BIOSIM™ BG NON-BIOLOGICAL AEROSOL SIMULANT

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A non-biological simulant has been developed for use as a safe effective alternative to *Bacillus globigii* (Bg). The simulant consists of one-micron polystyrene carrier beads with an aerodynamic diameter similar to anthrax spores, with total genomic Bg DNA attached to the surface. The DNA attachment to the bead is robust enough to withstand aerosolization and collection processes, yet reacts in a PCR-based detector using existing analytical protocols.

BioSim™ Bg can be used for hazard characterization, agent fate studies, detection equipment verification, training, and as an operator confidence check. The patent-pending delivery system allows the user to easily add a known dose of the simulant directly into a chamber, sampler inlet, or surface. Subsequent analysis with a PCR-based detector provides immediate operator feedback confirming that equipment and procedures are working properly.

INTRODUCTION

Historically, testing biological detectors or detection systems has required the use of biological organisms. Live pathogens can be used in a very limited number of laboratories. Attenuated strains or surrogate organisms such as *Bacillus globigii* (Bg) can be used in laboratory test chambers or in reasonably contained environments. Inactivated organisms such as irradiated organisms, killed whole-cell vaccines and toxoids have also been used to simulate biological material, and can be used within many laboratories meeting BioSafey Level 1 regulations. All of these materials of biological origin pose challenging logistics problems including safety issues, cost, decontamination and regulatory issues, thus limiting their use. Aerosol testing with biological organisms also requires sophisticated and complex laboratory setups and equipment in order to achieve reproducible and representative results and can usually only be done at or above BioSafety Level 2. Set-up time, test time and subsequent decontamination can be extensive and expensive.

With the proliferation of fielded biological detectors, there is an increasing need for biological test materials or alternatives for field applications. These test materials are needed for development and performance testing, acceptance tests, on-going operational evaluation particularly after a maintenance

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action, user training and for operator confidence checks. Live bio warfare agents are obviously out of the question for these applications, but even non-pathogenic and irradiated microorganisms are of concern when disseminated in the open air.

Sceptor Industries and Midwest Research Institute have a developed a non-biological simulant to address this critical need. This laboratory generated, reproducible test particle has the aerodynamic properties of anthrax and the biological properties of Bg without the unwanted properties of an organism, i.e. no virulence, non-reproducing, etc. Dispensed using a metered-dose dispenser, this simulant can be safely used for a variety of applications with aerosol collectors and PCR-based analytical methods.

METHODS

BioSim Bg particles are made using 1 micron nominal diameter polystyrene microspheres which were chosen to closely mimic the aerodynamic diameter of *Bacillus anthracis* endospores. Total genomic DNA was extracted from *Bacillus globigii* and attached to the surface of the polystyrene microspheres using biotin/streptavidin linkages.[1, 2] The microspheres were purchased pre-coated with streptavidin. Genomic DNA was biotinylated and then attached to the microspheres using protocols provided by two manufacturers (Bang's Laboratories and Pierce Laboratories) as shown in Figure 1. The resulting BioSim Bg particles were then subjected to dissemination, collection and analysis using fluorescence PCR-based methods.

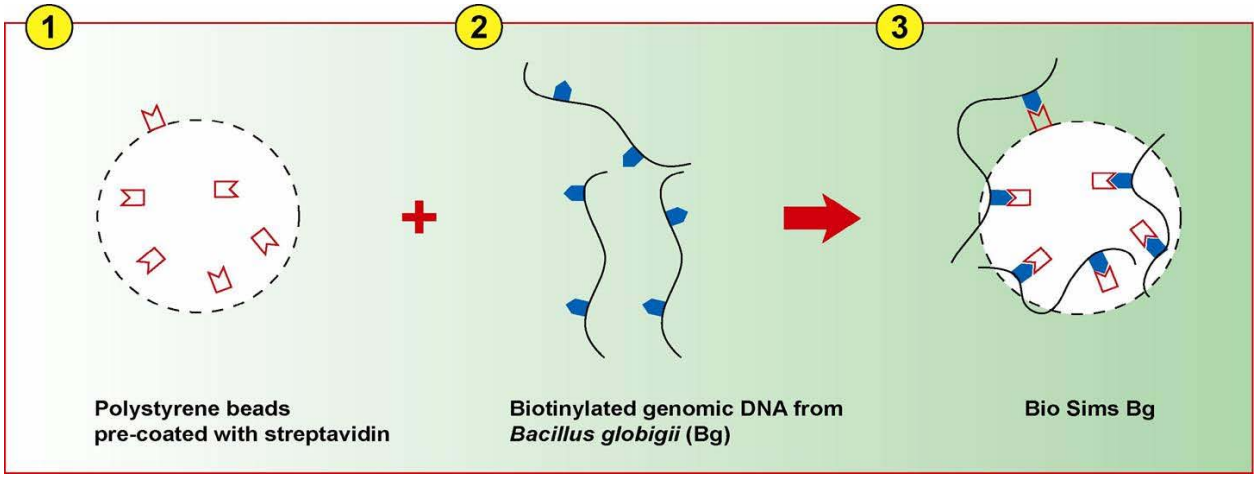


Figure 1. Illustration of biotin-streptavidin binding of genomic DNA from Bg to polystyrene beads.

The intent of the effort was also to dispense the BioSim beads using a simple, convenient method such as a metered-dose dispenser. The materials used in the BioSim Bg delivery system; therefore, must be physically, chemically, and biologically compatible with the BioSim particles. Specifically, the microspheres, the DNA and the streptavidin/biotin linkages must not be adversely affected by the component in the propellant formulation. Furthermore, the density of the propellant formulations must be similar to the BioSim Bg particles, to ensure homogenous distribution of the beads throughout the solution. The vapor pressure must be adequate for proper aerosolization when the metered-dose dispenser is actuated[3]. A list of candidate propellants is shown in Table 1.

TABLE 1. Candidate propellant blends evaluated and considered for BioSim delivery system.

Propellant	General Information	Vapor Pressure (psia@77° F)	Density (Liquid) (g/ml@77°F)
1,1,1,2-tetrafluoroethane	HFA 134a – Used in pharmaceutical inhalers	96.6	1.206
1,1,1,2,3,3,3-heptafluoropropane	HFA 227ea – Used in pharmaceutical inhalers Boiling point (2.5° F)	66.0	1.39
1,1,1,3,3,3-hexafluoropropane	HFA 236fa – New Dupont Dymel for pharmaceutical inhalers Higher boiling point (29.4° F)	39.5	1.36
1,1-difluoroethane	HFA 152a – Not used for pharmaceutical inhalers, is used for personal products Boiling point (-13° F)	63 (psig@70°F)	0.908
Isobutane	Flammable at high concentration	35	0.56
Acetone	Degrades DNA	4	0.79
Ethanol	Disrupts DNA bond to microsphere	0.5	0.79

An important criteria was that the propellant be non-flammable and non-toxic, and not contain ozone-depleting compounds. Potential propellants were evaluated simultaneously for safe use, dispensing system compatibility, and biodetection system compatibility. Some candidate propellant blend components, such as vinyl chloride, were eliminated from consideration due to health risks (VC is a known IARC-listed carcinogen). Others such as chlorofluorocarbons (CFCs, i.e. Freons) were not acceptable because they are harmful to the environment. CFCs are still conditionally approved by EPA and FDA for use in metered-dose inhalers, but are becoming costly and hard to obtain, and are known to harm the earth's ozone layer.

TESTING

The BioSim Bg was tested using an integrated bioaerosol collection and detection system as shown in Figure 2. The BioSim Bg was disseminated from a metered-dose dispenser as a dry aerosol into a heated flow tube, collected as an aerosol, concentrated into a liquid by the aerosol sampler (SpinCon PAS-450-10, Sceptor Industries, Inc. Kansas City, MO) and detected using a polymerase chain reaction (PCR) assay for *B. globigii* (GeneXpert, Cepheid Corporation, Sunnyvale, CA). Additionally, aerosolized beads were analyzed using a TSI 3317 UV-APS to measure the aerodynamic diameter.

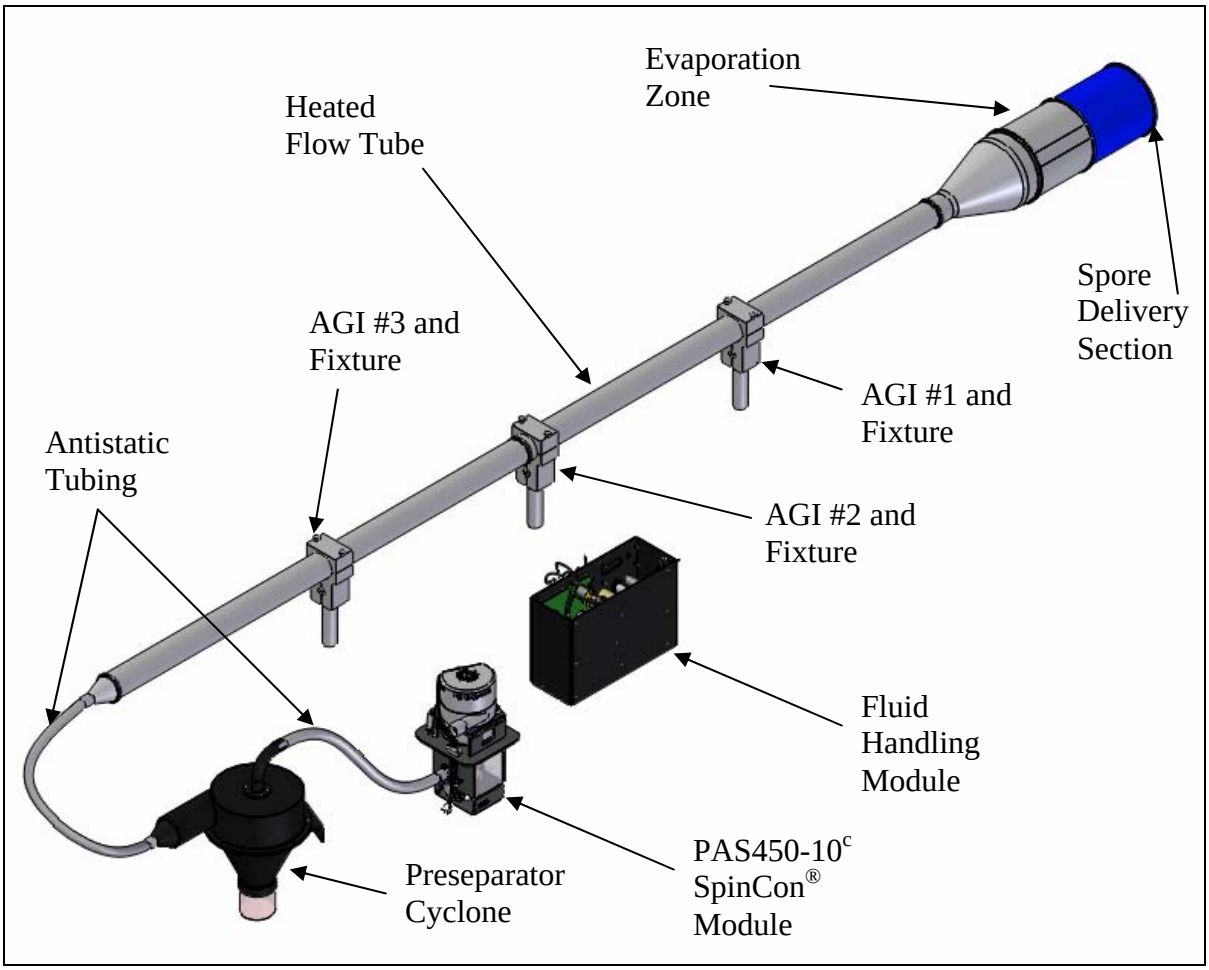


Figure 2. Bioaerosol dissemination and collection test setup used for BioSim evaluation.

RESULTS

Figure 3 shows a sample plot of the aerodynamic size distribution of BioSim Bg. The beads were injected into a warmed flow tube using a dispenser with a 0.48 mm nozzle and collected on a TSI 3317 UV-APS. The results show a fairly sharp distribution of beads with a peak just under one micron aerodynamic diameter (the actual bead diameter for the tested preparation was 0.95 microns). The beads are primarily monodispersed when dispensed and should behave very much the same as aerosolized anthrax spores. These observations were also confirmed using light microscopy to view the BioSim beads following DNA binding. Figure 4 shows a side-by-side visual comparison of BioSim Bg beads with *Bacillus anthracis* endospores, as viewed by light microscopy.

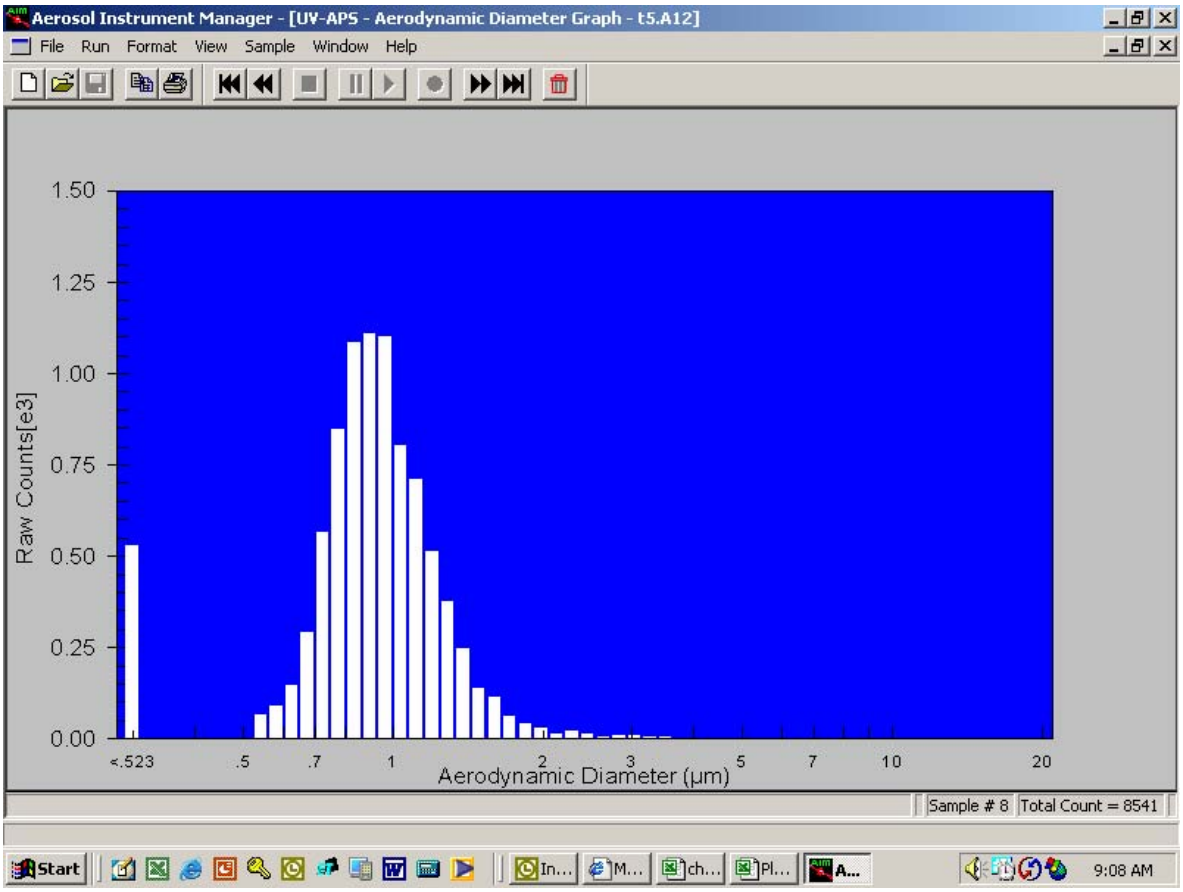


Figure 3. The BioSim beads are predominately monodispersed with an average aerodynamic diameter of approximately 1.0 micron.

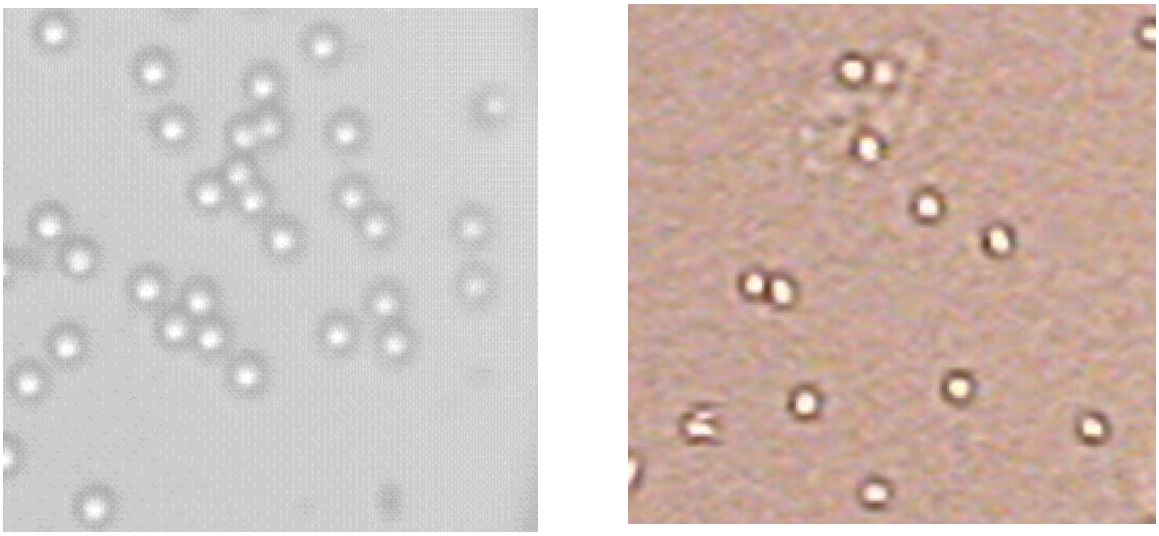
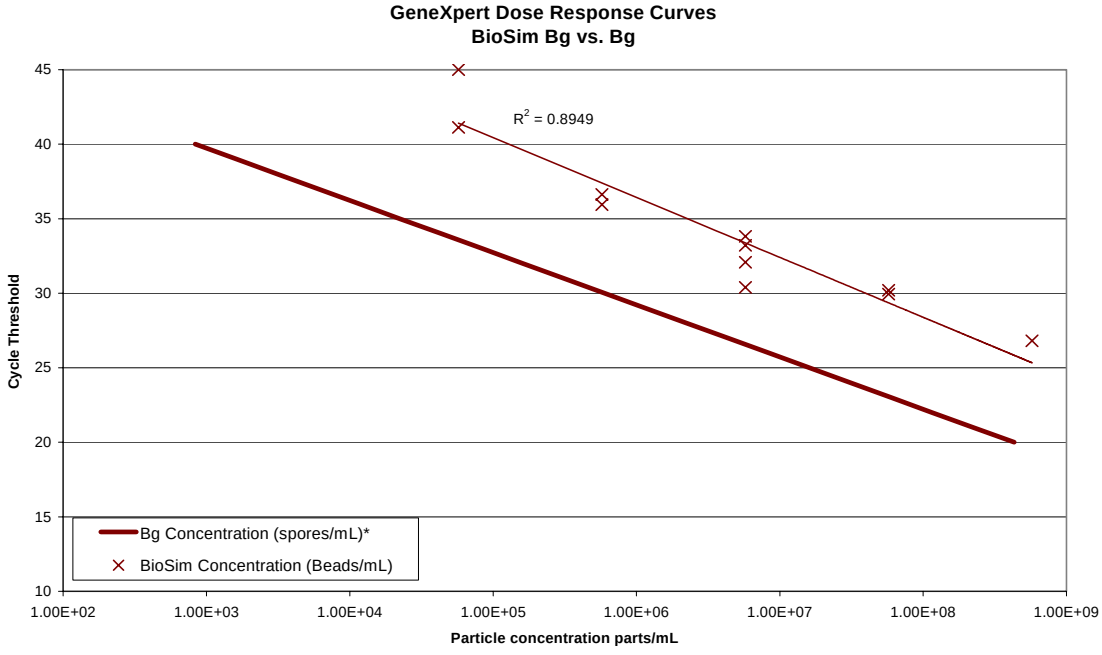


Figure 4. Side-by-side comparison of BioSim beads with endospores from *Bacillus anthracis*, viewed by light microscopy. As shown in the photographs, the BioSim Bg beads and *B. anthracis* endospores have the same approximate shape.

The BioSim beads were designed to mimic as closely as possible the *Bacillus globigii* endospore, the commonly used surrogate organism for *Bacillus anthracis*, the causative agent of Anthrax. An important aspect in the design of the beads is the determination of how many genome equivalents of Bg genomic DNA to attach to each bead. One endospore of Bg contains one genome of Bg DNA. This then would be a good theoretical target for the number of genome equivalents to attach to the beads. In practice the theoretical target for genome equivalents per bead was .83, however based on attachment efficiency, the effective equivalents is quite lower. This can be seen in the dose response curves comparing Bg to BioSim Bg in Figures 5 and 6. Note that these curves also include the effects of extraction efficiency, or in the case of the Taqman analysis shown in Figure 6, the efficiency reduction associated with amplifying DNA attached to a surface, therefore the exact genome equivalents cannot be determined directly using these graphs.



* Calculated per experimentally determined formula from "Bacillus globigii Standard Curve Analysis for the GeneXpert® Bio-Identification Module of the Biohazard Detection System." Smiths Detection, February 06, 2004

Figure 5: Dose response curves for BioSim Bg and a plot of a Bg dose response equation using the GeneXpert.

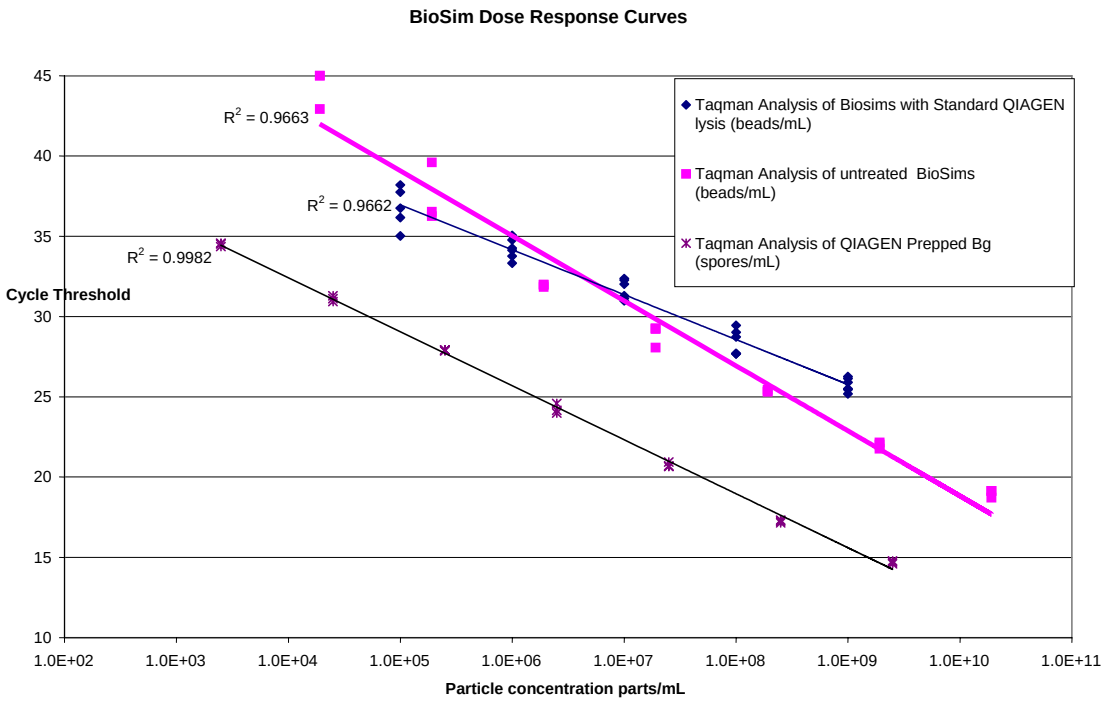


Figure 6: Dose response curves for BioSim Bg, QIAGEN extracted Bg spores, and QIAGEN extracted BioSim Bg using TaqMan® PCR analysis on an ABI 7700.

When Bg endospores are processed using the GeneXpert System, the endospores are collected on a filter, washed, subjected to sonication which breaks open the endospore and releases the DNA. After sonication any free DNA is rinsed from the filter into a PCR chamber for amplification. Therefore any free DNA present in the solution before sonication is rinsed to waste and does not contribute to the analysis. Only DNA that is present after lysis is analyzed. The data presented above in Figure 5 therefore show that BioSim can be subjected to sonication, that the DNA is removed during this treatment, and that this DNA is analyzable by the GeneXpert. In these experiments, the behavior of the BioSim mimics precisely the behavior of the endospores.

When the Bg endospores are processed using a conventional real-time PCR system, they need to be lysed prior to analysis to extract the DNA. A commercial DNA isolation kit from QIAGEN was used for lysis. When BioSim Bg are processed using a conventional real-time PCR system, they do not necessarily need to be lysed prior to analysis. Since the DNA is exposed on the surface of the polystyrene particles, the DNA may be amplified while attached, or optionally run through a QIAGEN lysis procedure as shown by the data presented above in Figure 6.

These particles, made from inert microspheres, are loaded with target DNA in a reliable, robust, and reproducible way. The DNA must be attached to the microsphere from the start, yet be released into the detector at the appropriate time as if it were part of a living organism. It is this functionality that makes the bio-analogous particles work as a safe and effective microorganism surrogate.

The materials used to construct the BioSim and the delivery system must be physically, chemically and biologically compatible. Depending on the biodetection system and the detection method used, the type and strength of the linkage between the particle and the DNA is defined and the attachment method is optimized. The density of the formulation must be very close to that of the

particle, to ensure uniform suspension of the particles prior to dispensing. The vapor pressure of the formulation must be high enough to aerosolize properly when actuated.

To develop BioSim for air sampling/PCR detection biological detection systems, propellant development tests were conducted by filling clear pressure jars with experimental propellant formulations to demonstrate the miscibility of components, chemical compatibility with polystyrene, and temperature stability from 0°C to 23°C. For the selected propellant blend, 10-mm polystyrene pellets were placed in a glass observation jar with the formulation and the pellets did not visibly change over the course of 8 weeks. Additionally, this blend was stored overnight in a freezer and did not separate.

Metered-dose dispensers were filled with BioSim Bg suspended in the formulation and then disseminated into the test rig. Samples were analyzed using a GeneXpert and a Bg assay, and gave positive results for Bg. These tests showed that the BioSim was not harmed by the propellant or the dispensing system, could be collected by the SpinCon Aerosol Collector and could be analyzed by GeneXpert. An on-going stability study (six months to date) is being conducted and continues to show consistent operation and function of the BioSim in the metered-dose dispensers. Stability data is shown in Figure 7. For each run plotted in Figure 7, the dosing was five sprays from a 100µL per spray metered-dose dispenser. Each spray for the metered-dose dispensers tested was approximately $6.3x10^4$ beads per spray based on calculations of the particle dilution in the propellant.

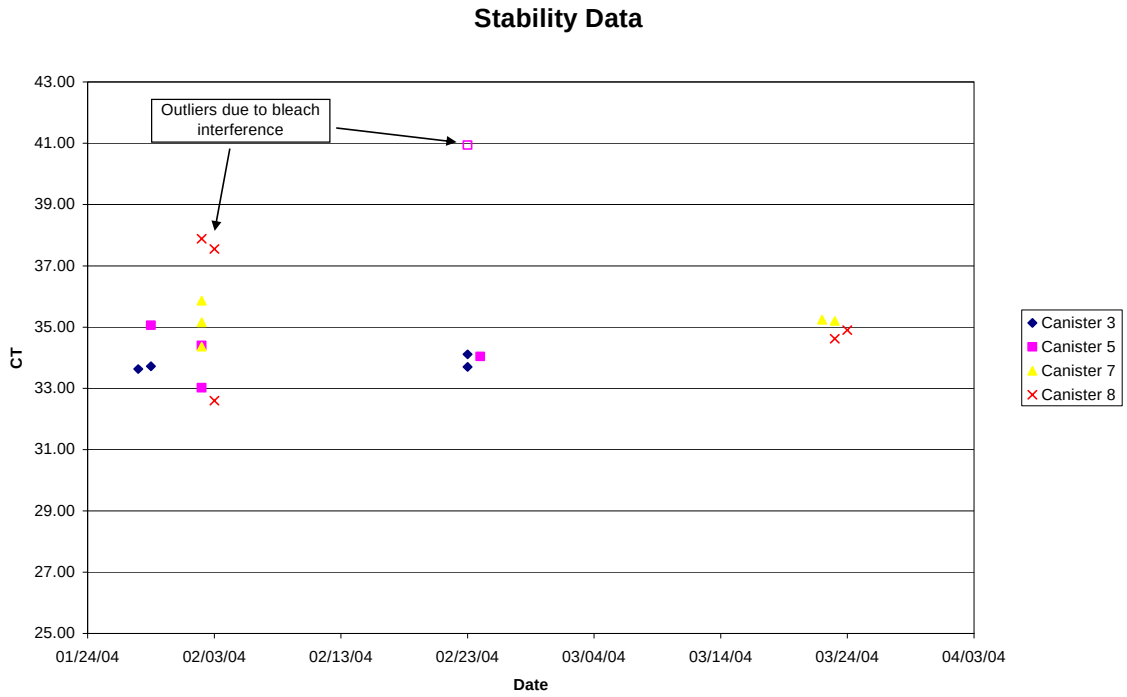


Figure 7. BioSim Bg stability analyses showing the baseline results at T=0 and runs up to 3 months from the first analysis

Between each of the test runs, bleach was used to clean the system and reach a clean baseline for each run. During this testing it became apparent how sensitive the particles were to any bleach residue in the system. As can be seen from the graph, there are a number of runs, particularly in the baseline runs which were apparently affected by bleach residues. This sensitivity makes sense due to the DNA in the product

being exposed on the surface of the beads; therefore, any small amount of cleaning agent greatly affects the DNA, and therefore the response of the product during analysis.

USES AND APPLICATIONS

BioSim Bg is envisioned to have a wide variety of uses and applications where the use of biological organisms is inappropriate or unsafe. The simulant can be used in a controlled aerosol in laboratory environment for equipment tests and evaluations or dispersed closed facilities for mock biological event exercises or agent fate and transport studies. The BioSim can be used for evaluation of air sampling and filtration equipment performance. The BioSim can be used in new product acceptance test as a cost effective alternative to the more costly live agent or Bg challenges. Lastly, the simple to use metered-dose dispenser shown in Figure 4, allows the BioSim to be used in the field as part of biological detection equipment or integrated biological detection systems for routine operation verification testing, maintenance verification procedures and as part of new equipment training and operator confidence checks.

Figure 7. The BioSim Bg can be easily dispensed using the metered-dose dispenser provided.

Currently all materials used in the BioSim except for the DNA itself have been registered with the EPA per TSCA regulations and are well within acceptable limits. Registration of the DNA itself, which EPA considers to be a chemical, is currently in progress to enable acceptance of the entire BioSim mixture. There are no known issues with exposure to genomic DNA. In addition, other DNA molecules currently registered with the EPA indicate no environmental or health issues. While there are no known safety issues with the product, Sceptor recommends that users administering doses of BioSim Bg wear standard dust masks during use of the product. This is only recommended for the user administering the product due to their potential exposure to the concentrated aerosol stream as it leaves the canister. This is a simple precaution that should be taken when testing with any particle in the respirable size range to minimize user exposure.

CONCLUSIONS

The BioSim is a safe, convenient and cost-effective simulant for use with aerosol collectors and PCR-based detection methods. This non-biological simulant has an aerodynamic diameter similar to anthrax and can be used for aerosol performance testing and verification and can be easily measured using simple PCR assays for Bg. The simulant is easily and reproducibly dispensed using convenient metered-dose dispensers and safe for most in-door and open air challenges. The BioSim can be used for equipment testing, operation verification and user training and confidence checks. Future embodiments will include fluorescent proteins for fluorescent trigger applications and common immunoassay antigens for use with hand-held immunoassay kits.

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