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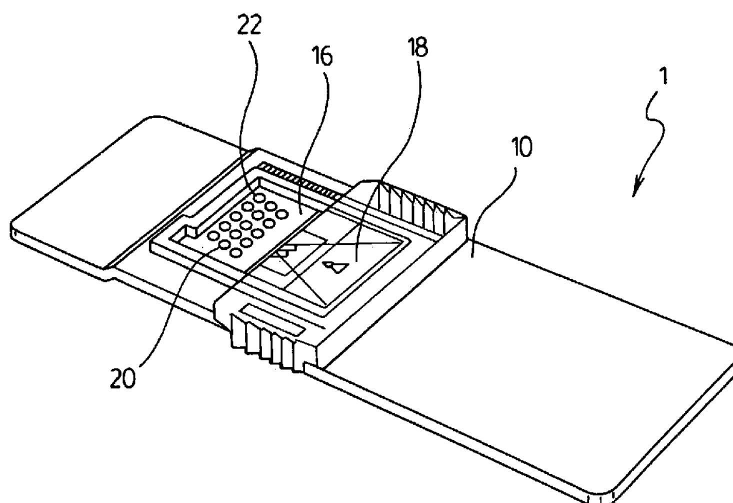
(54) **Title:** METHOD TO MEASURE DYNAMIC INTERNAL CALIBRATION TRUE DOSE RESPONSE CURVES



(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.
5 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.

10 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.
15 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.

20 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
25 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
30 interfere with the sample matrix to no longer reflect the content of the patient

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

5 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 10 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 15 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 20 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.

25 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 30 integrated to form the dynamic internal calibration true dose response curve. In a

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.

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.

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 5 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 10 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.

15 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 20 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, 25 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 30 for the purposes of the present invention.

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

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
5 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
10 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
15 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

20 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
25 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
30 mean values for each concentration tested (r=0.9786). Figure 2 is a graph that

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.

5 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 10 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 15 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.

20 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 25 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 30 Array printing technology on the chip assay device platform.

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
5 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
10 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.

15 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.

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.

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.

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.

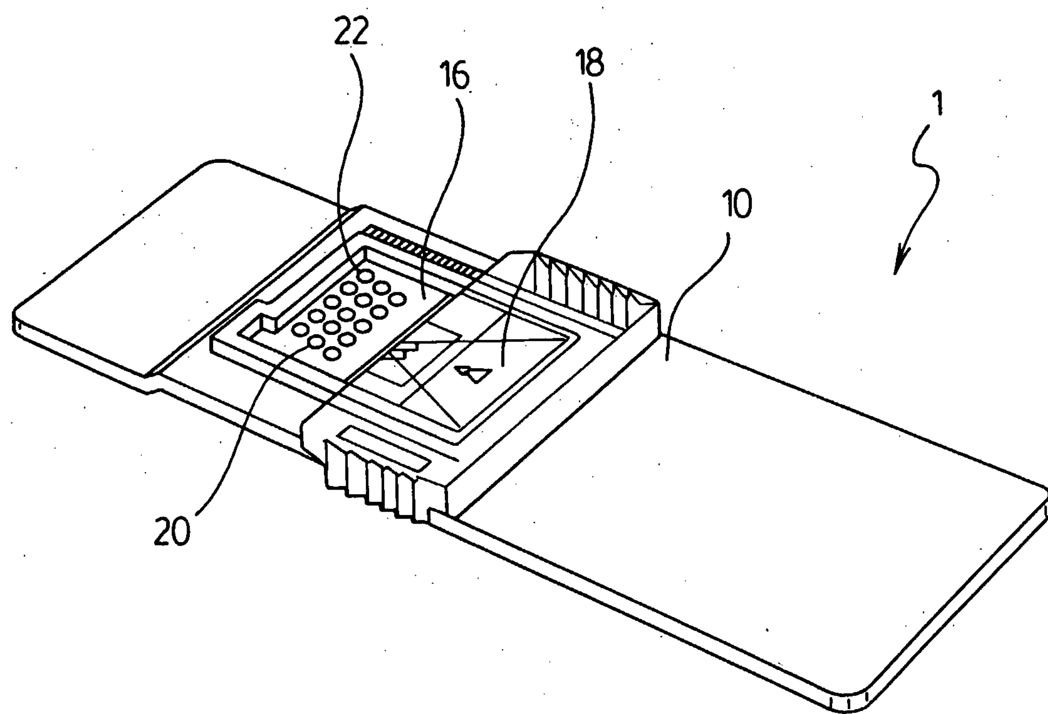

FIG.1.

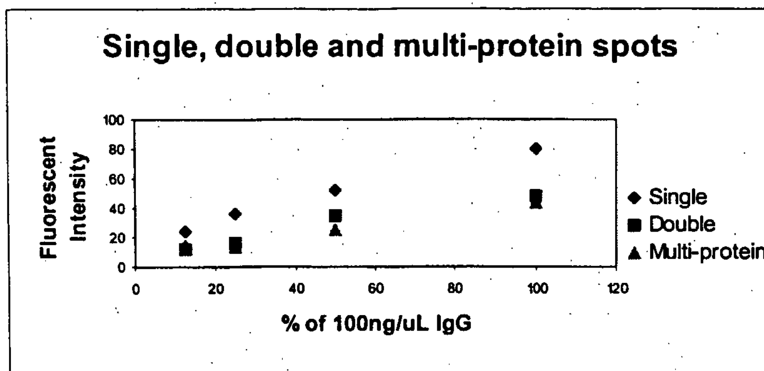

Figure 2

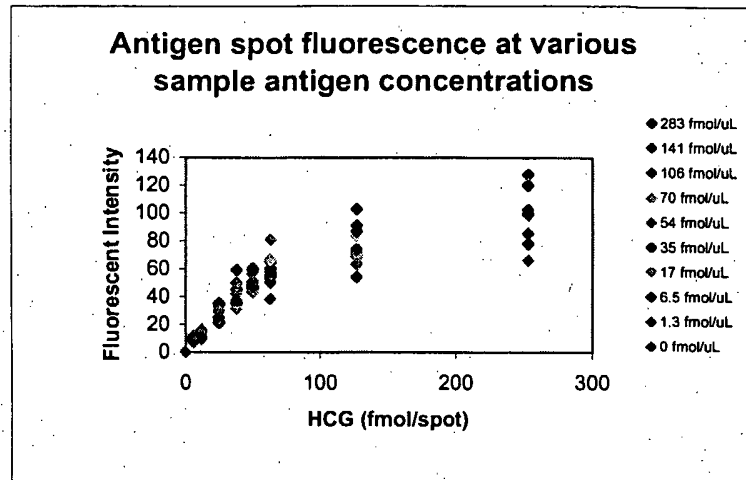

Figure 3

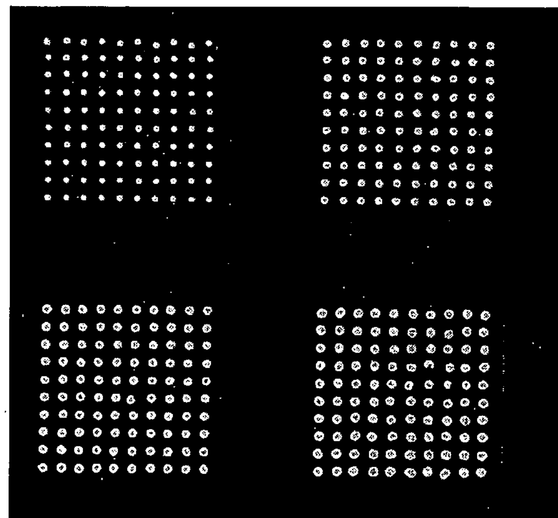

Figure 4

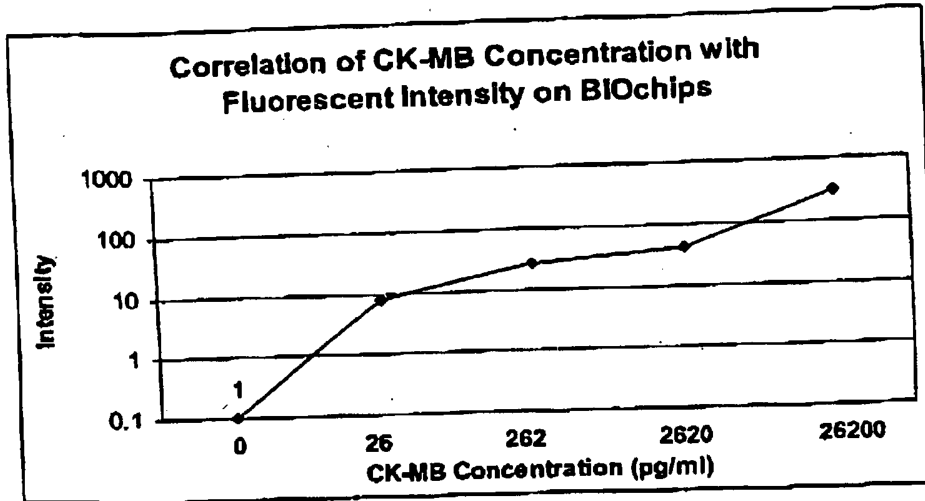

Figure 5

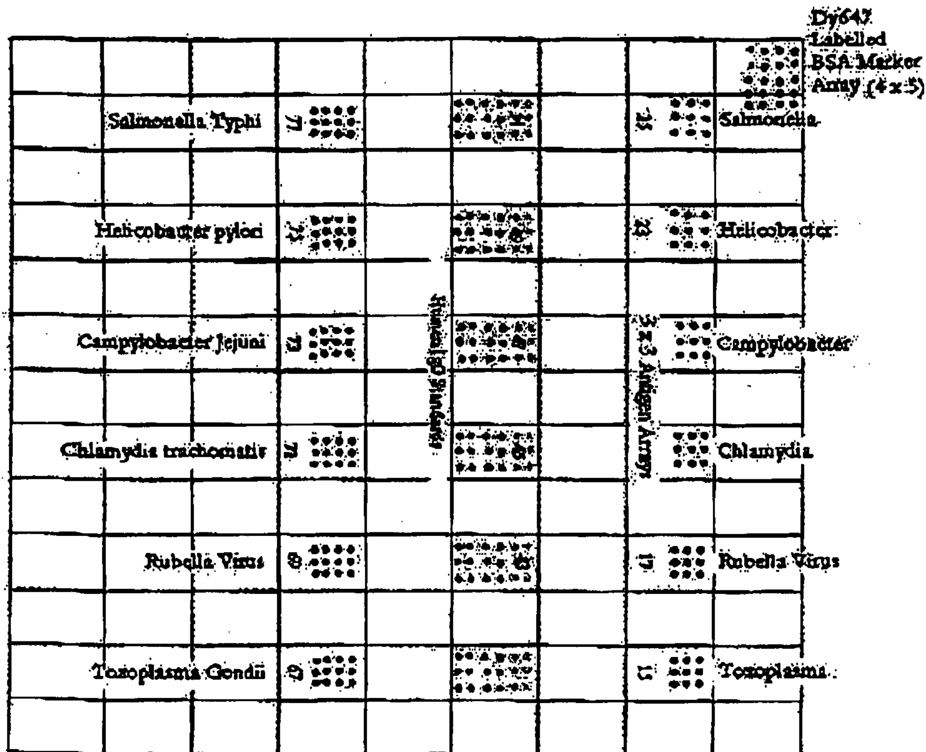

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
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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

"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
20 October 2005 (20-10-2005)	15 November 2005 (15-11-2005)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001(819)953-2476	David Boudreau (819) 997-2926

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2005/001147

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/CA2005/001147

Patent Document	Publication Date	Patent Family Member(s)	Publication Date
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