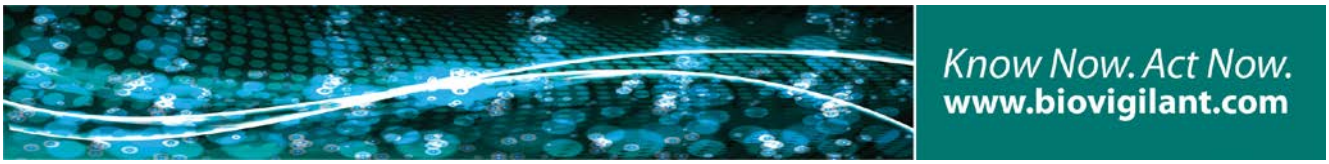

BioVigilant
Instantaneous Microbial Detection

IMD-A™ Model 600 Microbial Challenge and Interferent Test Results

Introduction

This fact sheet provides a summary of microbial and interferent test results obtained with the BioVigilant IMD-A-600 system. IMD-A-series systems are designed to continuously monitor air and detect the presence of both single and agglomerated microbes in real time. Testing was performed to demonstrate that the IMD-A-600 system is equivalent to, if not better than, the compendial microbiological method in the detection of four relevant microorganisms. Comparison of the IMD-A-600 system to the compendial culture-based method was assessed through the side-by-side testing of two IMD-A-600 systems and three air samplers commonly used in pharmaceutical manufacturing environment — the SAS Super 100, MAS 100NT, and SMA. In order to assess all systems stringently, a highly homogenous, state-of-the-art aerosol test chamber was employed to ensure that these systems were concurrently sampling the same aerosol environment throughout the extensive test trials. Information is presented on the IMD-A-600 system's accuracy, correlation to the compendial method, and linearity and specificity to microorganisms and interferent materials.

Background

The pharmaceutical industry recognizes a need to implement modern technologies to drive risk reduction and process control, as evident in guidance such as the FDA's 2004 Guidance for Industry document on Process Analytical Technology (PAT), ICH Guidelines Q8, Q9 and Q10, and the FDA's Pharmaceutical cGMPs for the 21st Century, which encourage the adoption of Quality by Design (QbD) principles and new technologies. The IMD-A system is an example of such a tool, based on modern technology, and developed for continuous environmental air monitoring.

The IMD-A system is a rapid, alternative microbiological method that can monitor the presence of particles and microorganisms continuously in an environment. The fundamental method of microbial detection is different between the traditional culture-based method and the method used by bio-fluorescent particle (BFP) light-induced fluorescence (LIF)-based technologies like the IMD-A-600 system. Traditional culture-based methods require cell proliferation, leading to the formation of a visually detectable colony-forming unit (CFU) to indicate microorganism presence. Yet, with media and incubation parameters typically utilized in industry, not all organisms are culturable. A BFP detection method does not require cell growth and is not restricted by limitations such as incompatible media or incubation conditions. The IMD-A-600 system offers the ability to perform continuous environmental monitoring, making the system an excellent tool for use in continuous manufacturing, risk reduction, investigations, and process control. To evaluate the system's microbial detection capability further, the IMD-A-600 system's sensitivity was assessed and compared to commonly used air samplers.

Test Parameters

Microbial Species Tested

Spore, gram positive	Bacillus subtilis
Vegetative, gram positive	Micrococcus lylae Staphylococcus epidermidis
Vegetative, gram negative	Escherichia coli 12 replicates tested at each of five concentrations, for all microbes
Facility	Azbil g-Lab, Fujisawa, Japan facility
Test Apparatus	2.9m^3 chamber specifically designed for aerosol studies Salter Laboratories nebulizer Kanomax 3900 particle counter
Systems Compared	IMD-A-600 system (2 units) Kanomax 3900 particle counter SAS Super 100 instrument MAS 100NT instrument SMA instrument
Auxiliary Test Site	Robustness and Specificity/2 tests were performed at Fujisawa Technology Center, Azbil Corporation

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Test Microbes

Four indicator microbes, those common to the pharmaceutical manufacturing environment, were chosen including gram negative and gram positive, vegetative bacteria, and spore-state bacteria, as noted earlier.

Test Facility

Biological challenge testing was performed at Azbil's Corporation g-Lab facility in Fujisawa, Japan. The general laboratory area provides a Grade B/ISO 7 environment with the capabilities in place to handle the aerosol testing of BSL-1 organisms. Laboratory personnel have a broad range of experience including microbiology, biochemistry, and industrial hygiene, and specific expertise in fundamental research on bacterial culturability and viability associated with biological aerosol testing.[1]

Test Apparatus and Instruments

Aerosol Test Chamber

All biological challenge testing was performed in a purpose-built, state-of-the-art aerosol test chamber located in the Azbil g-Lab facility (**Figure 1**). The development and engineering of this chamber was based on broad aerosol testing experience and knowledge Azbil BioVigilant has accumulated from extensive testing performed in previous years at Dugway Proving Ground, Eglin Air Force Base, Nelson Laboratories, AlburtyLab, Swedish Royal Institute of Technology, and the Kitasato Institute, among others.

The chamber contains a re-circulating HEPA filter with the ability to operate at 10m^3 per minute, which is equivalent to roughly three chamber air changes per minute. Sampling instruments are located outside of the chamber, providing the ability to change out media plates and perform maintenance without compromising the test environment within the chamber. The chamber floor contains five ports for inlet sampling tubes of instrumentation (reference particle counters, IMD-A systems), and four ports are located on the chamber sidewall with automated sanitary valves for air sampler interfacing. Furthermore, various sensors monitor chamber conditions and equipment including temperature (22±0.5°C), humidity (50±5% RH), and barometric pressure (<10Pa differential).

Nebulizer

A Salter Laboratories 8900-series nebulizer was located inside the test chamber and utilized to disseminate all bacterial suspensions during testing.

Figure 1: Aerosol test chamber in the Azbil g-Lab facility.

Particle Counter

An ISO-21501-4-compliant Kanomax 3900 particle counter was used as a reference instrument for microbial aerosol concentrations and to establish background particle count levels during testing.

Air Samplers (Compendial Method)

Three air samplers representing the traditional air sampling method were utilized during testing: the SAS Super 100, MAS 100NT, and SMA. These air samplers were chosen due to their prevalence in pharmaceutical manufacturing environments.

IMD-A-600 System

Testing was completed with two IMD-A-600 systems. The IMD-A-600 operates at an air flowrate of 4.1LPM with 2.83LPM of air analyzed by the system. The system has a particle size detection range from 0.5 micron to ≥ 5 micron in diameter and operates based on a Mie scatter detection method for particle sizing and enumeration, and intrinsic fluorescence detection for biologic classification.

Test Chamber Characterization

Significant testing was performed to characterize the aerosol test chamber and its performance for use in validation testing. It has been found that monodisperse bacteria are capable of survival in air, as determined through testing and detection by both air samplers and IMD-A systems; therefore, it was desirable to challenge and confirm the IMD-A systems' performance when exposed to monodisperse organisms, as these microbes are the most challenging to detect. Agglomerated microbes are often easier to detect due to their larger size and corresponding higher level of fluorescence. Consequently, testing to ensure the cleanliness and homogeneity of the chamber down to at least a 0.5-micron particle size was performed to ensure an adequate testing environment. These tests included hydrogen leak testing, repeatability testing, chamber uniformity testing, and cleanliness testing. Six mixing fans, strategically placed within the chamber, achieved a homogenous aerosol distribution.

[1] Hasegawa, N. et al., A study of bacterial culturability during bioaerosol challenge test using a test chamber, Journal of Aerosol Science, 42, 6, 397-407 (2011).

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Hydrogen Leak Test

Molecular hydrogen was utilized to perform a chamber leak test. Hydrogen was used as it is smaller than microbes, ensuring the leak tightness of the chamber. No leak was detected when testing the chamber door, connection points, and hardware locations (e.g. screws).

Repeatability Test

The coefficient of variation (CV) of the number of particles detected across 10 replicates was calculated for each of the seven sampling locations within the chamber. This value was confirmed to be less than 10% in all cases.

Uniformity Test

The chamber uniformity test involved nebulizing 0.8 micron PSL beads in the test chamber in accordance with the JIS 3836 *Annex 2* standard for evaluation of chamber uniformity.[2] A Kanomax 3900 particle counter was utilized to obtain 10, one-minute samples at each of seven locations within the chamber, including all five sampling locations in the base of the chamber and two air-sampler sampling port locations. The results of this test are shown in **Figure 2**. The mean number of particles detected at each of the seven locations was within a range of ± 10% of the average number of particles across all sampling locations.

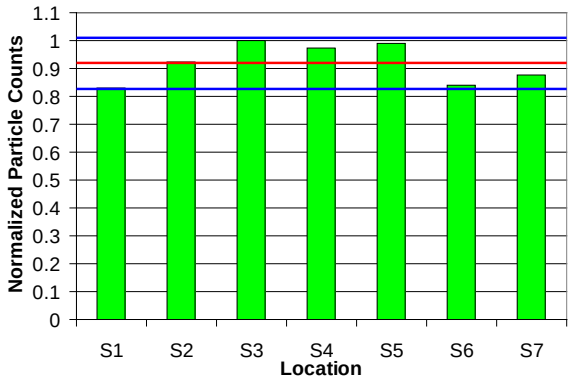

Figure 2: Aerosol test chamber static uniformity test. The red line marks the average normalized particle counts across all seven sampling locations. The blue lines represent plus and minus 10 percent of this average value.

Cleanliness Test

An evaluation of the level of particle and biologic cleanliness in the chamber was performed. It was confirmed that the chamber complied with ISO Class 4 particulate levels and contained less than 1 cfu/m^3.

Test Parameters

I. Microbial Species Tested

Four industry-relevant organisms were utilized to challenge the IMD-A-600 system and assess its detection performance as compared to a reference particle counter and the active air sampling method. These organisms include *Escherichia coli* (ATCC 10798), *Bacillus subtilis* (ATCC 6633), *Micrococcus lylae* (ATCC 27566), and *Staphylococcus epidermidis* (ATCC 12228). *E. coli* and *S. epidermidis* were purchased from the RIKEN Bio Resource Center, *M. lylae* was purchased from the American Type Culture Collection (ATCC) and the *B. subtilis* spore suspension was obtained from MesaLabs (Ref: SUS-1A-6). *B. subtilis* was in the spore state, while all other microorganisms were tested as vegetative cells.

II. Microbe Preparation

Vegetative organisms *E. coli* and *M. lylae,* were inoculated from their glycerol stock in Tryptic Soy Broth (TSB) and cultured aerobically overnight at 32°C while *S. epidermidis* was cultured aerobically for two days at 32°C. The bacteria were then streaked onto TSA and incubated at approximately 32°C for 20 to 24 hours for *E. coli*, and 20-48 hours for *M. lylae* and *S. epidermidis*, to achieve the stationary phase. The bacteria were then harvested in sterile, distilled water (DW) and washed once through centrifugation at 2,100g for three minutes. The supernatant was removed and the pellet was re-suspended in filtered DW. Optical density measurements at 600 nm (OD600) then were utilized to estimate concentration, followed by dilution with filtered DW to reach the target microbial concentration. This dilution was utilized in the system testing and plated on TSA to perform a final titer check. The B. subtilis spore suspension was prepared following a different procedure. B. subtilis spore suspensions were diluted with filtered DW directly from the stock suspension.

Five target concentrations were tested for each microorganism, with twelve replicates performed at each concentration tested. Concentrations were chosen to ensure that the IMD-A system and culture-based method yielded no more than two zero or, in the case of the culture-based method, two TNTC results per concentration tested.

[2] Japanese Industrial Standard: Testing methods for collection efficiency of airborne microbe samplers. JIS K 3836: 1995.

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Microbial Challenge Test Results

The IMD-A-600 systems were challenged with four microorganisms, over five concentrations, with twelve replicates performed at each concentration. The test was designed such that single cells, as opposed to agglomerates, were sampled by the IMD-A-600 systems and active air samplers to ensure sensitivity down to the level of intrinsic fluorescence emitted by single microbes. Furthermore, all sampling was performed concurrently with a reference aerosol particle counter.

I. IMD-A-600 Accuracy

Accuracy, as described in USP <1223> Validation of Alternative Microbiological Methods[3], is the equivalence of the test results obtained by the IMD-A system to those obtained by the compendial air sampler, with a goal of the alternative method (i.e. the IMD-A system) recovering at least as many microorganisms as the compendial method. The plots (**Figure 3**) show IMD-A-600 biologic counts per liter and air sampler CFU per liter versus the concentration of particles determined to be within the test chamber by a reference aerosol particle counter.

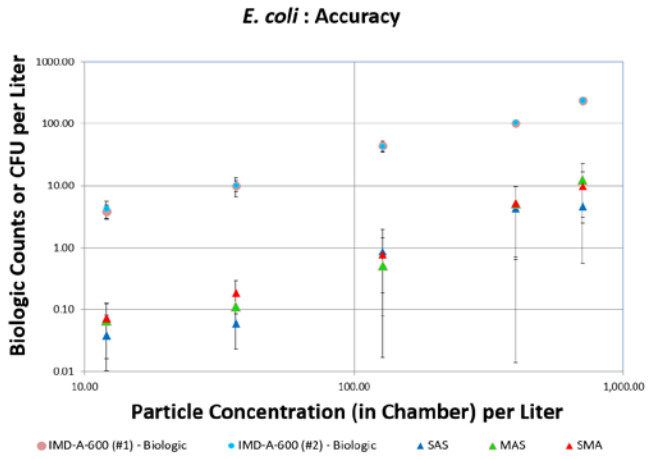
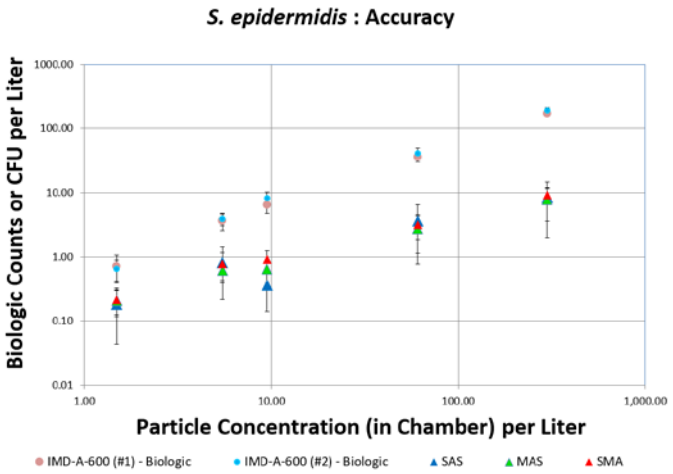
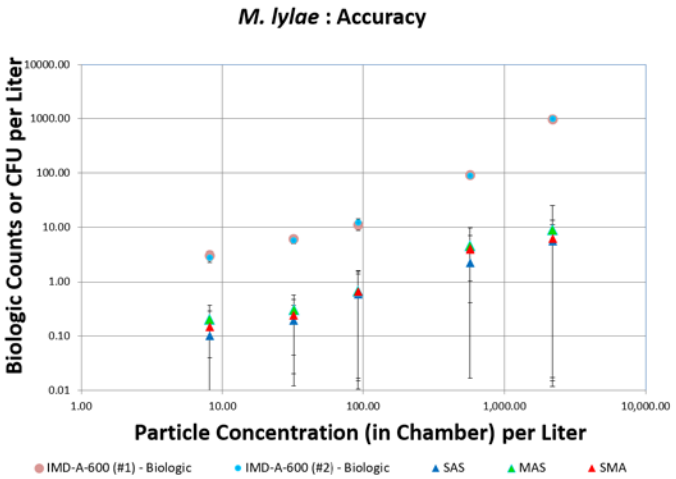
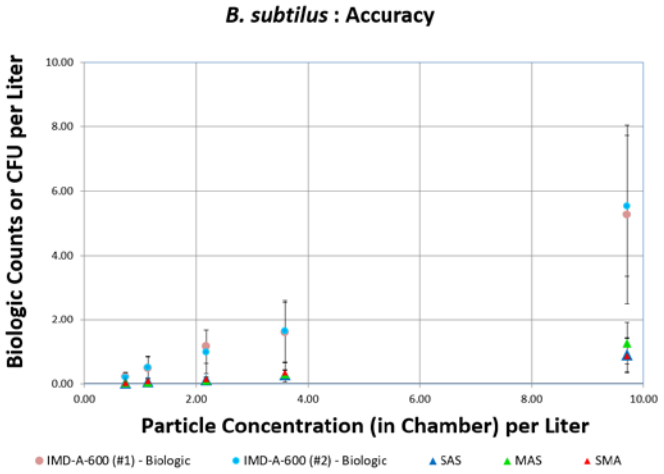


Figure 3: In an assessment of IMD-A accuracy, these four plots show average IMD-A-600 biologic counts per liter and average air sampler CFU per liter for the four microorganisms tested versus the number of particles in the test chamber as determined by a reference aerosol particle counter. The IMD-A-600 systems were capable of detecting a higher number of the microorganisms present in the chamber than the three air samplers tested, for all four microorganisms.







As mentioned above, the two IMD-A-600 systems, three air samplers and the reference particle counter were all sampling concurrently in the test chamber when each organism replicate was aerosolized. Since the IMD-A biologic counts are based on intrinsic fluorescence and

[3] USP. 2015. <1223> Validation of Alternative Microbiological Methods. *United States Pharmacopeial Convention.* USP 38/NF33:*1439*.

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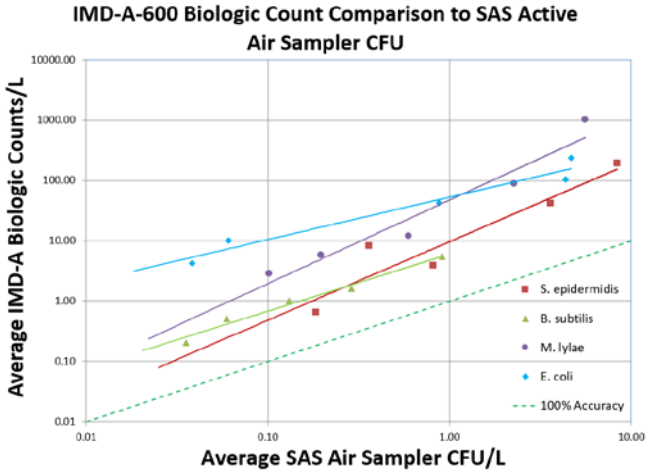
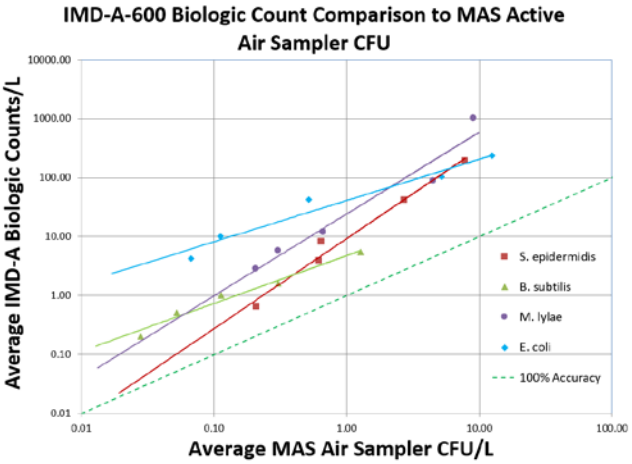
the air sampler CFU counts are based on growth, the number of microbes in the chamber was best approximated by the reference particle counter, given that minimal background counts in the chamber were confirmed before each test was performed. *E. coli S. epidermidis* and *M. lylae* are plotted on a log scale so that results from the broad range of concentrations tested can be compared on the same plot, for each organism (**Figure 3**). The *B. subtilis* plot uses a linear scale because the five concentrations tested span only approximately one log. As can be seen in the plots (**Figure 3**) both IMD-A-600 systems reported very similar counts across concentrations and microorganisms tested. Furthermore, the IMD-A-600 systems were capable of detecting a higher number of microorganisms present in the chamber than the three air samplers tested, for all four microorganisms. This testing shows that the IMD-A system has an acceptable accuracy with a detection sensitivity that is equivalent to or better than the compendial method for all organisms tested.

II. Correlation of IMD-A-600 Biologic Counts to the Reference Air Sampler CFU Counts

IMD-A-600 biologic count results were found to correlate well with reference air samplers' culture-based results for the organisms tested. Average IMD-A-600 biologic counts per liter, averaged over the twelve replicates performed at each concentration, are plotted on the y-axis while average air sampler CFU per liter are plotted on the x-axis. The green dotted line represents a 100% accuracy line. If results are on this line, it indicates that IMD-A biologic counts and traditional culture-based method CFU counts are equivalent. Organisms above this line showed higher biologic counts on the IMD-A-600 system than on the reference culture-based active air sampling method. As seen in **Figure 4**, all four organisms showed higher recoveries with the IMD-A-600 system than with the traditional culture-based method and counts correlate well across the range of concentrations and organisms tested.





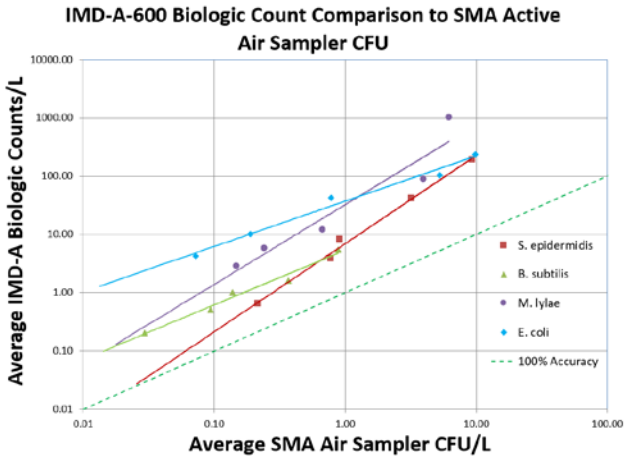


Figure 4: Average IMD-A-600 biologic counts per liter compared to the average air sampler CFU per liter for three reference air samplers. The dashed green line represents the 100% Accuracy Line. Organisms above this line showed higher counts with the IMD-A-600 system than with the reference air sampler.

III. IMD-A-600 Linearity

Linearity, as defined in USP<1223>, is the ability to produce results with the alternative method (i.e. the IMD-A system) that are proportional to the results obtained with the compendial method.[3] The correlation, or R^2 value, was determined between the log of the biologic counts from the IMD-A-600 system and the log of the CFU counts from the reference air samplers. The IMD-A-600 biologic count results were found to correlate well with the reference air sampler culture-based results for the organisms tested. This is seen in the high R^2 values shown in the table below. A value that is close to one indicates a high correlation in the results from both techniques, across the concentration range tested. However, the variability in recovery seen across organisms (**Figure 4**), due to differences in size, fluorescence and culturability of these organisms, make it such that a conversion factor between CFU and biologic counts cannot be applied to these fundamentally different detection methods.

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Microorganism Tested	IMD-A-600 vs SAS R^2	IMD-A-600 vs MAS R^2	IMD-A-600 vs SMA R^2
E. coli	0.9553	0.9492	0.9777
M. lylae	0.9352	0.9492	0.9101
S. epidermidis	0.9117	0.99	0.9966
B. subtilis	0.9743	0.9667	0.9622

Table 1: IMD-A-600 R^2 results.

Interferent Material Test Results

Specificity testing includes both the ability of a system to detect a range of challenge microorganisms (i.e. true positives), and an assessment of the likelihood of obtaining biologic counts from commonly used materials. Related to the IMD-A system, a "false positive" biologic result occurs when system noise is recorded as a biologic particle, even though particle fluorescence has not been detected by the system. An "interferent", in relation to the IMD-A system, is defined as an inert particle which imitates the fluorescence generated by biological particles when excited with 405nm radiation. The IMD-A system has two fluorescence detectors and advanced algorithms that better enable the differentiation of fluorescent microbes from fluorescent interferent materials, with a target max interferent rate of 10%.

The interferent material testing is utilized to test common cleanroom materials on the IMD-A-600 system that have the potential to elicit a fluorescent response and determine at what rate they are classified by the system as biologic. This is determined by looking at the percentage of total particles counted by the system that are reported as biologic counts. As a result, the IMD-A-600 system was challenged with eight common pharmaceutical cleanroom materials with the potential to elicit a fluorescence response on the system. These materials include PTFE (e.g. Teflon®), polypropylene (PP), low density polyethylene (LDPE), polyethylene terephthalate (PET), vial glass, sterile clean room garments, tryptic soy broth (TSB) and sterile 70% isopropyl alcohol (IPA). A sample of each material was created, placed in filtered DW, and aerosolized into the IMD-A system. In the case of the sterile cleanroom garments, the garment was torn and waved over the IMD-A inlet.

As can be seen in the results table below, only sterile cleanroom garments and TSB had a percent biologic above our target maximum interferent rate of 10%. TSB was expected to have a positive biologic count rate, greater than 10%, because of its significant fluorescence in 405nm light. The sterile cleanroom garments were found to pose an interferent risk with a percent biologic of 13%, a few percent above our target maximum interferent rate. However, it is important to note that the garments had to be torn and vigorously shaken to release a significant enough number of particles for testing. This is a very atypical operating condition. Overall, the positive count rate, or percent biologic, varies depending upon the material and all other materials tested had a positive biologic count rate far below our maximum target of less than 3% biologic. It is important for users to assess the interferent risk of materials commonly used in their manufacturing environment to gain a better understanding of the specific environment. Please contact BioVigilant for more information on the materials tested and testing procedure.

Materials tested	Positive Biologic Count Rate
PTFE	0.03%
PP	0.03%
LDPE	1.8%
PET	0.6%
Vial glass	2.4%
Sterile cleanroom garments	13%
Tryptic Soy Broth (TSB)	54%
Sterile Isopropyl alcohol 70%	2.9%

Table 2: IMD-A-600 interferent material testing results.

Conclusions

- Through this microbial challenge testing, the IMD-A-600 system was found to detect all four organisms tested with a sensitivity equivalent to or greater than the compendial air sampling method, indicating sufficient IMD-A-600 Accuracy.
- IMD-A-600 biologic counts were found to correlate well with air sampler CFU results across the range of five concentrations and four organisms tested. However, the variability in recovery seen across organisms (**Figure 4**), due to differences in size, fluorescence and culturability of these organisms, make it such that a conversion factor between CFU and biologic counts cannot be applied to these fundamentally different detection methods.
- The IMD-A-600 system showed excellent linearity across all organisms tested, as shown by the R^2 values (**Table 1)** that are all greater than a value of 0.9.
- Interferent testing found that six of the seven potential interferent materials tested did not pose a risk of percent biologic counts above the target for the IMD-A-600 system as values were all below 3% biologic. The seventh material, a cleanroom garment, did pose an interferent risk but only after the material was torn and waved over the IMD-A inlet in order to obtain adequate particulate. It is recommended that end users assess potential interferent risks within their unique environments.
- With an equivalent or better sensitivity (when compared to the compendial method) and the ability to monitor an environment continuously and in real time, the IMD-A-600 system is a powerful monitoring and trending tool that can be used to increase product quality assurance and process understanding.

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