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(54) Title: DIAGNOSTIC METHODS

(57) Abstract: The present invention provides a novel method that combines the features associated with a largely conventional FANA slide test and those of a largely conventional multiplex array assay into a single assay performed substantially simultaneously, with minimal modifications to each assay, to provide physicians and other users of the method with previously unknown advantages such as ease of use and enhanced assay speed that are useful, for example, for the diagnosis and assessment of autoimmune disorders.

DIAGNOSTIC METHODS

Cross-Reference to Related Applications

This application is a non-provisional application, which incorporates by reference herein and claims priority of US Provisional Application No. 61/429,892 and US Provisional Application No. 61/524,630.

Background of the Invention

Field of the Invention

Generally, the present invention relates to the field of diagnostic assay systems, and in particular is useful for diagnosing and assessing autoimmune disorders, and more specifically the invention relates to novel methods for performing auto-antibody diagnostic assays.

Background Art

Antibodies are proteins produced by the body in response to invading or infectious materials. They constitute one of the many means the body has to protect itself from disease. In normal circumstances, the body can recognize "foreign" from "self" tissues, and therefore only generates antibodies to those materials that are "foreign" to the body.

Autoimmunity develops when the body begins to produce antibodies to its own tissues. There are numerous types of autoimmune disorders; some systemic and others that are organ specific. The exact mechanism that initiates these diseases is not fully understood. Some may be easily treated with minimal patient impact while others can be quite severe and even fatal.

The symptoms associated with the onset of an autoimmune disorder can be varied. Also, any of the early symptoms can mimic those of many other diseases. Typically, if a patient visits a physician with symptoms remotely suggestive of an autoimmune disorder, the physician will usually suggest that an anti-nuclear antibody (ANA) test be performed. The ANA test is a good "first round" screening test to see if somebody has antibody to self tissues. There are many different versions of the ANA Screen test. Methodologies can range from western blot to sophisticated microparticle arrays; however, the most popular is probably the fluorescent anti-nuclear antibody assay (FANA).

At the present time, the most popular of the commercially available FANA tests is the so-called HEp-2 FANA test. HEp-2 is a continuous, human epithelial cell line that grows rapidly and readily attaches to solid surfaces such as tissue culture flasks and glass slides. The HEp-2 cell also has a large, well defined nucleus. The HEp-2 FANA test is made by growing HEp-2 cells on glass slides and fixing the cells permanently on the surface of the slide. The assay is performed by allowing a serum sample from a patient to react to the cells. If anti-nuclear antibodies are present, they will bind to the cells. The slides are washed and the antibody is labeled with anti-human Immunoglobulin (Ig) labeled with a fluorescent tag. When viewed using a properly equipped microscope, specific patterns of reactivity are observed which are associated with specific autoimmune disorders. Examples of these patterns are shown in Figure 1 of the drawings.

Auto-antigens, used in the ANA test, represent the majority, but not all, of known auto-antigens expressed on the HEp-2 cell. Some auto-antigens found on the HEp-2 cell are not present in the multiplex suspension. Consequently, the multiplex bead method, such as the AtheNA Multi-Lyte ANA Test System (commercially available from Zeus Scientific, Inc.,

Bridgewater, NJ), contains a bead mix that is built on the Luminex xMAP® platform (commercially available from Luminex Corporation, Austin, TX) using only a highly purified auto-antigen mixture conjugated onto separate microspheres and run as a multiplex assay. This system has most, but not all, of the auto-antigens found on the HEp-2 cell. For that reason, it would be desirable to include an actual HEp-2 cell along with the multiplex bead mix to ensure that the patient sample is tested for all potential autoantigens substantially simultaneously (all known and yet unknown autoantigens). However, while these types of sophisticated ANA tests are generally more specific than the traditional FANA using HEp2 cells, they can be perceived to be under-sensitive, since they incorporate a limited and finite number of antigens. Collectively, these auto-antigens represent the majority of known auto-antigens expressed in the HEp-2 cell; however, the natural HEp2 cell theoretically supplies any and all auto-antigens that could likely exist *in vivo*.

While the HEp-2 FANA method has become the reference method, the aforementioned AtheNA Multi-Lyte ANA Test System provides substantially equivalent results, and there are those that feel that the ANA multiplex arrays are adequate and there are those that feel that only the HEp2 cell is sufficient in screening for ANA. However, it is generally agreed that following an acceptable screening method, providing the specific data from a multiplex array would be of extreme value.

Summary of the Invention

In contrast to the conventional methodologies previously known, it is an object of the present invention to provide a novel method that combines the features associated with a largely conventional FANA slide test and those of a largely conventional multiplex array assay into a single assay performed substantially simultaneously, with minimal modifications

to each assay, to provide physicians and other users of the method with previously unknown advantages such as ease of use and enhanced assay speed that are useful, for example, for the diagnosis and assessment of autoimmune disorders. The present invention additionally provides novel and unexpected advantages over the methods described and claimed in published International application No. PCT/US2009/056307, commonly assigned herewith and the disclosure of which is hereby incorporated by reference in its entirety. In contrast to the inventions disclosed and claimed in the above-referenced International application, the present invention has been found to achieve substantially similar goals and results without the necessity of using multiplex beads or instrumentation as described therein, by employing, such as by printing, an array of antigen spots in proximity to cells that are fixed on a glass slide or other solid phase, and therefore the present invention is unexpectedly advantageous in that it does not require the involvement of multiplex bead arrays or instrumentation, such as the Luninex instrument referred to in said application.

Thus, it is an object of the invention to improve upon the technology disclosed in the art referenced above and upon the invention disclosed in the above-referenced PCT application, by combining in a novel way the aforementioned conventional FANA slide test and a multiplex array into a single assay, with minimal modifications to each. So far as is known, it has not yet been attempted to combine these two very different immunoassays into a single immunoassay.

In accordance with the invention, these advantages are unexpectedly achieved from a combination of substantially simultaneous performance of these two types of immunoassays.

Brief Description of the Drawings

Figure 1 shows examples of positive ANA reactions; specific patterns of reactivity are shown using HEp-2 FANA (performed using a conventional slide method).

Figure 2 shows a dual-spotted slide array for cells utilizing the methods of the present invention.

Figure 3 shows a slide and flexible grid used to form wells for performing the methods of the present invention.

Figure 4 shows a formation of wells on a slide useful for performing the methods of the invention, so that each well contains two areas; one with cells and one with a defined antigen array.

Detailed Description of the Invention

As discussed above, the symptoms associated with the onset of an autoimmune disorder can be quite varied. Also, any of the early symptoms can mimic those of many other diseases. The ANA screening test is widely recognized as a good "first round" screening test to see if a patient has antibody to self tissues, the currently most popular being the fluorescent anti-nuclear antibody assay (FANA), and the currently most popular of the FANA tests being the HEp-2 FANA. The assay is performed by allowing the patient serum to react to the cells. If anti-nuclear antibodies are present, they will bind to the cells. The patient antibody is then tagged with anti-human Ig labeled with a fluorescent moiety. When viewed using a properly equipped microscope, specific patterns of reactivity are observed (if present) and some have been highly associated with specific autoimmune disorders. Examples of some positive reactions appear in Figure 1 of the drawings.

Accordingly, the present invention, in preferred embodiments, provides novel methods for the substantially simultaneous analysis of autoantigens in a single sample from a subject by combining the known FANA slide tests and multiplex arrays into a single assay, with minimal modifications to each. The HEp-2 FANA test comprises a slide test using HEp-2 cells that have been allowed to grow in TC media on the surface of glass slides. The slides are then rinsed and the cells fixed with conventional organic solvents, thus permeabalizing the membrane and keeping the cells adhered to the glass slides. This assay can be performed, for example, in the small wells of a microscope slide.

Definitions:

1. Multiplex Array Platform: as used herein, a multiplex array platform is any platform that enables one to test for multiple analytes simultaneously. Common array systems involve spotting target molecules in precise locations on a solid support such as glass or plastic. A popular example of a widely used multiplex array platform is the system developed and commercially available based on the Luminex® xMAP® Technology (Luminex Corporation, Austin, TX). Other arrays are known to be fabricated with spots, wells, posts, beads, cantilevers, wires, electrodes or fiber optics (see Clinical Chemistry; 56:12 (2010)).
2. Reporter Molecule: as used herein means the reporter molecule is the fluorescent, visual or chemiluminescent tag that is bound to the detection molecule(s) in the assay. In the case of immunoassays designed to measure human antibody, the detection molecule may be goat anti-human IgG (for example) that is labeled with phycoerythrin (for example).

3. Cellular substrate: as used herein means any cellular material or tissue substrate that is generally adhered to a solid support (glass or plastic microscope slide) and then used as part of a biochemical assay for analysis of biomolecules such as antibody or antigens.

The combination assay as provided by the present invention therefore can be performed without the use of, instrumention such as for example, a conventional Luminex xMAP instrument, which is essentially a flow cytometer that has been modified for use of the Luminex polystyrene microspheres in a multiplex bead assay. The present invention is therefore greatly advantageous over know methods in the art for performing similar assays, in the aspect of simplicity and lack of need for expensive instrumentation, among other advantages which will be apparent to those skilled in the art.

In a preferred embodiment, the array in the case of the present invention comprises a series of purified antigens that have been spotted onto a glass slide in proximity to the cellular substrate containing cells that are of interest to analyze. The array can be as simple or as complex as desired, depending on the nature of the assay in question. For the ANA example, it is presently preferred that a minimum of 10 highly purified auto-antigens, and preferably a maximum of 20 to 25 highly purified auto-antigens, be used.

For example, the practice of the methods of the present invention typically begins with a glass slide. Preferably, a glass slide with two "areas" per "well" (see Figure 2 of the drawings) is used. The cells of interest are en applied, thereby creating a cellular substrate and fixed into the circular wells in the image shown in Figure 2. Then, the purified antigens are spotted in proximity to the substrate as an array in the square wells shown in Figure 2. This slide is thereafter packaged and provided to the user of the assay system, for further analysis of patient specimens in accordance with the user's needs. For analysis, the slide

may be modified as needed by the user so that wells are formed for each patient specimen. The wells can be configured, for example, so that each well contains one circle (HEp2 cells) and one square (purified antigen array). Figures 3 and 4 of the drawings depict the formation of these wells. This slide can then be used to evaluate patient specimens (for example, serum, urine, plasma, etc.) for reactivity to the combination of the cells and the antigen array, substantially all simultaneously, using suitable reporter molecules well known to those skilled in the art, and thereby providing a novel way to collect the combination of reactivity to naturally occurring biomolecules within fixed cells or tissues, as well as an array of highly specific, highly purified biomolecules.

In a preferred embodiment of the present invention, the HEp-2 FANA test comprises a slide test using HEp-2 cells that have been allowed to grow in TC media on the surface of the glass slide. They are rinsed and fixed with some organic solvents, which permeabalizes the membrane and keeps them adhered to the glass slide. This assay has conventionally been performed in tiny wells of a microscope slide. However, the array (in this case according to the invention) is a series of purified antigens that have been spotted onto a glass slide for reactivity to the combination of the cells and the antigen array all substantially simultaneously.

It is to be appreciated that in addition, while the above example focuses on measuring autoantibodies in patients with possible autoimmune disorders, the present invention could be applied to a myriad of other examples including autoimmune, infectious disease, cancer diagnostics, cytological studies, to name only a few. Other examples will be apparent to those skilled in the art.

It will also be appreciated that in addition to analysis of serum samples as described above, the methods and teachings of the present invention can be applied to analysis of any biological fluid that may be obtained from a subject; for example, blood, urine, cerebral spinal fluid and plasma, as well as other biological fluids as are well known to those skilled in the art, can all be suitable for obtaining samples upon which to perform the methods of analysis in accordance with the present invention.

Further, it is to be appreciated that many additional modifications and variations, that will be apparent to and appreciated by those skilled in the art in view of the disclosure herein, may be made in the specific embodiments of the invention as described herein, and that all such modifications are fully within the scope of the present invention, which is intended to be limited only by the claims appended hereto.

What Is Claimed Is:

1. A method for performing diagnostic assays on a biological sample, which method comprises combining a FANA slide test assay and a multiplex array assay to produce a single assay system.
2. The method of claim 1, wherein the assays comprise auto-antibody immunoassays.
3. The method of claim 1, wherein the assays comprise an array of antigen spots in proximity to cells that are fixed on a glass slide or other solid phase.
4. The method of claim 1, wherein the antigen spots are applied by printing.
5. The method of claim 1, wherein the assays are performed substantially simultaneously.
6. The method of claim 1, wherein the assay system comprises a slide and flexible grid used to form wells.
7. The method of claim 6, wherein the wells each contain two areas, one with cells and one with a defined antigen array.
8. The method of claim 1, wherein at least 10 highly purified auto-antigens are used in the assay system.
9. The method of claim 1, wherein the assay system is useful for the diagnosis and assessment of conditions selected from the group consisting of autoimmune disorders, infectious disease or cancer diagnostics, or for cytological studies.
10. The method of claim 1, wherein the sample is selected from the group consisting of serum, blood, urine, cerebral spinal fluid or plasma, as well as other biological fluids.

Fig. 1

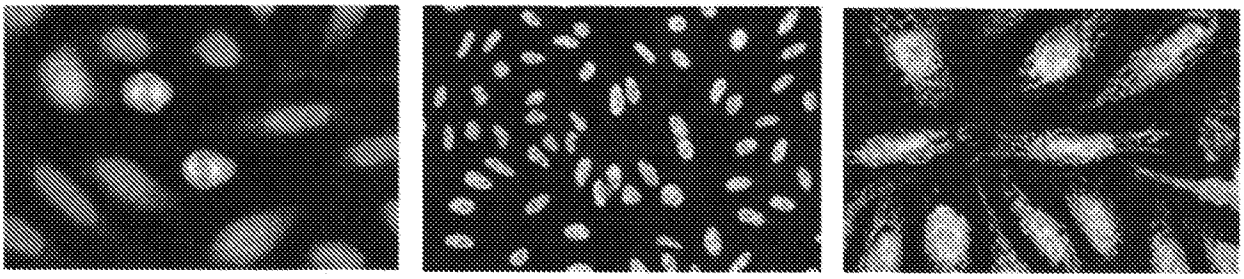


Fig. 2

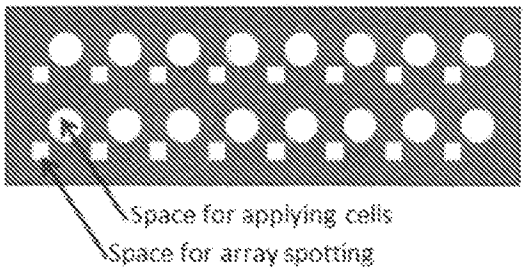


Fig. 3

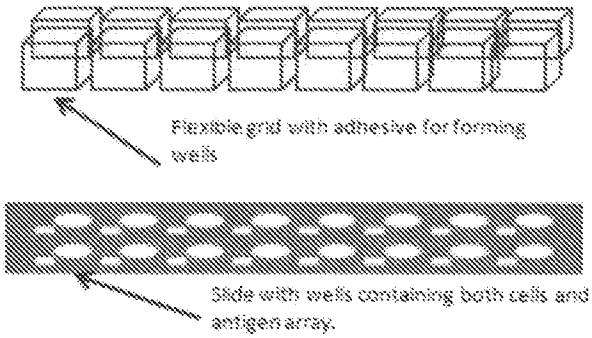
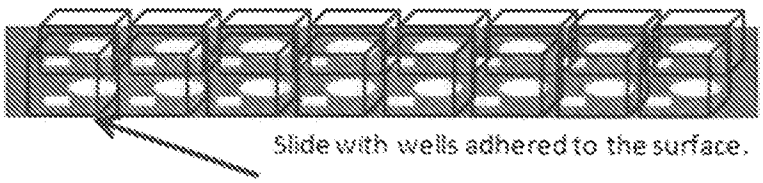


Fig. 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/020234

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53 (2012.01)
USPC - 435/7.1
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(8) - A61K 39/395; A61P/ 37/00; C12Q 1/68; G01N 33/50, 53 (2012.01)
USPC - 424/130.1; 435/6, 7.1, 7.9, 287.1, 287.2; 436/501

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)
PatBase, Orbit.com, Google Patents, Proquest

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/030624 A1 (KOPNITSKY) 18 March 2010 (18.03.2010) entire document	1-3, 5-10
Y		4
Y	US 2008/0254482 A1 (MATTOON et al) 16 October 2008 (16.10.2008) entire document	4
A	US 6,939,720 B2 (CHANDLER et al) 06 September 2005 (06.09.2005) entire document	1-10
A	US 2010/0285986 A1 (MENGES et al) 11 November 2010 (11.11.2010) entire document	1-10

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 April 2012	23 MAY 2012
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. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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