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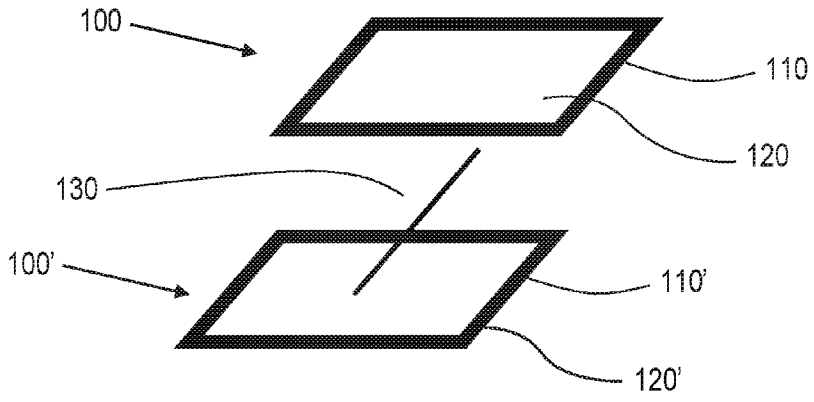
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(54) Title: LOW COST SYSTEM FOR TESTING ANTIBIOTICS

FIG. 1A



(57) Abstract: A system for evaluating susceptibility of microorganisms to a test compound includes a first porous sheet, a second porous sheet, wherein each of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent; and a porous filament treated with a test compound and having a length sufficient to span the at least one zone, wherein the first and second sheets and the filament are positioned in stacking arrangement to sandwich the filament between the first and second sheets.

LOW COST SYSTEM FOR TESTING ANTIBIOTICS

RELATED APPLICATION

[0001] This application claims the priority and benefits of U.S. provisional application 62,184,997, filed June 26, 2015, the contents of which are incorporated by reference in their entirety. The contents of all the patent, patent application, and journal publications referred to herein are incorporated by reference in their entirety.

TECHNICAL FIELD

[0002] This technology relates generally to antibiotic susceptibility. In particular, the technology relates to methods and kits for screening of antibiotic activity against microbes.

BACKGROUND

[0003] Different bacteria can be found from the depths of the oceans to the top of the mountains; even the air is more or less colonized by bacteria. Most bacteria are either harmless or even advantageous to human beings but there are also bacteria that can cause severe infectious diseases or spoil the supplies intended for human consumption. Therefore, it is vitally important not only to be able to detect and enumerate bacteria but also to assess their viability and possible harmfulness.

[0004] Antibiotic resistance is a globally growing threat for public health resulting in significant rates of morbidity and mortality. Due to this problem, there is an increasing trend of infections caused by pathogens. Microorganisms continuously develop new resistance to antibiotics making it essential to develop new treatment strategies. The mechanisms that are involved in antibiotic resistance or susceptibility are complex and not well understood.

[0005] To predict the effects of antibiotic agents *in vivo*, antibiotic susceptibility tests are routinely carried out. This approach provides an insight about the potential failure or success of the antibiotic candidates in a patient specific fashion. The existing techniques utilize expensive kits and specialized reagents and require highly trained personnel to evaluate the results. Currently, there is a lack of practical platforms to test antibiotic susceptibility or resistance especially in remote areas.

SUMMARY

[0006] This disclosure describes simple, flexible, and easily accessible platforms to test effectiveness of antibiotics against bacteria. In one aspect, a hybrid platform including paper and string is provided. In one aspect, a hybrid platform comprising paper and a string pair is provided. In another aspect, a method for evaluating the behavior, and in some embodiments, a dose response behavior, of antibiotics against bacteria is described.

[0007] In one aspect, a system uses an antibiotic infused string for testing of antibiotic susceptibility of microbes. Cells are cultured in paper scaffold and are contacted with antibiotic infused string. For example, an antibiotic-embedded cotton string is placed between two layers of paper, which contain a metabolic activity reagent for living cells, and bacteria in both layers. This assembly is cultured and after the culture is complete, the layers are separated and examined. Where antibiotic is effective, the paper remains blue. The intensity and size of the blue region is a measure of antibiotic effectiveness. The set up permits multiple screenings at the same time, is inexpensive and easy to use.

[0008] In one aspect, a system for evaluating susceptibility of microorganisms to a test compound includes a first porous sheet; a second porous cellulosic sheet, wherein each of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent; and a porous filament treated with a test compound, wherein the first and second sheets and the filament are positioned in stacking arrangement to sandwich the filament between the first and second sheets.

[0009] In one or more embodiments, the first and/or second sheets include cellulosic sheets.

[0010] In one or more embodiments, the system further includes a microorganism inoculated onto the first and second sheets.

[0011] In any of the preceding embodiments, both of the first and second sheets include at least one zone bounded by a fluid impermeable barrier.

[0012] In any of the preceding embodiments, both of the first and second sheets are treated with a metabolic activity reagent.

[0013] In any of the preceding embodiments, the first and second sheets include a plurality of zones bounded by a fluid impermeable barrier and the zones are positioned to be in stacking alignment.

[0014] In any of the preceding embodiments, the metabolic activity reagent is a colorimetric indicator.

[0015] In any of the preceding embodiments, the filament is of a length sufficient to span the at least one zone.

[0016] In any of the preceding embodiments, the first and second sheets may further include a hydrogel.

[0017] In any of the preceding embodiments, the test compound is an antibiotic.

[0018] In another aspect, a method of evaluating susceptibility of microorganisms to a test compound includes providing the system as described herein, wherein at least one of the first and second sheets is inoculated with a microorganism and the microorganism is in contact with the filament; culturing the microorganism-inoculated system, wherein the metabolic activity reagent undergoes reaction to provide a change indicative of metabolic activity; imaging the cultured microorganism-inoculated system after a preselected time, wherein the image provides an indication of metabolic activity level of the microorganism in the presence of the test compound.

[0019] In another aspect, a kit for evaluating susceptibility of microorganisms includes a first porous cellulosic sheet; a second porous cellulosic sheet, wherein each of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier; a porous filament having a length sufficient to span the at least one zone; and a metabolic activity reagent, wherein the first and second sheets and the filament are capable of positioning in stacking arrangement to sandwich the filament between the first and second sheets, wherein the metabolic activity reagent is in a form capable of application to one or both of the first and second sheets.

[0020] In any of the preceding embodiments, both of the first and second sheets include at least one zone bounded by a fluid impermeable barrier.

[0021] In any of the preceding embodiments, the first and second sheets include a plurality of zones bounded by a fluid impermeable barrier and the zones are aligned to be in stacking arrangement.

[0022] In any of the preceding embodiments, the metabolic activity reagent includes a colorimetric indicator.

[0023] In any of the preceding embodiments, the first and second sheets are suitable for cell culturing.

[0024] In another aspect, a system for evaluating susceptibility of microorganisms to a test compound includes a first porous cellulosic sheet, a second porous cellulosic sheet, wherein each of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent, a first porous filament treated with a test compound and having a length sufficient to span the at least one zone; and a second porous filament inoculated with a microorganism and having a length sufficient to span the at least one zone; wherein the first and second sheets and the first and filament are positioned in stacking arrangement to sandwich the filaments between the first and second sheets, and wherein the first and second filaments contact each other at least one point.

[0025] In another aspect, a method for testing compounds for antibacterial properties includes providing multiwelled cell plate, the multiwelled cell plate comprising first and second porous cellulosic sheets, wherein each of the first and second sheets comprise a plurality of wells bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent; introducing a known microorganism into the plurality of wells; positioning the first and second sheets in stacking arrangement to sandwich a plurality of filaments treated with a test compound between the first and second sheets; incubating the cells and observing a color change of the metabolic activity reagent, the color change correlated to antibiotic activity of the test compound.

[0026] In yet another aspect, a method for identifying an effective antibiotic for treatment of an infection includes providing multiwelled cell plate, the multiwelled cell plate comprising first and second porous cellulosic sheets, wherein each of the first and second sheets comprise a plurality of wells bounded by a fluid impermeable barrier and wherein at

least one of the first and second sheets is treated with a metabolic activity reagent; introducing a biological sample comprising an infectious microorganism into the plurality of wells; positioning the first and second sheets in stacking arrangement to sandwich a plurality of filaments treated with an antibiotic compounds between the first and second sheets; and incubating the cells and observing a color change of the metabolic activity reagent, the color change correlated to antibiotic activity of the antibiotic compounds on the infectious microorganism.

[0027] It is contemplated that any embodiment disclosed herein may be properly combined with any other embodiment disclosed herein. The combination of any two or more embodiments disclosed herein is expressly contemplated.

[0028] The invention will help developing effective methods of treatment for antibiotics and improve patient outcomes. By understanding the mechanisms involved in antibiotic susceptibility and/or resistance, and by providing a rapid and low cost method for testing for antibiotic susceptibility, new generation of therapeutics for infections can be developed to enhance global health.

[0029] These and other aspects and embodiments of the disclosure are illustrated and described below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] The invention is described with reference to the following figures, which are presented for the purpose of illustration only and are not intended to be limiting.

[0031] In the Drawings:

[0032] FIG. 1A is an exploded view and FIG. 1B is a cross-sectional view of a system for screening microorganisms for different activities according to one or more embodiments.

[0033] FIG. 2 is a schematic of the experimental procedure for testing the antibiotic activity according to one or more embodiments.

[0034] FIG. 3A is an exploded view of a system for screening microorganisms for different activities according to one or more embodiments.

[0035] FIGS. 3B-3D are a demonstration for the use of *E.coli* in string to generate patterns of bacteria on paper. The image for the control experiment is given in part a) indicating only Alamar Blue® on paper without bacteria. A cotton string that includes *E.coli* 11775 is incubated between two sheets of paper comprising Alamar Blue® for 8 h. The resulting images in FIG. 3C and FIG. 3D illustrates the patterns of *E.coli* on paper.

[0036] FIG. 4A represent the results for reproducibility of experiments, and FIG. 4B is a graph showing quantitative determination of color change after culturing the bacteria for 15 h against different antibiotics.

[0037] FIG. 5A is a schematic of the high-throughput experiment, which uses a wax-printed HT device to test 24 samples in parallel using a multizone culture format. FIG. 5B represents the images of the samples taken with a SLR camera and the quantified intensity of color in the images for each of the cell seeding zone.

[0038] FIGS. 6A-B represents the effect of initial seeding density of bacteria on the susceptibility behavior against 100 μg kanamycin. FIG. 6A is a simple schematic of the experiment, in which the paper was inoculated with different concentrations of *E.coli* 25922, and FIG. 6B is the quantified intensity of color from the images of SLR camera for each bacteria seeding density.

[0039] FIGS. 7A-7B are dose-response plot for high-throughput susceptibility analysis of *E.coli* 25922 against kanamycin. FIG 7A is a simple schematic of the experiment in which the paper samples were inoculated with bacteria and cultured with strings that included different concentrations of kanamycin (100, 10, 1, and 0.1 μg). FIG. 7B is the dose-response plot for *E.coli* against kanamycin from the quantified results using ImageJ.

[0040] FIGS. 8A-8C are comparisons of different imaging techniques to quantify the results for the susceptibility of *E.coli* 25922 against different concentrations (100-0.1 μg) of kanamycin The intensity of color determined by ImageJ for the images acquired by an SLR camera (FIG. 8A), a low-cost bench-top scanner (FIG. 8B), and a high-resolution scanner (FIG. 8C) (n=36 replicates).

DETAILED DESCRIPTION

[0041] The use of color transformation from a metabolic activity reagent to quantify antibiotic susceptibility against bacteria is described. The system provides an easy low cost method for testing antibiotics for effectiveness against microorganisms, and does not require the multiwell plates typically used to assess cell viability. In addition, testing and detection does not require additional equipment or analysis.

[0042] The system includes a pair of porous paper substrates containing a metabolic activity reagent and a microorganism. The metabolic activity reagent is selected to undergo a detectable change, e.g., a colorimetric change, in response to a change in the cellular metabolic process of the microorganism. The paper layer can include a water impermeable barrier to retain fluids and microorganisms within a selected region of the porous paper. The system also includes a string, yarn, filament or thread that is treated with a screening compound. The thread can be long enough to span a dimension of the test paper and has sufficient porosity or surface area to absorb and retain compounds for screening as antibiotics.

[0043] An exemplary embodiment of the invention is illustrated in FIGS. 1A-1B, shown in exploded view in FIG. 1A and assembled, cross-sectional view in FIG. 1B. The system includes two sheets 100, 100' of paper that can include a water-impermeable edge 110, 110'. The water impermeable edge can permeate the thickness of the paper and provide a barrier to further lateral flow (or wicking) of liquid. The porous paper can be any non-woven or woven sheet that can infuse culture medium and provide a support for cell growth. Exemplary paper includes lens paper (40 μm thick), printing paper (100 μm thick), filter paper (180 μm thick), chromatography paper (200 μm thick), blotting paper (3 mm thick), and cellophane (40 μm thick). Other non-limiting examples of suitable paper substrates include nitrocellulose and cellulose acetate paper, tissue paper, paper towels, and other grades and types of cellulosic paper.

[0044] The impermeable boundary can be a water-immiscible material, such as polymer or wax, which can be applied by screening, printing or stamping. In some embodiments, the barrier can be formed using wax printing methods, such as set out in International Application No. PCT/US10/25647, which is incorporated in its entirety by reference. For example, wax material is deposited by hand-drawing, printing or stamping, followed by

heating to spread the material into the thickness of the paper. The paper and the boundary materials are selected to be compatible with the microorganisms used in the screening assay.

[0045] The paper is treated with a metabolic activity reagent 120, 120'. The metabolic activity reagent can be infused into the pore space of the paper or it can be applied as a coating to the paper surface. A metabolic activity reagent is a reagent that is used to assess cell metabolic activity. As the microorganisms under evaluation grow, the metabolic activity reagent provides an indication of the innate metabolic activity of the cells or organisms. Typically, continued growth maintains a selected environment, reflected in some indicator, e.g., a color indicator. A reduction in growth causes the indicator to be transformed, resulting in a measurable or observable change in the observed system. A test material that functions as an effective antibiotic kills off or impairs microorganism growth, resulting in a reduction of metabolic activity and a change in the color indicator.

[0046] Exemplary indicators are dyes that undergo redox reactions with a corresponding change in colors. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) (sold commercially by Life Technologies under the name Alamar Blue® reagent) has been used to monitor cellular metabolic activity. It is a blue dye, itself weakly fluorescent until it is irreversibly reduced to the pink colored and highly red fluorescent resorufin. It is used as an oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells. Other assays can be used as well as long as they produce a significant color change and are detectable by naked eye. For example, Presto Blue reagent, another a resazurin-based solution sold by Life Technologies, and Cell Biolabs' CytoSelect™ WST-1 Cell Proliferation Assay Reagent can be used. Tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and related compounds such as XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), and WSTs (Water soluble Tetrazolium salts) may be used, but they would require an additional step to dissolve the insoluble formazan crystals that form after the reaction with metabolically active cells. Other indicators that provide changes notable by other techniques, such as fluorescence, UV or other detection means are also contemplated.

[0047] The system also includes as thread or filament that can be treated with a molecule or test compound to be screened for antibiotic activity. The thread can be hydrophilic for

easy loading of the test agent in liquid form. The thread can be cotton or synthetic, and can be made up of one or two or multiple strands. The thread diameter is selected to hold a selected amount of the antibiotic compound. In one or more embodiments, 100-200 micron diameter is suitable. The load of the test molecule depends on the stock concentration of the test solution. Threads as described herein can load a wide range of concentrations from milligrams to picograms.

[0048] In one or more embodiments, multiple individual test modules (or 'wells') can be formed on a single paper sheet. The use of multiple wells per sheet provides a high-throughput antibiotic susceptibility assay. The new high-throughput paper device allows the user to test multiple samples (*e.g.*, up to 24 have been demonstrated, but higher numbers are contemplated) at the same time.

[0049] FIG. 2 illustrates the assembly and operation of a system according to one or more embodiments. Starting with the illustration in the upper left hand corner and following the arrow flow, a sheet of paper is printed with an impermeable barrier to create a region of fluid permeability bounded by a fluid-impermeable boundary. The fluid-impermeable boundary defines a 'well' for culturing cells while conducting the assay. Although a single well is shown in this figure, it is possible to produce multiple wells in a single sheet of paper. This allows for high throughput screening and/or multiple runs to increase accuracy.

[0050] The printed test sheet is then decontaminated, *e.g.*, sterilized. Sterilization can be conducted using any conventional method, such as autoclaving or gamma ray sterilization techniques.

[0051] The sterile test sheet is then treated with a metabolic activity reagent, such as Alamar Blue® solution. For example, the paper test sheet can be treated by application of an Alamar Blue® solution and allowed to dry. In some embodiments, a solution of the metabolic activity reagent is applied by droplets, e.g., by pipette, onto the paper surface. In other embodiments, the paper can be soaked in the solution. Any method suitable for application onto paper is contemplated, including automated continuous coating processes.

[0052] Paper treated with metabolic activity reagent is subsequently seeded with microorganisms. Exemplary microorganisms can include bacteria (e.g. *Escherichia coli, Pseudomonas aeruginosa, Vibrio Cholerae, Salmonella Typhi, Bacillus subtilis,*

Streptococcus thermophiles, Helicobacter pylori, Yersinia pestis), archaea, fungi, algae, and viruses. For example, the paper can be inoculated with bacteria. Bacteria can be applied to the treated paper by adding a drop of bacteria suspension in media on to paper. Other ways can include the addition of bacteria in the lyophilized form, followed by the constitution with media. In other embodiments, the paper can be entirely soaked in bacteria suspension. In some embodiments, the bacteria are provided in lyophilized form, e.g., as part of a kit containing the paper assay device or obtained separately. The bacterial can be reconstituted in water or an appropriate solution, *e.g.*, physiological buffer. In other embodiments, the microorganism is cultured in nutrient broth at 37° C in a tube. In still other embodiments, the microorganisms can be obtained from a patient in the form of bodily fluids, e.g., blood, saliva, feces, urine, etc. A wide range of different loads of bacteria, e.g., from thousands to millions CFU/ml, can be used.

[0053] In some embodiments, the paper can be infused with a hydrogel before inoculating. For example, agar or a hydrogel precursor solution can be deposited on the paper scaffold after the decontamination step. Due to capillary wicking, the gel fills in the pores of the paper forming a flat substrate. After the gelation process, bacteria can be inoculated on the scaffold. The bacteria were found to be homogeneously distributed throughout the thickness of the paper. The viability of bacteria has been determined to be greater than 90% 1 day and 3 days following their culture in paper. See, International Application No. PCT/US2009/038566, which is incorporated by reference, for discussion of hydrogel impregnated paper culturing of cells.

[0054] A string or thread is then treated with an antibiotic or test molecule for screening of its antibiotic capabilities. For example, the thread can be treated by application of an antibiotic solution (or antibiotic suspension if the molecule is not water soluble), and allowing it to dry. In other embodiments, the antibiotic can be loaded in solid form or using vapor deposition. In one exemplary embodiment, 1-100 microgram antibiotic is loaded on the string, but milli- to –picogram levels are also contemplated. The load is selected to provide sufficient antibiotic to kill bacteria, e.g., to provide an observable color change.

[0055] Two sheets of paper containing a metabolic activity reagent and bacteria and one test thread are assembled as shown in FIG. 2. An antibiotic-embedded cotton string is placed between two layers of paper, which contained Alamar Blue® reagent and bacteria. Other

ways of assembling the system are contemplated. For example, a paper sheet may include one or more test threads in a single assembly. As noted above, a test sheet can include one or more 'wells' and the 'wells' can be tested under the same or different conditions. In FIG. 2, the antibiotic-impregnated string is sandwiched between two pieces of paper that contain the bacteria and Alamar Blue® reagent to monitor the metabolic activity.

[0056] In use, the system may be made available with one or the other or both of the metabolic activity reagent and microorganisms preloaded. In other embodiments, one or the other or both of the metabolic activity reagent and microorganisms are loaded by the user before running the test. In one embodiment, it is contemplated that the kit would be made commercially available without addition of bacteria or other microorganism.

[0057] Once assembled, the microorganisms are cultured. Culturing can be accomplished by sandwiching the assembly between two layers of wet blotting paper in a sterile petri dish. Time for culturing can vary depending on the specifics of the microorganism, however, typically the microorganisms can be cultured for overnight (15-18 h). During culturing, the AlamarBlue® viability indicator, which contains resazurin, is converted into fluorescent resorufin (bright pink) via the reduction reactions of metabolically active cells. As a result, the maintenance in the intensity of blue color in the string area corresponds to the effect of the antibiotic to the bacterial culture due to less viable cells in that area. After the culture is complete, the layers are separated and imaged, for example, by an SLR camera, laser scanners such as those designed for biomolecular imaging applications, *e.g.*, Typhoon scanner, and low-cost bench-top scanners. The samples can be scanned in either the wet or dry state.

[0058] The sandwich nature of the assembly provides two layers from each experiment, and both can be examined for information on microorganism susceptibility, providing confirmatory data from a single experiment. The devices can be photographed using a Nikon single-lens reflex camera (SLR) camera in joint photographic experts group (jpeg) or tiff format. The intensity of color in the pictures can be quantified using imaging software, such as NIH ImageJ Software. Each acquired image can be converted into HSB stack and its components can be separated in a free image processing software (NIH ImageJ). In order to quantify the intensity of color, the jpeg image can be converted into HSB stack using ImageJ and the "mean grey value" from the "Hue" component can be used. This value can be

normalized to the one obtained from a standard color strip that is used in every experiment as internal standard. These numbers can then be plotted against the type of the sample. The significant differences between results can be determined by GraphPad prism Software or any other commercially available statistical programs. The effect of the antibiotic is therefore quantified based on the change in the color intensity on the device by quantifying the "Hue" values.

[0059] The samples can be dried and stored at room temperature after the imaging process is complete.

[0060] Using the imaging process described above, it is possible to semi-quantify the susceptibility of a microorganism to a particular antibiotic. The quantifiable nature of the assay allows one to quantify antibiotic susceptibility against bacteria and to generate dose-response plots for antibiotics by varying their concentrations.

[0061] It is also contemplated to use bacteria in one string instead of one the paper. A second string containing no antibiotic or antibiotic is crossed with the first strings in between two pieces of paper with Alamar Blue®. This way, patterns of bacteria in paper can be generated. An exemplary embodiment of the invention is illustrated in exploded view in FIG. 3A. The system includes two sheets 300 and 300' of paper that can include a water impermeable edge 310 and 310' as described herein above. The porous paper can be any non-woven or woven sheet that can infuse culture medium and provide a support for cell growth.

[0062] The paper is treated with a metabolic activity reagent 320, 320'. Metabolic activity reagents as described herein above are suitable for use in this embodiment.

[0063] The system includes a first thread or filament 330 that can be seeded with microorganisms. Exemplary microorganisms can include bacteria (e.g. *Escherichia coli, Pseudomonas aeruginosa, Vibrio Cholerae, Salmonella Typhi, Bacillus subtilis, Streptococcus thermophiles, Helicobacter pylori, Yersinia pestis*), archaea, fungi, algae, and viruses. For example, the paper can be inoculated with bacteria. Bacteria can be applied to the treated paper by adding a drop of bacteria suspension in media on to paper. Other ways can include the addition of bacteria in the lyophilized form and then constitution with media. Or the paper can be entirely soaked in bacteria suspension. In some embodiments, the

bacteria are provided, e.g., as part of a kit containing the paper assay device or obtained separately, in lyophilized form. The bacterial can be reconstituted in water or the appropriate solution. In other embodiments, the microorganism is obtained cultured in nutrient broth at 37° C in a tube. In still other embodiments, the microorganisms can be obtained from a patient in the form of bodily fluids, e.g., blood, saliva, feces, urine, etc. A wide range of different loads of bacteria, from thousands to millions CFU/ml can be used.

[0064] The system also includes a second thread or filament 340 that can be treated with a molecule or test compound to be screened for antibiotic activity. Alternatively, the thread can contain no test compound. The thread can be hydrophilic for easy loading of the test agent in the liquid form. The thread can be cotton or synthetic, and can be made up of one or two or multiple strands. The thread diameter is selected to hold a selected amount of the antibiotic compound. In one or more embodiments, 100-200 micron diameter is suitable. The load of the test molecule depends on the stock concentration of the test solution. Threads as described herein can load a wide range of concentrations from milligrams to picograms.

[0065] FIGS. 3B-3D are a demonstration for the use of *E.coli* in string to generate patterns of bacteria on paper. The image for the control experiment is given in FIG. 3B indicating only Alamar Blue® on paper without bacteria. A cotton string that includes *E.coli* 11775 is incubated between two sheets of paper comprising Alamar Blue® for 8 h. The resulting images in FIG. 3C and FIG. 3D illustrate the patterns of *E.coli* on paper.

[0066] The assay can be used to identify new compounds for use as antibiotics. In one embodiment, a series of compounds whose antibiotic properties are being tested can be cultured with known microorganisms. The viability of the known microorganism in the presence of the compounds whose antibiotic properties are unknown provides information regarding the effectiveness of the compounds in treating infections.

[0067] In other embodiments, the assay can be used to determine the best course of antibiotic treatment for an infection. The test can be administered on site, for example, in a clinic, hospital or physician's office. A series of approved antibiotic can be cultured with a biological sample from a patient. The biological sample can be a bodily fluid, such as blood, saliva, urine, feces and the like. By testing the viability of the unknown infection in the presences of available antibiotics, an appropriate course of treatment can be developed. For

example, it is possible to determine whether the particular infection is an antibiotic resistant strain. One can also determine to which antibiotic an infection will respond better.

[0068] The invention is illustrated by reference to the following examples, which are provided for the purpose of illustration and are not intended to be limiting of the invention.

[0069] In these experiments, we demonstrated that we can simply use color transformation from a metabolic activity reagent to quantify antibiotic susceptibility against bacteria. The results were obtained for *E.coli* strains that have no antibiotic resistance. Our results establish that i) the images can be quantified by NIH ImageJ Software, which is a free and widely accessible program, ii) our experiments are reproducible, and iii) our paper-string hybrid platform is amenable to high-throughput sample preparation and analysis.

[0070] FIGS. 4A-4B illustrate the paper-string hybrid system for testing susceptibility of *E. coli* bacterium against different antibiotics. The images were evaluated to provide a quantitative comparison between the types of the antibiotics. We incubated a cotton string infused with an antibiotic (100 μg kanamycin, 100 μg carbenicillin, or 1 μg erythromycin) between two sheets of filter paper comprising Alamar Blue® and $5x10^4$ CFU of *E.coli* 25922 for 15 h. We removed the strings and imaged the samples using an SLR camera. The SLR images of the samples were provided above the x-axis of the plot. We evaluated the images to provide a quantitative comparison between positive and negative controls, and the type of the antibiotic. The color intensity was plotted against the cell density of the bacteria in a bar chart using Microsoft Excel. FIG. 4A illustrates the results of the positive (*E.coli* only, no antibiotic) and negative (no *E.coli*, no antibiotic) control experiments. The data points from different replicates provided consistent results (n=16). b) We determined the susceptibility of *E.coli* 25922 against carbenicillin, erythromycin, and kanamycin in the paper devices (Error bars: ± SD). *E.coli* 25922 was susceptible to carbenicillin and kanamycin at the given concentrations as indicated by the high intensity of blue color in the images. The growth of bacteria was not affected by erythromycin to a significant extent at the given concentration as shown by the high intensity of pink color compared to the positive control experiment.

[0071] A HT wax-printed paper device was fabricated to test 24 samples in parallel. The antibiotic susceptibility behavior of *E.coli* 25922 was tested against 100 μg kanamycin, 100 μg carbenicillin, and 1 μg erythromycin. The samples were prepared as noted above and were cultured for 15 hours. Twenty four replicates were performed. The color intensity in

the sample was determined by ImageJ measurements from the "Hue" component of the HSB stacks and results were plotted against type of the antibiotic. FIG. 5B shows the plots of color change for different antibiotics at 15 h. FIG. 5A shows a schematic of the experimental procedure. The paper sheet contained 24 seeding zones for bacteria that were separated by hydrophobic wax. We treated the paper with Alamar Blue® and seeded it with $2.5x10^4$ CFU *E.coli* 25922. We applied the antibiotic to the cotton string and placed it between two layers of the HT device consisting Alamar Blue® and bacteria. We cultured this assembly similar to the single samples as described in the Experimental section. (n=24, Error bars: ± SD) (FIG. 4B). We also performed negative (no bacteria, no antibiotic), and positive control (bacteria, no antibiotic) experiments. Photographs of the samples were provided above the x-axis of the plot. The blue color was preserved in the negative control experiments, while a full conversion to bright pink was achieved in the positive control experiments, as expected. The bacteria demonstrated susceptibility to carbenicillin and kanamycin while erythromycin was ineffective (resistant) to inhibit the growth of the bacteria at the given concentration. The HT paper device renders the experiments much simpler, and uses smaller amount of materials and cells compared to the single scaffolds.

[0072] The paper samples were inoculated with $0.25x10^4$ CFU, $2.5x10^4$ CFU, and $25x10^4$ CFU *E.coli* 25922 and cultured for 15 h at 37°C (FIG. 6A). We then determined the influence of initial seeding density of bacteria on the susceptibility behavior against 100 μg kanamycin (FIGS. 6A-6B). A schematic for these experiments is given in FIG. 6A. We provided the images of the samples above the x-axis of the plot (n=36 replicates, Error bars: ± SD). We used the last row of the HT paper devices as a positive control without using an antibiotic. We also performed negative control experiments without using bacteria or antibiotic. The growth of bacteria occurred as a function of the inhibitory effects of antibiotics as indicated by the presence of the blue color in the images. When the initial bacteria seeding density was $0.25x10^4$ CFU, the cells did not grow upon incubation with a string comprising 100 μg kanamycin. Because the cell density was low, the bacteria did not grow to a significant extent even when an antibiotic was not used. An initial bacteria seeding density of $2.5x10^4$ CFU allowed for bacteria to grow when they were incubated with the same concentration of kanamycin although the bacteria was susceptible to this antibiotic as indicated by the plot and the SLR image. In this case, there was a significant difference in the cell growth compared to the control case in which an antibiotic was not used. An

increasing initial bacteria seeding density of $25x10^4$ CFU provided an increase in the cell growth at the same concentration of kanamycin but the difference was not significant compared to that of $2.5x10^4$ CFU.

[0073] To determine the dose-response curves, we incubated strings that included different concentrations of an antibiotic (0.1, 1, 10, and 100 μg) on our high-throughput paper device comprising the metabolic activity agent and the bacteria for 15 h. We provide the dose-response plot for high-throughput susceptibility analysis of *E.coli* 25922 against different concentrations of kanamycin in FIGS. 7A-7B. A schematic for these experiments is given in FIG. 7A. We took images of these samples with and SLR camera after the culture period and provided them above the x-axis of the plot. We quantified the intensity of color to construct the dose-response plot for *E.coli* at the given concentrations of kanamycin (n=36 replicates, Error bars: ± SD). There was an increase in the growth of bacteria as a result of decreasing concentration of the antibiotic, as expected.

[0074] We compared different imaging techniques to quantify the results for the susceptibility of $150x10^6$ CFU/mL *E.coli* 25922 against different concentrations (100-0.1 μg) of kanamycin (n=36, Error bars: ± SD, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$) (FIGS. 7A-7B). We evaluated the change in color from the images obtained by a) an SLR camera, b) a low-cost bench-top scanner, and c) a high-resolution Typhoon scanner using ImageJ. The pictures of the samples corresponding to each measurement technique were provided above the x-axis of each plot. The trends in the intensity of color were similar using different methods of imaging. The SLR camera provided brighter images compared the low-cost flatbed bench-top scanner. The flexibility to use different methods to image samples makes our approach feasible for a wide range of research facilities including fully equipped laboratories to low-resource settings. The acquired images can alternatively be quantified with Photoshop, which is another approach to achieve quantitative evaluation of the results. The flexibility in imaging methods greatly enhances the feasibility of our approach. The acquired images can be quantified with standard methods such as NIH ImageJ Software or Photoshop. The ability to obtain images of qualitatively similar quality over a range of scanning methods provides versatility and portability of the device into inaccessible areas.

[0075] Unless otherwise defined, used or characterized herein, terms that are used herein (including technical and scientific terms) are to be interpreted as having a meaning that is

consistent with their accepted meaning in the context of the relevant art and are not to be interpreted in an idealized or overly formal sense unless expressly so defined herein. For example, if a particular composition is referenced, the composition may be substantially, though not perfectly pure, as practical and imperfect realities may apply; e.g., the potential presence of at least trace impurities (e.g., at less than 1 or 2%) can be understood as being within the scope of the description; likewise, if a particular shape is referenced, the shape is intended to include imperfect variations from ideal shapes, e.g., due to manufacturing tolerances. Percentages or concentrations expressed herein can represent either by weight or by volume.

[0076] Although the terms, first, second, third, etc., may be used herein to describe various elements, these elements are not to be limited by these terms. These terms are simply used to distinguish one element from another. Thus, a first element, discussed below, could be termed a second element without departing from the teachings of the exemplary embodiments. Spatially relative terms, such as "above," "below," "left," "right," "in front," "behind," and the like, may be used herein for ease of description to describe the relationship of one element to another element, as illustrated in the figures. It will be understood that the spatially relative terms, as well as the illustrated configurations, are intended to encompass different orientations of the apparatus in use or operation in addition to the orientations described herein and depicted in the figures. For example, if the apparatus in the figures is turned over, elements described as "below" or "beneath" other elements or features would then be oriented "above" the other elements or features. Thus, the exemplary term, "above," may encompass both an orientation of above and below. The apparatus may be otherwise oriented (e.g., rotated 90 degrees or at other orientations) and the spatially relative descriptors used herein interpreted accordingly. Further still, in this disclosure, when an element is referred to as being "on," "connected to," "coupled to," "in contact with," etc., another element, it may be directly on, connected to, coupled to, or in contact with the other element or intervening elements may be present unless otherwise specified.

[0077] The terminology used herein is for the purpose of describing particular embodiments and is not intended to be limiting of exemplary embodiments. As used herein, singular forms, such as "a" and "an," are intended to include the plural forms as well, unless the context indicates otherwise.

[0078] It will be appreciated that while a particular sequence of steps has been shown and described for purposes of explanation, the sequence may be varied in certain respects, or the steps may be combined, while still obtaining the desired configuration. Additionally, modifications to the disclosed embodiment and the invention as claimed are possible and within the scope of this disclosed invention.

CLAIMS

1. A system for evaluating susceptibility of microorganisms to a test compound, comprising:

a first porous sheet;

a second porous cellulosic sheet, wherein each of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent; and

a porous filament treated with a test compound, wherein the first and second sheets and the filament are positioned in stacking arrangement to sandwich the filament between the first and second sheets.

2. The system of claim 1, wherein the first and/or second sheets comprise cellulosic sheets.

3. The system of claim 1, further comprising a microorganism inoculated onto the first and second sheets.

4. The system of claim 1 or 3, wherein both of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier.

5. The system of claim 1 or 3, wherein both of the first and second sheets are treated with a metabolic activity reagent.

6. The system of claim 1 or 3, wherein the first and second sheets comprise a plurality of zones bounded by a fluid impermeable barrier and the zones are positioned to be in stacking alignment.

7. The system of claim 1 or 3, wherein the metabolic activity reagent comprises a colorimetric indicator.

8. The system of claim 1 or 3, wherein the filament is of a length sufficient to span the at least one zone.

9. The system of claim 1 or 3, wherein the first and second sheets further comprise a hydrogel.

10. The system of claim 1 or 3, wherein the test compound is an antibiotic.

11. A method of evaluating susceptibility of microorganisms to a test compound, comprising:

providing the system of any of claims 1-10, wherein at least one of the first and second sheets is inoculated with a microorganism and the microorganism is in contact with the filament;

culturing the microorganism-inoculated system, wherein the metabolic activity reagent undergoes reaction to provide a change indicative of metabolic activity;

imaging the cultured microorganism-inoculated system after a preselected time, wherein the image provides an indication of metabolic activity level of the microorganism in the presence of the test compound.

12. A kit for evaluating susceptibility of microorganisms, comprising:

a first porous cellulosic sheet;

a second porous cellulosic sheet, wherein each of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier;

a porous filament having a length sufficient to span the at least one zone; and

a metabolic activity reagent,

wherein the first and second sheets and the filament are capable of positioning in stacking arrangement to sandwich the filament between the first and second sheets; and

wherein the metabolic activity reagent is in a form capable of application to one or both of the first and second sheets.

13. The kit of claim 12, wherein both of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier.

14. The kit of claim 12, wherein the first and second sheets comprise a plurality of zones bounded by a fluid impermeable barrier and the zones are aligned to be in stacking arrangement.

15. The kit of claim 12, wherein the metabolic activity reagent comprises a colorimetric indicator.

16. The kit of claim 11, wherein the first and second sheets are suitable for cell culturing.

17. A system for evaluating susceptibility of microorganisms to a test compound, comprising:

a first porous cellulosic sheet;

a second porous cellulosic sheet, wherein each of the first and second sheets comprise at least one zone bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent;

a first porous filament treated with a test compound and having a length sufficient to span the at least one zone; and

a second porous filament inoculated with a microorganism and having a length sufficient to span the at least one zone;

wherein the first and second sheets and the first and filament are positioned in stacking arrangement to sandwich the filaments between the first and second sheets; and

wherein the first and second filaments contact each other at least one point.

18. A method for testing compounds for antibacterial properties, comprising:

providing multiwelled cell plate, the multiwelled cell plate comprising first and second porous cellulosic sheets, wherein each of the first and second sheets comprise a plurality of wells bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent;

introducing a known microorganism into the plurality of wells;

positioning the first and second sheets in stacking arrangement to sandwich a plurality of filaments treated with a test compound between the first and second sheets;

incubating the cells and observing a color change of the metabolic activity reagent, the color change correlated to antibiotic activity of the test compound.

19. A method for identifying an effective antibiotic for treatment of an infection, comprising:

providing multiwelled cell plate, the multiwelled cell plate comprising first and second porous cellulosic sheets, wherein each of the first and second sheets comprise a plurality of wells bounded by a fluid impermeable barrier and wherein at least one of the first and second sheets is treated with a metabolic activity reagent;

introducing a biological sample comprising an infectious microorganism into the plurality of wells;

positioning the first and second sheets in stacking arrangement to sandwich a plurality of filaments treated with an antibiotic compound between the first and second sheets; and

incubating the cells and observing a color change of the metabolic activity reagent, the color change correlated to antibiotic activity of the antibiotic compounds on the infectious microorganism.

FIG. 1A

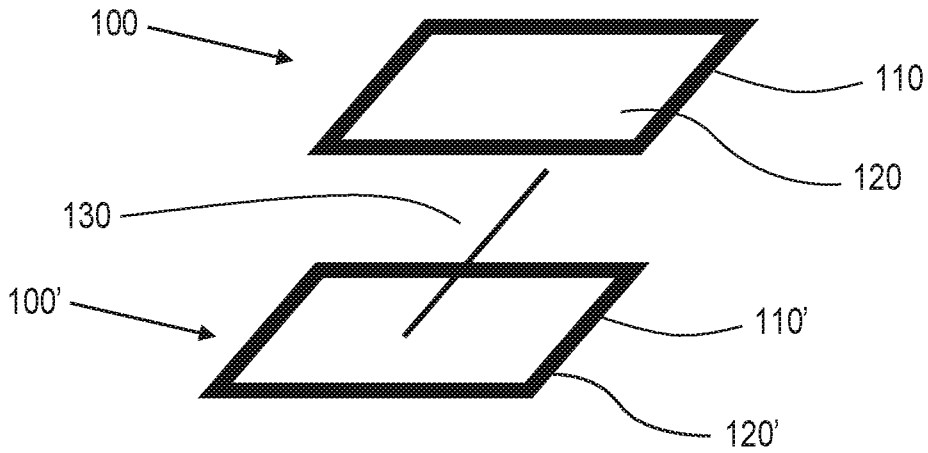


FIG. 1B



FIG. 2

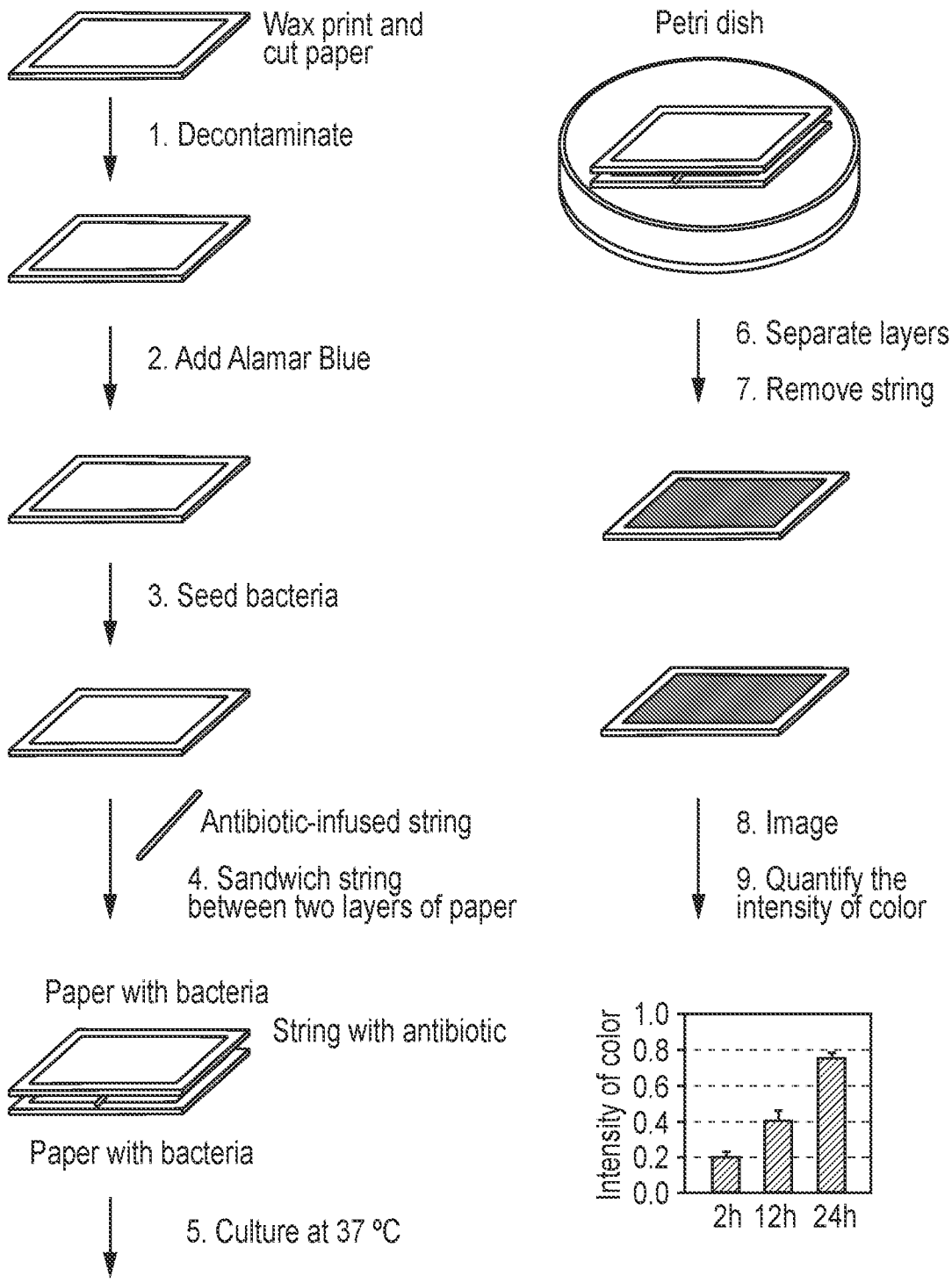
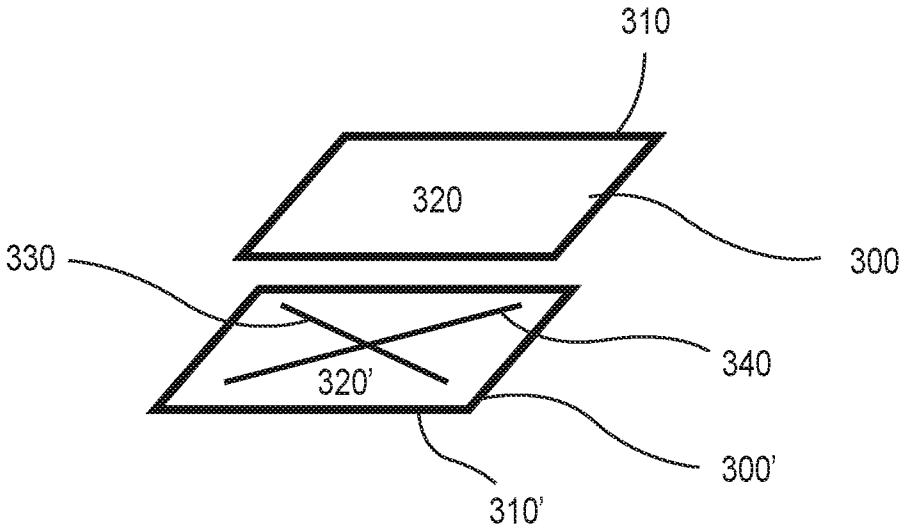

Wax print and cut paper
1. Decontaminate
2. Add Alamar Blue
3. Seed bacteria
Antibiotic-infused string
4. Sandwich string between two layers of paper
Paper with bacteria
String with antibiotic
Paper with bacteria
5. Culture at 37 °C
Petri dish
6. Separate layers
7. Remove string
8. Image
9. Quantify the intensity of color
Intensity of color
1.0
0.8
0.6
0.4
0.2
0.0
2h 12h 24h

FIG. 3A



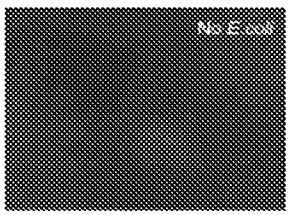


FIG. 3B

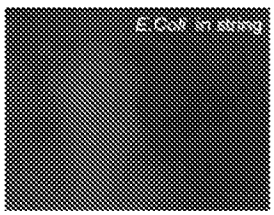


FIG. 3C

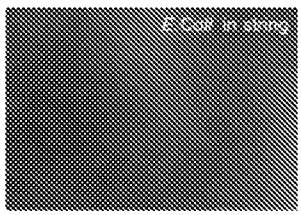


FIG. 3D

FIG. 4A

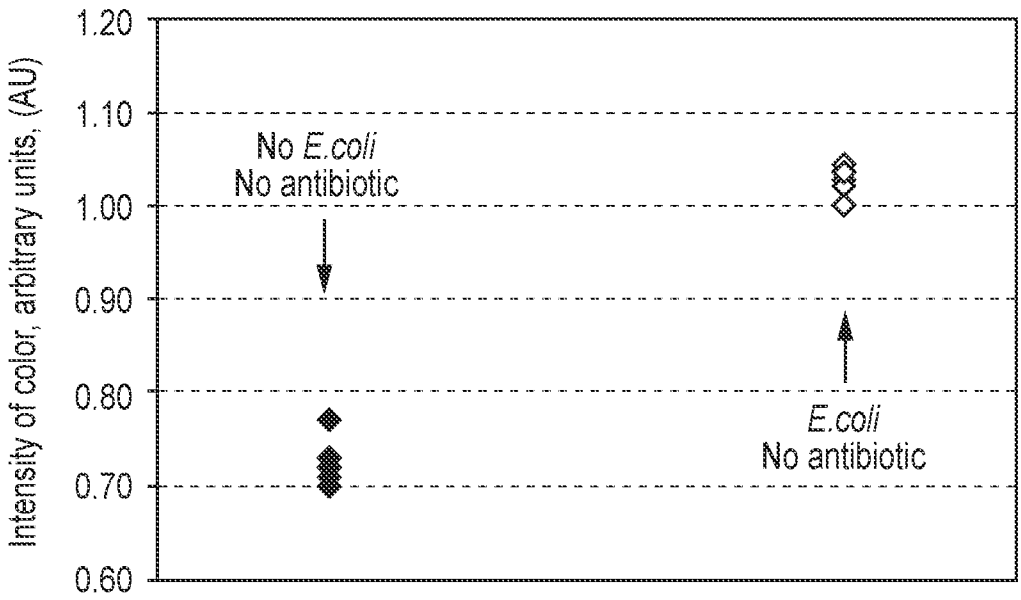

Intensity of color, arbitrary units, (AU)
1.20
1.10
1.00
0.90
0.80
0.70
0.60
No E.coli
No antibiotic
E.coli
No antibiotic

FIG. 4B

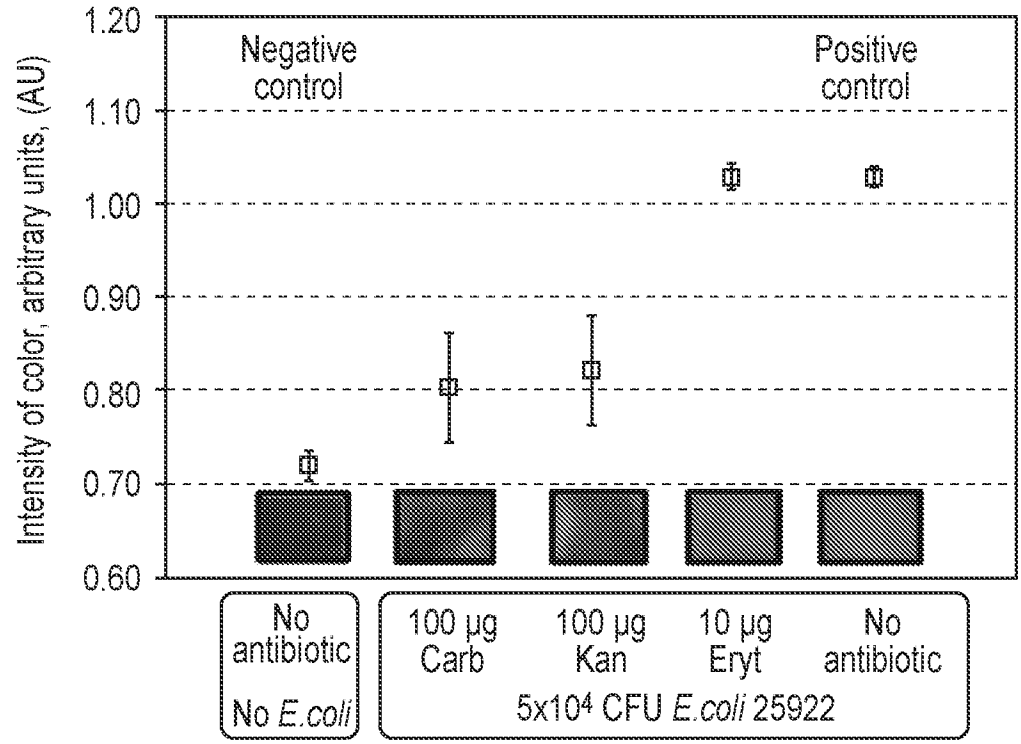
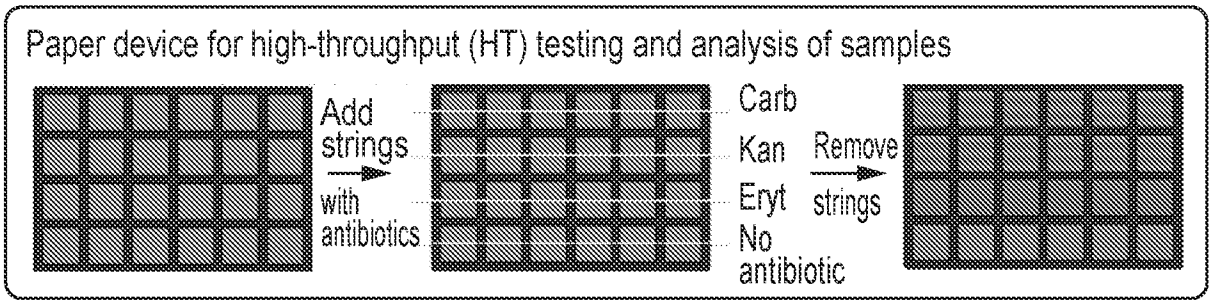

Intensity of color, arbitrary units, (AU)
1.20
1.10
1.00
0.90
0.80
0.70
0.60
Negative control
Positive control
No antibiotic
No E.coli
100 µg Carb
100 µg Kan
10 µg Eryt
No antibiotic
5x10⁴ CFU E.coli 25922

FIG. 5A


Paper device for high-throughput (HT) testing and analysis of samples
Add strings with antibiotics
Carb
Kan
Eryt
No antibiotic
Remove strings

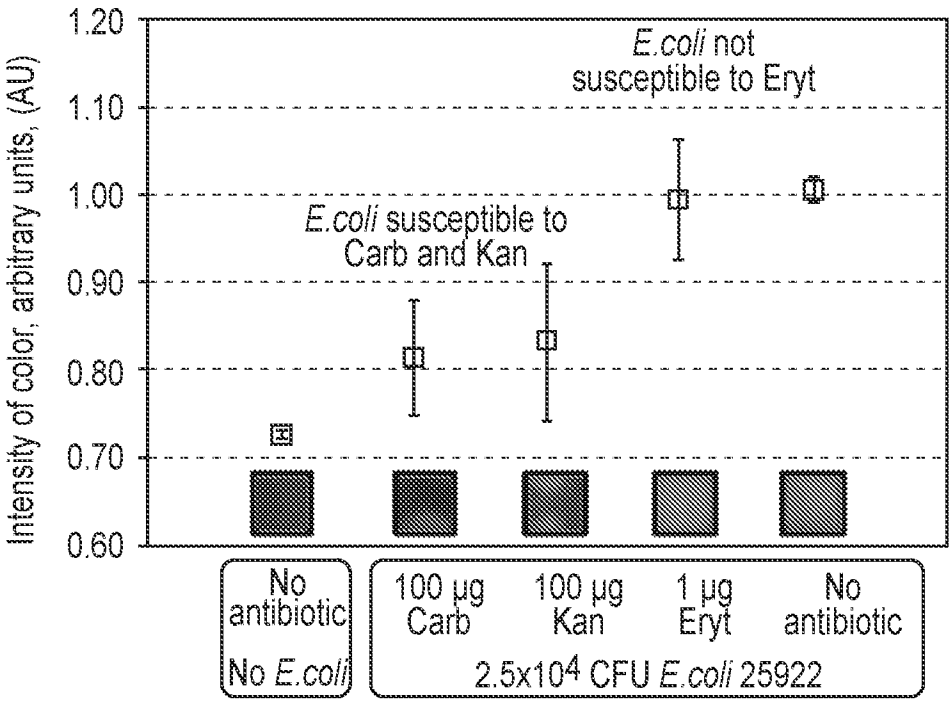

Intensity of color, arbitrary units, (AU)
1.20
1.10
1.00
0.90
0.80
0.70
0.60
E.coli not susceptible to Eryt
E.coli susceptible to Carb and Kan
No antibiotic
No E.coli
100 µg Carb
100 µg Kan
1 µg Eryt
No antibiotic
2.5x10^4 CFU E.coli 25922

FIG. 5B

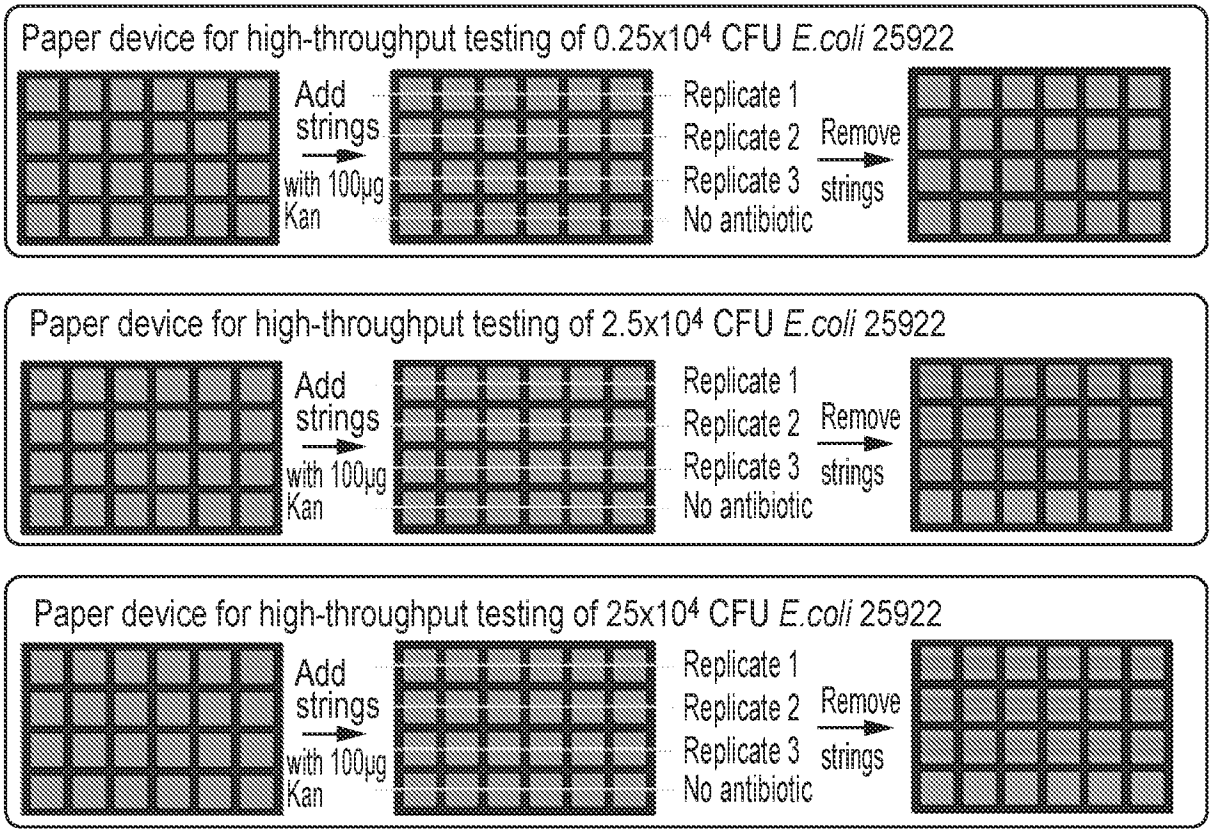

Paper device for high-throughput testing of 0.25x10⁴ CFU E.coli 25922
Add strings with 100µg Kan
Replicate 1
Replicate 2
Replicate 3
No antibiotic
Remove strings
Paper device for high-throughput testing of 2.5x10⁴ CFU E.coli 25922
Add strings with 100µg Kan
Replicate 1
Replicate 2
Replicate 3
No antibiotic
Remove strings
Paper device for high-throughput testing of 25x10⁴ CFU E.coli 25922
Add strings with 100µg Kan
Replicate 1
Replicate 2
Replicate 3
No antibiotic
Remove strings

FIG. 6A

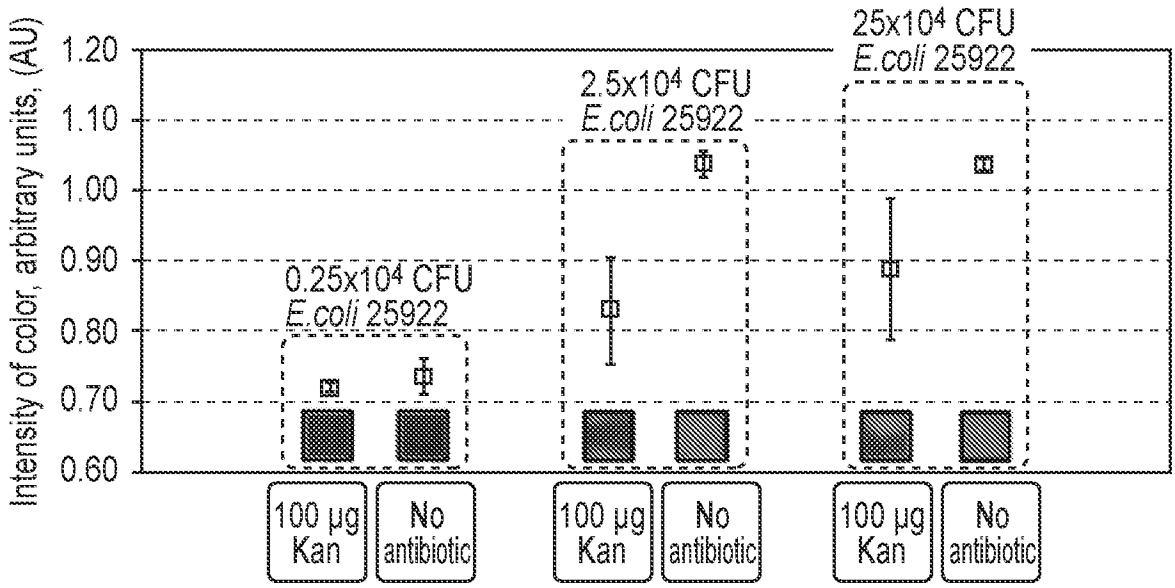
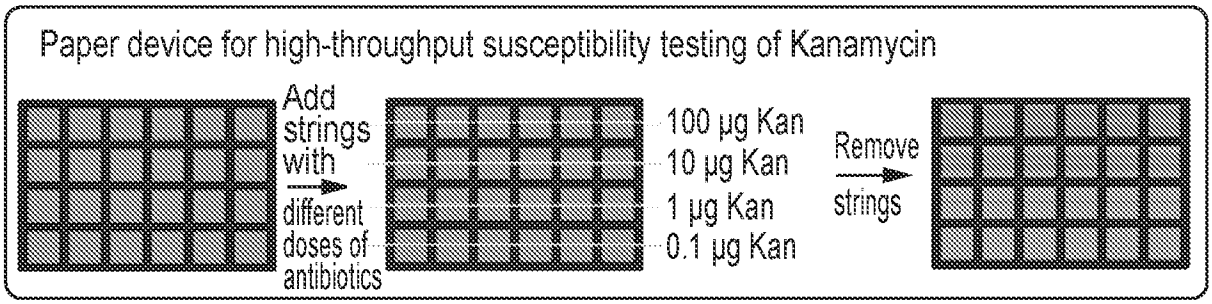

Intensity of color, arbitrary units, (AU)
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.25x10⁴ CFU E.coli 25922
2.5x10⁴ CFU E.coli 25922
25x10⁴ CFU E.coli 25922
100 µg Kan
No antibiotic
100 µg Kan
No antibiotic
100 µg Kan
No antibiotic

FIG. 6B

FIG. 7A


Paper device for high-throughput susceptibility testing of Kanamycin
Add strings with different doses of antibiotics
100 µg Kan
10 µg Kan
1 µg Kan
0.1 µg Kan
Remove strings

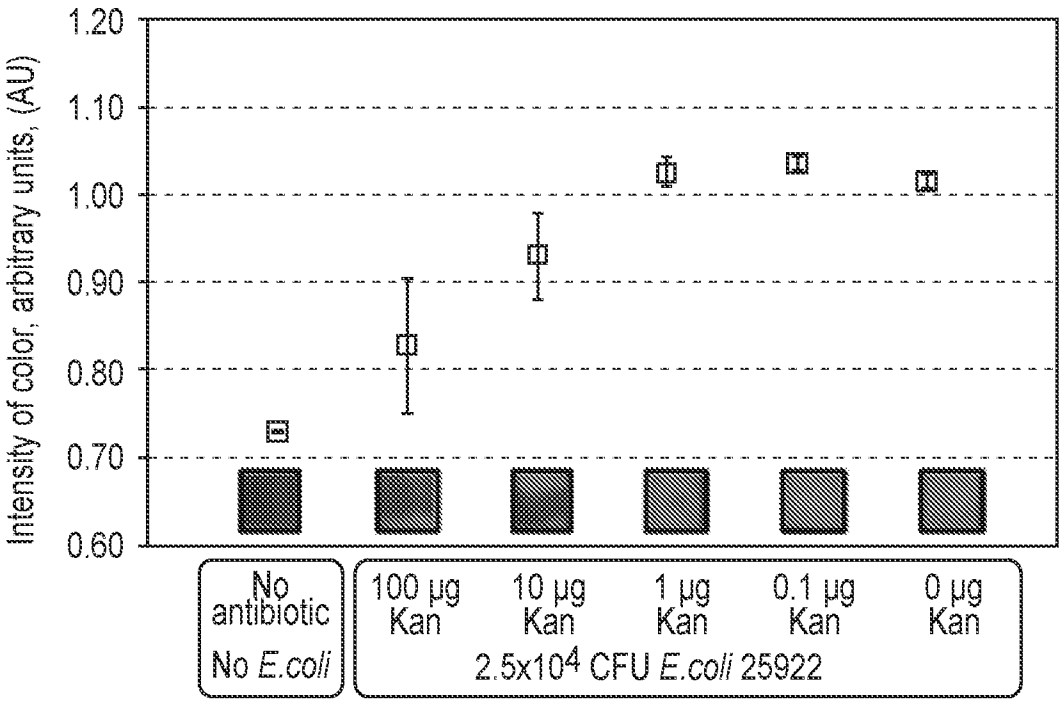

Intensity of color, arbitrary units, (AU)
1.20
1.10
1.00
0.90
0.80
0.70
0.60
No antibiotic
No E.coli
100 µg Kan
10 µg Kan
1 µg Kan
0.1 µg Kan
0 µg Kan
2.5x10^4 CFU E.coli 25922

FIG. 7B

FIG. 8A

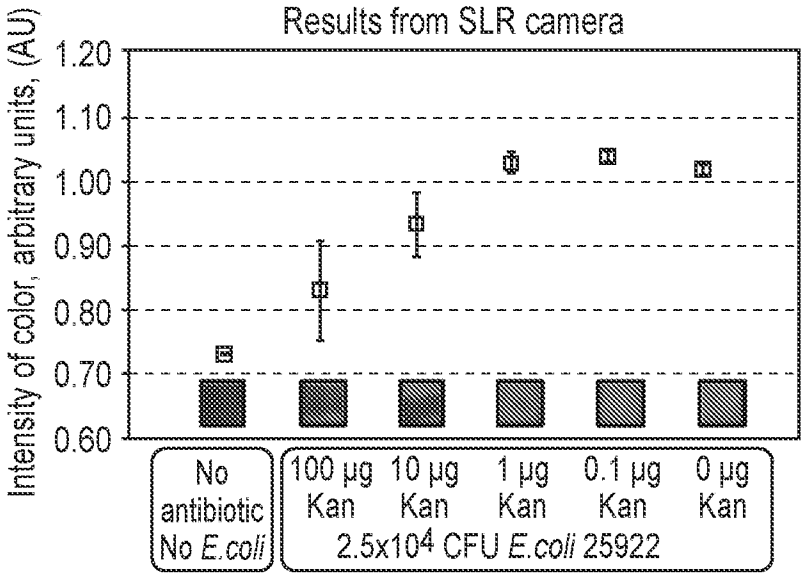


FIG. 8B

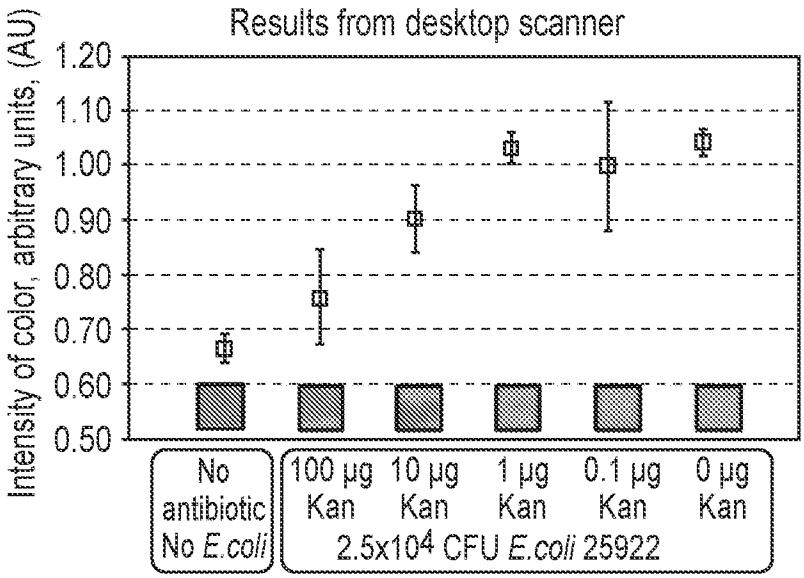
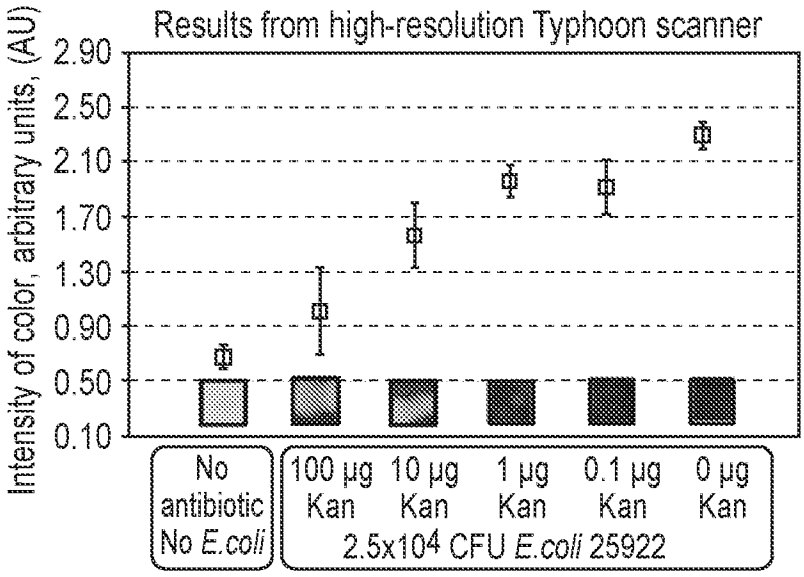


FIG. 8C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/038524

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

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

3. ☒ Claims Nos.: 11, 16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/038524

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 48/00; C12N 15/10; C12N 15/113; C12N 15/63 (2016.01)
CPC - A61K 48/00; C12N 9/22; C12N 15/1082; C12N 15/86 (2016.08)
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 - A61K 48/00; C12N 15/10; C12N 15/113; C12N 15/63
CPC - A61K 48/00; C12N 9/22; C12N 15/1082; C12N 15/86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/196; 435/462; 435/320.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Patbase, Google Patents, PubMed, Google
Search terms used: sheet cellulose impregnate porous filament zone antibiotic colorimetric dye resistance antibiotic culture bacteria

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0017629 A1 (THE GOVERNORS OF THE UNIVERSITY OF ALBERTA) 15 January 2015 (15.01.2015) entire document	1-10, 12-15, 17-19
Y	WO 1997/38312 A1 (SCRIPPS LABORATORIES INC) 16 October 1987 (17.10.1997) entire document	1-10, 12-15, 17-19
A	US 2011/0105360 A1 (DERDA et al) 05 May 2011 (05.05.2011) entire document	1-10, 12-15, 17-19
A	US 2013/0034869 A1 (WHITESIDES et al) 07 February 2013 (07.02.2013) entire document	1-10, 12-15, 17-19
A	US 2013/0316387 A1 (MACE et al) 28 November 2013 (28.11.2013) entire document	1-10, 12-15, 17-19

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent 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
26 August 2016	14 SEP 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774