improved by immunoassay where an antibody-antigen reaction is induced through specific binding to an antibody of Europium that a luminescent or fluorescent reporter. Further, more objectified,

highly sensitive, and accurate analysis can be accomplished through development of a TFR apparatus.

In one aspect, the present invention provides an immunoassay diagnostic device capable of qualitatively and quantitatively determining whether there is Chlamydia infection in a biological sample and the degree of the Chlamydia infection through quantification thereof. The present invention also provides a highly sensitive assay system, including but not limited to time-resolved fluorescent dye used in a lateral flow assay format, which ensures improved sensitivity while maintaining the beneficial aspects of lateral flow assay. The highly sensitive assay system is a chromatographic test system using Europium particles.

10 The assay system is characterized in rapidity, convenience, high sensitivity, and specificity.

[Brief Description of the Drawings]

Figure 1 shows scheme for preparing Europium encapsulated nanoparticle bound to antibody.

15 Figure 2 shows an outline for immunochromatographic assay.

Figure 3 shows details of lateral flow immunochromatographic assay

Figure 4 shows details of time-resolved fluorescence assay.

Figure 5 shows details of time-resolved fluorescence assay.

Figure 6 shows details of time-resolved fluorescence assay system.

20 Figure 7 shows details of time-resolved fluorescence assay system instrumentation.

Figure 8 shows details of time-resolved fluorescence assay system lens instrumentation.

[Detailed Description of the Invention]

In the present immunoassay diagnostic apparatus, an analyte may be without limitation an antibody and an antigen. A specific binding partner may be without limitation an antibody, an antigen, biotin, streptavidin or avidin.

- 5 The present invention is directed to a method for determining the presence of at least one type of an analyte in a sample comprising applying an amount of the sample to the apparatus described above. If at least one type of analyte is present in the sample, the sample migrates to a wicking membrane where a chemical reaction occurs. The presence of a signal indicates that the analyte is present in the sample, and the signal is a Europium combination.
- 10 The Europium particles may be Europium encapsulated carboxylate modified polystyrene particles. These particles are known as fluorescent nanomaterials with a superior fluorescent strength. The polystyrene particles are advantageous in nontoxicity, wide surface area, strong adsorption force, and antibody compatibility. In the polystyrene particles, polystyrene is modified to enhance efficiency for binding to an antibody, and encapsulated is Europium
- 15 which is a lanthanide element that can be observed with fluorescence. These particles excite at UV light (maximum absorbance wavelength: 333 nm) and emit at 613nm. These particles have a very long lifetime of approximately 0.5 milliseconds, which is about 10,000 to 100,000 times the lifetime of most fluorophores. Since their long lifetime allows time-resolved fluorescence to be applicable, light interference is reduced. Thus, these particles
- 20 may be used in an ultrasensitive specific system.

In the present invention, a sample may be a biological sample. Further, the sample may be without limitation blood, serum, plasma, urine, saliva, sweat, and liquid media processed from a biological or environmental sample.

An apparatus used in the method may be without limitation a lateral flow assay format apparatus, and the analyte specifically reacts to Chlamydia. The present invention is also directed to a kit comprising a compartment that contains the apparatus described above, and instructions for using the apparatus as described above. Time-resolved fluorometry
5 technology is a system based on Europium embedded micro particles conjugated with antigens and antibodies. This technology may be used in Chlamydia markers that require highly sensitive tools for their detection.

Europium may be used as fluorescent labels to form labeled reagents by adsorbing or covalently binding other proteinaceous compounds such as antibodies or antigens to the
10 surface of the polymeric latex beads.

Antibodies may be adsorbed to particles modified to polystyrene (PS) or carboxyl group by hydrophobic bond or ionic bond. Further, the antibodies may be adsorbed to the particles by bonding amide to an amino group of the antibodies through activation of the carboxyl group of the particles with water-soluble carbodiimide 1-ethyl-3-(3-
15 dimethylaminopropyl) carbodiimide (EDAC). This is a kind of covalent bond which is stronger than hydrophobic bond or ionic bond. In this covalent bond, a Europium-antibody conjugate may be maintained at a more stable state. A Europium-antibody conjugate is completed based on the foregoing, and high sensitivity is reproduced by comparison and examination of the Europium-antibody conjugate.

20 As used herein, "polymeric particle" refers to a spherical or near-spherical polymer particle at various sizes. Preferably, the size is about 0.05-0.5 μm in diameter. However, it is understood that the invention is not limited to the use of any particular type of polymeric

particle. In its broadest sense, any substance or particle that can encapsulate the fluorescent dye is encompassed by the present invention.

As used herein, "tag" refers to a substance that is labeled to the specific binding reagent, which is labeled with time-resolved fluorescent dye. The tag specifically reacts to
5 specific binder immobilized on solid phase. In particular, the tag may be biotin when the specific binder immobilized on solid phase is avidin or streptavidin.

The inventive test may be performed on-site by a lay person with minimal training while rapid results are obtained after adding one or two drops of sample to a disposable test device or card or strip. However, it is to be understood that the assay is not limited to an
10 antigen/antibody complex.

The present invention adopts a lateral-flow immunochromatography method for a rapid chromatography test which is capable of diagnosing *Chlamydia trachomatis* infection. A lateral flow test kit roughly comprises a membrane and a pad. The membrane and the pad are various in material, dimension, and pore size and were selected according to diagnosis
15 field and kit formation. In the present invention, the membrane and the pad are being tested considering compatibility with reagents and chemicals used in kit formation, such as a Chlamydia sample, a Chlamydia antibody, a Europium conjugate, and a fixed material, for diagnosis of Chlamydia. Each of the most compatible materials was processed. A wicking pad was attached on a top surface of the membrane to fix materials which constitute a control
20 line capable of capturing antibodies and a test line capable of capturing *Chlamydia trachomatis* and induce and control a flow of fluid. A pad for fixing a nanoparticle-antibody conjugate, i.e., dye was attached to a bottom surface of the membrane, which allowed the dye to migrate onto the membrane by binding to antigens. Thus, a target antigen reacts to a

labeled specific antibody contained in the dye pad when liquid is absorbed/flows along a card. The materials flow into a film fixed to at least one distinct site of an unlabeled antibody group. An antigen-labeled antibody complex is maintained and captured to form a predetermined line for reading. The predetermined line may be detected by a TRF-mounted
5 reader or any suitable detector.

As used herein, "time-resolved fluorometer" refers to a tool for measuring time dependence of fluorescence intensity after a short excitation pulse which can also be made as a function of emission wavelength.

In the time-resolved fluorometer, a wavelength of a light source is 365 nm and power
10 consumption is about 2.5 W. A CCD has 2048 x 2048 pixels each having a size of 7.4um x 7.4um and 12 bits of resolution. In order to increase sensitivity, 4 x 4 pixels are merged into a single pixel before being used. Accordingly, the final resolution uses 512 x 512 pixels. The CCD is turned on after 263 um have passed since an LED was turned off. Each pixel has 12 bits of resolution, but uses only 8 bits of resolution for the purpose of convenience. If a
15 silicon detector (Si-Detector) and a 12-bit analog-digital converter (12BIT ADC) are used, they exhibit sensitivity of 12 dB or higher as compared to the CCD. Therefore, it will be estimated that they have a sufficiently assayable value with respect to even a sample diluted 10,000 times or more. Further, if 340 nm that is an optimal wavelength to fluorescence is used in the silicon detector and an optical system design is optimized to limit a light-taken
20 region of a source-sample-detector to 1mm x 1mm, it will be estimated that a signal of 6 dB or higher is obtained as compared to conventional methods. Optical loss was measured at each wavelength band in the ultraviolet-visible-infrared (UV-Vis-IR) region of a manufactured optical fiber. A result of the measurement was that light transmissivity of the

optical fiber was about 89%. This makes it possible to overcome disadvantages of conventional time-resolved fluorescence (TRF) implementing methods (e.g., a signal including much noise is detected due to deficiency in the amount of light in case of an image sensor such as a CCD or a CMOS).

What is claimed is:

1. An analyte detection apparatus system comprising:

lateral flow pad,

5 lanthanide encapsulated particle, and

time-resolved fluorometer mounted reader positioned so as to be able to read the lateral flow pad.

2. The apparatus system according to claim 1, wherein a detector for the time-resolved
10 fluorescence assay system is silicon detector.

3. The apparatus system according to claim 1, wherein the lanthanide is Europium.

4. The apparatus system according to claim 1, wherein the particle is nanoparticle.
15

5. The apparatus system according to claim 1, wherein the ligand is antibody.

6. The apparatus system according to claim 1, wherein the specific partner is specific for Chlamydia antigen.
20

7. The apparatus system according to claim 1, wherein the particle is composed of a polymer.

8. The apparatus system according to claim 7, wherein the particle is composed of polystyrene.
25

9. The apparatus system according to claim 1, wherein the particle displays carboxyl group on its surface.

10. The apparatus system according to claim 1, wherein the lateral flow pad comprises a test line and control line, wherein the test line comprises lanthanide encapsulated particle attached to a specific binding partner of the analyte.

5 11. A method of detecting analyte in a sample comprising:

applying sample to the lateral flow pad according to claim 1, wherein the sample migrates to wicking membrane where a chemical reaction occurs thereby generating a signal;

detecting by time-resolved fluorometer the area where the chemical reaction occurs, wherein presence of a signal generated by lanthanide(III) in a particle bound to a specific

10 partner to the analyte, is in complex with said analyte, indicates that the analyte is present in the sample.

12. The apparatus system according to claim 11, wherein a detector for the time-resolved fluorescence assay system is silicon detector.

15

13. The apparatus system according to claim 11, wherein the lanthanide is Europium.

14. The apparatus system according to claim 11, wherein the particle is nanoparticle.

20 15. The apparatus system according to claim 11, wherein the ligand is antibody.

16. The apparatus system according to claim 11, wherein the specific partner is specific for Chlamydia antigen.

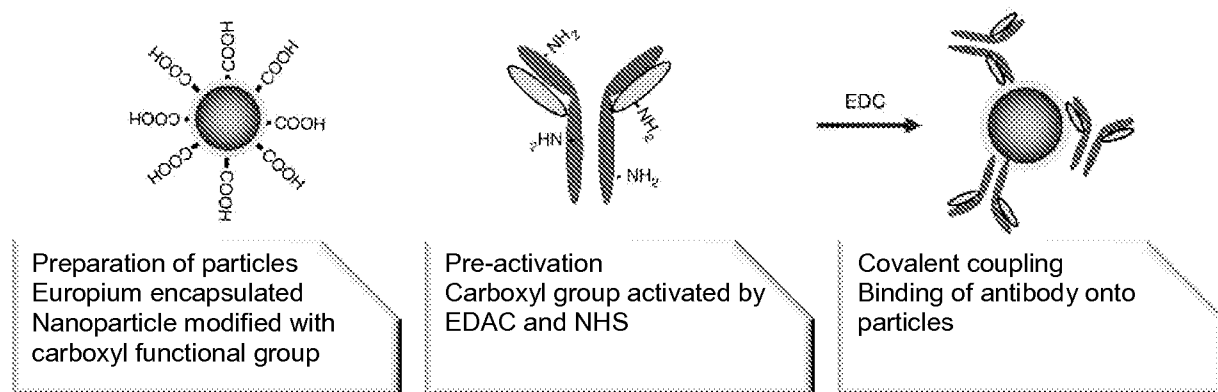
25 17. The apparatus system according to claim 11, wherein the particle is composed of a polymer.

18. The apparatus system according to claim 17, wherein the particle is composed of polystyrene.

30

19. The apparatus system according to claim 11, wherein the particle displays carboxyl group on its surface.

20. The apparatus system according to claim 11, wherein the lateral flow pad comprises a test
5 line and control line, wherein the test line comprises lanthanide encapsulated particle attached to a specific binding partner of the analyte.

FIGURE 1

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FIGURE 2

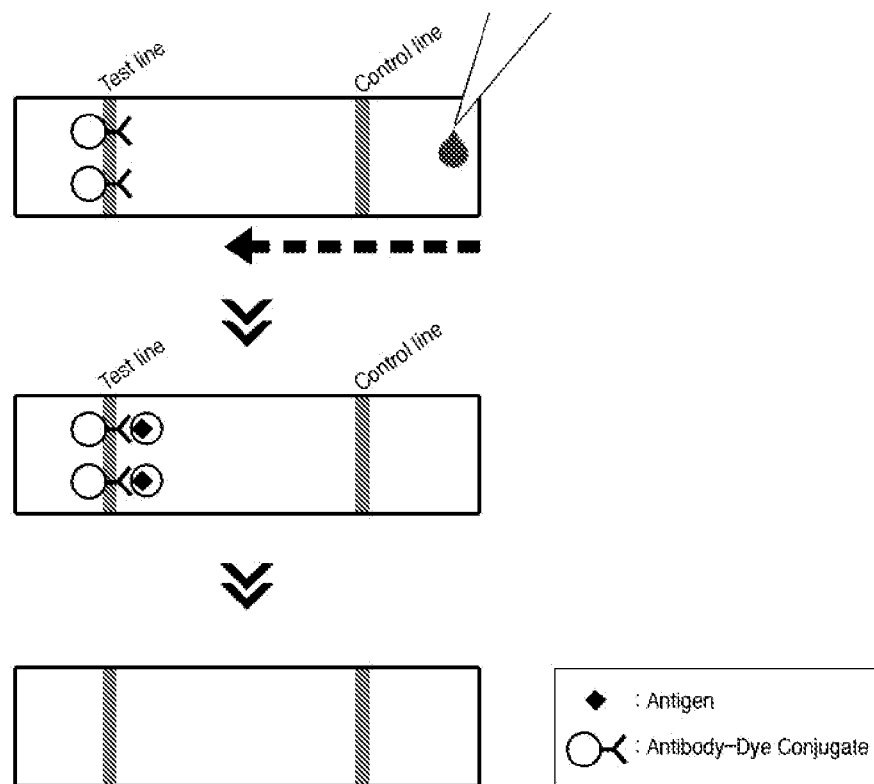
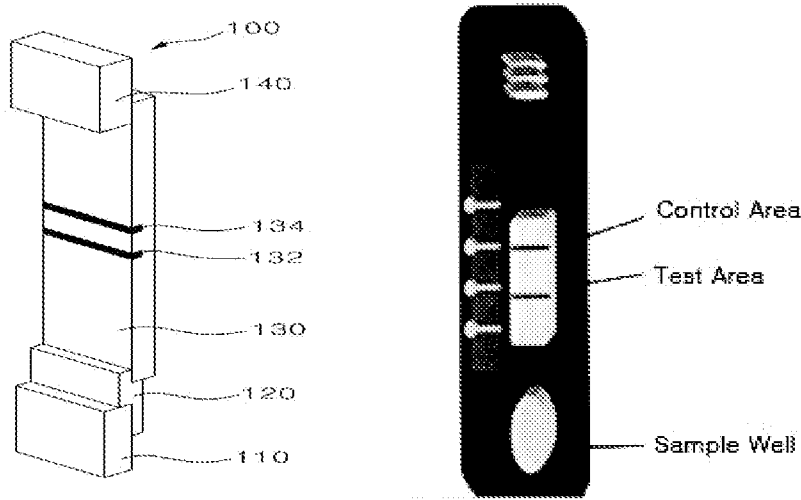
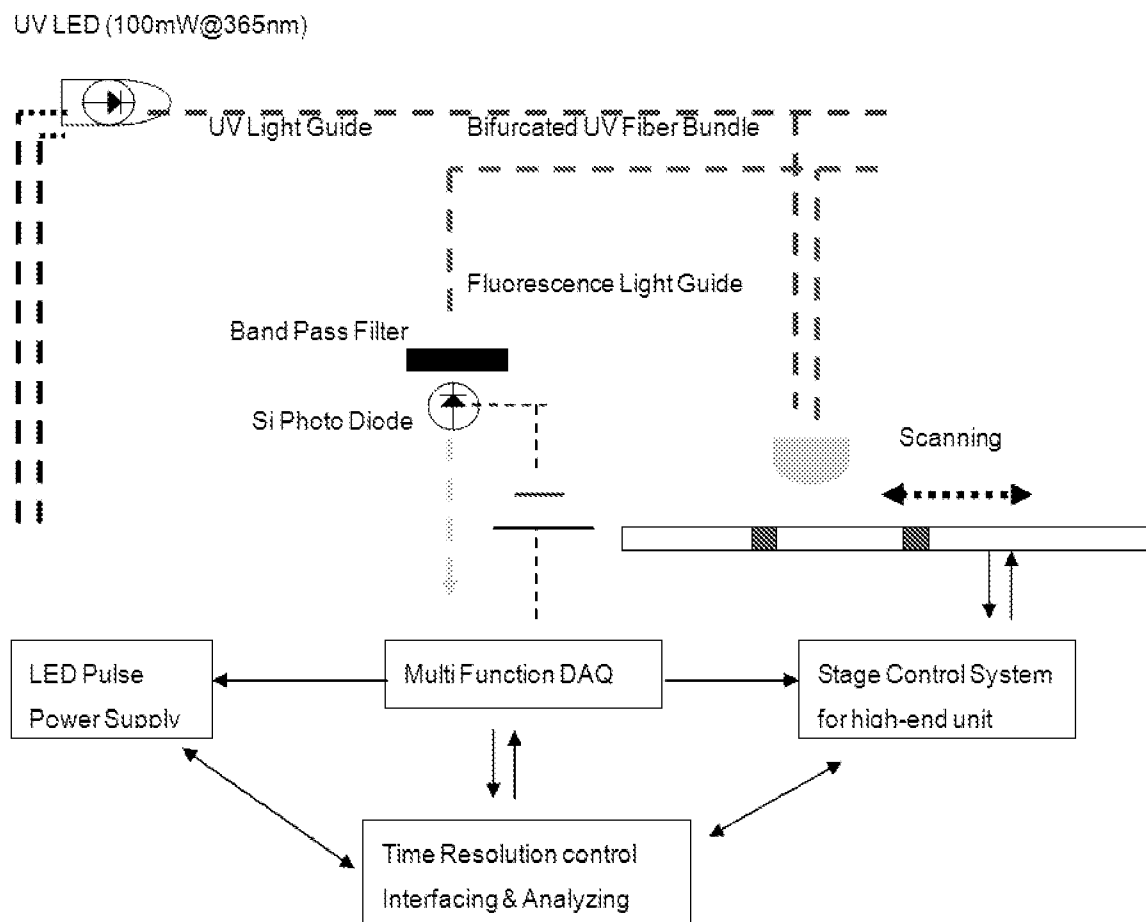


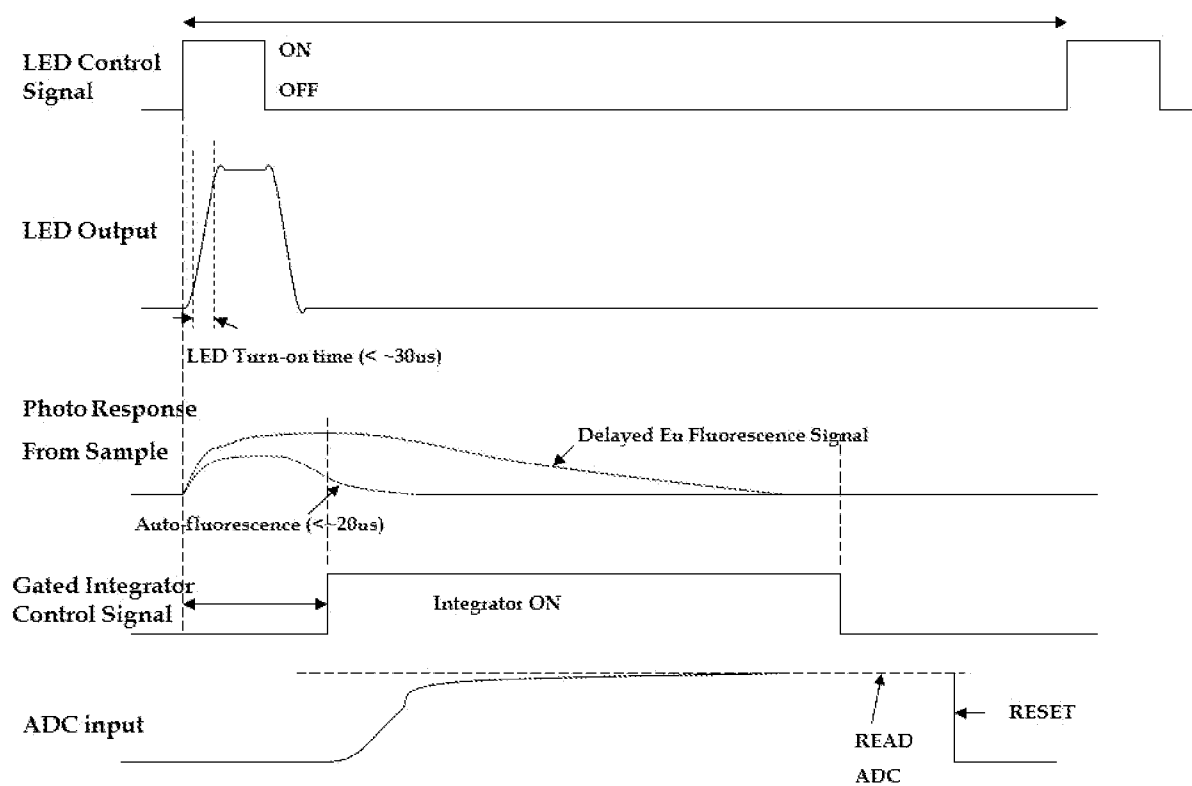
FIGURE 3



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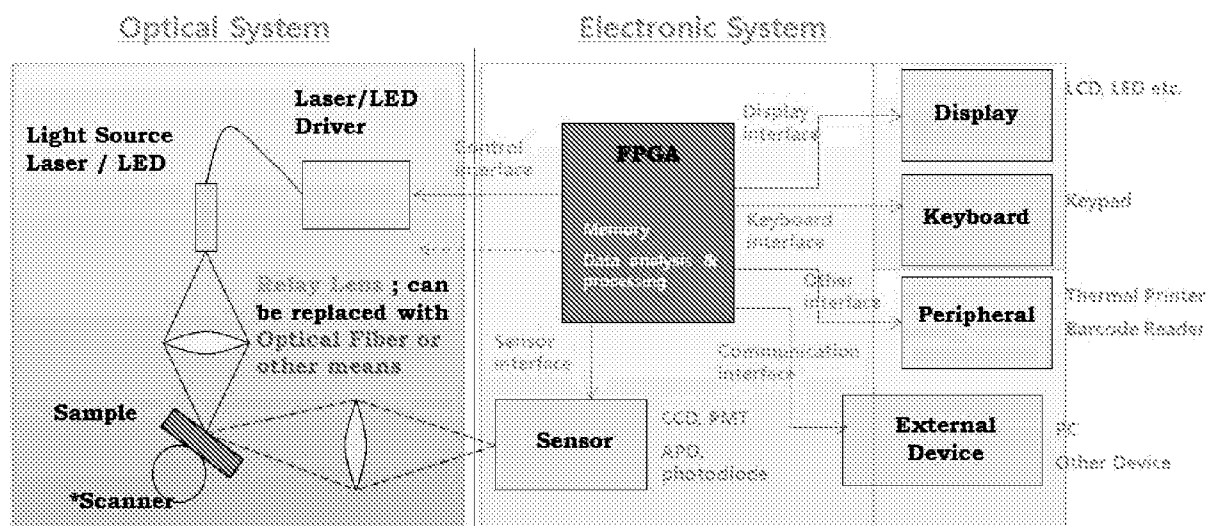
FIGURE 4

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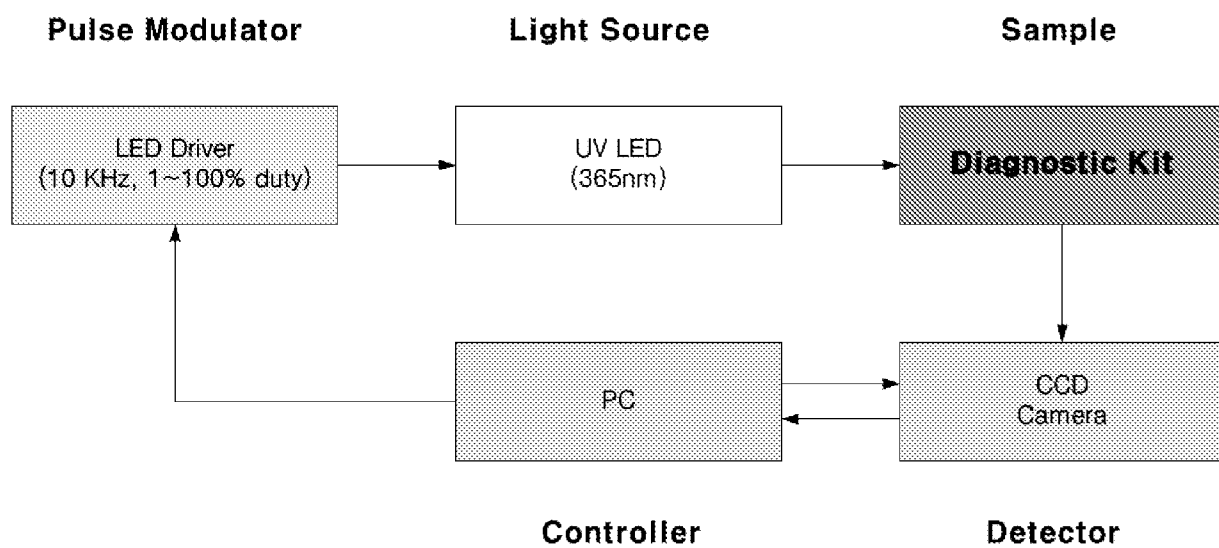
FIGURE 5

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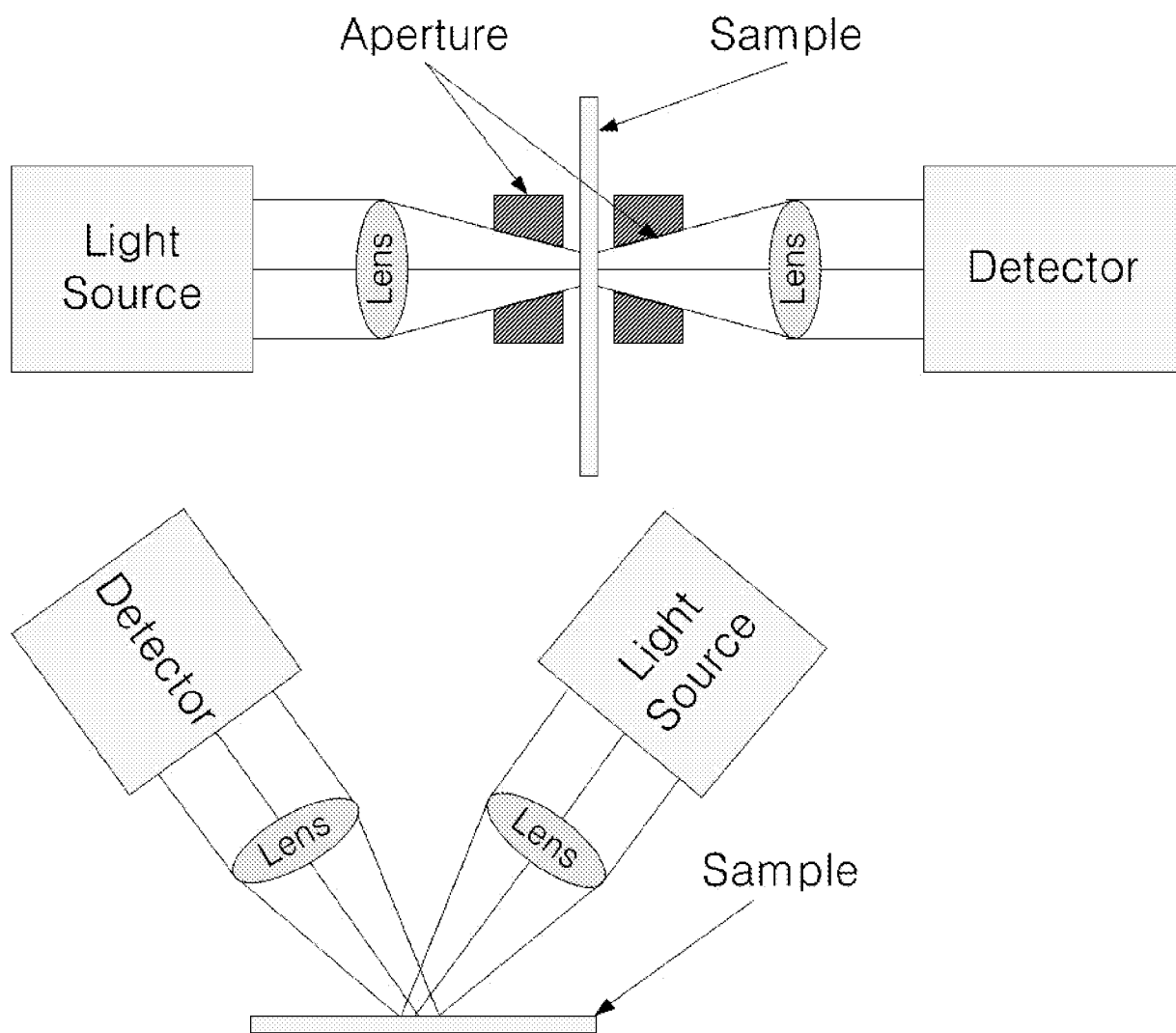
FIGURE 6



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FIGURE 7

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FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/47737

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - G01N 33/558; G01N 33/543 (2012.01)
 USPC - 436/514, 436/518

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)
 USPC-436/514

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 USPC-436/518

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 PubWest (PGPB, USPT, USOC, EPAB, JPAB, DWPI, TDBD), FreePatentsOnline (US Pat, PgPub, EPO, JPO, WIPO, NPL),
 GoogleScholar (PL, NPL); search terms: analyte detection apparatus system lateral flow pad lanthanide encapsulated particle time-
 resolved fluorometer mounted reader silicon detector Europium nanoparticle antibody Chlamydia antigen polystyrene

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/0219690 A1 (Choi et al.) 4 November 2004 (04.11.2004) para [0009], [0027], [0059]-[0064]	1-5, 7, 9-11, 13-15, 17, 19-20
X	US 2008/0199851A1 (Egan et al.) 21 August 2008 (21.08.2008) para [0008], [0031], [0041], [0092], [0129], [0180], [0207], [0274]	1-2, 6, 8, 11-12, 16, 18
A	US 2003/175812 A1 (Reppy et al.) 18 September 2003 (18.09.2003) [0044]-[0049], [0064]-[0071],	1-20

☐ Further documents are listed in the continuation of Box C.

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Date of the actual completion of the international search

01 October 2012 (01.10.2012)

Date of mailing of the international search report

16 OCT 2012

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