PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12M 1/20 | A1

(11) International Publication Number: **WO 99/32601**

(43) International Publication Date: 1 July 1999 (01.07.99)

(21) International Application Number: PCT/US98/08092

(22) International Filing Date: 22 April 1998 (22.04.98)

(30) Priority Data:
08/997,337 23 December 1997 (23.12.97) US

(71) Applicant: MINNESOTA MINING AND MANUFACTURING COMPANY [US/US]; 3M Center, P.O. Box 33427, Saint Paul, MN 55133–3427 (US).

(72) Inventors: WICKERT, Peter, D.; P.O. Box 33427, Saint Paul, MN 55133–3427 (US). WILLIAMS, Michael, G.; P.O. Box 33427, Saint Paul, MN 55133–3427 (US).

(74) Agents: ROGERS, James, A. et al.; Minnesota Mining and Manufacturing Company, Office of Intellectual Property Counsel, P.O. Box 33427, Saint Paul, MN 55133–3427 (US).

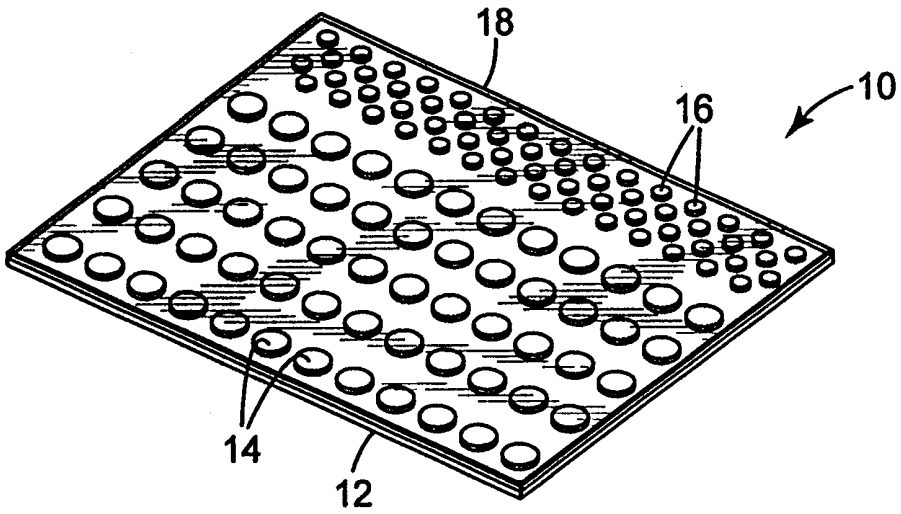
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: DISC ASSAY DEVICES AND METHODS OF USE

(57) Abstract

A culture device having hydrophilic liquid retaining discs for use in detecting and enumerating microorganisms. Methods of use therefor are also disclosed.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

DISC ASSAY DEVICES AND METHODS OF USE

This application is a continuation in part of U.S. Patent Application Serial No. 08/838,397, filed April 9, 1997, now pending.

This invention relates to disc devices and methods of use for partitioning biological samples into microvolume aliquots, and detecting and enumerating microorganisms present within the samples.

The detection and enumeration of microorganisms is practiced in numerous settings, including the food-processing industry (testing for the contamination of food by microorganisms such as *E. coli* and *S. aureus*), the health care industry (testing of patient samples and other clinical samples for infection or contamination), environmental testing industry, the pharmaceutical industry, and the cosmetic industry.

Growth-based detection and enumeration of microorganisms is commonly practiced using either liquid nutrient media (most probable number analysis (MPN)) or semi-solid nutrient media (agar petri dishes). Enumeration using the liquid MPN method is typically achieved by placing serial 10-fold dilutions of a sample of interest in replicate sets of tubes containing selective media and chemical indicators. The tubes are incubated at elevated temperature (24-48 hours) followed by examination for growth of organisms. A statistical formula, based on the volume of sample tested and the number of positive and negative tubes for each set, is used to estimate the number of organisms present in the initial sample.

This method of performing MPN analysis has several disadvantages. It is labor intensive because of the multiple diluting and pipetting steps necessary to perform the analysis. In addition, in practice it is only practical to use replicate sets of about three to five tubes for each dilution. As a result, the 95% confidence limits for an MPN estimate for microbial concentration are extremely wide. For example, a three tube MPN estimate of 20 has 95% confidence limits ranging from 7 to 89. Furthermore, results typically are not obtainable in less than twenty-four hours.

In contrast to the method described above, a direct count of viable

microorganisms in a sample can be achieved by spreading the sample over a defined area using nutrient media containing a gelling agent. The gelling agent (agar) prevents diffusion of the organisms during incubation (24-48 hours), producing a colony in the area where the original organism was deposited. There is, however, a limit to the number of colonies that can fit on a given area of nutrient media before fusion with neighboring colonies makes counting difficult. This usually necessitates performing several dilutions for each sample. In addition, the classes of chemical indicator molecules that can be used for identifying individual types of microorganisms present within a mixed population are limited to those that produce a product that is insoluble in the gelled media. Furthermore, rapid detection, i.e., in less than about twenty-four hours, and enumeration is not feasible using this method.

The present invention addresses the disadvantages of the prior art. The invention provides devices and methods for the rapid detection and enumeration of microorganisms. In one aspect, the invention provides a device that is capable of retaining microvolumes of sample. The device has a substrate with a relatively hydrophobic surface. Within or on the surface are hydrophilic sample retaining discs. The discs may comprise hydrophilic fiber material projecting from the assay surface. The discs may be constructed of a variety of materials, including cellulosics, polyesters, polyolefins, and polyamides.

A sample that is suspected of having microorganisms present is placed on the assay surface of the device. The hydrophobic/hydrophilic interaction between the discs and the substrate permits for rapid inoculation of the discs such that the sample is substantially contained on the discs and substantially excluded from the substrate. Sample may be poured or otherwise delivered to the device, such as, for example, by dipping the device into sample. The interaction acts to contain the sample on the discs and substantially exclude the sample from the substrate. This interaction also helps to prevent cross contamination on the discs that may occur if microorganisms were permitted to migrate from disc to disc. The device is then incubated to permit growth of the suspected microorganisms.

The discs have media provided thereon to facilitate growth of the suspected microorganisms. The media may be selective for one or more types of microorganisms. The discs are biocompatible with the microorganisms such that the

materials do not substantially interfere with the growth or detection of the microorganisms.

Appropriate indicator substances may be coated or deposited on the discs, or may be mixed with the sample to be inoculated onto the discs. Suitable indicators include without limitation chromogenic indicators, fluorescent indicators, luminescent indicators and electrochemical indicators. In a preferred embodiment, the indicators are fluorescent.

The discs may be of uniform size, with each disc having a liquid retention capacity of about 0.01 to about 25 microliters, more preferably about 1 to about 2 microliters.

The culture device can have, for example, about 10 to about 10,000 discs, more preferably about 400 to about 600 hydrophilic liquid-retaining discs.

In an alternative embodiment, the culture device may comprise a plurality of sets of hydrophilic liquid-retaining discs, each of the sets having discs of uniform size, the sets varying in liquid retention capacity, and the device having at least two sets of discs. In a preferred embodiment, the device has 100 discs total, with a set of 50 having discs with a volume retention of about 2 microliters and another set of 50 having discs with a volume retention of about 20 microliters. This device allows for a device having a relatively small number of discs but with a wide counting range.

In another aspect, the invention provides methods of delivering sample to the devices. The sample is partitioned into the discrete discs based in part on the hydrophobic/hydrophilic interactions between the substrate and discs and in part on the absorbency of the discs.

As used herein, the term “microvolume” refers to a volume of less than about 25 microliters, and includes volumes in the sub-microliter range. The term "microorganism" includes all microscopic living organisms and cells, including without limitation bacteria, mycoplasmas, rickettsias, spirochetes, yeasts, molds, protozoans, as well as microscopic forms of eukaryotic cells, for example single cells (cultured or derived directly from a tissue or organ) or small clumps of cells. Microorganisms are detected and/or enumerated not only when whole cells are detected directly, but also when such cells are detected indirectly, such as through detection or quantitation of cell fragments, cell-derived biological molecules, or cell

by-products. The terms "hydrophobic" and "hydrophilic" are herein given the meanings commonly understood in the art. Thus, a "hydrophobic" material has relatively little or no affinity for water or aqueous media, while a "hydrophilic" material has relatively strong affinity for water or aqueous media. The relative hydrophobicities and hydrophilicities of the devices described herein are such as to ensure partitioning of liquid samples substantially into the described hydrophilic liquid-retaining discs upon application of the sample. The required levels of hydrophobicity and hydrophilicity may vary depending on the nature of the sample, but may be readily adjusted based on simple empirical observations of the liquid sample when applied to the devices. The term "electrochemical" means a chemical indicator that changes the resistance or conductance of the sample upon reaction with the microorganism.

The devices and methods provide a system for the detection and enumeration of microorganisms and other biological materials that solves the problems associated with currently used systems. The system is a liquid-based system, allowing efficient and effective partitioning of the sample into discrete microvolumes for testing, and allows for rapid detection and enumeration.

In the case of MPN analysis for the detection and enumeration of microorganisms, the approaches described herein allow for the use of water-soluble indicator species, and reduce or eliminate the need for the several dilutions typically required in current MPN analysis.

Figure 1 is a perspective view of one embodiment of a microcompartment culture device.

Figure 2 is a perspective view of a microcompartment culture device having two sets of different volume discs and a coversheet.

Figure 3 is a perspective view of a microcompartment culture device having discs within wells.

This invention relates to disc devices and methods of use thereof for partitioning biological samples into microvolume liquid sample aliquots and conducting signal-based detection and enumeration of microorganisms in such samples.

Among the problems encountered in the art relating to the testing of liquid

samples for microorganisms are relatively lengthy incubation times, the need to undertake multiple pipetting operations for aliquots being tested, and the need for a relatively large volume of sample for testing.

This invention addresses the difficulties in the prior art by providing a device that is easily inoculated with microvolumes of sample. The invention provides absorbent disk materials that are absorbent yet are biocompatible. Furthermore, these materials are compatible with fluorescent indicator systems. The materials lend themselves easily to the manufacturing process.

The methods and devices of the present invention provide for efficiently partitioning a liquid sample into microvolume compartments of a test device, with only minimal manipulation of the liquid sample required of the laboratory technician or other operator. The present inventors have discovered that the use of microvolumes in signal-based detection of microorganisms in liquid samples results in remarkably shorter incubation times required to produce a detectable signal. Because shorter incubation times are highly desirable in this field, this feature of the invention provides a distinct advantage.

In addition to achieving shorter incubation times, the use of microvolumes in the testing of liquid samples may allow for the use of substantially smaller test samples. Very small volume test samples are sometimes necessary due to very small volume sample sources. Small volume liquid test samples are also sometimes desirable, for example to ease handling or transport of the sample to a testing facility.

The present inventors have developed novel devices and methods for partitioning biological liquid samples into discrete microvolumes within liquid-retaining discs. Advantageously, the devices allow for the testing of liquid samples using microvolume aliquots in a single device, eliminating the need for separate vessels in such testing. A test sample may be distributed among hundreds or even thousands of discrete liquid-retaining discs, substantially increasing the number of data points in a test of the liquid sample.

A particularly useful application of these methods and devices is in the growth-based detection and enumeration of microorganisms in liquid test samples. Such growth-based detection and enumeration is very important in the testing of food, environmental, clinical, pharmaceutical, cosmetic, and other samples for

contamination by microorganisms. The methods and devices of this invention allow for the efficient, accurate, convenient, and cost-effective testing of such samples. A preferred use of the methods and devices of this invention in such microbiological testing is in MPN. In traditional MPN, a sample of interest is serially diluted (10 fold) and pipetted in equal amounts into replicate sets of tubes containing selective growth media and chemical indicators. The tubes are incubated at elevated temperature for about 24-48 hours followed by examination for growth of organisms. A statistical formula, based on the volume of the sample and the number of positive and negative tubes for each set, is used to estimate the number of organisms present (per volume) in the initial sample. As currently used, this traditional method has several disadvantages. It is labor intensive because of the multiple diluting and pipetting steps required to perform the analysis. As a practical matter, only replicate sets of about three to five tubes for each dilution are commonly used. As a result, the 95% confidence limits for an MPN estimate of microbial concentration using this method are extremely wide. For example, a nine tube (3 ten-fold dilutions) MPN estimate of 20 has 95% confidence limits ranging from 7 to 89.

The use of the methods and devices of the present invention in MPN analysis overcomes several of the above-noted disadvantages. The amount of labor is greatly reduced because no pipetting into individual tubes is necessary, and very little or no agitation or other manipulations are required. Instead, the liquid sample is distributed to microvolume liquid-retaining discs by simply contacting the liquid sample with the device. In addition, fewer sample dilutions are necessary when large numbers of liquid-retaining discs are present in the device. The relatively large number of liquid-retaining discs also provides a more accurate estimate of microbial concentration. This is because the correspondingly larger number of data points provides a correspondingly narrower confidence limit interval.

Accordingly, the present invention provides a method for detecting (including enumerating) a microorganism in a liquid test sample. The method involves distributing microvolumes of the test sample to a plurality of hydrophilic liquid-retaining discs of an assay device. The assay device may be any device that includes an assay surface having a plurality of hydrophilic liquid-retaining discs, where each disc has a microvolume capacity of liquid retention. The device also

includes a land area between the discs that is hydrophobic and remains substantially free of liquid after the biological sample has become distributed into the liquid-retaining discs. Non-limiting examples of such assay devices include those described herein.

The liquid-retaining discs in the assay device preferably are of uniform size and each disc has a liquid-retention capacity of about 0.01 to about 25 microliters of the liquid sample. Preferably, each disc has a liquid retention capacity of about 0.1 to about 10 microliters, and more preferably about 1 to about 2 microliters. The assay device preferably contains between 1 and about 100,000 liquid-retaining discs, more preferably about 10 to about 10,000 discs, even more

preferably about 200 to about 5,000 discs and most preferably about 400 to about 600 discs.

The device may also preferably contain sets of discs with differing volumes. In this preferred embodiment, the device preferably has 100 discs. Preferably, 50 discs have a volume of about 20 microliters and 50 discs with a volume of about 2 microliters.

The device is particularly useful in the context of testing a liquid sample for microorganism concentration using MPN. Certain regulatory requirements may dictate that a testing method must be able to detect one microorganism in a one-to-five-milliliter sample. Such a sample size is standard in the food processing industry for microbiological testing. Thus, for example, an assay device having 500 hydrophilic liquid-retaining discs, where each disc has a liquid capacity of about 2 microliters, would be very useful for testing a 1-ml sample. A liquid-retention disc having a capacity of 2 microliters allows for rapid development of a detectable signal in accordance with the invention, and the use of about 400 to about 600 discs provides a sufficiently large number of data points to substantially improve the confidence interval for an MPN calculation. In addition, it is feasible to perform a manual count of liquid-retaining discs testing positive for the microorganism. Use of devices having substantially more than 400 liquid-retaining discs may require, as a practical matter, instrument-assisted or automated counting.

The discs may be attached to the substrate by various means known in the art, including without limitation, by using adhesives. Preferred adhesives include water-insoluble isooctyl acrylate adhesives as disclosed in U.S. Patent No. 5,409,838, the disclosure of which is incorporated herein by reference.

The liquid test sample may be any sample of interest, from any source. The sample may be distributed to the plurality of liquid-retaining discs directly, or the sample may be diluted before distribution to the discs. The determination as to whether sample dilution is necessary will depend on a variety of factors such as sample source and age, and such determination is a routine matter to those of skill in the art.

The liquid test sample may include selective nutrient growth media for the microorganism of interest, and/or an indicator substance that produces a signal in the

presence of the growing microorganism. Optionally, the nutrient medium may include a gelling agent that assists in "encapsulating" the growing microorganisms. Such gelling agents are known to those of skill in the art, and include any water-absorbing material that becomes a gel upon addition of an aqueous liquid.

In any event, nutrient growth media is present as a coating or other deposition within or on the liquid-retaining disc, in amounts sufficient to achieve desired concentrations when a microvolume of the liquid test sample is distributed into the disc. Such a coating may be achieved, for example, by placing or distributing a solution of the nutrient medium (with or without gelling agent) onto the disc and drying the solution to produce a coating or deposition of the nutrient medium on the disc. Components of the media may be present in the adhesive or other substance that binds the discs to the substrate (if applicable). The media ultimately diffuses into the disc material.

A wide variety of selective growth media for a wide variety of microorganisms of interest is known, as is a wide variety of indicator substances for a wide variety of microorganisms, and any of these media or indicator substances is suitable for use in the method of the invention. An advantage of the present invention is that soluble indicators can be used, since diffusion is prevented by confinement of the aqueous biological sample liquid in the hydrophilic liquid-retaining discs.

Various methods may be employed to distribute a liquid test sample to the liquid-retaining discs. More than one method may be applicable to a particular device, although the preferred method may depend to some extent on the configuration of a particular assay device. The sample may be poured or pipetted over the device and the sample spread to the liquid-retaining discs by tilting or rocking the device. The hydrophilic/hydrophobic interaction acts to retain the sample on the discs and substantially excluding the sample from the substrate. Alternatively, the assay surface of the device can be immersed in the sample. Upon removal of the assay surface from the liquid sample, liquid is retained in the hydrophilic liquid-retaining discs and is likewise substantially excluded from the hydrophobic land area.

After the sample is distributed to the hydrophilic liquid-retaining discs of the assay device, various assays may be carried out depending on desired uses. For

microbial detection or enumeration, the assay device may be incubated for a time sufficient to permit at least one cell division cycle of the microorganism. For these purposes, the device is generally incubated at about 25°C to about 45°C, more preferably at about 30°C to about 37°C. The incubation time for bacterial detection will vary. The detection time will also vary depending on the growth rate and the number of microorganisms present in the sample. Taking into account these considerations, detection time for purposes of enumeration may be as little as about 10 hours. This relatively short incubation time represents a distinct advantage over detection methods currently used, which typically require incubation times of about 24 hours or more.

Following incubation of the assay device, the presence or absence of the microorganism in discs (and thus in the liquid test sample) is detected. The mode of detection depends on the type of indicator substance used in the method. Any indicator substance that is capable of providing a detectable signal may be used. Such indicators include but are not limited to fluorescent, chromogenic, luminescent, and electrochemical indicators. The presence or absence of a microorganism in a disc can be visually detected, with the naked eye or microscopically, if a chromogenic or luminescent indicator is used. The indicator may be coated or otherwise incorporated into the discs. The indicators may also be included in the adhesive or other substance that binds the discs (if applicable) to the substrate. In this instance, the indicator ultimately diffuses into the disc material. If a fluorescent indicator substance is used, equipment and methods for detecting a fluorescent signal may be employed for detection. There are numerous indicator substances and signal detection systems, including systems for detecting electrochemical changes, known in the art for detecting microorganisms, and any such substance or system may be used in accordance with the present invention.

In the present invention, fluorescent indicators are preferred because they may be detected at relatively low concentrations. Suitable indicators include 4-methylumbelliferyl phosphate, and 4-methylumbelleferyl -B-D-glucopyranoside, L-phenylalanine-7-amido-4-methylcoumarin. Others may include 4-methylumbelliferyl acetate and 4-methylumbelliferyl sulfate.

The detection of microorganisms in the liquid sample may further involve

the enumeration of a microorganism count in the liquid test sample. In a preferred embodiment, the enumeration is performed using MPN. Once the number of liquid-retaining discs containing the microorganism of interest is determined, an MPN calculation can be made using known MPN techniques. If desired, the number of microorganisms in an individual disc can then be determined using known techniques, for example, signal intensity compared to a known standard, or by plating the contents of the disc. Advantageously, the large number of liquid-retaining discs used in the method of the invention allows for narrower intervals for the 95% confidence limits in an MPN analysis of a liquid test sample.

Because of the large number of liquid-retaining discs that may be manufactured in a single device, it is possible to use a single device in the detection and enumeration of multiple microorganisms of interest, while retaining the advantages of the invention. For example, a single liquid test sample can be tested for the presence or concentration of *E. coli* and *S. aureus*. One portion of an assay device can contain hydrophilic liquid-retaining discs for the detection and enumeration of one of these microorganisms, while a second set of discs can be directed to detection and enumeration of another microorganism of interest. This is accomplished, for example, by including microorganism-specific nutrients and/or indicator substances in the respective sets of liquid-retaining discs. Alternatively, all liquid-retaining discs can contain assay reagents designed for the simultaneous detection of multiple microorganisms. For example, *E. coli* can be detected with a fluorescent indicator substance while, at the same time, other coliforms are detected with a chromogenic indicator substance.

Subsequent tests may be conducted. For example, the discs can be removed from the device and transferred into a test tube to differentiate the specific microorganisms growing thereon.

In another embodiment, the distribution step can involve distributing aliquots of the liquid test sample to a plurality of hydrophilic liquid-retaining discs of an assay device, wherein the assay device includes a plurality of sets of discs. Each set has discs of uniform size, and the device has at least two sets of discs. For example, the assay device can include a plurality of lanes, with the hydrophilic liquid-retaining discs in a particular lane having the same liquid-retention capacities. This

feature allows for the distribution of the liquid test sample into different test volume sizes within a single assay device. In MPN, this feature provides a significant advantage in that, for a highly concentrated sample, an appropriate volume size may be selected and MPN analysis performed using a single distribution step in a single device without the need for serial dilutions.

As stated above, the methods of this invention may be practiced using any assay device containing hydrophilic liquid-retaining discs and a hydrophobic land area, depending on the particular embodiment being practiced. The present inventors have developed several novel devices suitable for use in the methods of this invention. The following are non-limiting examples of such devices.

Referring to Figure 1, a device 10 comprises a substrate 12 having a plurality of hydrophilic liquid-retaining discs 14. In a preferred embodiment, the discs 14 are hydrophilic and absorbent. The discs 14 may be constructed from a variety of materials, including cellulosics, polyolefins, polyesters, and polyamides, with cellulosics being preferred. Suitable cellulosics include paper, wood pulp and rayon and may include chemically modified cellulosics, such as cellulose esters. Suitable polyolefins include hydrophilic polyethylene or hydrophilic polypropylene fibers. Suitable polyamides include nylon. Suitable polyesters include polylactic acid.

The materials of the present invention 10 are biocompatible and may be used with fluorescent indicators. The materials do not exhibit significant inherent fluorescence that would interfere with the use of the indicators. In addition, the discs 14 do not exhibit significant absorption at the emission wavelength of the indicators.

The substrate 12 can be fabricated from any material that is relatively hydrophobic and provides a suitable surface or support for the discs 14. Substrate 12 can be fabricated, for example, from polymeric films or other appropriate materials. Appropriate polymers include without limitation polyethylene, polypropylene, polyimides, fluoropolymers, polycarbonates, polyurethanes, and polystyrenes. Should a particular polymer not be sufficiently hydrophobic, it can be treated to impart hydrophobicity. For example, a thin layer of acrylated silicone or other hydrophobic material may be added to the substrate. The film substrate 12 should not exhibit fluorescent or light-absorbing properties that would interfere with any

fluorescent indicator system that is used. Those skilled in the art will recognize other means to impart surface hydrophobicity.

The device 10 can include any desired number of discs 14. Additionally, the device 10 can include relatively large reservoirs or other compartments adapted to hold larger volumes of liquid for maintenance of an appropriate humidity level within the device 10. Although the number of discs 14 can be relatively small (e.g., 2-50) for certain applications such as preliminary screening, the small sizes of the microvolume discs 14 allow relatively large numbers of discs 14 to be on a single device 10. The device 10 can have a population of uniformly sized discs 14, although the discs need not be of uniform size. For example, referring to Figure 2, the device can have sets (e.g., rows) of microvolume discs 14, 16 in which volumes are constant within a set, but vary between sets. The volumes can vary incrementally over an array of sets of discs 14, 16, with the smaller discs 14 holding sub-microliter volumes and the larger discs holding microliter volumes 16. It is even possible for the largest discs in a device such as depicted in Figure 2, to include discs 16 that would not be classified as microdiscs. Such discs 16 might have a liquid-retention capacity, for example, of substantially more than 25 microliters.

Optionally, the device 10 may include a coversheet 18 to protect the discs 14 from contamination or desiccation once sample has been added to the device 10. The coversheet 18 may further be sealed to the device along its edges with a pressure sensitive adhesive.

In an alternative embodiment, as depicted in Figure 3, the device 10 may include discs 14 contained in microwells 22 that have been made in the substrate 12 of the device 10. As with other embodiments, the numbers and size of discs may be varied.

The discs may be of any shape. For example, the discs may be of circular, oval, square, or polygonal shape or other appropriate shapes.

Assay reagents are coated or otherwise deposited within the liquid-retaining discs of the assay devices. Such assay reagents include without limitation nutrients for growth of microorganisms. Other reagents may include, without limitation, gelling agents and indicator substances such as chromogenic indicators, fluorescent indicators, luminescent indicators, and electrochemical indicators. The

assay reagents can be immobilized in the liquid-retaining discs by any of numerous methods for immobilizing assay reagents on solid substrates known to those of skill in the art. Such methods include for example drying down assay reagent-containing liquids in the discs, as well as other methods for noncovalently attaching biomolecules and other assay reagents to a solid substrate. Alternatively, various methods may be employed to covalently attach assay reagents to the discs by methods well known to those of skill in the art.

As discussed above, the presence of hydrophilic liquid-retaining discs with microvolume liquid-retention capacity in an assay device allows for separation of a liquid test sample into a relatively large number of test volumes. The ability to separate a liquid sample into microvolume aliquots and to perform MPN or other assays without cross-contamination between aliquots is an advantage of the present method and devices.

All references and publications cited herein are expressly incorporated herein by reference into this disclosure. Particular embodiments of this invention will be discussed in detail and reference has been made to possible variations within the scope of this invention. There are a variety of alternative techniques and procedures available to those of skill in the art which would similarly permit one to successfully practice the intended invention.

The following examples are offered to aid in understanding of the present invention and are not to be construed as limiting the scope thereof. Unless otherwise indicated, all parts and percentages are by weight.

Example 1

Absorbent Disc Culture Devices

Absorbent disc culture devices containing a plurality of hydrophilic absorbent discs arrayed on a hydrophobic surface and capable of being used for the detection and enumeration of microorganisms in a liquid test sample were constructed as described in this example.

A. Culture Devices Constructed with Absorbent Paper Discs

A sheet of absorbent material (Schleicher & Schuell Grade 903 Paper; absorbs about 4.5 g of water/100 cm^2) was laminated to a Rexam silicone-coated film (Grade #15819 D 2MIL CL PET MM34P/000 having a clear 2-mil thick polyester

film as a substrate, Rexam Release, Oak Brook, IL) with an acrylate pressure sensitive adhesive (PSA) containing the chromogenic indicator 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC) (Amresco, Solon, OH). The material was saturated with tryptic soy broth (TSB) growth nutrient containing 0.5% of the fluorescent indicators 4-methylumbelliferyl phosphate (100 μg/ml, Sigma, St. Louis, MO) and 4-methylumbellifery-α-D-glucoside (50 μg/ml, Sigma), wiped with a wire-wound rod, and dried at 110°C for 10 minutes. Circular discs approximately 0.635 cm in diameter were punched out of the laminate and the silicone-coated film backing removed. The discs with PSA were then adhered to another sheet of Rexam silicone-coated film so that the discs were patterned in equally spaced parallel rows. The film and discs assemblies were gamma irradiated to a level of 8.9 kGy, cut to size, and then taped into a petri dish such that each dish contained a piece of film with 20 discs. Based on gravimetric measurements, each disc in the resulting culture devices had a capacity to retain about 40 μl of liquid.

B. Culture Devices Constructed with Various Polymeric Absorbent Disc Materials

Silicone-coated polyester release liner (as described in Example 1A) and biaxially-oriented polypropylene (BOPP) film (1.6-mil thickness, 3M Co., St. Paul, MN) were cut into 7.6-cm x 10.2-cm rectangular pieces. Pieces of each material were joined at one end with SCOTCH™ brand double-coated adhesive tape (No. 665, 3M Co.) with the silicone-coated side of the release liner oriented toward the BOPP film. The release liner functioned as the base of the culture device and the BOPP film functioned as the top film.

Sheets of the following polymeric absorbent materials were laminated onto separate layers of an acrylate adhesive (No. Y966, 3M Co.): Product No.10201-9 cellulose (Dexter, Windsor Locks, CT), Grade 903 cotton lint paper (Schleichter & Schuell, Keene, NH), Product No. P-110 Supersorbent polyolefin (3M Co.), Product No. 9208283 polyester (Veratec, Walpole, MA), Spunbond Nylon (4 ounces per square yard) polyamide (Cerex Advanced Fabrics, Cantonment, FL), and polylactic acid polyester [absorbent nonwoven meltblown web prepared from polylactic acid pellets (HEPLON™, Chronopol, Inc., Golden, CO) as described in U.S. Patent 5,230,701, which herein is incorporated by reference]. Circular discs (approximately

0.64-cm diameter) were punched out of the resulting laminates and adhered to the silicone-coated side of the polyester release liner. Each culture device contained 12 discs equally spaced in a 3 x 4 array of parallel rows. After construction was completed, the culture devices were gamma irradiated to a level of 8 kGy. Each disc had the capacity to retain about 10 µls.

Example 2

Method of Inoculation

(Method Utilizing Absorbent Disc Culture Devices)

The method of inoculating absorbent disc culture devices containing a plurality of microvolume liquid-retaining discs with bacteria-containing media was demonstrated in this example. The inoculated devices constructed with absorbent paper discs were utilized to detect and enumerate *E. coli* bacteria.

A. Microbial Assay Using Culture Devices Constructed with Absorbent Paper Discs (from Example 1A)

A culture of *E. coli* ATCC 51813 was diluted to produce suspensions containing about 10 CFU/ml and 1 CFU/ml. Samples (1 to 2 ml) of the suspensions were applied by pipette to the absorbent disc culture devices described in Example 1A. Excess liquid sample was poured off, thereby leaving about 0.8 ml retained on the device (20 discs, about 40 µl of liquid per disc). The inoculated devices were incubated at 35°C for 23 hours and inspected under ultraviolet light. The number of discs exhibiting fluorescence was counted for each device and most probable number (MPN) values calculated using the formula MPN = N ln (N/N-X) where N is the total number of inoculated discs and X is the total number of discs showing a positive reaction. The MPN per milliliter was calculated by dividing the value obtained by the total volume of the sample (0.8 ml). Results are provided in Table 2A and are compared with counts obtained from standard testing with Coliform Count PETRIFILM™ Plates (3M Co.). The fluorescent discs often showed the red TTC color, usually as discrete spots within the discs. No cross-contamination between absorbent discs was observed.

Table 2A
Enumeration of Microorganisms (*E. coli*)

Bacterial Suspension (~ CFU/ml)	Positive Discs (Out of 20)	MPN (CFU/ml)	Coliform Count PETRIFILM ™
10	17	47	22
10	19	74	24
1	2	2.6	5
1	3	4.1	4

The results of this example show that absorbent disc culture devices having a plurality of absorbent discs arrayed on a hydrophobic film can be easily inoculated with bacteria-containing liquid samples and that the inoculated devices can be utilized for the detection and enumeration of *E. coli*, with the values obtained being comparable with those obtained from commercial Coliform Count PETRIFILM ™ Plates.

B. Microbial Assay Using Culture Devices Constructed with Various Polymeric Absorbent Disc Materials (from Example 1B)

Cultures of different bacterial strains (Table 2B) were grown overnight at 35°C in 5 ml of TBS media. A 0.01-ml volume of each culture was diluted into 99 ml of sterile Butterfield's diluent (Fisher Scientific, Pittsburgh, PA), to obtain initial 10^{-4} dilutions of the original bacterial suspensions. Three subsequent 10-fold dilutions (10^{-5}, 10^{-6}, and 10^{-7}) of the bacterial suspensions were made in Standard Methods Broth containing the following ingredients: Pancreatic Digest of Casein (10.0 g/l, Difco Labs), Yeast Extract (5.0 g/l, Difco Labs), Glucose (2.0 g/l, Becton Dickinson and Co., Cockeysville, MD), and the fluorescent indicator 4-methylumbelliferylphosphate (0.05 g/l, Biosynth International). With the top covers of the culture devices (from Example 1B) raised, three 0.01-ml aliquots of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were transferred by pipette onto nine individual discs on each of the devices. An equivalent volume of sterile medium was transferred to the remaining three discs on each device to serve as sterility controls. The top covers of the inoculated culture devices were closed, and the devices placed into GLAD-LOCK® ZIPPER™ storage bags (First Brands Corp., Danbury, CT), each containing a moistened paper towel. The bags were placed in a 35°C incubator for 24 hours, after which the culture devices were examined under a long-wave ultraviolet light source. Positive growth and detection was evidenced by a bluish fluorescence. Results are provided in Table 2B.

Table 2B

Growth and Detection of Bacteria on Various Disc Materials					
Bacterial Strain	Disc Material	No. of Postive Discs (at designated dilutions)			
		10^{-5}	10^{-6}	10^{-7}	Control
Escherichia coli P18 (Clinical isolate; obtained from Centers for Disease Control and Prevention, Atlanta, GA)	Cellulose (Dexter)	3	3	3	0
	Paper (S & S)	3	3	3	0
	Polyolefin (3M)	3	3	3	0
	Polyester (Veratec)	0	0	0	0
	Polyamide (Cerex)	3	3	3	0
Bacillus sp. L11 (Food isolate)	Cellulose (Dexter)	3	3	0	0
	Paper (S & S)	3	2	0	0
	Polyolefin (3M)	0	0	0	0
	Polyester (Veratec)	0	0	0	0
	Polyamide (Cerex)	1	0	0	0
	Polylactic Acid Polyester (Chronopol)	2	0	1	0
Streptococcus faecium P92 (Clinical isolate; obtained from Centers for Disease Control and Prevention)	Cellulose (Dexter)	3	1	0	0
	Paper (S & S)	3	0	0	0
	Polyolefin (3M)	0	0	0	0
	Polyester (Veratec)	0	0	0	0
	Polyamide (Cerex)	3	0	0	0
	Polylactic Acid Polyester (Chronopol)	3	1	0	0

Hafnia alvei 3026 (Obtained from the University of Minnesota)	Cellulose (Dexter)	3	3	1	0
	Paper (S & S)	3	2	0	0
	Polyolefin (3M)	1	0	0	0
	Polyester (Veratec)	0	0	0	0
	Polyamide (Cerex)	3	1	0	0
	Polylactic Acid Polyester (Chronopol)	3	3	0	0

The results of this example show that culture discs constructed with an array of discs made from different absorbent materials can be utilized for the detection of various bacterial strains. Especially effective in this example were absorbent discs made from cellulosic, polyamide, and polyolefin materials.

Various modifications and alterations of this invention will be apparent to those skilled in the art without departing from the scope and spirit of this invention, and it should be understood that this invention is not limited to the illustrative embodiments set forth herein.

What is claimed is:

1. A culture device for detection or enumeration of microorganisms, said device comprising a substrate having liquid retaining discs wherein said substrate is hydrophobic relative to said liquid retaining discs and wherein the discs have media for growth of microorganisms and said discs have a microvolume capacity of liquid retention.

2. The culture device of claim 1 wherein said discs are constructed at least in part of a material selected from the group consisting of cellulosics, polyolefins, polyamides and polyesters.

3. The culture device of claim 2 wherein the discs are constructed at least in part of alpha cellulose.

4. The culture device of claim 2 wherein the discs are constructed at least in part of rayon.

5. The culture device of claim 2 wherein the discs are constructed at least in part nylon.

6. The culture device of claim 2 wherein the discs are constructed at least in part of polylactic acid.

7. The culture device of claim 1, wherein each said disc has a liquid retention capacity of about 1 to about 2 microliters.

8. The culture device of claim 1, having an indicator substance on said discs.

9. A method for partitioning an aqueous liquid sample into discrete microvolumes, comprising:

a) providing a device as set forth in claim 1 for culturing a

microorganism, said device having an assay surface, said assay surface comprising hydrophilic liquid-retaining discs and a hydrophobic land area between said discs, each said disc having a microvolume capacity of liquid retention and having media for growing microorganisms; and

b) contacting said liquid sample with said assay surface such that said liquid sample is partitioned into said hydrophilic liquid-retaining discs.

10. The method of claim 14, wherein the discs are constructed of a material selected from the group consisting of cellulosics, polyolefins, polyamides and polyesters.

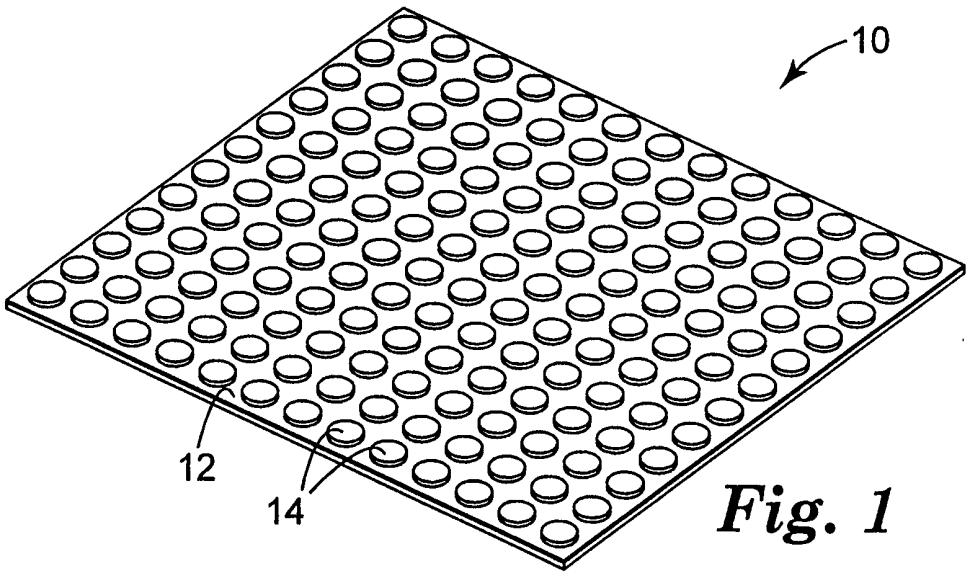


Fig. 1

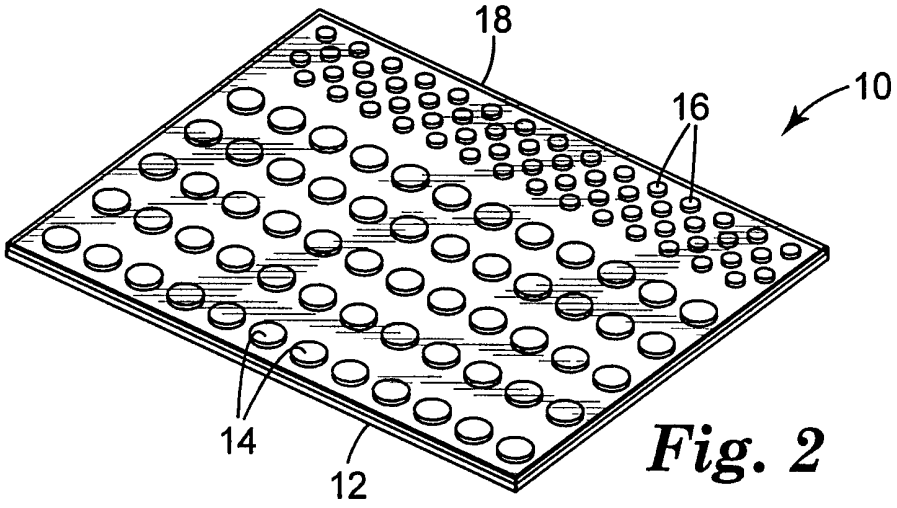


Fig. 2

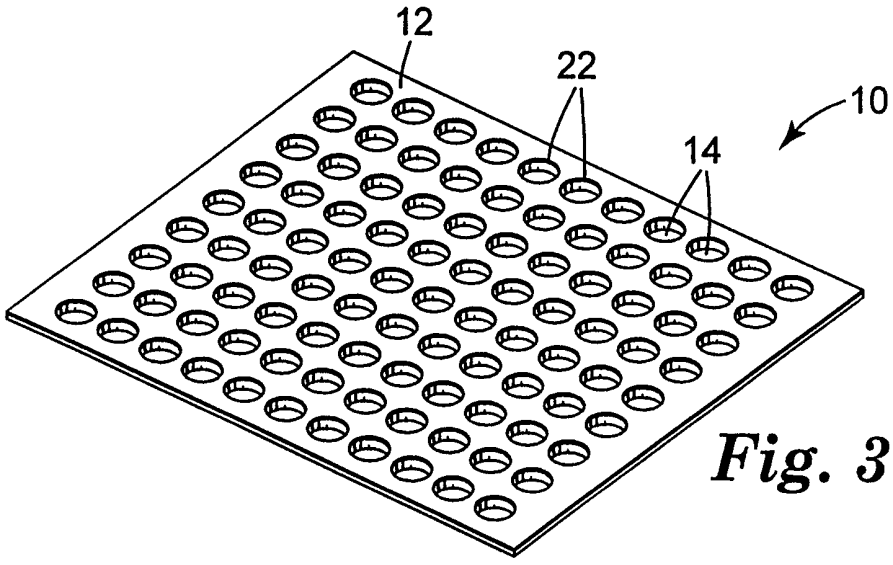


Fig. 3

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 98/08092

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12M1/20

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 6 C12M

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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 656 420 A (SHOWA PHARM CHEM IND) 7 June 1995	1-3,7-10
Y	see claims; figures	4-6
X	EP 0 795 600 A (SHOWA PHARM CHEM IND) 17 September 1997	1-5,7-10
Y	see claims; figures	4-6
X	US 3 010 880 A (M.L. LITTMAN ET AL.) 28 November 1961	1-3,7-10
Y	see claims; figures	4-6
Y	DE 42 18 917 A (SCHMITZ KLAUS PETER DR ING HAB ;BEHREND DETLEF DR ING (DE); DITTRI) 16 December 1993 see claims	6

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

☒ Patent family members are listed in annex.

° Special categories of cited documents :

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

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 July 1998	20/07/1998

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	Coucke, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter: nal Application No
PCT/US 98/08092

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0656420 A	07-06-1995	WO 9404703 A	03-03-1994
		JP 6181745 A	05-07-1994
		US 5728350 A	17-03-1998
EP 0795600 A	17-09-1997	CA 2207590 A	27-06-1996
		WO 9619565 A	27-06-1996
		JP 8224078 A	03-09-1996
US 3010880 A	28-11-1961	NONE	
DE 4218917 A	16-12-1993	NONE	