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(54) Title: INSTANT READ-OUT BIOLOGICAL INDICATOR WITH GROWTH CONFIRMATION

(57) Abstract: A self-contained sterilization process biological indicator is provided. The indicator includes a cuvette having at least one liquid-impermeable wall that forms an opening into a compartment, a nucleic acid-interacting dye disposed in the compartment, a predetermined number of sterilization process-resistant spores disposed in the compartment, a first liquid medium disposed in the compartment, and a nutrient composition that facilitates germination and/or outgrowth of a viable spore. The spores in the self-contained sterilization process biological indicator are contacting the dye. The nutrient composition is disposed in the compartment and isolated from the spores. The opening is part of a pathway that permits passage of a sterilant gas into the compartment. The nucleic acid-interacting dye is fluorescent when it interacts with DNA or RNA. A method of using the self-contained sterilization process biological indicator to determine the effectiveness of a sterilization process is also provided.

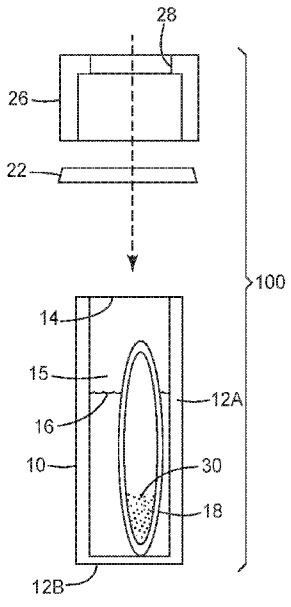


FIG. 1

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# INSTANT READ-OUT BIOLOGICAL INDICATOR WITH GROWTH CONFIRMATION

## Background

**[0001]** Monitoring the effectiveness of processes used to sterilize equipment such as medical devices, instruments and other non-disposable articles is routinely carried out in health care as well as various industrial settings. An effective sterilization process is expected to completely destroy all viable microorganisms, including microorganism forms such as viruses and spores. Hospitals, as a standard practice to assay the lethality of a sterilization process, include a sterility indicator with a batch of articles to be sterilized. Both biological and chemical sterility indicators have been used.

**[0002]** A standard type of biological sterility indicator includes a known quantity of test microorganisms, for example *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) or *Bacillus atrophaeus* (formerly *Bacillus subtilis*) spores, which are many times more resistant to a sterilization process than most contaminating organisms. After the indicator is exposed to the sterilization process, the spores are incubated in nutrient medium to determine whether any of the spores survived the sterilization process, with spore growth indicating that the sterilization process was insufficient to destroy all of the microorganisms. In another example, after being subjected to a sterilization process, the activity of an enzyme, which can be correlated with spore viability, is determined. Although advances have been made; the time required for determining with certainty whether the sterilization process was effective can be undesirably long.

**[0003]** Available chemical sterility indicators can be read immediately at the end of the sterilization process. However, the results indicate only that a particular condition was present, such as the presence of a particular chemical or a temperature for a certain period of time.

**[0004]** It is generally considered that the response of living organisms to all conditions actually present is a more direct and reliable test for how effective a sterilization process is in achieving sterilization. Accordingly, there is a continuing need for biological sterility indicators which can indicate the effectiveness of a sterilization process without an excessive delay after completion of the sterilization process.

## Summary

**[0005]** A self-contained sterilization process biological indicator is now provided, therein with everything needed to rapidly assess the effectiveness of a sterilization process and subsequently to detect germination and/or outgrowth of viable spores, if present, after exposing the self-contained sterilization process biological indicator to a sterilant gas. Advantageously, this discovery provides its user with the ability to confirm the rapid assessment (by measuring fluorescence of a nucleic acid-interacting dye) of inactivation of the spores by the sterilant gas. Thus, the aforementioned self-

contained sterilization process biological indicator can be used in a rapid method of determine effectiveness of a sterilization process. Because the rapid method does not require any germination or growth of the test microorganism, the effectiveness of the sterilization process can be determined almost immediately after the sterilization process has been completed. The optional confirmation can be conducted if the rapid result indicates all of the spores are inactivated or if the rapid result indicates not all of the spores are inactivated.

**[0006]** In one aspect, the present disclosure provides a self-contained sterilization process biological indicator. The indicator can comprise a cuvette having at least one liquid-impermeable wall that forms an opening into a compartment, a nucleic acid-interacting dye disposed in the compartment, a predetermined number of sterilization process-resistant spores disposed in the compartment, a first liquid medium disposed in the compartment, and a nutrient composition that facilitates germination and/or outgrowth of a viable spore. The spores in the self-contained sterilization process biological indicator are contacting the dye. The nutrient composition is disposed in the compartment and isolated from the spores. The opening is part of a pathway that permits passage of a sterilant gas from outside the cuvette into the compartment. The nucleic acid-interacting dye is fluorescent when it interacts with DNA or RNA. In any embodiment, the self-contained sterilization process biological indicator further can comprise a container disposed in the compartment, wherein the nutrient composition is disposed in the container.

**[0007]** In any of the above embodiments, a detection reagent, an enhancer reagent, or a collisional quenching reagent can be disposed in the compartment. In certain of the above embodiments, the liquid medium can be disposed in the container.

**[0008]** In any of the above embodiments, the nucleic acid-interacting dye can be selected from the group consisting of Acridine Orange, SYTO 9, SYTO 16, DAPI, propidium iodide, and a combination of any two or more of the foregoing nucleic acid-interacting dyes. In any of the above embodiments, the spores can be produced by a species of microorganisms selected from the group consisting of *Geobacillus stearothermophilus, Bacillus atrophaeus, Bacillus megaterium, Clostridium sporogenes, Bacillus coagulans,* and a combination of any two or more of the foregoing species.

**[0009]** In another aspect, the present disclosure provides a method of determining effectiveness of a sterilization process. The method can comprise positioning the self-contained sterilization process biological indicator of any one of the above embodiments in a sterilization chamber; while the indicator is positioned in the sterilization chamber, exposing the indicator to a sterilant gas; contacting the spores with the liquid medium; contacting the spores with the nutrient composition; after contacting the spores with the nutrient composition, incubating the indicator at a predetermined temperature for a period of time sufficient to permit germination and at least one cell division of a germinated spore; after the exposing the sterilization process biological indicator to the sterilant gas and after the contacting the spores with the liquid medium and before the incubating the indicator at the predetermined temperature, measuring a first fluorescence intensity emitted by the nucleic acid-

interacting dye in the sterilization process biological indicator; comparing the first fluorescence intensity to a reference fluorescence intensity to determine whether the exposing the sterilization process biological indicator to the sterilant gas was effective to kill all of the spores; and after the incubating the sterilization process indicator at the predetermined temperature, detecting a presence, an absence, or a quantity of a parameter associated with a germinated spore.

**[0010]** In any of the above embodiments of the method, the liquid medium can be contained in an container that is disposed in the container. The container is impermeable to the aqueous liquid medium. After the exposing the self-contained sterilization process biological indicator to the sterilant gas, the method further can comprise disintegrating the container to contact the spores with the liquid medium.

**[0011]** In any of the above embodiments, the method further can comprise prior to the exposing the indicator to the sterilant gas, positioning an article to be sterilized in the sterilization chamber.

**[0012]** Additional details of these and other embodiments are set forth in the accompanying drawings and the description below. Other features, objects and advantages will become apparent from the description and drawings, and from the accompanying claims.

**[0013]** Herein, the terms " biological sterilization process indicators", “sterilization process biological indicator”, “sterilization process indicator”, “biological indicator”, “BI”, “indicator”, “self-contained biological indicator”, and “SCBI” are used interchangeably.

**[0014]** Also herein, in the written description and the claims, the phrases "substantially dry", "substantially water- free" or the like refer to a composition or a coating which has a water content no greater than about the water content of the dehydrated coating once it has been permitted to equilibrate with the ambient environment.

**[0015]** The numbers, E5, E6, and E7 are used interchangeably herein with $10^5$, $10^6$, and $10^7$, respectively.

**[0016]** The term "comprising" and variations thereof (e.g., comprises, includes, etc.) do not have a limiting meaning where these terms appear in the description and claims.

**[0017]** As used herein, "a", "an", "the," "at least one, "and "one or more" are used interchangeably, unless the context clearly dictates otherwise.

**[0018]** Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 500 to 7000 nm includes 500, 530, 551, 575, 583, 592, 600, 620, 650, 700, etc.).

**[0019]** The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments.

## Brief Description of Drawings

**[0020]** FIG. 1 is a partially-exploded, cross-sectional schematic view of one embodiment of a self-contained sterilization process biological indicator according to the present disclosure.

**[0021]** FIG. 2 is a top view of the self-contained sterilization process biological indicator of FIG. 1.

**[0022]** FIG. 3 is a partially-exploded, cross-sectional schematic view of an alternative embodiment of a self-contained sterilization process biological indicator according to the present disclosure.

## Detailed Description

**[0023]** As indicated above, self-contained biological sterilization process indicators are now provided which can detect an indication of viable spores almost immediately upon removal of the biological sterilization process indicators from a sterilization process. Advantageously, the near-immediate detection of an indication of viable spores (after removal of the BI from a sterilizer) eliminates undesirably-long waiting periods to determine whether a sterilization process was effective. In addition, the indication of viable spores present in the biological sterilization process indicators can be detected automatically, thereby eliminating a potential human error in deciding whether microbial growth or enzyme activity is present after the biological sterilization process indicators have been exposed to a sterilization process. Furthermore, the rapid result (either indicating sterility or non-sterility) subsequently can be confirmed (e.g., by detecting germination and growth of a viable spore) using a self-contained biological sterilization process indicator of the present disclosure according to a method of the present disclosure.

**[0024]** For certain embodiments, including any one of the biological indicator or method embodiments described herein, the at least one nucleic acid-interacting dye can interact with nucleic acids present in the spores, resulting in an increase in fluorescence intensity in the biological indicator. The interaction of the dye with the nucleic acids while the spores are exposed to a sterilant or shortly thereafter provides an early indication of viable spores, if present in the biological indicator after the exposure.

**[0025]** For certain embodiments, including any one of the biological indicator or method embodiments described herein, the at least one nucleic acid-interacting dye can interact with nucleic acids present in the plurality of spores to produce a measurable fluorescence intensity that corresponds to killed spores. Without being bound by theory, the investigators believe this is due to the ability of the dye to penetrate spores that have increased permeability (e.g., due to poor integrity of the spore coat, the spore cortex, and/or the spore membrane(s) (e.g., spore inner membrane and/or spore outer membrane) after exposure to a sterilant gas) of the nonviable spores. This effect is not seen in vegetative cells. In contrast to the differential reaction of SYTO®9 dye with live versus dead spores shown herein, the SYTO9 nucleic acid-interacting dye can penetrate both live and dead vegetative cells, as evidenced by the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit available from Molecular Probes, Inc. (Eugene, OR). Selective

interaction of the dye with nucleic acids present in the nonviable spores results in lower fluorescence when viable spores are present. Thus, an absence of viable spores in the biological indicator after exposure to a sterilant gas results in a correspondingly higher fluorescence intensity.

**[0026]** A number of sterilization process are presently known and in use, including, for example, exposure to steam, dry heat, gaseous or liquid agents such as ethylene oxide, hydrogen peroxide, peracetic acid, and ozone, and radiation. The plurality of sterilization process resistant spores may be selected according to the sterilization process to be used. Any spores may be used as long as they provide sufficient resistance to the sterilization process conditions, such that the spores are more resistant to the sterilization process conditions than most microorganisms encountered in natural contamination. For example, for a steam sterilization process, *Gb. stearothermophilus* (formerly *Bacillus stearothermophilus)* may be used. In another example, for an ethylene oxide sterilization process, *B. atrophaeus* (formerly *B. subtilis)* may be used. For certain embodiments, including any one of the above process indicator and method embodiments, the plurality of sterilization process resistant spores is selected from the group consisting of *Geobacillus stearothermophilus, Bacillus atrophaeus, Bacillus megaterium, Clostridium sporogenes, Bacillus coagulans,* and a combination thereof. For certain of these embodiments, the plurality of sterilization process resistant spores is selected from the group consisting of *Gb. stearothermophilus, B. atrophaeus, B. megaterium,* and a combination thereof.

**[0027]** By way of example only, the present disclosure describes the microorganisms used in the biological sterilization indicator as being "spores;" however, it should be understood that the type of microorganism (e.g., spore) used in a particular embodiment of the biological sterilization indicator is selected for being highly resistant to the particular sterilization process contemplated. Accordingly, different embodiments of the present disclosure may use different microorganisms, depending on the sterilization process for which the particular embodiment is intended.

**[0028]** Using nucleic acid-interacting fluorescent dyes, the present biological indicators and methods can detect the presence of nucleic acids within the spores, without the need for germination and/or outgrowth. In any embodiment of the method disclosed herein, a sub-lethal concentration of nucleic acid-interacting dye is used so that germination and/or outgrowth of the spores can be done to confirm that the microorganisms in the biological indicators of the present invention have been inactivated (e.g., killed). Accordingly, for certain embodiments, including any one of the above indicator and method embodiments, the concentration of the nucleic acid-interacting fluorescent dye in the medium is not more than 0.10 mM, 0.05 mM, or 0.01 mM. For certain of these embodiments, the concentration of the dye is not less than 0.0001 mM, 0.0005 mM, or 0.001 mM.

**[0029]** In some, if not all, of the sterilization processes in use, an elevated temperature, for example, 50° C, 100° C, 121° C, 132° C, 134° C, 134° C, or the like, is included or may be

encountered in the process. Accordingly, for certain embodiments, including any one of the above biological indicator and method embodiments, the nucleic acid-interacting fluorescent dyes are stable at sterilization temperatures.

**[0030]** For certain embodiments, including any one of the above biological indicator and method embodiments, nucleic acid-interacting fluorescent dye is stable at a temperature up to at least 121° C. For certain of these embodiments, the nucleic acid-interacting fluorescent dye is stable at a temperature up to at least 132° C. For certain of these embodiments, the nucleic acid-interacting fluorescent dye is stable at a temperature up to at least 134° C. For certain of these embodiments, the nucleic acid-interacting fluorescent dye is stable at a temperature up to at least 135° C. For certain of these embodiments, the nucleic acid-interacting fluorescent dye is stable when exposed to temperatures between 121-135° C for periods of time that are customary for sterilization processes.

**[0031]** For certain embodiments, including any one of the above biological indicator or method embodiments, the liquid medium is essentially free of any background fluorescence at emission and excitation wavelengths used to detect the fluorescence intensity of the nucleic acid-interacting dye when it is interacting with nucleic acid in the biological indicator. This may provide an improved sensitivity because any background level of fluorescence occurring at the same wavelengths as the emission and excitation of the dye interacting with a nucleic acid, is minimized.

**[0032]** For certain embodiments, including any one of the above biological indicator or method embodiments, the medium is essentially free of any nucleic acids other than nucleic acids present in and produced by the plurality of spores. This may provide an improved sensitivity because any baseline level of fluorescence resulting from the dye interacting with any nucleic acids not present in the plurality of spores is minimized.

**[0033]** The nucleic acid-interacting dye has a lower level of fluorescence at the emission wavelength (or wavelength range) when not interacting with a nucleic acid and a higher level fluorescence at this wavelength when interacting with a nucleic acid. For example, in certain embodiments, when not interacting with a nucleic acid the nucleic acid-interacting dye has a fluorescence quantum yield of less than 0.1, preferably less than 0.05, more preferably not more than 0.01. In certain of these embodiments, when interacting with a nucleic acid the nucleic acid-interacting dye has a fluorescence quantum yield of at least 0.1, preferably at least 0.2, more preferably at least 0.4.

**[0034]** For certain embodiments, including any one of the above biological indicator or method embodiments, the nucleic acid-interacting fluorescent dye is a dye which interacts with DNA, RNA, or DNA and RNA. The interaction of the dye with DNA and RNA may be the same or different. The dye interacting with DNA may have a different excitation and/or emission maximum than the same dye interacting with RNA.

**[0035]** For certain embodiments, including any one of the above biological indicator or method embodiments, the nucleic acid-interacting fluorescent dye is a dye which interacts with the nucleic acids in a variety of ways know in the art, including intercalation, electrostatic attraction, charge interaction, hydrophilic-hydrophobic interaction, or a combination thereof. As indicated above, this interacting or binding of the dye with nucleic acids, which include total cellular nucleic acids, such as DNA, RNA (mRNA, rRNA, tRNA), and extrachromosomal nucleic acids, causes a relatively large increase in fluorescence from the dye. For certain of these embodiments, the nucleic acid-interacting fluorescent dye is selected from the group consisting of acridine orange, a substituted unsymmetrical cyanine dye, and salts thereof, and combinations thereof.

**[0036]** Acridine orange bound to a DNA has an excitation maximum at about 490 nm and an emission maximum at about 520 maximum, but when bound to an RNA about 530 nm and 620 nm, respectively. See MacInnes, J. W and McClintock, M., Differences in Fluorescence Spectra of Acridine Orange-DNA Complexes Related to DNA Base Composition, Biopolymers. Communications to the editor, Vol 9, Pages 1407-1411 (1970). For certain embodiments, fluorescence at the emission maximum of the dye bound to RNA can be used to indicate a presence of viable spores.

**[0037]** Suitable examples of substituted unsymmetrical cyanine dyes include dyes available under the trade name, SYTO (Invitrogen Corp., Carlsbad, Calif.). SYTO dyes are permeable to many, if not all, cell membranes but may differ from each other, for example, in degree of permeability through intact spore coats and/or spore cortexes, and/or spore membranes; amount of fluorescence intensity increase when bound to a nucleic acid; excitation and emission maxima; selectivity in binding to DNA and RNA; and binding affinity to DNA and RNA. See Tarnok, Cytometry Part A, 73A, 477-479 (2008). Suitable nucleic acid-interacting dyes of the present disclosure include those nucleic acid-interacting dyes that are substantially excluded from viable spores (i.e., spores that are capable of germination to form vegetative cells that are capable of reproduction) but are not excluded from nonviable spores (e.g., spores that have been exposed to a lethal sterilization process. The determination of selective permeability of any given nucleic acid-interacting dye is easily performed by a person having ordinary skill in the art by separately contacting similar numbers of viable and nonviable spores of a given species with the nucleic acid-interacting dye in a microwell plate according to the method described in Example 2 of U.S. Provisional Patent Application No. 62/785,386, filed on even date herewith and entitled "INSTANT READ-OUT BIOLOGICAL INDICATOR", which is incorporated herein by reference in its entirety.

**[0038]** Suitable nucleic acid-interacting dyes include, but are not limited to Acridine Orange, SYTO9, SYTO16, DAPI, propidium iodide, and a combination of any two or more of the foregoing nucleic acid-interacting dyes. For certain embodiments, preferably the substituted unsymmetrical cyanine dye is SYTO9 or SYTO 16.

**[0039]** Preferred nucleic acid-interacting dyes penetrate the spore coat and/or spore cortex and/or spore membranes substantially better after the spores have been exposed to a gas sterilant under conditions that result in the disruption and/or activation of substantially all of the spores. Without being bound by theory, it is believed the disruption of the spore coat and/or spore cortex and/or spore membrane(s) facilitates penetration of the dye into the spore, thereby also facilitating interaction of the dye with nucleic acids disposed in and/or released from the disrupted spores.

**[0040]** The nucleic acid-interacting dyes can be used in a self-contained biological sterilization process indicator at concentrations of about 0.05 μM to about 50 μM.

**[0041]** In certain preferred embodiments, the nucleic acid-interacting dye differentially penetrates (and interacts with the nucleic acid in) inactivated (killed) spores relative to viable spores. Thus, the higher the number of spores inactivated by the sterilant gas, the greater the interaction between the nucleic acid-interacting dye and the nucleic acid in the spores. Accordingly, the higher the number of spores inactivated by the sterilant gas, the greater the intensity of fluorescent signal emitted by the dye-bound nucleic acid from the spores in the sterilization process indicator.

**[0042]** The at least one nutrient composition of the present disclosure induces germination and may also provide for outgrowth of the spores, if viable, with the simultaneous production of nucleic acids. Moreover, it has been found that the presence of the nutrient composition with the dye reduces the effective toxicity of the dye with respect to the spores. The nutrient composition includes one or more sugars, for example, glucose, fructose, cellibiose, or the like. The nutrient composition may also include a salt such as potassium chloride, calcium chloride, or the like. The nutrient composition may also include at least one amino acid, for example, at least one of methionine, phenylalanine, and tryptophan. The germination medium may also include one or more other materials with the nutrient composition. The quantities of such nutrient compositions and materials as well as other nutrient compositions known in the art for inducing germination and initial outgrowth of the sterilization process resistant spores may be used. Media components and concentrations are known and described, for example, WO 99/05310 (Tautvydas) and Zechman et al., J. Food, Sci., 56, 5, p. 1408-1411 (1991), referred to as Zechman and Pflug, 1991.

**[0043]** For certain embodiments, including any one of the biological indicator or method embodiments described herein, the biological indicator further comprises a collisional quenching component disposed in the compartment (e.g., in the liquid medium. Such components reduce the background fluorescence signal of free (unbound to nucleic acid) nucleic acid-interacting dye through collisional quenching. Examples of species known to collisionally quench fluorescence include organic compounds such as purines, pyrimidines, aliphatic amines, and nitroxides, certain ions, for example, nitrate anions and dissolved metal ions. Other species known to

collisionally quench fluorescence are described, for example, in Principles of Fluorescence Spectroscopy, Chapter 9, Joseph R. Lakowicz, Plenum Press, 1983.

**[0044]** For certain embodiments, including any one of the biological indicator or method embodiments described herein, the biological indicator further comprises at least one reference dye disposed in the compartment (e.g., in the liquid medium). The reference dye does not bind to nucleic acids but responds similarly to nucleic acid-interacting dyes to changes in temperature or changes in the medium induced by spore germination and outgrowth, for example an increase or decrease in pH, ionic strength, or concentration change in metabolic by-products that alter the fluorescent signal. By monitoring the reference dye, signal from the nucleic acid-interacting dye binding to nucleic acid can be distinguished from signal produced from a change in temperature or a change in the media. The reference dye preferably fluoresces at a different wavelength than the nucleic acid-interacting dye.

**[0045]** As indicated above, the sterilization process indicator provided herein comprises a predetermined number of sterilization process resistant viable spores disposed in a compartment of a cuvette. The predetermined number of spores can comprise at least 10, at least $10^2$, at least $10^3$, at least $10^4$, at least $10^5$, at least $10^6$, or at least $10^7$ viable spores. In certain embodiments, of the present disclosure has a predetermined number of about $10^6$ viable spores disposed therein.

**[0046]** The germination medium and the sterilization process resistant spores are kept separate but in close proximity to each other for ease of combining the medium and the spores when desired, for example, after exposure to a sterilization process and before detection of a first fluorescence intensity; and then incubating spores with the germination medium to confirm whether or not any viable spores are present as would be indicated by a lower than expected first fluorescence intensity. Accordingly, for certain embodiments, including any one of the above indicator and method embodiments, the plurality of sterilization process resistant spores and the germination medium are separate from each other and adjacent each other in the chamber of the process indicator.

**[0047]** For certain embodiments, including any one of the above indicator and method embodiments, the germination medium is an aqueous solution or suspension. The concentration of the at least one nucleic acid-interacting fluorescent dye in the medium is dependent on the minimum level needed to provide a detectable first fluorescence intensity after the process indicator is exposed to a sterilant in a sterilization process, and on the maximum level that can be tolerated by the spores without a significant adverse effect on the germination and/or growth of the spores. Examples of dye concentrations that may be used are described above. When viable spores are present the detectable first fluorescence intensity can be measured in less than 30 minutes, preferably less than 15 minutes, more preferably in less than 10 minutes, and even more preferably in less than 5 minutes after completion of exposure of the process indicator to the sterilant.

**[0048]** In one alternative, for certain embodiments, the germination medium is in a dry form. The at least one nucleic acid-interacting fluorescent dye and the at least one nutrient composition can be dried together or separately to form a film or a layer on a support film or on a carrier material, optionally in a desired shape, or compounded together or separately as dry solids to form a tablet(s), caplet(s), or capsule(s). Any of these forms can be kept adjacent the spores, and water or an aqueous buffer can be added at an appropriate time to incubate the spores with the resulting medium suspension or solution. When resuspended or dissolved, the resulting medium can be a liquid or a gel. Additional embodiments of a medium in a dry form, which can be used in the indicator and method embodiments described herein are described in PCT Application Publication No. WO2010045138A2.

**[0049]** When incubating the spores with the germination medium, an incubation temperature above room temperature may be used. For certain embodiments, the incubation temperature is at least 37° C. For certain embodiments, preferably the incubation temperature is at least 50° C. For certain embodiments, the incubation temperature is 50 to 60° C.

**[0050]** As indicated above, the sterilization process indicator provided herein comprises a predetermined number of sterilization process resistant viable spores disposed in an compartment of a cuvette. The predetermined number of spores can comprise at least 10, at least $10^2$, at least $10^3$, at least $10^4$, at least $10^5$, at least $10^6$, or at least $10^7$ viable spores. In certain embodiments, of the present disclosure has a predetermined number of about $10^6$ viable spores disposed therein.

**[0051]** In certain embodiments, the spores may be disposed on a carrier. For certain embodiments, the carrier is a sheet material such as paper, woven cloth, nonwoven cloth, plastic, a polymeric material, a microporous polymeric material, metal foil, glass, porcelain, ceramic, or the like, or a combination thereof. For certain embodiments, the sheet material is water-absorbent or can be wetted to aid in quickly bringing the liquid medium in intimate contact with the spores at the appropriate time.

**[0052]** In certain embodiments, the sterilization process indicator also comprises a container, which contains the liquid medium and, optionally, the nutrient composition, without allowing the sterilant or any microorganism to enter the container. As indicated above, the carrier is adjacent to the container for ease of contacting the spores, supported by the carrier, with the liquid medium and the germination medium when incubation is to be initiated. The container can be readily opened to contact the spores with the medium by expelling a plug, crushing or puncturing the container, or the like. The container can be equipped with a plug, or at least a portion of the container can be a breakable material, such as glass (e.g., glass ampoule) or other material, which can be breached by physical pressure but sufficiently tough to remain intact during manufacturing, storage, shipping, and sterilization conditions.

**[0053]** Known biological sterilization process indicator constructions such as those described, for example, in U.S. Pat. No. 5,073,488 (Matner et al.) and in U.S. Patent No. 10,047,334

(Chandrapati et al.) may be adapted to construct a biological indicator of the present disclosure. FIGS. 1 and 2 show one embodiment of a sterilization process indicator 100 according to the present disclosure. The sterilization process indicator 100 includes a cuvette 10 having at least one liquid impermeable wall (e.g., side wall 12A and bottom wall 12B) that forms an opening 14 into a compartment 15. The cuvette 10 is shown as a circular tube, but other known configurations can be used. The at least one wall is preferably transparent or translucent to the extent that electromagnetic radiation of a particular wavelength (e.g., u.v.-visible) and intensity sufficient to cause a nucleic acid-interacting dye to fluoresce can be transmitted therethrough. In addition, the walls are preferably transparent or translucent to the extent that electromagnetic radiation at a particular wavelength (e.g., in the visible wavelength range) emitted by the nucleic acid-interacting dye can be transmitted through the wall and be measured. Suitable materials for the walls may include glass, polycarbonate, polypropylene, polyester, and the like. For certain embodiments, the at least one wall of the cuvette transmits at least 90% of incident electromagnetic radiation within a wavelength range of at least 500 to 700 nm, preferably at least 500 to 675 nm.

**[0054]** In any embodiment, at least a portion of the cuvette can be configured (e.g., shaped and dimensioned) to be received into an instrument capable of detecting fluorescence emitted by the nucleic acid-interacting dye. That is, the instrument is configured to illuminate the portion of the cuvette with electromagnetic radiation of an appropriate first wavelength to cause fluorescence of the nucleic acid-interacting dye when the dye is bound to DNA or RNA. Moreover, the instrument is configured to detect and quantify electromagnetic radiation of a second wavelength emitted by fluorescence of the nucleic acid-bound nucleic acid-interacting dye. In certain embodiments, the instrument is also capable of measuring absorbance or scattering of electromagnetic radiation (e.g., at wavelengths typically absorbed or scattered by spores and/or microorganisms) passed through the portion of the cuvette. That is, the instrument is configured to pass the radiation through the portion of the cuvette and also detect an amount of radiation that passed through the cuvette. In certain alternative embodiments, at least a portion of the cuvette is configured (e.g., shaped and dimensioned) to be received into a second instrument, the second instrument being capable of measuring the absorbance of electromagnetic radiation passed through the cuvette.

**[0055]** Disposed in the compartment 15 is a liquid medium 16, a nucleic acid-interacting dye (not shown), and a predetermined number of sterilization process-resistant spores (not shown). Suitable nucleic acid-interacting dyes are disclosed hereinabove. The liquid medium 16 can be a liquid (e.g., an aqueous liquid) in which the nucleic acid-interacting dye is suspended and/or dissolved. In certain embodiments, the nucleic acid-interacting dye is disposed in the liquid medium. In certain embodiments, the spores are disposed in the liquid medium. In certain embodiments, such as the illustrated embodiment of FIGS. 1 and 2, the spores and the nucleic acid-interacting dye are disposed in the liquid medium 16.

**[0056]** In use, the sterilization process indicator 100 is exposed to a sterilant gas (e.g., in the sterilization chamber of an automated sterilizer). The sterilant gas (not shown) passes through the closure member 22 into the compartment 15 where it contacts the liquid medium in which the spores and the nucleic acid-interacting dye are disposed. As the sterilant gas inactivates the spores, the nucleic acid-interacting dye is able to penetrate the spore and interact with the nucleic acid therein.

**[0057]** In certain embodiments, the liquid medium, whether present in the process indicator in the compartment or in a container as described herein, has a volume of not less than 10 microliters, not less than 20 microliters, not less than 50 microliters, not less than 100 microliters, or not less than 150 microliters. In certain embodiments, the liquid medium, whether present in the process indicator in the compartment or in a container as described herein, has a volume of not more than 1000 microliters, not more than 500 microliters, not more than 400 microliters, not more than 300 microliters, not more than 250 microliters, or not more than 200 microliters. In certain embodiments, the volume of the liquid medium is about 10 microliters to about 1000 microliters. In certain embodiments, the volume of the liquid medium is about 10 microliters to about 500 microliters. In certain embodiments, the volume of the liquid medium is about 100 microliters to about 250 microliters.

**[0058]** The opening 14 to compartment 15 is provided with a gas-transmissive, microorganism-impermeable closure member 22, which may be adhered to cuvette 10 by an adhesive, a heat seal, or the like. Alternatively, closure member 22 may be held on to opening 14 with a cap 26 having an aperture 28. During exposure to a sterilant gas, the sterilant gas is directed through the closure member 22, enters the compartment 15, and contacts the spores. In certain preferred embodiments, the pathway that permits passage of the sterilant gas into the cuvette also resists or prevents passage into the container of bacteria from outside the sterilization process indicator. A nonlimiting example of suitable materials for closure members include microporous materials such as a filter membrane. Alternative embodiments for the above structures are shown in U.S. Patent Publication No. 20150165082 (Chandrapati et al.), filed Oct. 17, 2008, entitled Biological Sterilization Indicator, System, and Methods of Using Same. A skilled artisan will recognize alternative structures (e.g., a tortuous path) that may be suitable for providing passage of a sterilant gas into the compartment 15 while resisting or preventing passage of bacteria into the compartment.

**[0059]** A container 18, which holds the nutrient composition (dry powder 30), is shown within compartment 15. In an alternative embodiment (not shown), the container can be positioned outside of and the adjacent compartment, with a conduit providing fluid communication between the container and the compartment. Container 18, which is sealed, can be a breakable ampoule, but could alternatively be a container equipped with a plug, or other mechanism which when activated allows the nutrient composition to contact carrier 16 and the spores supported thereon.

Container 18 is shown as an elongated ampoule, but other known configurations can be used as well. Optionally, the dry powder 30 further can comprise the collisional quenching component and/or detection reagent disclosed herein.

**[0060]** In certain embodiments, the aqueous liquid medium, whether present in the process indicator in the compartment or in a container as described herein, has a volume of not less than 10 microliters, not less than 20 microliters, not less than 50 microliters, not less than 100 microliters, or mot less than 150 microliters. In certain embodiments, the aqueous liquid medium, whether present in the process indicator in the compartment or in a container as described herein, has a volume of not more than 1000 microliters, not more than 500 microliters, not more than 400 microliters, not more than 300 microliters, not more than 250 microliters, or not more than 200 microliters. In certain embodiments, the volume of the aqueous liquid medium is about 10 microliters to about 1000 microliters. In certain embodiments, the volume of the aqueous liquid medium is about 10 microliters to about 500 microliters. In certain embodiments, the volume of the aqueous liquid medium is about 100 microliters to about 250 microliters.

**[0061]** The opening 14 to compartment 15 is provided with a gas-transmissive, microorganism-impermeable closure member 22, which may be adhered to cuvette 10 by an adhesive, a heat seal, or the like. Alternatively, closure member 22 may be held on to opening 14 with a cap 26 having an aperture 28. During exposure to a sterilant gas, the sterilant gas is directed through the closure member 22, enters the compartment 15, and contacts the spores. A nonlimiting example of suitable materials for closure members include microporous materials such as a filter membrane. Alternative embodiments for the above structures are shown in U.S. Patent Publication No. 20150165082 (Chandrapati et al.), filed Oct. 17, 2008, entitled Biological Sterilization Indicator, System, and Methods of Using Same. A skilled artisan will recognize alternative structures (e.g., a tortuous path) that may be suitable for providing passage of a sterilant gas into the compartment 15 while preventing passage of bacteria into the compartment.

**[0062]** In certain embodiments, the container is opened (e.g., by crushing) prior to exposing the sterilization process indicator to the sterilant gas. In certain embodiments, the container is opened (e.g., by crushing) after exposing the sterilization process indicator to the sterilant gas. Opening the container places the nutrient composition in contact with the aqueous liquid medium, thereby dissolving the nutrient composition into the medium and placing the nutrient composition in fluid contact with the spores.

**[0063]** In any embodiment of a sterilization process indicator of the present disclosure, the sterilant gasses include, but are not limited to, water vapor (i.e., steam), ethylene oxide, hydrogen peroxide, and ozone.

**[0064]** FIG. 3 shows an alternative embodiment of a sterilization process indicator 200 according to the present disclosure. The indicator comprises a cuvette 10 having at least one liquid impermeable wall (e.g., side wall 12A and bottom wall 12B) that forms an opening 14 into a

compartment 15, each as described hereinabove. Disposed in the compartment 15 is a liquid medium 16, a nucleic acid-interacting dye and a predetermined number of sterilization process-resistant spores 20, each as described hereinabove. In the illustrated embodiment of FIG. 3, the liquid medium 16 is contained in a container 18. The sterilization process indicator 200 also includes a gas-transmissive, microorganism-impermeable closure member 22 and a cap 26, each as described hereinabove.

**[0065]** In certain embodiments of the process indicator 200, such as the illustrated embodiment of FIG. 3, the liquid medium 16 in the container 18 can have the nutrient composition (not shown) suspended and/or dissolved therein. Thus, the nutrient composition is isolated from the spores. In certain embodiments (not shown), aqueous liquid medium further can have a portion or all of the collisional quenching component and or a portion or all of the detection reagent suspended and/or dissolved therein.

**[0066]** The container 18, which holds the liquid medium 16, is shown within compartment 15. Container 18, which is sealed, can be a breakable (e.g., plastic or glass) ampoule, but could alternatively be a container equipped with a plug, or other mechanism which when activated (e.g., opened inside the compartment 15) allows the liquid medium 16 to contact the spores 20. Container 18 is shown as a frangible, elongated ampoule, but other known configurations can be used as well.

**[0067]** In any embodiment of a process indicator according to the present disclosure, the spores are disposed (e.g., in a substantially water-free layer or coating) on a surface in the compartment. For example, the spores may be suspended in a suitable liquid, which is then deposited onto the surface and subsequently dried (e.g., by evaporation). In certain embodiments (not shown), the surface can be a portion of the inner surface of one of the at least one walls (e.g., the bottom wall) of the cuvette.

**[0068]** Referring back to FIG. 3, the predetermined number of process-resistant spores 20 is disposed (e.g., as a layer or a substantially water-free coating) on an optional carrier 24. In any embodiment, the spores 20 can be disposed in a composition comprising a plurality of sterilization process-resistant microbial spores and a nucleic acid-interacting dye, wherein the composition is substantially water-free, as described in U.S. Provisional Patent Application No. 62/785,386, filed on even date herewith and entitled "INSTANT READ-OUT BIOLOGICAL INDICATOR". Optionally, in certain embodiments, the enhancer reagent (not shown, described herein) and/or the nucleic acid-interacting dye (not shown) can be disposed in the container 15 with the spores (e.g., in the layer or substantially water-free coating). The carrier 24 can be any suitable material onto which the spores 20 can be disposed (e.g., by a coating process) wherein the material does not substantially hinder or prevent 1) contact between the spores and a sterilant gas, 2) contact between the spores and the nucleic acid-interacting dye (e.g., by adsorbing and/or sequestering the dye from contact with the spores), and/or 3) detection of fluorescence by the nucleic acid-interacting dye when it is bound to DNA or RNA (e.g., by substantially absorbing the electromagnetic radiation used to excite the

fluorescent dye and/or by substantially absorbing the electromagnetic radiation emitted by the nucleic acid-bound dye). Suitable materials for carriers 24 include, but are not limited to paper, glass, or a polymeric sheet or film.

**[0069]** In any embodiment, a self-contained sterilization process biological indicator can comprise an enhancer reagent disposed in the compartment. In certain embodiments, the enhancer reagent contacts the spores (e.g., it may be deposited on the inner surface of the wall or on the carrier with the spores). Additionally, or alternatively, in certain embodiments, the enhancer reagent is disposed in the liquid medium and contacts the spores when the liquid medium contacts the spores. The enhancer reagent, when present in the sterilization process indicator, improves the consistency of the sterilization of the spores and/or the detection of viable spores after the sterilization process indicator is exposed to the sterilant gas. Without being bound by theory, it is believed the enhancer reagent promotes stability of viable spores during storage and/or facilitates the resuspension of dried spores into the liquid medium and/or facilitates penetration of the nucleic acid-interacting dye into the spores.

**[0070]** Nonlimiting examples of suitable enhancer reagents include glycerol, sucrose, trehalose, polyvinyl pyrrolidone, and a combination of any two or more of the foregoing enhancer reagents.

**[0071]** In any embodiment, a self-contained sterilization process biological indicator of the present disclosure can comprise a buffering agent disposed in the compartment. In certain embodiments, the buffering agent can be disposed in the liquid medium. Additionally, or alternatively, the buffering agent can be disposed with the spores (e.g., in a dried coating as discussed above). The buffering agent can serve to buffer the liquid medium at a pH that facilitates fluorescence of the nucleic acid-interacting dye. Suitable buffering agents include, but are not limited to HEPES buffer (e.g., 50 mM), Tris-EDTA (e.g., 50 mM Tris, 10 mM EDTA) buffer, Tris buffer (e.g., 50 mM). The buffer can be selected for use at a neutral pH (e.g., about 6.0-8.0).

**[0072]** In certain embodiments, a self-contained sterilization process biological indicator of the present disclosure can comprise a collisional quenching component, as described hereinabove, disposed in the compartment. The collisional quenching component may be dissolved and/or suspended in the liquid medium, for example.

**[0073]** In use, the sterilization process indicator 200 is exposed to a sterilant gas (e.g., in the sterilization chamber of an automated sterilizer). The sterilant gas (not shown) passes through the closure member 22 into the compartment 15 where it contacts the spores, the nucleic acid-interacting dye and/or the enhancer reagent. As the sterilant gas inactivates the spores, the nucleic acid-interacting dye can penetrate the spore and interact with the nucleic acid therein. In certain embodiments, the container is opened (e.g., by crushing) prior to exposing the sterilization process indicator to the sterilant gas. In certain embodiments, the container is opened (e.g., by crushing) after exposing the sterilization process indicator to the sterilant gas.

Opening the container places the spores in fluid contact with the aqueous liquid medium containing the nutrient composition.

**[0074]** In certain embodiments, a self-contained biological indicator of the present disclosure can comprise a nucleic acid-interacting dye that, when bound to a nucleic acid can cause a change in the electrical conductance (or resistivity) of an aqueous mixture in which the dye and the nucleic acid are disposed. In some embodiments, this change can be due to a redox reaction in which the nucleic acid-bound dye can participate. Thus, the presence or absence of the dye bound to nucleic acid can be detected electrochemically (e.g., by measuring conductance of the aqueous medium. A non-limiting example of such a nucleic acid-interacting dye is Hoechst 33258 DNA binding dye (available from VWR International, Radnor PA).

**[0075]** The present disclosure additionally provides a method. The method can be used to determine the effectiveness of a sterilization process. The method includes positioning any embodiment of a self-contained sterilization process biological indicator according to the present invention in a sterilization chamber. The sterilization chamber can be a sterilization chamber of a sterilizer (e.g., a commercially-available automated sterilizer), the sterilization chamber being typically sized to contain a plurality of articles to be sterilized and equipped with a means of evacuating air and/or other gases from the chamber and adding a sterilant gas to the chamber. The biological sterilization indicator can be positioned in the most difficult location (e.g., above the drain) in the sterilizer to achieve proper sterilization conditions (e.g., temperature, pressure). Alternatively, the biological sterilization indicator can be positioned adjacent an article to be sterilized when placed in the sterilization chamber. Additionally, the biological sterilization indicator can be adapted into routinely-used process challenge devices before positioning it in the sterilization chamber.

**[0076]** While the self-contained sterilization process biological indicator is positioned in the sterilization chamber, the method includes a step of exposing the sterilization process indicator to a sterilant gas (e.g., during a sterilization process). The sterilization process indicator is exposed to the sterilant gas in the sterilization chamber. In certain embodiments, exposing the sterilization process indicator to a sterilant gas comprises exposing the sterilization process indicator to steam. In certain embodiments, exposing the sterilization process indicator to a sterilant gas comprises exposing the sterilization process indicator to ethylene oxide. In certain embodiments, exposing the sterilization process indicator to a sterilant gas comprises exposing the sterilization process indicator to a peroxide. In certain embodiments, exposing the sterilization process indicator to a sterilant gas comprises exposing the sterilization process indicator to ozone.

**[0077]** Because a sterilization process indicator according to the present disclosure comprises an opening that is part of a pathway that permits passage of a sterilant gas from outside the cuvette into the chamber of the indicator; exposing the sterilization process indicator to the sterilant gas

comprises exposing the spores disposed in the compartment of the indicator to the sterilant gas. Typically, exposing the sterilization process indicator to the sterilant gas comprises exposing the spores to the sterilant gas under conditions (e.g., time, temperature, pressure, and concentration of sterilant gas) selected to be sufficient to inactivate (e.g., render nonviable and/or non-cultivable) all of the spores of the predetermined number of sterilization process-resistant spores disposed in the sterilization process indicator.

**[0078]** The sterilant gas can be added to the sterilization chamber after evacuating the chamber of at least a portion of any air or other gas present in the chamber. Alternatively, the sterilant gas may be added to the sterilization chamber without evacuating the chamber. A series of evacuation steps is often used to assure that the sterilant gas reaches all areas within the sterilization chamber and contacts all areas of the article(s) in the sterilization chamber to be sterilized. When the sterilant gas is added to the sterilization chamber, the sterilant gas also contacts the spores under conditions (e.g., temperature, pressure, concentration) where the sterilant gas reaches all areas within the sterilization chamber.

**[0079]** The method of the present disclosure further comprises contacting the spores with the nucleic acid-interacting dye. The spores are contacted with the nucleic acid-interacting dye in the compartment of the sterilization process indicator. In certain embodiments, contacting the spores with the nucleic acid-interacting dye comprises contacting the spores with the nucleic acid-interacting dye while exposing the sterilization process indicator to the sterilant gas. In these embodiments, if the nucleic acid-interacting dye is disposed in the sterilization process indicator in a container (e.g., in an aqueous liquid medium in the container as described hereinabove), the container is opened (e.g., by fracturing, crushing or otherwise disintegrating the container) prior to the exposing the indicator to the sterilant gas (e.g., before the indicator is positioned in the sterilization chamber).

**[0080]** The method of the present disclosure further comprises contacting the spores with the liquid medium. In certain embodiments of the method, the self-contained sterilization process biological indicator comprises a container disposed in the compartment, wherein the container contains the liquid medium. In these embodiments, after the exposing the self-contained sterilization process biological indicator to the sterilant gas, the method further comprises disintegrating the container to contact the spores with the aqueous liquid medium. Thus, in these embodiments, the spores are not in contact with the aqueous liquid medium while the spores are exposed to the sterilant gas.

**[0081]** The method of the present disclosure further comprises contacting the spores with the nutrient composition. In any embodiment wherein the nutrient composition is disposed (either in dry form or liquid form) in a container within the sterilization process biological indicator, the method further comprises disintegrating the container to contact the spores with the aqueous nutrient composition.

**[0082]** After contacting the spores with the nutrient composition, the method comprises incubating the sterilization process indicator at a predetermined temperature for a period of time sufficient to permit germination and at least one cell division of a germinated spore. When incubating the spores in fluid communication with the nutrient composition, an incubation temperature above room temperature may be used. For certain embodiments, the incubation temperature is at least 37° C. For certain embodiments, preferably the incubation temperature is at least 50° C. For certain embodiments, the incubation temperature is 50 to 60° C.

**[0083]** The sterilization process indicator can be incubated for a period of time sufficient to permit germination and at least one cell division of a germinated spore. In certain embodiments, the incubation can be not less than 15 minutes, not less than 30 minutes, not less than 1 hour, not less than 2 hours, not less than 4 hours, not less than 6 hours, not less than 8 hours, or not less than 12 hours. In certain embodiments, the incubation can be up to 30 minutes, up to 1 hour, up to 2 hours, up to 4 hours, up to 6 hours, up to 8 hours, up to 12 hours, or up to 24 hours.

**[0084]** After the incubating the indicator at the predetermined temperature, the method comprises measuring a parameter associated with a germinated spore. Parameters associated with a germinated spore include, but are not limited to, metabolic activity (e.g., catabolic consumption of nutrients to produce cellular energy, anabolic production of biomolecules, production of metabolic byproducts (e.g., $CO_2$, organic acids, alcohols), and enzyme activities found in germinated spores and microbial vegetative cells.

**[0085]** In certain embodiments of the method, measuring the parameter associated with a germinated spore comprises measuring a second amount of absorbance and/or scattering of an electromagnetic radiation as the electromagnetic radiation is directed through the nutrient composition. Measurement of the second amount of absorbance or light-scattering is generally related to particles (e.g., germinated spores and vegetative cells) in the liquid medium of the sterilization process indicator. Spores that have survived the exposure to the sterilant gas are able to germinate and reproduce using the nutrient composition in the sterilization process indicator, thereby increasing the absorbance and/or scattering of light directed through the sterilization process indicator. If the measured absorbance/light-scattering value is greater than a predetermined value (e.g., an arbitrary threshold or a value measured in a "control" sterilization process indicator in which all of the spores have been killed), it can be concluded that at least one spore in the sterilization process indicator survived the exposure to the sterilant gas.

**[0086]** Measuring a first or second amount of absorbance and/or scattering of an electromagnetic radiation as the electromagnetic radiation is directed through the nutrient composition can be done using any wavelength of electromagnetic radiation typically used to detect turbidity that is due to microorganisms. In certain embodiments, the wavelength can be about 420 nm, 600 nm, or 660 nm. In certain preferred embodiments, the wavelength is about 420 nm to about 660 nm. In certain preferred embodiments, measuring the amount of absorbance and/or scattering of an

electromagnetic radiation as the electromagnetic radiation that is directed through the aqueous liquid medium can be performed as described hereinabove

**[0087]** In certain embodiments, the second amount of absorbance or light-scattering can be monitored and measured continuously or intermittently while incubating the spores with the liquid medium comprising the nutrient composition.

**[0088]** In certain embodiments of the method, detecting the parameter associated with a germinated spore comprises detecting and/or measuring an enzyme activity associated with a germinated spore. Suitable enzyme activities to detect germinated spores of *Geobacillus stearothermophilus* include, but are not limited to, alpha-D-glucosidase, beta-D-glucosidase, alkaline phosphatase, acid phosphatase, butyrate esterase, caprylate esterase, lipase, leucine aminopeptidase, chymotrypsin, phosphohydrolase, alpha-D-galactosidase, beta-D-galactosidase, alanine aminopeptidase, tyrosine aminopeptidase, phenylalanine aminopeptidase, and fatty acid esterase. Suitable enzyme activities to detect Bacillus atrophaeus include, but are not limited to, alpha-L-arabinofuranosidase, beta-D-glucosidase, N-acetyl-μ-glucosaminidase, beta-D-cellibiosidase, alanine aminopeptidase, leucine aminopeptidase, and phenylalanine aminopeptidase. The enzyme activity of the germinated spore(s) can be detected and/or measured, for example, by detecting a product (e.g., a colored or fluorescent product) of the enzyme reaction. Thus, in certain embodiments, the sterilization process monitor of the present disclosure comprises a detection reagent comprising a fluorogenic or chromogenic enzyme substrate, as described in U.S. Patent No. 6, 623,955, entitled "RAPID READ-OUT BIOLOGICAL INDICATOR", which is incorporated herein by reference in its entirety.

**[0089]** In certain embodiments of the method, detecting the parameter associated with a germinated spore comprises detecting and/or measuring a product of microbial metabolism. The product of microbial metabolism can be, for example, an organic acid produced by incomplete oxidation of one of the components of the nutrient composition. The accumulation of the product in the liquid medium can be detected, for example, by including a detection reagent such as a pH indicator (e.g., bromcresol purple) into the sterilization process monitor. In certain embodiments, the pH indicator can be incorporated into the liquid medium or the nutrient composition. In these embodiments, detecting a parameter associated with a germinated spore comprises detecting a change in a pH indicator from a first state (e.g., first color) to a second state (e.g., second color) in the liquid medium of the sterilization process indicator.

**[0090]** The present method, which uses the process indicators described above, can, therefore, be sufficiently sensitive to the presence of viable spores to provide an indication thereof in a short period of time. In addition, the indication can be provided even when the number of viable spores present is relatively low. Moreover, the rapid indication of spore viability (e.g., as evidenced by a first fluorescent intensity that is higher than the reference fluorescence intensity)

can be confirmed by the subsequent incubation and detection of a parameter associated with a germinated spore, as discussed herein.

**[0091]** After the step of exposing the sterilization process indicator to the sterilant gas and after the contacting the spores with the liquid medium and before the incubating the indicator at the predetermined temperature, the method of the present disclosure comprises measuring a first fluorescence intensity emitted by the nucleic acid-interacting dye in the self-contained sterilization process biological indicator. Measuring the first fluorescence intensity is performed when the spores, the liquid medium, and the nucleic acid-interacting dye are all in fluid communication. Thus, in some embodiments wherein the liquid medium is disposed in a container, the container is opened as described herein before the first fluorescence intensity is measured. Opening the container places the spores in contact with the nutrient composition disposed in the container. Measuring the first fluorescence intensity can comprise illuminating at least a portion of the cuvette with electromagnetic radiation of a first (excitation) wavelength and intensity sufficient to cause the nucleic acid-interacting dye that is bound to nucleic acid to fluoresce at a second (emission) wavelength. The first fluorescence intensity is measured (e.g., in a fluorometer adapted to receive the cuvette) at the second wavelength and is an indication of the effectiveness of the inactivation of the spores in the sterilization process indicator, as discussed hereinabove.

**[0092]** After the measuring the first fluorescence intensity, the method also comprises comparing the first fluorescence intensity to a reference fluorescence intensity to determine whether the exposing the sterilization process biological indicator to the sterilant gas was effective to kill all of the spores. In certain embodiments, the reference fluorescence intensity is a measured intensity of fluorescence (i.e., a second fluorescence intensity) emitted by a "control" sterilization process indicator (e.g., of a "positive control", such as an identical sterilization process indicator that has not been exposed to a sterilant gas and, thus, the spores are substantially all viable; or a "negative control", such as an identical sterilization process indicator that has been treated (e.g., in an "overkill" sterilization process) so that substantially all of the spores are nonviable). In certain embodiments, the reference fluorescence intensity is a measured intensity (i.e., a second fluorescence intensity) emitted by a "fluorescence control" (e.g., a solution or a solid state material that emits a predetermined fluorescence intensity used to set a threshold intensity value to which the first fluorescence intensity can be compared to determine whether or not all of the spores in the sterilization process monitor that was exposed to the sterilant gas have been inactivated. In certain embodiments, the reference fluorescence intensity can be an arbitrary fluorescence intensity value, for example, in a printed publication such as instructions for use that can be used by the operator to make a comparison with the first fluorescence intensity, or in an electronic data set that can be used (e.g., by a fluorimeter or a microprocessor) to make a comparison with the first fluorescence intensity.

**[0093]** Comparing the first fluorescence intensity to a reference fluorescence intensity comprises determining whether the first fluorescence intensity is less than, equal to, or greater than the reference fluorescence intensity. In certain embodiments, a first fluorescence intensity that is greater than the reference intensity indicates the sterilization process was effective to inactivate all of the predetermined number of spores. In certain embodiments, a first fluorescence intensity that is greater than or equal to the reference intensity indicates the sterilization process was effective to inactivate all of the predetermined number of spores. In certain embodiments, a first fluorescence intensity that is less than the reference intensity indicates the sterilization process was not effective to inactivate all of the predetermined number of spores. In certain embodiments, a first fluorescence intensity that is less than or equal to the reference intensity indicates the sterilization process was not effective to inactivate all of the predetermined number of spores.

**[0094]** In any of the above embodiments, before the incubating the sterilization process indicator at a predetermined temperature, a method according to the present disclosure optionally can comprise measuring a first amount of absorbance and/or scattering of electromagnetic radiation when the electromagnetic radiation is directed through the aqueous liquid medium in the cuvette. Preferably, the first amount absorbance and/or scattering is measured after the exposing the sterilization process indicator to the sterilant gas. The wavelength of electromagnetic radiation used to measure the absorbance and/or scattering can be, for example, a wavelength used in the art to assess the optical density of spores suspended in a liquid medium. Nonlimiting examples of suitable wavelengths for measuring the absorbance and/or scattering are those wavelengths in the range of about 500nm to about 600nm. The amount of absorbance and/or scattering of the electromagnetic radiation by the liquid medium is a further indication of the inactivation (e.g., disintegration) of the spores by the sterilization process. In certain embodiments, the method further includes a step of calculating a ratio of the first fluorescence intensity to the measured amount of electromagnetic radiation absorbance and/or scattering.

**[0095]** In certain preferred embodiments, measuring the amount of absorbance and/or scattering of an electromagnetic radiation as the electromagnetic radiation that is directed through the aqueous liquid medium can be performed using a device (e.g., a spectrophotometer, a colorimeter) capable of receiving the sterilization process indicator and measuring the absorbance and/or light-scattering of the liquid contents therein.

**[0096]** The ratio of the first fluorescence intensity to the measured first amount of electromagnetic radiation absorbance and/or scattering can be used to calculate and adjusted first fluorescent intensity ($I_A$) according to the following formula:

$$I_A = I/O.D.,$$

wherein I = the first fluorescent intensity and O.D. is the measured absorbance and/or lights scattering (i.e., optical density) of the spores in the liquid medium after the sterilization process indicator has been exposed to the sterilant gas.

**[0097]** In any embodiment of a method according to the present disclosure, the method further can comprise agitating the self-contained sterilization process biological indicator for a period of time. Agitation can be used to mix the contents present in the chamber of the indicator. The indicator can be agitated, for example, to suspend the spores in the liquid medium (e.g., after a container has been actuated to release the liquid medium into the chamber). Additionally, or alternatively, the indicator can be agitated before measuring the first fluorescence intensity and/or before measuring the amount of absorbance and/or scattering of electromagnetic radiation as the electromagnetic radiation is directed through the aqueous liquid medium in the cuvette. The indicator can be agitated manually (e.g., by swirling, tapping, or shaking) or it can be agitated using an sample-vortexing machine.

**[0098]** After the measuring the first fluorescence intensity, the method of the present disclosure comprises contacting the spores with the nutrient composition. Contacting the spores with the nutrient composition comprises opening the container (containing the nutrient composition in dry form or in the liquid medium) to place the spores in fluid communication with the nutrient composition. The container can be opened as discussed hereinabove.

**[0099]** For certain embodiments, including any one of the above method embodiments, the method further comprises placing an article to be sterilized along with the sterilization process indicator in the sterilization chamber. For certain of these embodiments, the method further comprises determining whether or not the sterilization process was effective for sterilizing the article. An indication of no viable spores may be used to determine that the sterilization process was effective for sterilizing the article, whereas an indication of viable spores may be used to determine that the process was not effective. Thus, an assessment of the sterility of an article subjected to a sterilization process may be made in a relatively short time using the indicator and method embodiments described above by directly measuring production of nucleic acids in any viable spores.

**[00100]** In certain alternative embodiments of a method according to the present disclosure, detection of the nucleic acid-interacting dye with a nucleic acid after a sterilization process can be effected by measuring the electrical conductance of a liquid medium in which the nucleic acid-interacting dye with the nucleic acid, as described, for example, in Examples 7 and 8 herein.

## Examples

**[00101] Example 1. Assembly of a Self-Contained Sterilization Process Biological Indicator.**

**[00102]** Spores of *Geobacillus stearothermophilus* can be produced in liquid sporulation medium. The spores can be washed in sterile deionized water and resuspended in sterile deionized water to achieve a concentration of about $10^8$-$10^9$ spores/mL.

**[00103]** Biological indicators (part number 1292) can be obtained from 3M Company (St. Paul, MN). Caps on the biological indicators can be removed and the contents (glass ampule, spore strip, and nonwoven packing adjacent the spore strip in the bottom of each tube) can be removed. The

inside of the tubes and the outside of the glass ampules can be rinsed with sterile deionized water. The nonwoven sheet in the cap of each tube can be left in place. The caps (with the nonwoven sheets therein) can be replaced on the tubes and each assembled tube can be sterilized in a steam sterilizer.

**[00104]** The spore suspension can be diluted in a sterile solution of SYTO 9 dye (Invitrogen, Carlsbad, CA) to obtain a final spore suspension having about $2 \times 10^8$/mL in a solution of 40 μM SYTO9 dye. Ten-microliter aliquots of the resulting spore suspension can be pipetted onto polyethylene film discs (about 6 mM diameter). The spore-coated discs can be dried at 50 degrees C for about 30 minutes.

**[00105]** The self-contained sterilization process indicators can be assembled by removing the caps from the sterilized tubes, placing one of the spore-coated discs and one of the washed glass ampules into the bottom each of the sterilized tubes, and replacing the caps on the tubes.

**[00106]** **Reference Example 1 – Spore Suspension**

**[00107]** *Sample Preparation*:

**[00108]** *Geobacillus stearothermophilus* ATCC 7953 spores were suspended in sterile $H_2O$ ($3.4x10^6$ spores/mL) and aliquoted (2 mL volumes) into six glass test tubes. Table 9 shows the six different steam-exposure times at 121°C in a dynamic-air removal steam sterilization process in a MidMark M9 sterilizer. Commercial 1492V biological indicators (3M Company, St. Paul, MN) were used as growth controls as described below.

**[00109]** Table 1. Samples and steam SFPP exposure times

| Sample # | MidMark M9 121°C exposure |
|---|---|
| 1 | T0 of exposure |
| 2 | 2 Minutes of exposure |
| 3 | 4 Minutes of exposure |
| 4 | 6 Minutes of exposure |
| 5 | 8 Minutes of exposure |
| 6 | No Exposure |

**[00110]** *Sample Analysis:*

**[00111]** After the tubes containing the spore suspensions were exposed to the sterilization processes, Syto9 (3.0 μM) nucleic acid binding dye (100 μL) was then added to aliquots of each test sample (100 μL) and fluorescence intensity was immediately measured in a BIOTEK 96-well plate reader at room temperature. The excitation wavelength used in the plate reader was 480 nm and the emission wavelength measured was 510 nm. In addition, the optical density (600 nm) was also measured in the BIOTEK plate reader. The colony forming units for each test sample was determined by diluting the spore suspensions and surface-plating the dilutions onto Tryptic Soy Agar followed by incubation of the plates at 60°C.

**[00112]** *Results*

**[00113]** The data for this example are shown in Table 2. The RFU/OD600 value shown in Table 2 correlated well with the length of exposure of the spores to the steam sterilization process. 1492V commercial biological indicator controls were all growth negative at exposure times greater than 2 minutes of exposure in this experiment.

**[00114]** Table 2. Reference Example 1 Data. The OD600, Syto9 (RFU), and Syto9 (RFU)/OD600 reported in this table are the average of the triplicate technical replicates for each condition. The CFU/mL is the average of duplicate technical replicates for each condition.

| | CFU/mL | OD600 | Syto9 (RFU) | Syto9 (RFU)/OD600 |
|---|---|---|---|---|
| Positive control | $3.40 \times 10^6$ | 0.38 | 5703 | 15155 |
| T0 Exposure | $1.76 \times 10^6$ | 0.32 | 24283 | 76499 |
| 2 Min | $7.00 \times 10^2$ | 0.28 | 25065 | 89198 |
| 4 Min | $0.00 \times 10^0$ | 0.26 | 26777 | 101264 |
| 6 Min | $0.00 \times 10^0$ | 0.25 | 27424 | 108519 |
| 8 Min | $0.00 \times 10^0$ | 0.25 | 26850 | 109146 |

**[00115]** **Example #2 – Dried spores - DNA dye added to spore coating solution**

**[00116]** *Sample preparation:*

**[00117]** *Geobacillus stearothermophilus* ATCC 7953 spores were suspended in an aqueous trehalose solution (25% w/v) with and without syto9 dye (10μM) and spotted (20μL) onto circular polypropylene film carriers to yield approximately $>10^6$ spores/carrier. The coated carriers were then dried at 60°C for 15 minutes. The spore-coated and dried carriers were then exposed to a 6-minute steam sterilization process at 121°C in a dynamic-air removal steam sterilization cycle using a MidMark M9 sterilizer. Samples were prepared and tested in duplicate.

**[00118]** *Sample analysis:*

**[00119]** Following sterilization of the samples, 200 μL of $H_2O$ was added to the samples that already had syto9 dried with the spores prior to sterilization. For samples that had syto9 added post sterilization, 20 μL of syto9 (10 μM) was added to the wells followed by 180 μL of $H_2O$. Therefore, all samples had a final concentration of 1 μM syto9 at the time of the reading. The samples were pipetted up and down approximately 5 times and fluorescence intensity and optical density of each well was immediately measured in a BIOTEK 96-well plate reader at room temperature as described in Reference Example 3.

**[00120]** *Results:*

**[00121]** The data for this example are shown in Table 3, 4 and 5. The exposure time indicates the length of exposure to steam at 121C in a Midmark M9 commercial sterilizer. Syto9 pre/post sterilization indicates whether the sample had syto9 dye added to the coating solution (Pre-

sterilization), or after the sterilization process (Post). The RFU/OD600 values shown in Table 3 correlated well with the length of exposure of the spores to the steam sterilization process. The observed fluorescent signal in the steam-exposed samples was higher when the dye is added prior to sterilization.

**[00122]** Table 3. Pre-sterilization addition of Syto9 dye.

| Sample # | Exposure Time | Relative fluorescence units (RFU) | OD600 | RFU/OD600 |
|---|---|---|---|---|
| 1 | No exposure | 6592 | 0.80 | 8225 |
| 2 | 6 Min. | 15502 | 0.42 | 37361 |

**[00123]** Table 4. Post-sterilization addition of Syto9 dye

| Sample # | Exposure Time | Relative fluorescence units (RFU) | OD600 | RFU/OD600 |
|---|---|---|---|---|
| 3 | No exposure | 13796 | 1.08 | 12756 |
| 4 | 6 Min. | 9613 | 0.46 | 20807 |

**[00124]** Table 5. Pre-sterilization addition of Syto9 dye – dye only control (no spores)

| Sample # | Exposure Time | Relative fluorescence units (RFU) | OD600 | RFU/OD600 |
|---|---|---|---|---|
| 5 | No exposure | 1369 | 0.10 | N/A |
| 6 | 6 Min. | 1483 | 0.11 | N/A |

**[00125]** **Example #3 – Dried spores in biological indicator**

**[00126]** *Sample Preparation*

**[00127]** *Geobacillus stearothermophilus* ATCC 7953 spores were suspended in a trehalose solution (25% w/v) with syto9 dye (10μM) and spotted (20μL) on the inside of the wall of polycarbonate housings from 3M™ ATTEST™ 1295 biological indicators. Each resulting biological indicator contained approximately $>10^6$ spores coated on inside of the wall of the housing. The coated BI sleeves were then dried at 56°C for approximately 1 hour. SRBI caps were then placed on the coated BI sleeves. The Samples were then exposed to steam at 121°C in a dynamic-air-removal benchtop commercial sterilization vessel at 2 different exposure points (T0 exposure and 6 minutes exposure). Samples were prepared in duplicate.

**[00128]** *Sample analysis:*

**[00129]** $H_2O$ (220 μL) was added to each BI. The BIs were then vortexed for 3 seconds. Aliquots were then pulled (200 μL) from each BI and the fluorescence and optical density were immediately

measured in a BIOTEK 96-well plate reader at room temperature as described in Reference Example 3.

**[00130]** *Results:*

**[00131]** The data for example #3 are shown in Table 6. Note that the BIs that were aborted at T0 of exposure were exposed to the pre-conditioning phase on the commercial sterilization cycle. The data show higher values of fluorescence in the BI's exposed to steam for 6 minutes.

**[00132]** Table 6. Example #3 Data.

| Sample # | Exposure time 121°C | Relative fluorescence units (RFU) | OD600 |
|---|---|---|---|
| 1 | Aborted at T0 of exposure | 1538 | 0.14 |
| 2 | Aborted at T0 of exposure | 1842 | 0.11 |
| 3 | 6 Minutes | 3945 | 0.16 |
| 4 | 6 Minutes | 4471 | 0.14 |

**[00133]** **Example #4 – Creation of Reference Standard Curve**

**[00134]** *Sample Preparation*

**[00135]** Spores were produced in liquid cultures. The spores were harvested by centrifugation, washed several times in sterile water, and resuspended in a sufficient volume of sterile water to achieve a concentration of $2.0x10^8$ CFU/mL). 150uL of the resulting suspension was placed in a 1.5 mL micro-centrifuge tube and centrifuged at 3400 RPM for 5 minutes in a micro-centrifuge. The supernatant was removed.

**[00136]** Preparation of coating solution (12% PVP W/V, and 2.5% Trehalose W/V in $H_2O$): Mix 3 mL of $H_2O$ with Polyvinyl Pyrrolidone (0.36 g) and Trehalose (0.075 g).

**[00137]** The spore pellet in the microcentrifuge tube was then resuspended with the coating solution (1 mL). Four µl of Syto9 (5000 µM stock) was then added to the suspension to yield a 20 µM concentration. The suspension was then coated onto polypropylene film carriers. Carriers were dried for 25 minutes at 56°C. The biological indicators were then placed in an H&W 105 resistometer, where they were exposed to an ISO 132°C Pre-vacuum sterilization cycle for various periods of 132°C exposure ( 30 seconds, 1.5 minutes, 2 minutes, 2.5 minutes, and 3.5 minutes).

**[00138]** *Sample Analysis*

**[00139]** After exposure to the sterilization process, sterile $H_2O$ (220 µL) was added to each Biological indicator and the indicators were vortexed briefly to resuspend spores. The resuspended spores were then transferred to a clear bottom, black walled 96-well plate. Plates were read at room temperature in the BioTek plate reader at 480nm excitation/520nm emission, and the OD600 was measured. Readings were performed as quickly as possible from the time of resuspension to reading in the plate reader. Following the fluorescence reading, nutrient media (containing bromocresol

purple) that supports spore germination and outgrowth was added to the wells. The plate was then placed in a 60°C incubator for a minimum of 24 hours, after which the media was examined for indications (e.g., turbidity, pH change) of spore germination and growth.

**[00140]** *Results*

**[00141]** Table 7 and 8 show the fluorescence and growth data respectively for this example. The values shown in Table 7 are relative fluorescence units (RFU) for five biological indicator replicates across 5 exposure times. The RFU values shown in Table 7 are with the background (from unexposed BI's) subtracted. Table 8 shows the growth result with bromocresol purple and media that supports spore germination and outgrowth.

**[00142]** These data indicate that a threshold value could be set that indicates where positive and negative fluorescence could be established based on how it correlates to spore growth. For example anything below 8000 RFU would be assigned a fluorescent positive value (Growth positive) and anything above 8000 RFU would be assigned a fluorescent negative value (Growth negative). This data set indicates an example reference standard curve data set that could be used to assign fluorescent designation for near immediate monitoring of sterilization cycles.

**[00143]** Table 7. Example #4 Fluorescence Data following exposure in ISO 132°C cycle.

| Exposure time (Min.) | 0.5 | 1.5 | 2 | 2.5 | 3.5 |
|---|---|---|---|---|---|
| Replicate 1 | 2596 | 3270 | 4129 | 7083 | 10310 |
| Replicate 2 | 3843 | 3825 | 5579 | 7967 | 10767 |
| Replicate 3 | 2611 | 5116 | 6114 | 6224 | 10387 |
| Replicate 4 | 3365 | 4869 | 6977 | 7373 | 11629 |
| Replicate 5 | 3193 | 3912 | 5549 | 7476 | 12968 |

**[00144]** Table 8. Example #4 Growth Data (P = growth positive, N = Growth negative) following exposure in ISO 132°C cycle.

| Exposure time (Min.) | 0.5 | 1.5 | 2 | 2.5 | 3.5 |
|---|---|---|---|---|---|
| Replicate 1 | P | P | N | N | N |
| Replicate 2 | P | P | P | N | N |
| Replicate 3 | P | P | N | N | N |
| Replicate 4 | P | P | P | P | N |
| Replicate 5 | P | P | P | N | N |

**[00145]** **Example #5 – Hydrogen Peroxide Monitoring**

**[00146]** *Sample Preparation*

**[00147]** Six 3M™ Attest™1295 biological indicators were placed into a Tyvek™ polyethylene fiber pouch that was exposed to $H_2O_2$ vapor for 90 seconds of exposure in an otherwise empty Sterrad®

100NX sterilizer. Preliminary experiments showed that a 90-second exposure in the sterilizer was sufficient to kill all of the spores in the biological indicators.

**[00148]** The carriers were then removed from 3 of the $H_2O_2$-exposed biological indicators and 3 unexposed (control) biological indicators and the carriers were placed in separate borosilicate glass vials with 1 mL of Butterfields buffer. The vials were then vortexed briefly and sonicated for 15 minutes in a water bath sonicator.

**[00149]** *Sample Analysis*

**[00150]** Duplicate samples (100 µL) of the buffer from each sonicated tube were then placed into separate wells of a clear bottom 96-well plate. 100µL of Propidium iodide (PI) only (30 µM) or Syto9 (10µM)+PI(30µM) was added to individual wells as shown in Table 9. Samples were mixed and immediately transferred to a 96-well plate for analysis using a BIOTEK synergy4 plate reader. The plate was sealed using an adhesive seal (Bio-Rad Life Sciences; Hercules, CA) The Optical density of each sample was measured at 600 nm. The Syto9 fluorescence was measured according to Reference Example 3. Propidium iodide fluorescence was measured using an excitation wavelength of 490 nm and an emission wavelength of 635 nm.

**[00151]** *Results*

**[00152]** The Optical Density (600 nm) and fluorescence (at 520nm and/or at 635 nm as indicated in Table 9) were analyzed using the BioTek plate reader described above. The results for are shown in Table 9. Note the increase in signal from live (unexposed) to dead ($H_2O_2$-exposed) for the syto9+PI samples. In addition, the response can be measured by taking the ratio of Syto9+PI/OD600 (using the 490ext/635em values for syto9+PI).

**[00153]** Table 9. Example #5 Data for hydrogen peroxide monitoring.

| | | OD600 | | 480ext/520em | | 490extt/635em | |
|---|---|---|---|---|---|---|---|
| Sample # | Description | Unexp[A] | Exp[B] | Unexp[A] | Exp[B] | Unexp[A] | Exp[B] |
| 1 | PI | 0.414 | 0.256 | 15 | 17 | 2691 | 2831 |
| 1 | PI | 0.414 | 0.263 | 14 | 17 | 2829 | 2972 |
| 2 | Syto9+PI | 0.397 | 0.258 | 808 | 660 | 4639 | 7258 |
| 2 | Syto9+PI | 0.392 | 0.26 | 824 | 679 | 4438 | 7308 |
| 3 | Syto9+PI/OD600 | 0.397 | 0.258 | N/A | N/A | 11685 | 28132 |
| 3 | Syto9+PI/OD600 | 0.392 | 0.26 | N/A | N/A | 11321 | 28108 |
| 4 | No Dye | 0.335 | 0.242 | 10 | 8 | 4 | 5 |

[A] – Unexposed to sterilant

[B] – Exposed to sterilant

**[00154] Example #6 – Detection using Electrochemical Methods – DNA Solutions Mimicking Different Levels of Spore DNA Available for Detection after Sterilization**

**[00155]** *Sample preparation:*

**[00156]** A sample stock solution of DNA (Lambda DNA available from Invitrogen, Waltham, MA) is prepared in a 50 mM phosphate buffer (40 mM $K_2HPO_4$ and 10 mM $KH_2PO_4$, pH 7.0) to a DNA concentration of 100 μg/mL. The stock solution is diluted with additional buffer to make a range of sample solutions with DNA concentrations of: 10, 1 and 0.1 μg/mL. A control sample solution containing no DNA is also prepared. The DNA sample solutions simulate samples of spore DNA that is released as a result of a sterilization process. The varying concentrations of DNA in the sample solutions simulate exposures to the sterilant for different exposure times. Hoechst 33258 DNA binding dye (available from VWR International, Radnor PA) is added to the DNA sample solutions as well as the control solution, to give a concentration of 1 μM for the Hoechst binding dye. The Hoechst binding dye concentration is constant (1 μM) for all DNA sample solutions as well as the control solution.

**[00157]** *Sample analysis:*

**[00158]** Because Hoechst 33258 is irreversibly oxidized, an oxidative linear sweep voltammetry (LSV) with scan is used for the electrochemical analysis of the DNA sample solutions. LSV is performed using a Metrohm AUTOLAB potentiostat (available from Metrohm, Riverview, FL) in the voltage range from 0 to 1.0 V at a sweep rate of 100 mV/s. The electrochemical cell consists of a Metrohm DropSense screen printed three electrode cell (DropSense 220AT available from Metrohm, Riverview, FL). The electrochemical cell has gold working and auxiliary electrodes and a silver reference electrode. The working electrode is 4mm in diameter. The DNA sample solutions are tested by placing a 100 μL drop of the sample solution onto the screen-printed electrochemical cell covering all three electrodes. After a five-minute equilibration at 0 volts, the LSV described above is initiated. The LSV analysis is repeated for the control solution containing no DNA. For each LSV analysis a new electrochemical cell is used.

**[00159]** *Results:*

**[00160]** The LSV curves will show oxidation of the Hoechst binding dye at 0.6V. The peak current measured in μA at this oxidation potential is always lower for the control solution in comparison to the DNA sample solutions. The ratio of the peak current at the oxidation potential for the series of DNA sample solution divided by the peak current of the control solution at that same potential increases proportionally with increasing DNA concentration in the sample solution series.

**[00161]** **Example #7 – Detection using Electrochemical Methods – Spore Suspension**

**[00162]** *Sample Preparation:*

**[00163]** *Geobacillus stearothermophilus* ATCC 7953 spores are suspended in sterile $H_20$ ($3.4x10^6$ spores/mL) and aliquoted (2 mL volumes) into multiple glass test tubes. This experiment is performed in duplicate. Table 10 provides the six different exposure times at 121°C in a dynamic-air removal steam sterilization cycle (MidMark M9 available from MidMark, Traverse City, MI). Commercial 1492V biological indicators (available from 3M, St. Paul, MN) are used as a control.

**[00164]** Table 10. Samples and steam SFPP exposure times

| Sample # | MidMark M9 121°C exposure |
|---|---|
| 1 | T0 of exposure |
| 2 | 2 Minutes of exposure |
| 3 | 4 Minutes of exposure |
| 4 | 6 Minutes of exposure |
| 5 | 8 Minutes of exposure |
| 6 | No Exposure |

**[00165]** *Sample Analysis:*

**[00166]** Hoechst 33258 (available from VWR International, Radnor, PA) nucleic acid binding dye is added to aliquots of each test sample and LSV is immediately conducted. LSV is performed using a Metrohm AUTOLAB potentiostat (available from Metrohm, Riverview, FL) in the voltage range from 0 to 1.0 V at a sweep rate of 100 mV/s. The electrochemical cell consists of a Metrohm DropSens screen printed three electrode cell (DropSense 220AT available from Metrohm, Riverview, FL). The electrochemical cell has gold working and auxiliary electrodes and a silver reference electrode. The working electrode is 4mm in diameter. The DNA sample solutions are tested by placing a 100µL drop of the test solution onto the screen-printed electrochemical cell covering all three electrodes. After a five-minute equilibration at 0 volts, the LSV described above is initiated. The colony forming units for each test sample was determined by diluting the spore suspensions and surface-plating the dilutions onto Tryptic Soy Agar followed by incubation of the plates at 60°C.

**[00167]** *Results*

**[00168]** The LSV curves show oxidation of the Hoechst binding dye at 0.6V. Sample 6 results the lowest peak current at the oxidation potential. Sample 1 results in a peak current greater than sample 6 at the oxidation potential. This is because, even though the sterilization exposure is T0 (meaning the cycle is stopped at the very beginning of the sterilization exposure phase of the cycle) the preconditioning portion of the cycle results in a very small amount of released DNA available for detection. The peak current for samples 2-5 is significantly larger than for sample 6, indicating greater amounts of DNA detected electrochemically. The peak current for samples 4 and 5 is comparable, indicating that for exposure times greater than 6 minutes all the possible DNA generated as a result of the sterilization cycle is detected electrochemically. 1492V commercial biological indicator controls are all growth negative at 2 minutes of exposure in this experiment.

**[00169]** **Example #8 – Detection using Electrochemical Methods – Hydrogen Peroxide Monitoring**

**[00170]** *Sample Preparation*

**[00171]** Commercial 3M™ Attest™1295 biological indicators (available from 3M, St. Paul, MN) are exposed to VH202 in an incomplete Standard Cycle for the Sterrad 100NX (available from ASP, Irvine, CA) sterilizer providing a total of 90 seconds of exposure. Six of the BI's from this lot are packaged in a TYVEK® polypropylene fiber pouch and placed in an otherwise empty chamber. This cycle is run in order to kill the BI's.

**[00172]** The carriers are then removed from 3 of the exposed (Dead) and unexposed (Live) BI's and placed in separate borosilicate glass vials with 1 mL of Butterfields buffer. The vials are vortexed briefly and sonicated for 15 minutes in a water bath sonicator.

**[00173]** *Sample Analysis*

**[00174]** 100µL of each sample is placed in a microcentrifuge tube. To each sample, 100µL of a 200uM Hoechts 33258 DNA binding dye solution is added. In addition, a control sample with no Hoechts dye added is prepared for both Dead and Live samples. Samples are mixed and immediately tested using LSV. LSV is performed using a Metrohm AUTOLAB potentiostat (available from Metrohm, Riverview, FL) in the voltage range from 0 to 1.0 V at a sweep rate of 100 mV/s. The electrochemical cell consists of a Metrohm DropSens screen printed three electrode cell (DropSense 220AT available from Metrohm, Riverview, FL). The electrochemical cell has gold working and auxiliary electrodes and a silver reference electrode. The working electrode is 4mm in diameter. The DNA sample solutions are tested by placing a 100µL drop of the test solution onto the screen-printed electrochemical cell covering all three electrodes. After a five-minute equilibration at 0 volts, the LSV described above is initiated.

**[00175]** *Results*

**[00176]** Except for the control samples, the LSV curves show oxidation of the Hoechst binding dye at 0.6V. The control samples do not have an oxidation peak at 0.6V and result in negligible current flow at that potential. The peak current for the Dead samples is significantly larger than for Live samples, indicating greater amounts of DNA detected electrochemically. Various modifications and alterations to this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention. It should be understood that this invention is not intended to be unduly limited by the illustrative embodiments and examples set forth herein and that such examples and embodiments are presented by way of example only with the scope of the invention intended to be limited only by the claims set forth herein

**What is claimed is:**

1. A self-contained sterilization process biological indicator, comprising:

a cuvette having at least one liquid-impermeable wall that forms an opening into a compartment;

a nucleic acid-interacting dye disposed in the compartment;

a predetermined number of sterilization process-resistant spores disposed in the compartment, wherein the spores are contacting the dye;

a first liquid medium disposed in the compartment; and

a nutrient composition that facilitates germination and/or outgrowth of a viable spore, wherein the nutrient composition is disposed in the compartment and isolated from the spores;

wherein the opening is part of a pathway that permits passage of a sterilant, but not bacteria, from outside the cuvette into the compartment;

wherein the nucleic acid-interacting dye is fluorescent when it interacts with DNA or RNA.

2. The self-contained sterilization process biological indicator of claim 1, further comprising a container a container disposed in the compartment, wherein the nutrient composition is disposed in the container.

3. The self-contained sterilization process biological indicator of claim 1 or claim 2, further comprising a detection reagent disposed in the compartment.

4. The self-contained sterilization process biological indicator of any one of claims 2 or 3, wherein the liquid medium is disposed in the container.

5. The self-contained sterilization process biological indicator of claim 4, wherein the spores are disposed on a surface in a substantially water-free layer, wherein the surface is disposed in the compartment.

6. The self-contained sterilization process biological indicator of any one of the preceding claims, further comprising an enhancer reagent disposed in the compartment, wherein the enhancer reagent contacts the spores.

7. The self-contained sterilization process biological indicator of any one of the preceding claims, wherein the spores are produced by a species of microorganisms selected from the group consisting of *Geobacillus stearothermophilus, Bacillus atrophaeus, Bacillus megaterium,*

*Clostridium sporogenes, Bacillus coagulans,* and a combination of any two or more of the foregoing species.

8. The self-contained sterilization process biological indicator of any one of the preceding claims, wherein the nutrient composition comprises a nutrient selected from the group consisting of glucose, fructose, cellibiose, potassium chloride, calcium chloride, an amino acid, methionine, tryptophan, phenylalanine, and a combination of any two or more of the foregoing nutrients.

9. A method of determining effectiveness of a sterilization process, the method comprising:

positioning the self-contained sterilization process biological indicator of any one of the preceding claims in a sterilization chamber;

while the indicator is positioned in the sterilization chamber, exposing the indicator to a sterilant gas;

contacting the spores with the liquid medium;

contacting the spores with the nutrient composition;

after contacting the spores with the nutrient composition, incubating the indicator at a predetermined temperature for a period of time sufficient to permit germination and at least one cell division of a germinated spore;

after the exposing the sterilization process biological indicator to the sterilant gas and after the contacting the spores with the liquid medium and before the incubating the indicator at the predetermined temperature, measuring a first fluorescence intensity emitted by the nucleic acid-interacting dye in the sterilization process biological indicator;

comparing the first fluorescence intensity to a reference fluorescence intensity to determine whether the exposing the sterilization process biological indicator to the sterilant gas was effective to kill all of the spores; and

after the incubating the sterilization process indicator at the predetermined temperature, detecting a presence, an absence, or a quantity of a parameter associated with a germinated spore.

10. The method of claim 9, wherein the sterilizer is a steam sterilizer, wherein exposing the sterilization process indicator to a sterilant gas comprises exposing the sterilization process indicator to steam.

11. The method of claim 9 or claim 10, wherein contacting the spores with the nucleic acid-interacting dye comprises contacting the spores with the nucleic acid-interacting dye while exposing the sterilization process indicator to the sterilant gas.

12. The method of claim 9 or claim 10,

wherein the aqueous liquid medium is contained in a container that is disposed in the compartment;

wherein the container is impermeable to the aqueous liquid medium;

wherein, prior to the exposing the self-contained sterilization process biological indicator to the sterilant gas, the method further comprises disintegrating the container to contact the spores with the aqueous liquid medium.

13. The method of claim 9 or claim 10,

wherein the liquid medium is contained in an container that is disposed in the container;

wherein the container is impermeable to the aqueous liquid medium;

wherein, after the exposing the self-contained sterilization process biological indicator to the sterilant gas, the method further comprises disintegrating the container to contact the spores with the aqueous liquid medium.

14. The method of any one of claims 9 through 13, further comprising:
before incubating the indicator at a predetermined temperature, measuring a first amount of absorbance and/or scattering of an electromagnetic radiation when the electromagnetic radiation is directed through the aqueous liquid medium in the cuvette.

15. The method of any one of claims 9 through 14, wherein the reference fluorescence intensity is obtained by measuring a second fluorescence intensity.

16. The method of any one of claims 9 through 15, wherein the parameter associated with a germinated spore is cell division, wherein measuring the parameter associated with a germinated spore comprises measuring a second amount of absorbance and/or scattering of an electromagnetic radiation as the electromagnetic radiation is directed through the nutrient composition.

17. The method of any one of claims 9 through 16, wherein the parameter associated with a germinated spore is microbial metabolism, wherein measuring the parameter associated with a germinated spore comprises measuring a product of microbial metabolism.

18. The method of claim 17, wherein the product of microbial metabolism is a product of an enzyme-catalyzed reaction.

19. The method of any one of claims 9 through 18, further comprising:
prior to exposing the sterilization process indicator to the sterilant, positioning an article to be sterilized in the sterilizer.

20. A method of determining effectiveness of a sterilization process, the method comprising:

positioning the self-contained sterilization process biological indicator of any one of the preceding claims in a sterilization chamber;

while the indicator is positioned in the sterilization chamber, exposing the indicator to a sterilant gas;

contacting the spores with the liquid medium;

contacting the spores with the nutrient composition;

after contacting the spores with the nutrient composition, incubating the indicator at a predetermined temperature for a period of time sufficient to permit germination and at least one cell division of a germinated spore;

after the exposing the sterilization process biological indicator to the sterilant gas and after the contacting the spores with the liquid medium and before the incubating the indicator at the predetermined temperature, measuring a first electrical conductance of the liquid medium;

comparing the first electrical conductance to a reference electrical conductance to determine whether the exposing the sterilization process biological indicator to the sterilant gas was effective to kill all of the spores; and

after the incubating the sterilization process indicator at the predetermined temperature, detecting a presence, an absence, or a quantity of a parameter associated with a germinated spore.

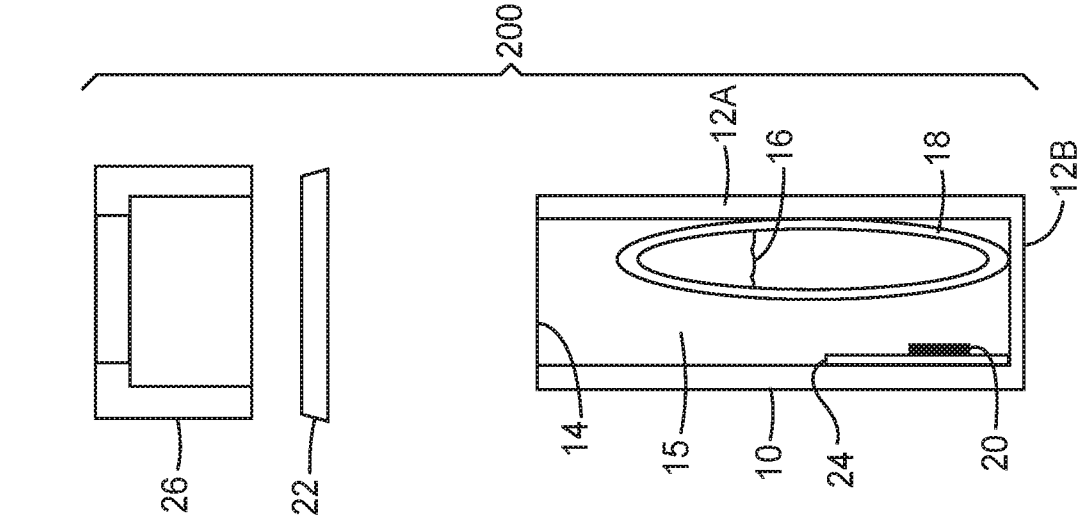


FIG. 3

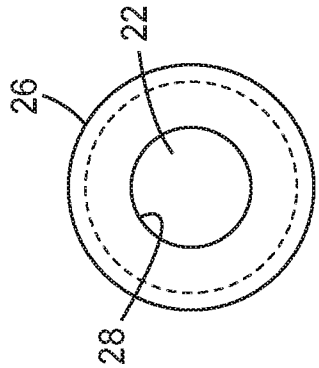


FIG. 2

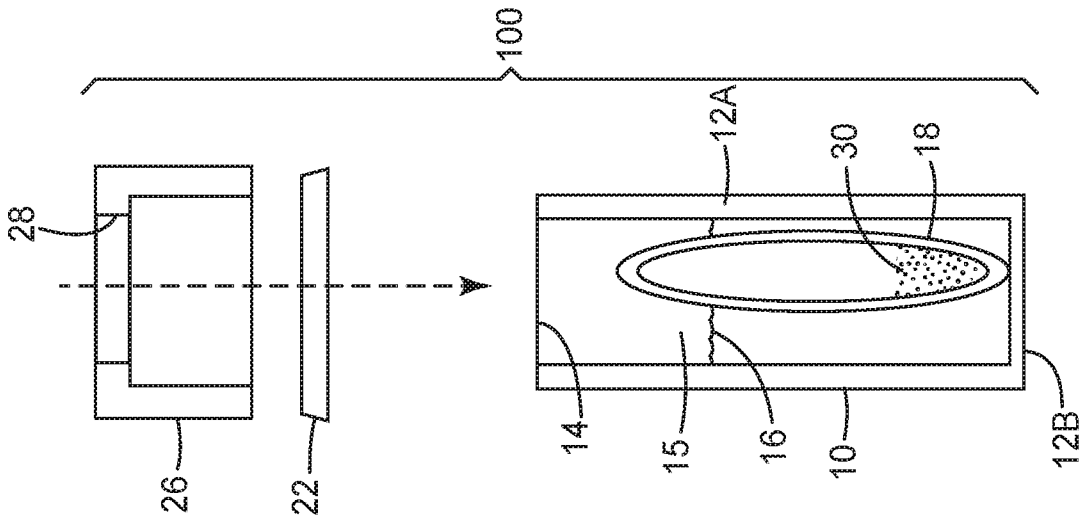


FIG. 1

# INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2019/061398

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/22
ADD.

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)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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|---|---|---|
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 27 March 2020 | 08/04/2020 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | C.F. Angioni |

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| Y | US 2017/292143 A1 (COTE MINDY A [US] ET AL) 12 October 2017 (2017-10-12) the whole document See in particular: paragraphs 21-73 and 163 (in particular 46); Example 5; Claims 1-29. | 1-20 |
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