CLIMET®
CLIMET INSTRUMENTS COMPANY

Best Practices:
Viable Air Monitoring

Application Note: 240123-A

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Climet Instruments Company, a Division of Venturedyne, Ltd.
www.climet.com
+1 (909) 793.2788

This document hopes to convey what regulatory agencies have in the past considered when examining drug producers. It is intended to provide a non-comprehensive general overview of applicable standards, and will provide a number of industry best practices used in biopharmaceutical industrial manufacturing and research.

This document is intended to provide a very basic and fundamental instructional guide. Actual practices at a facility may differ from the information presented herein due to managerial decisions and prerogatives, or on product or substance being manufactured and/or individual risk assessments.

PURPOSE & GOAL

Goal of Viable Environmental Monitoring

The ***purpose or objective of environmental monitoring*** of an operational cleanroom or clean zone is to provide evidence that the required level of cleanliness is achieved at critical control points.[1]

Another definition is provided in EU GMP, Annex 1:2022, §9.4, which states the purpose of the environmental monitoring programme, is to:

i Provide assurance that cleanrooms and clean air equipment continue to provide an environment of appropriate air cleanliness, in accordance with design and regulatory requirements; and

ii Effectively detect excursions from environmental limits triggering investigation and assessment of risk to product quality.

The ***goal of microbiological environmental monitoring*** is to reproducibly detect microorganisms for purposes of monitoring the state of environmental control.[2] Monitoring shall also be performed routinely in the operational state according to the selected Formal System.[3]

"Microbial monitoring cannot and need not identify and quantify all microbial contaminants in controlled environments." It is a semiquantitative exercise because a truly quantitative evaluation of the environment is not possible given sampling equipment, collection media, and collection methods. [4]

[1] ISO 14644-1:2015, B.3.1.1
[2] FDA, Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing - cGMP (2004). §A(B), page 35.
[3] ISO 14698-1:2003, 5.3.1
[4] USP <1116>, Microbial Control and Monitoring of Aseptic Processing Environments. pp 786 August 1, 2013

RISK ASSESSMENT – ENVIRONMENTAL MONITORING

Monitoring plans incorporate information from a documented **risk assessment**, a written plan designed to account for the required levels of air cleanliness, critical locations and performance attributes of the clean area. Like any business practice, this plan should undergo periodic evaluation and reviews, with ***improvements being implemented as appropriate***.[5]

Risk assessment plans for environmental monitoring are typically developed using a team based and scientific approach. This team often includes professionals from various fields such as Microbiologist, Quality Assurance, Quality Control, Manufacturing, Facilities, and Engineering. Additionally, a consultant familiar with FDA/GMP regulations is frequently part of the team.

There have been numerous FDA warning letters and citations recommending, and in some cases requiring, the use of a knowledgeable consultant. Consultants, with their significant FDA audit experience, can prove to be invaluable. Please contact Climet if you need a referral.

According to EU GMP, Annex 1:2022, §9.4, "*Risk assessments should be performed in order to establish this comprehensive environmental monitoring programme, i.e. sampling locations, frequency of monitoring, monitoring methods and incubation conditions (e.g. time, temperature(s), aerobic and/or anaerobic conditions).*

These risk assessments should be conducted based on detailed knowledge of; the process inputs and final product, the facility, equipment, the criticality of specific processes and steps, the operations involved, routine monitoring data, monitoring data obtained during qualification and knowledge of typical microbial flora isolated from the environment.

The risk assessment should include the determination of critical monitoring locations, those locations where the presence of microorganisms during processing may have an impact upon product quality, (e.g. grade A, aseptic processing areas and the grade B areas that directly interface with the grade A area). Consideration of other information such as air visualisation studies should also be included. These risk assessments should be reviewed regularly in order to confirm the effectiveness of the site's environmental monitoring programme. The monitoring programme should be considered in the overall context of the trend analysis and the CCS for the site."

Useful Sources:

- International Conference Harmonization (ICH) Q9 – Quality Risk Management
- European Medicines Agency (EMA)

[5] ISO 14644-2: 2015, 4.1.

- EudraLex – Volume 4, Good Manufacturing Practices (GMP) Guidelines

  Guide Presented in 3 parts:
  - Part I covers GMP Principles for the manufacture of medicinal products
  - Part II covers GMP for active substances used as starting materials
  - Part III is intended to host a collection of GMP related documents, which are not detailed guidelines on the principles of GMP laid down in the directives (EU Commission Directive 2003/94/EC and 91/412/EC)
- FDA:Q9 (R1) Quality Risk Management – Guidance For Industry: https://www.fda.gov/media/167721/download
- TRS 981 – Annex 2: WHO guidelines on quality risk management: https://www.who.int/DOCS/DEFAULT-SOURCE/MEDICINES/NORMS-AND-STANDARDS/GUIDELINES/PRODUCTION/TRS981-ANNEX2-WHO-QUALITY-RISK-MANAGEMENT.PDF

INTRO TO COLLECTION EFFICIENCY

The collection efficiency of microbial air samplers can be considered in two ways: physical efficiency and biological efficiency.[6]

> **Physical efficiency** is the ability of the sampler to collect various sizes of particles. Physical efficiency is the same whether the particle is a microorganism, carries a microorganism or is an inert particle.
>
> **Biological efficiency** is the efficiency of the sampler in collecting microbe-carrying particles. Biological efficiency will be lower than physical efficiency for a number of reasons, such as the survival of the microorganisms during collection and the ability of the collection medium to support their growth.

Since 1995, we have known that as a microbial air sampler's physical collection efficiency approaches 100%, an increase in the impaction velocity produces a significant decline in the percentage of microorganisms recovered.[7]

Additionally, we know that a cleanroom's only significant source of biocontamination is its personnel.[8]

According to Parenteral Drug Association : [9]

> *"In the microbial contamination of pharmaceutical products, human skin is an important source of contaminants."* This article continues, *"It has been suggested that up to 80% of the species associated with the skin are recoverable by culture method."*

Therefore, not all microorganisms can be cultured and identified. This is compounded further by the lower percentage of gut bacteria that can be cultured.

The experimental method for determining the 'Collection Efficiency' of an air sampler is discussed in ISO 14698-1:2003, Annex B; and is in Climet's opinion, poorly conceived. The experimental method in ISO 14698 does not measure 'collection efficiency,' but rather it employs a flawed 'comparative' methodology. Simply, the method consists of taking the '*sampler under-test'*, and placing it into an isolation chamber along with another microbial air sampler, i.e., '*reference sampler*.' Then, running a side-by-side comparison test, with the results defined as a 'Collection Efficiency.'

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[6] ISO 14698-1:2003, Annex B
[7] Stewart, Grinshpun, Terzieva, Ulevicius and Donnelly. *Effect of Impact Stress on Microbial Recovery on an Agar Surface.* Applied and Environmental Microbiology, Vol. 61, No. 4, Apr 1995, p. 1232-1239. American Society for Microbiology.
[8] USP <1116>
[9] Parenteral Drug Association, 'Microbial Control and Identification Strategies,' 2018, page 15.

The problem with this method is two-fold. First, it is very well-known that among the numerous air samplers on the market today, sampling efficiency and technology can differ substantially.[10] For example, let's say you have an apple. It would probably compare very favorably to the rotten apple in the image below, left. However, it might not compare that well to the apple on the right.

The second problem is there is no test methodology in the standard that informs the end-user how many viable particles were missed and not counted, which is a critical part of an evaluation. (Yao, 2006)

In response to these shortcomings, many astute microbiologists have referred to an article written in 2006 by Yao, et al. [11] This article discusses the concept of a **d50**. It is based upon a Computational Fluid Dynamics (CFD) equation for the *inlet velocity* of the airstream through a microbial air sampler.

Impact velocity of the air hitting the culture medium must be high enough to allow the entrapment of viable particles down to approximately 1 µm.

ISO 14698-1:2003, §A.3.4.2

This equation calculates an inlet velocity, taking into consideration the diameter and number of holes in the sample head. It also applies a Stokes number, with higher values given to larger particles and lower values given to smaller particles.

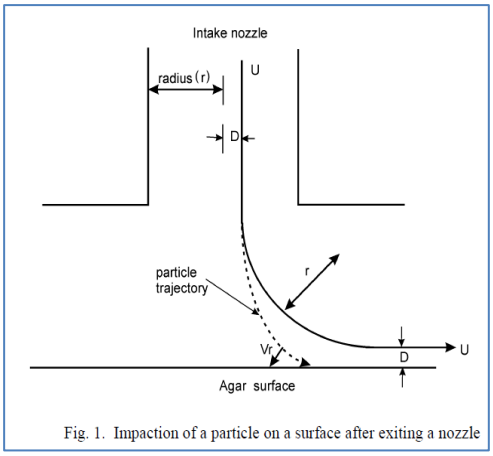

Fig. 1. Impaction of a particle on a surface after exiting a nozzle

The 'd50' is defined as, ***the particle size at which the air sampler has a 50% collection efficiency***.

For example, let's assume a microbial air sampler has a d50 value of 2 µm. This means that particles smaller than 2 µm have less than a 50% probability of impacting the collection media. Conversely, airborne particles larger than 2 µm have a greater than 50%

[10] Prog Health Sci (2011), Vol 1, No 1 Fungal air pollution different samplers. **Analysis of fungal air pollution using different samplers** Lukaszuk C., Krajewska-Kułak E., Kraszyńska B., Gniadek A., Chadzopulu A., Theodosopoulou E., Bousmoukilia S., Terovitou Ch., Amanatidou A., Danilidis D., Adraniotis J.

[11] Yao, Mainelis. *Investigation of Cut Off Sizes and Collection Efficiencies of Portable Microbial Samplers*. Aerosol Science and Technology, 40:595-606 (2006). American Association of Aerosol Research.

probability of impacting the collection media. This concept is fairly intuitive, as one would expect larger airborne particles, which have more mass, to be easier to collect.

When the d50 principle is applied to ISO 14698, the industry has broadly accepted that a d50 value of about 1 µm is acceptable. Per ISO 14698-1:2003, A.3.4.2(a)(1): "**Impact velocity of the air hitting the culture medium must be high enough to allow the entrapment of viable particles down to approximately 1 µm**."

BS EN 17141 (§E.5.2) provides a looser but more specific standard, whereas ***a d50 value smaller than 2 µm is considered appropriate***.

The *experimental* d50 is vastly more important than the *theoretical* d50.

Yao, et al., 2006

$$d_{50} = \sqrt{\frac{40 \times Dh}{U}}$$

This standard implements a simplified equation for calculating a microbial air sampler's d50 value (shown here). *Dh* is the diameter of the inlet in millimeters, and *U* is the inlet velocity.

In 2006, Yao, et al. demonstrated that only 7 of the 12 microbial air samplers tested had a calculated or *theoretical* d50 < 2 µm. Of those tested through an experimental method only 2 of these 7 samplers had actual or *experimental* d50 value less than 2 µm. [12] Also, it was shown that there is little correlation between the theoretical (math calculation) and experimental d50 values, and that an experimental d50 is vastly more meaningful.

It should be noted that there are some microbial air samplers on the market today that have a d50 = 18 µm, which suggests a very undesirable collection efficiency. [13]

The d50 also has weaknesses:

1. The d50 only addresses physical collection efficiency and does not measure biological collection efficiency.

2. The theoretical d50 (math equation) fails to take into consideration key factors. For example, the height of the agar (or collection media) in relation to the bottom of the nozzle in the sample head. This has a significant effect on impact velocity and physical collection efficiency.

[12] Yao, Mainelis. *Investigation of Cut Off Sizes and Collection Efficiencies of Portable Microbial Samplers*. Aerosol Science and Technology, 40:595-606 (2006). American Association of Aerosol Research.
[13] Yao, Mainelis. *Investigation of Cut Off Sizes and Collection Efficiencies of Portable Microbial Samplers*. Aerosol Science and Technology, 40:595-606 (2006). American Association of Aerosol Research.

3. The theoretical d50 (math equation) does not consider poor design, which has inherently shown that the mathematical/theoretical d50 value is virtually meaningless.

From a biological collection perspective, if the impaction velocity is too high, the sampler will kill or mechanically damage the microorganism, preventing it from being cultured.[14]

Similarly, if the impaction velocity is too low, the microorganism will not sufficiently embed itself into the agar, and cannot be cultured. Also, when the velocity is too low, there is a much higher probability that the MCP could remain entrained in the air stream and fail to impact onto the agar.

One common design fault is that the exit velocity of the sampler's exhaust may cause a pressure differential (back pressure) that could potentially destroy viable microorganisms, resulting in reduced biological collection efficiency.

Once more, when evaluating a microbial air sampler, end-users should not simply evaluate an instrument's computational or ***theoretical d50***, but rather the ***experimental*** d50 which is vastly more important.

When the experimental d50 value is greater than the theoretical d50, that is d50(e) > d50(t), this would suggest design inefficiencies, and as discussed above, would generally suggest biological inefficiencies exist.

In most cases, one would expect the experimental d50 value to be lower than or equal to the theoretical d50.

[14] Stewart, Grinshpun, Terzieva, Ulevicius and Donnelly. *Effect of Impact Stress on Microbial Recovery on an Agar Surface.* Applied and Environmental Microbiology, Vol. 61, No. 4, Apr 1995, p. 1232-1239. American Society for Microbiology.

CLEANROOM MICROBIOLOGY

Cleanroom contamination, both nonviable and viable, can originate from various sources. These include people, water, air and ventilation, surfaces, and transport of items into or out of a clean zone, which can lead to cross-contamination. However, the majority of contamination within a pharmaceutical cleanroom can typically be traced to humans working within these cleanrooms. [15] [16]

Humans shed a high number of skin cells, primarily in the form of skin flakes. The cleanroom garments worn by personnel cannot completely contain all human detritus.

Microbes in the air of occupied rooms are derived from personnel who disperse skin cells that may carry microorganisms and several hundred Microbe Carrying Particles (**MCPs**) per minute pass through cleanroom clothing and into cleanroom air. Microorganisms are usually found in cleanrooms attached to skin particles (and to a much lesser extent to clothing fibers). Most of the MCPs found in skin fragment experiments were reported to be greater than 5.0 µm.[17] Indeed, "While airborne microorganisms are not free-floating or single cells, they frequently associate with particles of 10 µm to 20 µm in size." [18]

From a microbiological perspective, the human body is an intricate system that hosts trillions of microbial cells, which also impact the external environment as they are shed through the skin or deposited through various orifices. In fact, the skin's outer surface alone can host up to one million microorganisms per square centimeter. [19]

The Human Microbiome Project (HMP) is a U.S. National Institute of Health initiative that has shown that there are approximately 1,200 species of bacteria from 19 phyla on human skin. Of these, most bacteria (> 90%) can be categorized into four phyla: [20]

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[15] Hyde, W. (1998). Origin of bacteria in the clean room and their growth requirements. *PDA J Sci Technol*; 52:154–164

[16] USP <1116>

[17] Eaton, Davenport and White. "Airborne microbial monitoring in an operational cleanroom using an instantaneous detection system and high efficiency microbiological samplers." European Journal of Parenteral & Pharmaceutical Sciences: 2012; pages 61-9; and Microbial Control and Identification Strategies, PDA, 2018, page 15-16.

[18] <USP 1116>, pp 699
https://dcvmn.org/wp-content/uploads/2015/07/usp_1116_em_for_aseptic_processing_copy.pdf

[19] Sandle, Tim. (2014). People in Cleanrooms: Understanding and Monitoring the Personnel Factor. Journal of GXP Compliance. 18. 1-5.
https://www.researchgate.net/publication/299507535_People_in_Cleanrooms_Understanding_and_Monitoring_the_Personnel_Factor

[20] In biology, a **phylum** (/ˈfaɪləm/; plural: **phyla**) is a taxonomic rank below kingdom and above class

1. **Actinobacteria** (51.8%): A group of Gram-positive[21] bacteria, such as Micrococcus, Corynebacteria and Propionibacteria, have a high guanine and cytosine content,.

2. **Firmicutes** (24.4%): Most of these bacteria have Gram-positive cell wall structure and belong to the genera Clostridia and Bacillus. Many Firmicutes produce endospores, which are resistant to desiccation and can survive extreme conditions. Firmicutes make up the largest portion of the mouse and human gut microbiome. The division Firmicutes as part of the gut flora has been shown to be involved in energy resorption, and potentially related to the development of diabetes and obesity.

3. **Proteobacteria** (16.5%): A major phylum of bacteria that includes a wide variety of pathogens such as Escherichia, Salmonella, Vibrio, Helicobacter, and many other notable genera.

4. **Bacteroidetes** (6.3%): This group is composed of three large classes of Gram-negative, nonspore-forming, anaerobic or aerobic, and rod-shaped bacteria that are widely distributed in the environment.

Examples of microbial divergence include Propionibacterium and Staphylococci species, which dominate the sebaceous areas with a high oil content. On dry, calloused areas such as the arms and legs, Gram-positive cocci (primarily the Micrococcaceae) are found. Gram-positive rods are found in high numbers on the torso. Staphylococci and Corynebacterium, along with some Gram-negative bacteria, are found in moist areas.

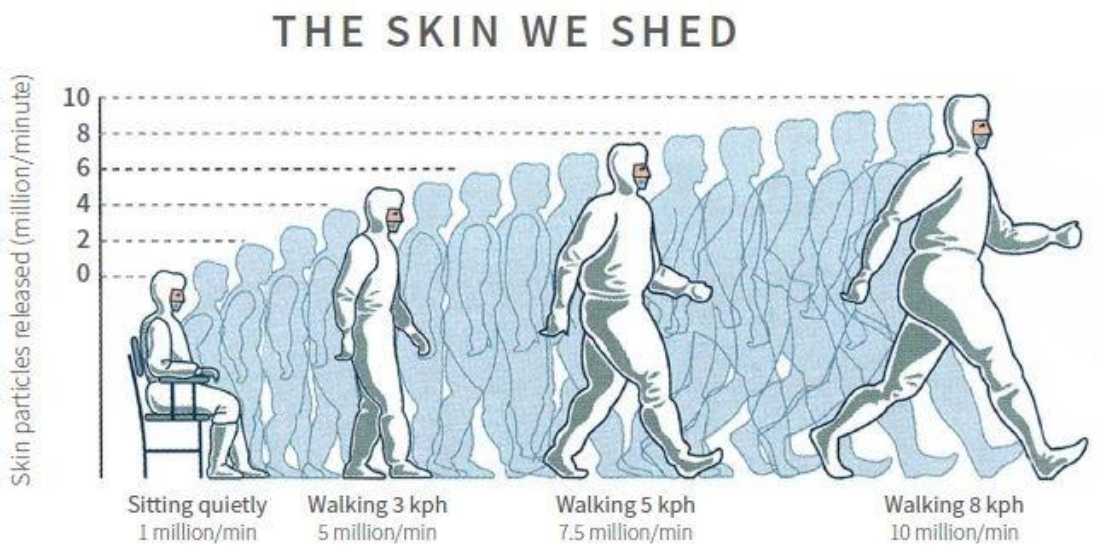
These types of microorganisms generally correspond to those recovered from cleanrooms**.**[22]

According to the Parenteral Drug Association, "*In the microbial contamination of pharmaceutical products, human skin is an important source of contaminants*." It continues, "*It has been suggested that up to 80% of the species associated with the skin are recoverable by culture method, which is much higher than other sites like the gut*." [23]

Up to 80% of the species associated with the skin are recoverable by culture method

Microbial Control and Identification Strategies, PDA: 2018

[21] **Gram-positive bacteria** are bacteria that give a positive result in a Gram stain test, while **Gram-negative bacteria** are a group of bacteria that do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation.
[22] Grice, E.A., Kong, H.H., Conlan, S. et al (2009). Topographical and Temporal Diversity of the Human Skin Microbiome, *Science*, 324: 1190 – 1192
[23] PDA's "Microbial Control and Identification Strategies," 2018, page 15



Another aspect of best practice is instructing personnel in the appropriate behaviors within the cleanroom. The generation of contamination is proportional to the activity conducted. Per the image above, a person with a head, arms, and body moving can generate about 1 million particles ≥ 0.5 µm/min. A person who is walking can generate about 5 million particles ≥ 0.5 µm/min. However, a person in a motionless position can generate only 100,000 particles ≥ 0.5 µm/min. In addition, personnel should reduce activities like talking, singing, whistling, coughing, sneezing, etc., especially when being close to the handled products and production equipment.

As previously discussed, a term commonly used to describe skin flakes and spittle with adhered microorganisms is **"MICROBE CARRYING PARTICLES"** or MCPs. "The only significant cause of biocontamination in a pharmaceutical clean area is its personnel." [24]

Research suggests that a typical person sheds 1 billion skin cells per day of a size 33µm x 44µm x 4µm, which is equivalent to a rate of 30,000 to 40,000 every minute. Of these skin cells, there are 4 microorganisms per skin cell on average. This research (Whyte) indicates that 10% of "*all particles*" (not just skin cells) carry microorganisms.[25] These facts are significant as they suggest people are not only the source of biological and foreign particulate matter contamination but also an agent for cross-contamination to locations that could pose a product risk. When MCPs gravitate towards a product or critical area, they may present a significant risk.[26]

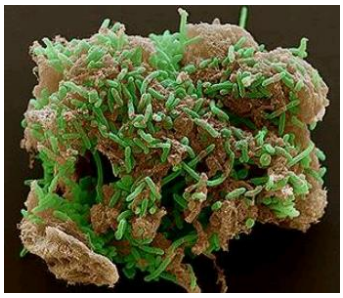
[24] USP <1116>

[25] Whyte, W. (1981) Setting and impaction of particles into containers in manufacturing pharmacies, *J. Paren. Sci. Technol*., 36: 255-268

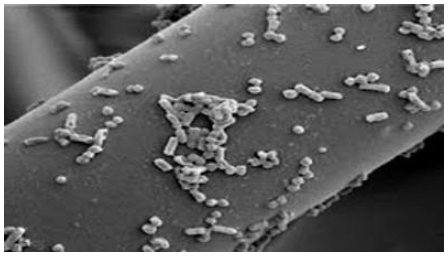
[26] Tim Sandle, R Vijakumar (2014) Cleanroom Microbiology DHI/PDA ISBN:1933722843.

People working in cleanrooms and other forms of controlled environments must be physically healthy. Diseases in the upper respiratory tract, as well as stomach disorders, can create problems in hygienic applications.

Another factor that can impact the environment is the number of people in the cleanroom. Only a necessary and limited number of persons should be allowed in a cleanroom at the same time. The more persons simultaneously present in a cleanroom, the higher the contamination level will be, i.e., the higher concentration of particles in the air). This is particularly important in relation to changing rooms.



Microbe Carrying Particle



Bacteria being transported on a lint particle

During a sneeze, millions of tiny droplets of water and mucus are expelled at about 200 miles per hour (100 m/s). The droplets initially are about 10-100 microns in diameter, but they dry rapidly **to** droplet nuclei of 1-4 microns, containing virus particles or bacteria. This is a major means of transmission of several diseases in humans.

CHARACTERISTICS OF AIRBORNE PARTICLES

Microorganisms tend to form in chains, clusters, and pairs.[27] Airborne microorganisms are neither single cellular, nor generally free-floating.[28] To become airborne, viable microorganisms must attach themselves to a nonviable particle. These Microbe-Carrying-Particles (MCPs) are on average 10 µm to 20 µm in size. [29]

According to one study, the probability of an MCP in a cleanroom given a particular size is a follows: [30]

Equivalent particle diameter (µm)	>1	>4	>12	>20	>50
Probability of occurrence	99%	75%	50%	25%	5%

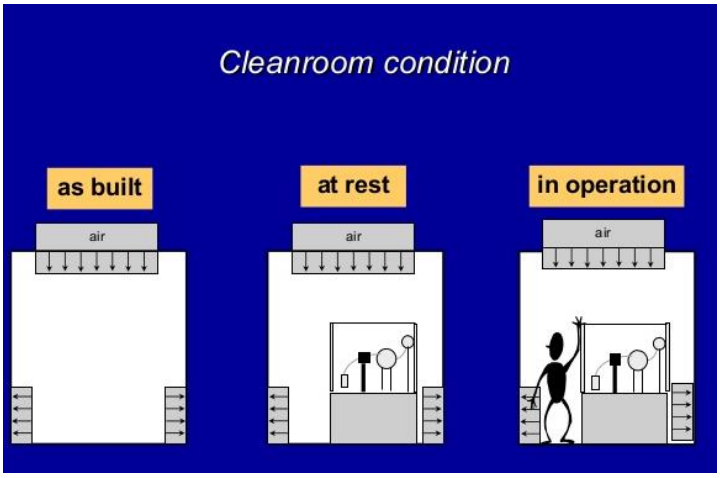
This reinforces the importance of monitoring nonviable particle in the > 5 µm particle channel for regulated life science applications.

[27] USP <1116>, Microbial Control and Monitoring of Aseptic Processing Environments. August 1, 2013
[28] USP <1116>, Microbial Control and Monitoring of Aseptic Processing Environments. August 1, 2013
[29] USP <1116>, Microbial Control and Monitoring of Aseptic Processing Environments. August 1, 2013
[30] Whyte, Green and Albisu. *Collection Efficiency and Design of Microbial Air Samplers*. Department of Mechanical Engineering, University of Glasgow, Scotland. 20 May 2014.

CLEANROOM STATES

The **qualification** study should include sufficient replicates under conditions both "at rest" and "dynamic" (aka "in operation") to allow identification of sites that provide useful information.

This differs from **routine monitoring**, as all standards require samples to be taken "in operation." (per PCI/S, EU GMP, FDA 2004)



It should be clarified that the term "useful information" does not refer to "those sites that yield the most desirable counts." Instead, it refers to those sites that either provide the highest counts (i.e., serve as the most sensitive measure of the state of control of the room) or were shown to be appropriately placed to herald a problem in the room. The number of sites in a room or zone should similarly be driven by data generated during this study.

Both the number and location of routine monitoring sites for each clean room or zone should be justified in the risk analysis report from this qualification study.

It is important to differentiate here between validation and routine monitoring. When validating a cleanroom, we are interested in validating the entire space. However, when performing routine monitoring, we are primarily concerned with the air around the process.

The following section (X.1.A) from the FDA guidance is relevant for consideration here:

> "*All environmental monitoring locations should be described in SOPs with sufficient detail to allow for reproducible sampling of a given location surveyed. Written SOPs should also address elements such as (1) frequency of sampling, (2) when the samples are taken (i.e., during or at the conclusion of operations), (3) duration of sampling, (4) sample size (e.g., surface area, air volume), (5) specific sampling equipment and techniques, (6) alert and action levels, and (7) appropriate response to deviations from alert or action levels*."

Per EU GMP, Annex 1: 2022, §4.31, Qualification should include both “at rest” and “in operation” states.

Per EU GMP, Annex 1:2022, §9.5, “*Routine monitoring of cleanrooms, clean air equipment and personnel should be performed in operation throughout all critical stages of processing, including equipment set-up*.”

Additionally, per EU GMP, Annex 1:2022, §9.23, “*Viable particle monitoring should also be performed within the cleanrooms when normal manufacturing operations are not occurring (e.g. post disinfection, prior to start of manufacturing, on completion of the batch and after a shutdown period), and in associated rooms that have not been used, in order to detect potential incidents of contamination which may affect the controls within the cleanrooms*.”

STANDARDS FOR VIABLE MONITORING

While particle counters have normative class limits mentioned in ISO 14644 and EU GMP, Annex 1; the standards for microbial samplers largely have only '*recommended*' class limits.

Regarding those standards that provide for maximum limits, for *qualification* purposes, EU GMP, Annex 1, §4.31 (2022) provides the following maximum permissible limits:

Table 2: Maximum permitted microbial contamination level during qualification

Grade	Air sample CFU/m^3	Settle plates (diameter 90 mm) CFU/4 hours [a]	Contact plates (diameter 55 mm) CFU/plate
A	No growth		
B	10	5	5
C	100	50	25
D	200	100	50

EU GMP, Annex 1, §9.30 (2022) also provides maximum action limits for *viable monitoring*, which mirror the above qualification limits:

Table 6: Maximum action limits for viable particle contamination

Grade	Air sample CFU /m^3	Settle plates (diam. 90 mm) CFU /4 hours[a]	Contact plates (diam. 55mm), CFU / plate[b]	Glove print, Including 5 fingers on both hands CFU / glove
A	No growth[c]			
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

Note, the PIC/S standard (August 2023) mirrors EU GMP, Annex 1.[31]

Per EU GMP, Annex 1: 2022, §4.31, Qualification should include both “at rest” and “in operation” states. Monitoring should occur in the “in operation” state. Additionally, per EU GMP, Annex 1:2022, §9.23, “Viable particle monitoring should also be performed within the cleanrooms when normal manufacturing operations are not occurring (e.g. post disinfection, prior to start of manufacturing, on completion of the batch and after a shutdown period), and in associated rooms that have not been used, in order to detect potential incidents of contamination which may affect the controls within the cleanrooms.”

[31] https://picscheme.org/docview/6608

CLEAN AREA CLASSIFICATIONS

In pharmaceutics, the grades of cleanroom areas are defined in the PIC/S (Pharmaceutical Inspection Convention / Scheme); and European Union, GMP, Annex 1.

Grade A	The local zone for high risk operations, such as the filling zone, stopper bowls, open ampoules and vials, making aseptic connections. Normally such conditions are provided by a laminar air flow workstation. Laminar air flow systems should provide a homogeneous air speed in a range of 0.36 – 0.54 m/s (as a guidance value) at the working position in open clean room applications. The maintenance of laminarity should be demonstrated and validated. Unidirectional airflow and lower velocities may be used in closed isolators and glove boxes.[32]
Grade B	For aseptic preparation and filling, this serves as the background environment for the grade A zone. [33]
Grade C & D	Clean areas are designated for carrying out less critical stages in the manufacture of sterile products such as in hallways and gowning rooms. [34]

Depending on what's being manufactured, the risk assessment may alternatively be categorized according to ISO classifications provided in ISO 14644-1.

[32] EU GMP, Annex 1
[33] EU GMP, Annex 1
[34] EU GMP, Annex 1

AGAR MEDIA & INCUBATION

What is the best collection media? This is a common question among microbiologists and often a topic of debate. According to the FDA, the microbiological culture media used in environmental monitoring should be validated. It must be capable of detecting fungi (i.e., yeasts and molds) as well as bacteria and incubated under appropriate conditions of time and temperature. [34]

The most ubiquitous propagated of agars are Tryptone Soya Agar (TSA) for aerobic bacteria and Sabouraud Dextrose Agar (SDA)/Sabouraud Maltose Agar (SMA) for Fungi (yeasts and molds). [35]

One source highlights the benefits from using **TSA with 1% glucose,** incubated at 25°C for 5 days, as an *all-purpose medium* for environmental monitoring of both bacteria and fungi (including molds and yeasts). [36] [37] The study concluded that SDA alone is not suitable as a general-purpose environmental medium and should be used to quantify molds and yeasts. However, **TSA with the inclusion of 1% glucose***,* seems to enhance fungal recovery to the same level as SDA. The use of a single medium also offers practical advantages in busy pharmaceutical applications. If there is a change in agar, validation studies should be conducted.

It is probably intuitive that whatever culture media is used, it should be validated to support the growth of whatever organisms you are trying to culture.

According to the World Health Organization, environmental monitoring samples should be incubated at a minimum of two temperatures to detect both bacteria and fungi. In practice, incubating samples at 20 °C to 25°C for 3 days to 5 days, followed by an additional 2 days to 3 days at 30 °C to 35°C, has been shown to be sufficient to detect most bacteria and fungi. The method chosen by each manufacturer should be carefully validated and standardized. Alternative methods are acceptable when high recoveries (>90%) of microorganisms of interest can be consistently demonstrated.[38]

[34] FDA, Sterile Drug Products Produced by Aseptic Processing — Current Good Manufacturing Practice , §X(B), pg. 35 (Sept 2004)
[35] European Journal of Parenteral & Pharmaceutical Sciences 2016: 21(2): 50-55. Pharmaceutical and Healthcare Sciences Society. http://www.climet.com/library/app_notes/Best_Practices_Viable/The_use_of_a_single_growth.pdf
[36] European Journal of Parenteral & Pharmaceutical Sciences 2016: 21(2): 50-55. Pharmaceutical and Healthcare Sciences Society. http://www.climet.com/library/app_notes/Best_Practices_Viable/The_use_of_a_single_growth.pdf
[37] TSA = Trypticase Soy Agar or Tryptone Soya Agar
[38] World Health Organization: Environmental Monitoring of Cleanrooms in Vaccine Manufacturing Facilities (Nov 2012), no. 62 https://nclb.dra.gov.pk/images/who%20env_monitoring_cleanrooms_final.pdf

SAMPLE SITE SELECTION

Sampling for routine environmental monitoring should begin in locations that are under the greatest control first (i.e., Grade A). Subsequently, sampling should be conducted in lesser controlled areas, specifically those areas outside the workstation but still within the room.

FDA Form 483 was issued on 2/22/2013, for not including specific sampling locations, such as BSC surfaces and air samples.

The PDA provides useful guidance: Factors to consider in selecting sites for routine surveillance are:[39]

1. At which sites would microbial contamination most likely have an adverse effect on product quality?

2. What sites would most likely demonstrate the heaviest microbial proliferation during actual production?

3. Should site selection involve a **statistical design**, e.g., following the calculations in Federal Standard 209E (now deprecated), or should site selection be made on the basis of **grid profiling** (recommended)?

   If grid profiling, routine monitoring would sample at the same sites as a non-viable particle counter, referring to ISO 14644-1: 2015, §A.4 (Also, refer to Climet's online calculator for the number of locations [40]).

   Should some sites for routine monitoring be rotated? [Note from author: *As 209e has been withdrawn in favor of ISO 14644, the answer is No*].

4. What sites would represent the most inaccessible or difficult areas to clean, sanitize, or disinfect?

5. What activities in the area contribute to the spread of contamination?

6. Would the act of sampling at a given site disturb the environment sufficiently to cause erroneous data to be collected or contaminate product?"

It is important that locations posing the most microbiological risk to the product be a key part of the program. It is especially important to monitor the microbiological quality of the critical area to determine whether or not aseptic conditions are maintained during filling and closing activities. Air and surface samples should be taken

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[39] Parenteral Drug Association, Technical Note 13: Fundamentals of an Environmental Monitoring Program (2014), §4.2, page 12.
[40] http://www.climet.com/toolbox/locations-4-validation/index.php

at the locations where significant activity or product exposure occurs during production. Critical surfaces that come in contact with the sterile product should remain sterile throughout an operation. When identifying critical sites to be sampled, consideration should be given to the points of contamination risk in a process, including factors such as difficulty of setup, length of processing time, and impact of interventions.[41]

The EU guidance document provides some site selection guidance:[42]

> "18. Where aseptic operations are performed, monitoring should be frequent using methods such as settle plates, volumetric air and surface sampling (e.g., swabs and contact plates). Sampling methods used in operation should not interfere with zone protection."

Similarly, guidance in the most recently proposed revision to USP <1116> (USP 2007) is of general interest:

> "Microbiological sampling sites are best selected when human activity during manufacturing operations are considered. Careful observation and mapping of a clean room during the qualification phase can provide information concerning the movement and positioning of personnel within these rooms. Such observation can also yield important information about the most frequently conducted manipulations and interventions.
>
> Other areas of concern relative to the introduction of contamination into clean rooms are at entry points where equipment and materials move from areas of lower classification to those of higher classification. Therefore, areas within and around doors and airlocks should be included in the monitoring scheme."

[41] FDA, cGMP, 2004, §X(A)(1).
[42] European Union, GMP, Annex 1, no. 18.

SAMPLE FREQUENCY

According to the Parenteral Drug Association: [43]

Viable Monitoring Guidance	U.S. FDA Aseptic Processing Guidance	USP <1116>	EU Annex 1, PIC/S, WHO Annex 4	Japan Aseptic Processing Guidance	JP XVI
Frequency	Class 100: Each production shift Other classes not specified	ISO 5: Each production shift ISO 7: Each operating shift ISO 8: Twice per week	Grade A: In operation, continuous monitoring required for critical operations. Frequent viable sampling. GRADE B: In operation, frequent particle monitoring is required. GRADE C/D: Monitoring on risk basis.	GRADE A/B: Each operating shift for airborne micro, surfaces, and personnel; continuous particulate monitoring GRADE C/D: Airborne micro twice per week; airborne particulate once per month; personnel not required	A: Each operating shift B: Each operating shift C/D (Potential product/container contact): Twice per week C/D (no potential product/container contact): Once per week.

According to the World Health Organization: [44]

Microorganism in-operation (dynamic) routine monitoring frequencies	
Classification	Volumetric
Grade A (Filling operations)	Once per shift (1)
Grade B (Unidirectional Air Flow)	Once per shift
Grade B	Daily
Grade C	Weekly
Grade D	Monthly

(1) The practice of air sampling at the start, middle, and end of filling operations provides better environmental monitoring and facilitates investigations related to filling batch release.

For critical areas, Climet microbial samplers can operate at 25 LPM or 1 CFM. Also, Climet conducted a Desiccation Study (Technical Note: TN 36), which demonstrated that a standard petri dish with a custom prefill of 32mL- 40mL can be used for up to 3 hours, and perhaps longer with a custom 40mL prefill.

[43] Parenteral Drug Association, Technical Note 13: Fundamentals of an Environmental Monitoring Program (2014), §3.0, Table 3.0-2, Page 9.
[44] World Health Organization: Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities, Nov. 2012.

Per EU GMP, Annex 1:2022: 9.22, "*Where aseptic operations are performed, microbial monitoring should be frequent using a combination of methods such as settle plates, volumetric air sampling, glove, gown and surface sampling (e.g. swabs and contact plates). The method of sampling used should be justified within the CCS and should be demonstrated not to have a detrimental impact on grade A and B airflow patterns. Cleanroom and equipment surfaces should be monitored at the end of an operation*."

Per EU GMP, Annex 1:2022: 9.24, "*Continuous viable air monitoring in grade A (e.g. air sampling or settle plates) should be undertaken for the full duration of critical processing, including equipment (aseptic set-up) assembly and critical processing. A similar approach should be considered for grade B cleanrooms based on the risk of impact on the aseptic processing. The monitoring should be performed in such a way that all interventions, transient events and any system deterioration would be captured and any risk caused by interventions of the monitoring operations is avoided*."

SAMPLE VOLUME & DURATION

For Grade A critical areas, the EU GMP, Annex 1, *DIS*, states:

> *9.27: Continuous viable air monitoring in the **Grade A zone** (e.g., air sampling or settle plates) should be undertaken for the full duration of critical processing, including equipment (aseptic set-up) assembly and filling operations. A similar approach should be considered for **Grade B** cleanrooms based on the risk of impact on the aseptic processing.* The monitoring should be performed in such a way that all interventions, transient events and any system deterioration would be captured and any risk caused by interventions of the monitoring operations is avoided.
>
> *9.30(a): Settle plates should be exposed for the duration of operations and changed as required after 4 hours (exposure time should be based on validation including recovery studies and it should not have any negative effect on the suitability of the media used). Individual settle plates may be exposed for less than 4 hours.*
>
> *9.30(b): any growth in a Grade A should result in an investigation.*

CLIMET RECOMMENDATIONS:

When the risk assessment calls for continuous viable monitoring in critical areas, Climet recommends a 25 LPM microbial air sampler. Referring to Climet Technical Note (TN 036), the longest time we can expect to sample the air with the 1 CFM CI-95A with commercially available pre-filled dishes is 90 minutes. That 90-minute limit applies to the dishes we purchased from our supplier, the temperature and humidity of the room at the time of exposure, and other unspecified variables we don't control. In order to be certain that enough water remains in the media to support growth in incubation, we must advise the customer to conduct a validation study in their environment. Otherwise, a safety margin of 30 minutes is recommended, reducing that 90 minutes to 1 hour. However, it is further possible to **sample for up to 3 hours, possibly 4 hours**, with a petri dish having a custom prefill of 40 mL TSA agar. If you plan to conduct extended sampling, please contact Climet's sales team to ask for a copy of our Technical Note: TN-036.

Sample volumes of ≥1 cubic meter should be taken for each measurement. In the case where this sample size results in an unreadable number of colonies, reduced volumes may be employed to monitor Class C and D areas if properly justified. [45]

Sample Volume Uncertainty

Unfortunately, there are no standards for microbial sampler flow rate to address *sample volume uncertainty*. Subsequently, pharmaceutical or biotechnology producers frequently follow that described in ISO 21501-4, Section 3.7, which states the flow rate must be within the nominal flow rate ±5%.

The uncertainty in sample volume plays a crucial role in environmental monitoring (EM) and is closely linked to both the flow rate and stability of the microbial sampler. We must keep in mind that an active air impaction sampler's collection efficiencies (physical and biological) are influenced by the flow rate and collection geometry.

It has been well established for decades that collection efficiency is partially a function of flow rate. It is therefore of paramount importance the microbial air sampler provide a stable and consistent impaction velocity to ensure both the physical collection and culturability of viable micrograms on the collection media.

Imbalanced Sampling is defined as variations in sample data, caused by variations in, for example, flow instability that causes variations in sample volume. Examples of Flow Instability include:

(i) Low flow rates have adverse effects on both physical and biological collection efficiency . A low flow rate will result in under sampling of the sample volume. Furthermore, Microbe Carrying Particles (MCPs) may remain entrained in the airstream and will (with a higher probability) either fail to impact onto the agar, or will not adequately embed into the agar.

(ii) High flow rate will result in improved physical collection efficiency; however, the increased impaction velocity will frequently result in the destruction or mechanical damage of the microbe-carrying particle, preventing it from being cultured. This will result in a significantly reduced biological collection efficiency.

[45] World Health Organization: Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities, Nov. 2012. Page 26

Automatic Flow Control

Today, no self-respecting Quality Manager in a regulated industry would permit the use of a particle counter used for non-viable monitoring into their cleanroom unless it had a validated flow rate, i.e., Automatic Flow Control. This technology automatically adjusts flow rate to ensure compliance with ISO 21501-4:2018, Section 6.6, which stipulates that the flow rate must be within ±5% of the nominal flow rate.

Yet, there seems to be a paradox among some drug manufacturers when it comes to aseptic monitoring, which many find difficult to understand. Microbial air sampling is typically performed in aseptic areas, which, to a large extent, are critical areas. We know there is a preponderance of evidence, both academic and industry, to confirm maintaining a flow rate is critical to achieving optimal physical and biological collection efficiencies. However, many drug manufactures continue to use microbial air samplers that have no validated flow rate (or Automatic Flow Control), and are inadequate for the commercial purpose intended.

EXPERIMENT

You can verify if your microbial air sampler has Automatic Flow Control by placing your hand over the sample head inlet holes while the instrument is turned ON. If the air sampler has flow control, you will hear the revolutions per minute increase within seconds in order to draw more air into the instrument.

Similarly, after a second or two, remove your hand and you should hear the revolutions of the blower decrease.

*Automatic flow control is also tied to **flow alarms**, which are included on particle counters. Next, using your hand, cover the sample head to block all air flow. The instrument should trigger a flow alarm similar to your particle counter after a few seconds.*

Ironically, when asked why they use a particular air sampler, drug manufacturers often respond that it is simply what was in use when they were hired. This answer may not be satisfactory in an audit. Indeed, all quality management systems should periodically evaluate and review their monitoring plan, and ***improvements should be implemented where appropriate***.[46]

Most air samplers available today, especially the less expensive ones, lack flow control. The ones that do possess this feature often integrate a delicate anemometer or other circuitry. If these instruments are dropped, they can fall out of calibration and result in an expensive deviation report and failure investigation.

Climet integrates an ultra-low pressure drop air flow meter in each microbial air sampler, which self-regulates the flow rate. This has also been referred to by some sources as a hot-wire anemometer and referred to by Climet as **Automatic Flow Control**. The advantage of this integrated technology is that there are no moving

[46] ISO 14644-2: 2015, 4.1.

parts that might potentially contribute to the particle burden of the cleanroom. Moreover, it is time-proven technology known to be extremely rugged and durable. A video of Climet's microbial air sampler drop testing is provided on our website.[47]

Climet's **Automatic Flow Control** ensures the flow rate of the microbial air sampler will remain within its nominal flow rate of ± 5%. The technology automatically adjusts the blower's revolutions per minute (RPM) to ensure a constant and stable flow rate is provided to ensure physical collection efficiency and biological collection efficiency are always optimized. If the flow rate is out-of-tolerance for 3 seconds, an alarm will trigger. Moreover, Climet uniquely drop tests all particle counters and air samplers to ensure they remain in calibration.

Longer Sample Times or Volumes - Problems

Does operating a microbial air sampler for a longer period of time, for example, 1.1 or 1.2 cubic meters instead of the compulsory 1 cubic meter, satisfactorily resolve sample volume uncertainty concerns?

The short answer is, not when we understand how an active air impaction sampler operates, and if we have a clear understanding of the standards.

Unfortunately, there is no standard for microbial samplers with regards to sample time, or how to address sample time uncertainty. Using the common methodology of augmenting microbial standards with particle counting standards, the sampling time for particle counters is addressed in ISO 21501-4:2018, section 6.7, which states:

> "The Maximum Permissible Error (MPE) in the duration of the sampling time shall be 0.01 (corresponding to 1 %) of the preset value"

For example, if using a 100 LPM microbial air sampler, it will take 10 minutes to complete a full cubic meter sample. According to the standard, your MPE for the sample time is *10 minutes x 101% = 10.1 minutes*, or 10 minutes and 6 seconds. Sampling for an additional 1-2 minutes grossly exceeds the maximum permissible error permitted by the standard, and could result in an unnecessary excursion that exceeds the recommended class limit.

To avoid oversampling, it is wise to adopt the application flow standards mentioned in ISO 21501-4.

Case Study

We heard a field report several years ago that another manufacturer's microbial air sampler had a flow rate

[47] https://www.climet.com/vid/CI-97-Drop-TestingV2-pc.mp4

was out of tolerance.[48] They continued to use the instrument, assuming that sampling for a longer period of time was acceptable. This assumption erroneously presumes little to no degradation in physical or biological collection efficiency. Again, academic and industry sources confirm that variations in flow rate can significantly impact both physical and biological collection efficiencies. (Stewart, et al., 1995; Yao, et al., 2006; and Whyte, et al., 2007).

[48] Climet microbial air samplers, like our particle counters, have **Automatic Flow Control**, and will not permit an instrument to be used when the flow rate is out of tolerance.

SAMPLE HEIGHT

All regulatory standards are silent in this area as it pertains to microbial air sampling.

Regulatory agencies have subsequently applied particle counter practices for viable routine monitoring. That is, it's good practice to:

1. Take the viable active air sample from **work height** and about 1 meter above the floor (recommend 1.2m), and point it in a direction that maximizes the probability of detecting particles.[49] This would typically be from a countertop or often from a cart. According to USP <1116>, the only significant source of bioburden is cleanroom personnel. Sampling at a height well above these factors will provide a false sense of comfort regarding the compliance of your cleanroom.

2. A poor practice is placing the microbial sampler on an elevated stand or tri-pod. The only logical explanation for sampling in this fashion is to perform a HEPA filter scan. In such cases, one would normally be performing an overlapping sweeping scan of the entire filter with a particle counter – not a microbial sampler. Simply, mounting a microbial sampler in an elevated position is a wasted effort. The World Health Organization states in a section pertaining to unilateral or laminar airflow zones, "[..] probes should be directed towards the area surrounding the product and not towards clean air flowing directly out of the HEPA filter." [50] Additionally, "It may not be appropriate to locate a sample probe directly under a HEPA filter in a non-unidirectional area because such a location may not be representative of the cleanroom or clean zone, and may prevent detection of contamination events in operation."[51] The same principle would equally apply to an active air microbial sampler.

There was a Form FDA 483, issued 5/3/2019, for sampling under a HEPA filter, and not at work height. This same 483 also mentioned that their SOP was deficient in establishing what specifically constitutes an 'operational height.'

[49] World Health Organization. “Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities.” no. 23, page 20.
[50] World Health Organization. “Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities.” no. 23, page 20.
[51] ISO 14644-2:2015, A.4.5, NOTE.

CALIBRATION INTERVAL

There are no standards for the calibration intervals of microbial samplers, and sources frequently refer to the risk assessment or monitoring plan.

Subsequently, biopharmaceutical research and production labs frequently follow that described in ISO 14644-1, Section A.2.2, which states:

> The particle counter shall have a valid calibration certificate with the frequency and method of calibration based upon current accepted practices as specified in ISO 21501-4.

This standard ISO 21501-4:2018, §6.9 states :

> The calibration should be conducted at an interval equal to or shorter than one year.

Generally speaking, the calibration interval should be established to ensure, with a high level of confidence, that the instrument will be in-tolerance at the interval date.

The issue of **criticality of use** is also an important consideration for regulated industries. Given the sensitive nature of aseptic areas in pharmaceutical production, many manufacturers calibrate their microbial samplers more frequently. Some drug manufacturers calibrate their microbial air samplers every six months, while other parenteral drug manufacturers (for example) calibrate their microbial samplers onsite before and after each production run.

MICROBIAL AIR SAMPLERS

For critical areas and applications, the calibration interval should be at least every six months. Otherwise, ISO 21501-4 recommends a minimum of 12 month intervals. The risk assessment often requires more frequent calibration intervals.

Climet recommendation

REMOTE SAMPLING

In cleanroom environments, airborne microorganisms typically adhere to air particles, commonly originating from human skin cells or flakes. These particles typically measure between 10 µm – 20 µm in size.[52] Subsequently, the use of isokinetic probes for *viable monitoring* is generally not recommended due to the substantial >5 µm particle loss from transport tubing.

According to the CDC, viable active air sampling in remote applications, such as a biological safety cabinet, isolator, or RABS should be taken using a remote sample head.[53]

The remote sample head would be mounted inside the enclosure, while the active air microbial sampler would be placed outside and to the side of the enclosure. [54] Autoclavable Silicon Braided tubing would be threaded through a hole in the enclosure. One end of the tubing connected to the exhaust port of the remote sample head, and the other to an adapter installed on the active air sampler. When taking a viable sample, only a covered petri dish or RODAC plate should be brought inside the enclosure, and placed into the remote sample head.

NOT USING A CLIMET AIR SAMPLER & IMPROPER METHODS

Inside a Grade C cleanroom, there is a Grade B (ISO 5) BSC. The analyst takes a plastic microbial air sampler into the BSC to conduct viable monitoring. Introducing the air sampler into a cleaner environment in this manner introduces cross-contamination that may exist on the exterior of the instrument. This also breaks the fragile air curtain, further increasing the probability of air cross-contamination. The instrument is laid on the deck of the BSC, once more increasing the risk of cross-contamination. The air sampler is turned on. The exhaust of the air sampler disrupts the interior laminar flow of the BSC, once more increasing the risk of cross-contamination. The air sampler does not have a HEPA filtered exhaust – nonviable and viable particles are subsequently reaspirated into the environment, almost certainly increasing the particle-burden of the clean area, and also potentially increasing the bioburden of the BSC if viable particles are in the interior of the air sampler. When the sample is complete, the plastic air sampler is once more removed from the BSC, breaking the air curtain for a second time and for the sixth time, increasing the risk of cross-contamination. Furthermore, the air sampler has a small scuff or scratches on the exterior plastic enclosure, and the Quality Manager has not conducted a *risk evaluation and determination study*.[55] The air sampler is known by the Quality Manager to be out of tolerance 14% of its interval

[52] USP <1116>
[53] CDC, Biosafety in Microbial and Biomedical Laboratories, 5th Edition (2009).
[54] CDC, Biosafety in Microbial and Biomedical Laboratories, 5th Edition (2009).
[55] Ref. FDA Warning Letter dated May 16, 2023 to a medicinal drug manufacturer.

calibrations, and subsequently, the Total Cost of Ownership is greater than $25,000 over a 10-year period.

The above example violates numerous CDC BMBL recommendations for BSCs, ISO 14698-1:2003, and numerous industry best practices.[56] A Warning letter was issued on 5/16/2023 for not conducting a *risk evaluation and determination study* on laboratory equipment that had scratches. Also, if we (i) know the initial purchase price, (ii) assume the cost for a simple failure investigation is $14,000 (2023 dollars), and (iii) use the law of continuous probabilities - - we can very conservatively calculate the Cost of Poor Quality (COPQ) of the air sampler over a 10 year period is with a high probability over $25,000. As of the date of this publication, Climet's interval calibration OOT% is 0.00% since product release in 2005. Indeed, the main cost driver for air samplers is not the initial purchase price, but COPQ associated with failure investigations and deviation reports. Competitor could give their equipment away for free, and Climet would still be cheaper to operating in a regulated environment.

A) Tubing Lengths

When performing remote sampling, flow restriction can occur. This happens when the tubing diameter is too small and/or the tubing length is too long. For remote monitoring using a Climet remote sample head, it is advisable to use autoclavable 3/4" ID silicon braided tubing with a maximum tubing length of 8 feet. For longer length runs, please contact an Application Engineer. Particle loss with tubing used in remote sampling is not a concern since the petri dish is situated within the remote sample head, which is positioned inside the BSC, isolator, or RABS.

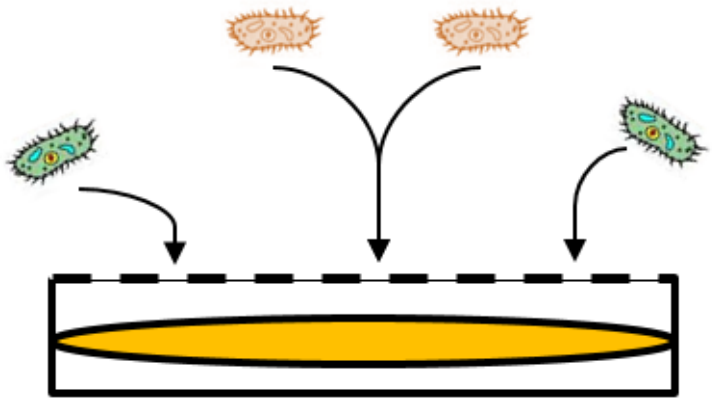
[56] Center for Disease Control (CDC). Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition, App A. [Online] https://www.cdc.gov/biosafety/publications/bmbl5/bmbl5_appendixa.pdf

ENUMERATION – FELLER CORRECTION

The Feller Correction Table is based on Probability Theory developed by William Feller in 1968, and was first conceived for microbial air samplers by Janet Macher in 1989.[57][58] Macher's Feller Correction is intended for multi-jet impactors typically with 200 to 400 holes, and not slit-to-agar sample heads.

$$P_r = N\left[\frac{1}{N} + \frac{1}{N-1} + \frac{1}{N-2} + \ldots + \frac{1}{N-r+1}\right]$$

What is a Feller Correction, and what's it for? Essentially, there is a difference between the actual CFU (colony forming unit) count, and the real number of colonies growing on the agar. When using a microbial air sampler, there is a statistical probability that one or more viable microorganisms enter the same hole in the sample head, and are impacted in the agar, forming only 1 colony forming unit, rather than 2 colony forming units.



Feller Correction

Possible to collect four microorganisms, and
only three colony forming units are enumerated

After incubation, when CFUs are enumerated, a "Feller Correction" is applied to account for this statistical anomaly.

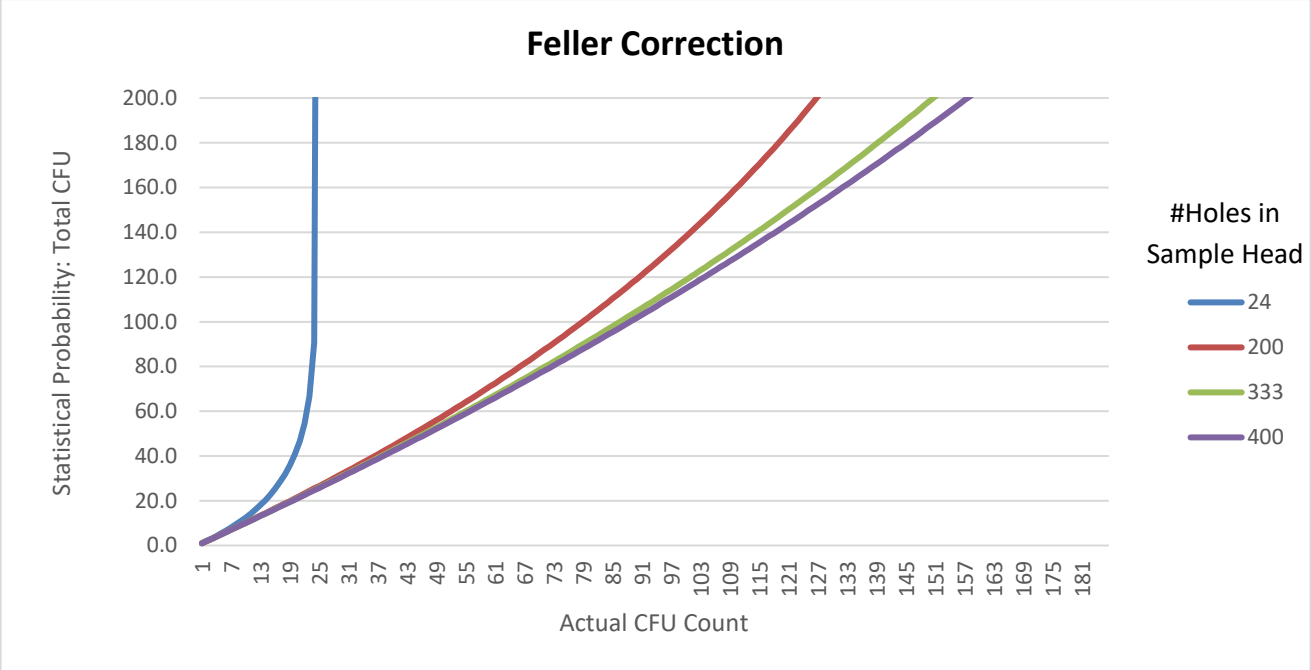
[57] http://www.climet.com/library/app_notes/Best_Practices_Viable/Feller-1968.pdf
[58] http://www.climet.com/library/app_notes/Best_Practices_Viable/Multiple_Jet_Impactors.pdf

The **main premise** behind the Feller Correction is that the greater the number of holes and the smaller the diameter of holes, the lower the probability of two microorganisms entering the same inlet of the sample head.

> ***About Climet****: The standard 100 LPM Climet microbial sample heads have 333 holes at 57.15 mil in diameter, which provide an inlet velocity of 19.5 m/s.*

So, how does it work? Per the chart below, slit to agar and other sample heads with less than 200 holes are susceptible to very high feller correction adjustments. Also, the higher the observed cfu count, the larger the applied Feller Correction.

Climet's standard sample head has 333 holes. There's no difference between a 333- and 400-hole sample head in Class A (No Growth) or Class B (limit of 10 CFUs) clean areas. As can be seen below, Class C (limit of 100 CFUs) and Class D (limit of 200 CFUs) the variance is negligible.



The above chart has an upper limit of 200 CFUs, as per EU GMP, Annex 1:2022 (Tables 2 and 6 and §9.9), the maximum permitted microbial contamination for an Air Sampler during cleanroom qualification or monitoring is 200 CFU/m^3. EU GMP, Annex 1 is mirrored by PCI/S, which is the pharmaceutical standard used in the United

States. Moreover, CFU counts above 250 are considered *Too Numerous To Count* (TNTC) because it is impossible to tell whether colonies are separated. [59]

Are there shortcomings? The Feller Correction assumes the physical and biological collection efficiencies of all microbial air samplers are identical, and that no design inefficiencies exist. We know this assumption is abjectly false (*Yao, et al*., 2006; *Whyte, et al*., 2007; *Lukaszuk, et al*., 2011; *PDA*, TN 13, §5.5.2.3, 2014).

It also assumes all viable microorganisms collected can be cultured. Again, according to studies conducted by the Parenteral Drug Association:

> *"In the microbial contamination of pharmaceutical products, human skin is an important source of contaminants." And, "It has been suggested that up to 80% of the species associated with the skin are recoverable by culture method."* [60]

In summary, no two microbial air samplers are the same, and design choices can significantly affect operational quality.

What is the Impact in a biopharmaceutical cleanrooms? The Feller Correction for most microbial air samplers will generally not start making adjustments until 18 to 21 colony-forming units are enumerated, depending on the manufacturer. Grade A and B clean areas generally have recommended CFU limits of <1 or 10 respectively. If CFU counts in these clean areas approach the Feller Correction limits, you obviously have a much larger problem than a Feller Correction. USP <1116> states that an excursion of > 15 CFU in a single ISO Class 5 sample indicates a significant loss of control that should immediately instigate a prompt, careful, and thorough investigation. Per the new BS EN 17141: 2020, Annex B, medical device application:

[59] Oregon State University. Microbiology Writing Guide: Presenting Data, https://wic.oregonstate.edu/microbiology-writing-guide-presenting-data/ , and The Microbiology Network, Inc.

[60] Parenteral Drug Association, 'Microbial Control and Identification Strategies,' 2018, page 15.

Table B.1 — Recommended limits for microbiological contamination monitoring for clean controlled environments during medical devices manufacture

Category	Air sample cfu/m³	Settle plates (diameter 90 mm) cfu/4h [a]	Contact plates (diameter 55 mm) cfu/plate	Glove print 5 fingers cfu/glove
1 [b]	< 1	< 1	< 1	< 1
2 [b]	10	5	5	5
3	100	50	25	N/A
4	200	100	50	N/A

Appropriate alert and action levels should be set for microbiological monitoring based on a risk assessment and environmental monitoring results over time. If these levels are exceeded operating procedures should specify the corrective action required.

Endotoxins and Pyrogens, while not in the scope of this standard, should be considered during risk assessment of certain medical devices such as sterilised implantable devices.

NOTE These are average values of culturable viable microorganisms.

[a] Individual settle plates may be exposed for less than 4 h, based on a risk assessment.

[b] Limits, for this category are relevant to aseptic processing of sterile medical devices like tissue products. Refer to EN ISO 13408-7 [46].

Warning

Beware of marketing hype concerning the Feller Correction.

1. Efficiency Claims: Asserting the Feller Correction has anything to do with physical or biological collection efficiency, or measurement accuracy and repeatability is a false marketing claim. If you understand the science behind a microbial air sampler, as well as variables used to calculate the Feller Correction, you quickly realize this claim is, let's say, highly inaccurate.

2. Slit-to-agar or sample heads with less than 200 holes: Macher's Feller Correction table, contrary to some marketing claims, does not apply to slit-to-agar microbial air samplers, or other impaction-type samplers with typically have less than 200 holes. The Feller Correction equation is first discussed in an article entitled "*Positive-Hole Correction of Multiple-Jet Impactors for Collecting Viable Microorganisms*." [61] The first sentence in the abstract defines "Multiple-jet impactors" as sample heads with "***… typically 200 to 400 holes."*** Quite simply, slit-to-agar and other sample heads with fewer than 200 inlets should intuitively tell you the probability of having two or more viable microorganisms enter the area is extremely high, and they are potentially subject to gross undercounting. Indeed, in Macher's article, she compared a multi-jet impactor with a slit-to-agar sample head and found that the slit-to-agar sample head routinely undercounted compared to the multi-jet impactor unless the concentration limit was over 1,400 CFU per cubic meter. This well exceeds the pharmaceutical limit of 250 CFU as *Too Numerous to Count* (TNTC).

[61] http://www.climet.com/library/app_notes/feller-correction/Multiple_Jet_Impactors.pdf

ALERT & ACTION LEVELS

Alert and action levels have been eliminated from USP <1116> with support from both regulatory and industry representatives. This may indicate a paradigm shift from alert and action levels to incident rate. However, at this time, companies are advised to monitor both parameters as official regulatory guidance from the European Union and the United States still retain GMP requirements for alert and action levels, which are different from incident rates outlined in USP <1116>. [62]

Alert and Action Level information is covered in detail, Climet Application Note 170712, which can be downloaded by CLICKING HERE.

Alternatively, you can login to Climet's Tech Library at http://www.climet.com/library/

[62] Parenteral Drug Association, Technical Note 13: Fundamentals of an Environmental Monitoring Program (2014), page 14.

PLASTIC SAMPLE HEADS

Air velocity of a microbial air sampler is calculated based on a fluid dynamics equation (i.e., d50), which takes into account the inlet geometry (i.e., number and size of holes) of the sample head. Any variation in this inlet geometry will result in sample volume uncertainty.

EXPERIMENT

If you are using plastic sample heads with a microbial air sampler that does not have a validated flow control, you can conduct a fairly easy experiment to validate sample volume uncertainty.

Equipment needed:
1x air sampler without flow control
1x air flow meter and calibration adapter head.
10x plastic sample heads
10x plastic petri dishes

Automatic Flow Control? If your air sampler does NOT have Automatic Flow Control:

1. ***Per ISO 21501-4, the flow rate of the air sampler must be within ± 5%. For example, at 100 LPM, the flow rate must be within 95–105 LPM. For 25 LPM, the measured flow rate must be within 23.75–26.25 LPM***

2. ***Using a flow meter, measure the flow rate of the air sampler using ten (or more) different plastic sample heads.***

   You can expect a variance in flow rate upwards of 5% to 8% by simply swapping out plastic sample heads. It would also not be uncommon to see the instrument fall in and out of calibration, depending on the plastic sample head installed.

3. ***Next, using the same sample head, test the flow rate with ten (or more) different petri dishes installed.***
   Once more, you can expect a variance in flow rate upwards of 3% by simply changing petri dishes.

4. ***If using a mix of 90mm petri dishes and RODAC plates, take at least 10 measurements with each and calculate the variances.***

You will be amazed, likely shocked, at the results.

Plastic sample heads are manufactured from hot liquid plastic, which is poured into a mold, and left to cool giving its final shape. Variations in the hole-size can occur during manufacture due to variations in temperature, cooling settlement times, and any number of environmental factors.

Some manufacturers offer autoclavable plastic sample heads with their instruments. Plastics have poor memory, and subsequently heating and cooling plastic sample heads more than a few times will cause variations in the dimensional size of the holes.

Another problem with plastics is ***electromagnetism***. Plastics can carry a rather large negative static charge that will attract airborne particles of all sizes, including aerosolized microbe carrying particles. Once you understand electromagnetism, you can quickly see that plastic sample heads may be biocontamination magnets that attract airborne particles that are not part of the sample, potentially resulting in false positive counts and a regulatory excursion. Electromagnetism may also cause airborne particles to adhere to the sample head, rather than remaining entrained in the airstream. As you can see, it is of paramount importance that sample heads be manufactured of static-neutral (or near static-neutral) materials such as aluminum or, preferably, stainless steel.

Climet highly recommends the use of stainless steel or aluminum sample heads.

CROSS-CONTAMINATION MITIGATION

When transporting microbial samplers between different clean zone classifications, companies must demonstrate the effectiveness of the measures taken to prevent cross-contamination. [63] [64]

A strategy to mitigate cross-contamination involves starting the day in the cleanest area, and progressively moving into an area with higher ISO classifications or GMP grade zones, which are typically dirtier.

Regarding particle counters and microbial air samplers:

A) VHP Decontamination

Both the FDA and MCA have in the past issued citations and findings against biopharmaceutical manufacturers for failure to sanitize the interior of a particle counter. Concerns cited stem from the potential of cross-contamination. [65]

In order to eliminate or mitigate cross-contamination, it is an industry best practice to periodically decontaminate microbial air samplers and particle counters in order to ensure viable growth is mitigated on the ***interior*** of the instrument, including its HEPA filtered exhaust. A best practice is to sanitize using noncondensing VHP every 1-3 months, and Climet recommends monthly while the instrument is in operation.

B) HEPA Filtered Exhaust

The FDA has in the past issued observations against biopharmaceutical manufacturers for using instruments without HEPA-filtered exhaust. Concerns cited stem from the quality of the air exhausted from the unit. [66]

In addition to infectious biohazard concerns, all laboratory equipment that creates air movement (instruments that incorporate centrifuges, fans, vacuum pumps, etc.) have internal components that create mechanical friction. When mechanical friction occurs, inert particles are generated and expelled through the exhaust. These particles on **new equipment** are generally, at a minimum, in the thousands at >5 µm and in the tens of thousands at 0.5 µm and above. **Used instruments** even a few years old can

[63] World Health Organization (WHO). "Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities." Section 3.2.3, no. 18, page 18 (2012).
[64] ISO 14698-1:2003, Annex A, section A.3.2(i)6 and sentence below it.
[65] Pharmaceutical Microbiology Form Newsletter, Volume 11(1), page 3: http://www.microbiologyforum.org/content/file/PMFNews.11.01.04.pdf
[66] Pharmaceutical Microbiology Form Newsletter, Volume 11(1), page 3 http://www.microbiologyforum.org/content/file/PMFNews.11.01.04.pdf

generate particles well into the hundreds of thousands. These particles can spread widely through production areas.[67]

Standards[68] require a HEPA-filtered exhaust for both particle counters and microbial air samplers. This is because they contribute to the bioburden (cross-contamination) and particle burden (foreign particulate matter) in a cleanroom. They produce nonviable and potentially infectious air through exhaust emissions, which are widely expelled throughout the cleanroom.

The CDC states that best practices are to insist the device's exhaust air is HEPA filtered or that the exhaust be removed from the laboratory.[69]

This is further confirmed twice in ISO 14698-1:2003:

- "The sampling plan shall take into account the cleanliness level of the risk zone and the degree of biocontamination control required for the activity being conducted, to protect individuals, the environment, the process and the product. Elements to be considered include, but is not limited to the impact of operations, personnel and equipment in risk zones which contribute to biocontamination, **such as monitoring/measuring devices**".[70]

- The exhaust air from the sampling apparatus should not contaminate the environment being sampled or be reaspirated by the sampling device. [71]

According to the World Health Organization,

> "When a process generates particles or microorganisms, it may be difficult or even impossible to demonstrate compliance with Environmental Monitoring requirements. In such cases a detailed validation study should be conducted that demonstrates that the nature of the product alone is responsible for these results. This may take the form of repetitive simulation studies (e.g., using an innocuous replacement of product such as growth media) where all Environmental Monitoring results are found to be acceptable." [72]
>
> Otherwise, it's just easier to use monitoring equipment (i.e., particle counters and microbial air samplers) that have a HEPA filtered exhaust.

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[67] World Health Organization (WHO). "Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities.", no2, page 4
[68] Center for Disease Control (CDC). "Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition", Appendix A.; and ISO 14698-1:2003(E), Section 5.3.2.4(h)(4); and ISO 14698-1:2003(E), Section A.3.2, last paragraph.
[69] Center for Disease Control (CDC). Biosafety in Microbiological and Biomedical Laboratories (BMBL) 6th Edition, Appendix A. https://www.cdc.gov/labs/bmbl.html
[70] ISO 14698-1: 2003, Section 5.3.2.4(h)(4)
[71] ISO 14698-1: 2003, Section A.3.2
[72] World Health Organization (WHO). "Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities." No. 73, page 34.

ISOKINETIC SAMPLING – NOT RECOMMENDED

Isokinetic sampling for viable routine monitoring is a very poor sampling technique subject to significant undercounting of viable microorganisms.

We know that airborne microbe-carrying-particles are on average 10-20 µm in size.[73] We also know that at 100 LPM flow rate, there is significant macro particle (>5 µm) loss from transport tubing. At 100 LPM, the 5 µm particle loss on transport tubing after 1 meter is nearly 50%. Macro particles 4 to 8 times larger than 5 µm are going to be significantly higher than this percentage, and would require a validation study.

In rare instances, remote sampling may not be practical. For example, when the risk management plan calls for a sample location in the middle of a fill station, and personnel must reach over vials to swap out a petri dish. In this case, reaching over vials to change-out a petri dish will substantially increase the risk of biocontamination, and would disqualify this method of sampling.

In these situations, we would generally recommend a settlement plate. However, when there is no other workaround, isokinetic sampling might be the only alternative.

As every configuration will be different, a validation report is required by regulators, and it should address factors affecting a risk analysis. The risk analysis should at least consider the following factors:

- The higher the flow rate, the greater amount of macro particle loss due to inter-tubular turbulence, impaction, and sedimentation forces. It is *recommended to use a 25 LPM flow rate*, which reduces tubing loss to 38% over one meter.

- Laminar flow reduces the risk of viable contamination. *If isokinetic sampling is necessary, we recommend it only be employed in laminar flow areas*.

- The longer the tubing, the higher the particle loss. It is recommended to use no more than 3 feet or 1 meter maximum tubing length. If possible, shorter lengths are preferred.

- Bends in the tubing will increase macro particle loss. Avoid 90 degree bends that go horizontally, as they create an impaction zone, especially for large macro particles. It is recommended to have no bends.

Climet recommends stainless steel sampling probes to mitigate macro particle tubing loss (see below).

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[73] USP <1116>

Climet enclosure probes for biological safety cabinets, isolators, or RABS.

DISINFECTING / SANITIZING

A) Enclosure (Exterior and Interior)

On May 16, 2023, the FDA issued a warning letter to a compounding site in Woburn, MA (USA). One of the issues mentioned was "scratched decks [work surfaces] on several ISO 5 clean hoods," for which there was no "**documented justification/risk evaluation**."

What's important here is that there is at least one FDA Auditor who's looking for damaged laboratory equipment that could potentially contribute to viable contamination risk. Climet technicians inspect enclosures and, where appropriate, will recommend a replacement. Given the current situation, Climet recommends that customers conduct at least an annual inspection of laboratory instrumentation enclosures, including but not limited to particle counters and microbial air samplers, as well as laboratory equipment work surfaces to ensure no scuffs or scratches. If any issues are identified, appropriate corrective action should be taken.

If a Climet enclosure is damaged or scratched, the stainless steel enclosure can be easily replaced. This simple repair is significantly cheaper and easier than a *documented justification and risk evaluation*.

The exterior enclosure of Climet microbial air samplers is stainless steel. Climet recommends the instrument be wiped down with an IPA spray to disinfect the ***exterior*** – while the sampler is NOT in operation. Caution should be exercised when sanitizing with bleach, as bleach will cause stainless steel to rust. If using a diluted bleach solution, it is best practice to do a second wipe-down with sterilized water to remove any bleach residue.

To disinfect the ***interior*** of a microbial air sampler, the instrument should be exposed to VHP and turned ON, which allows the instrument interior to be exposed to the gas. NOTE: Not all microbial air samplers are VHP compatible. Climet's air samplers have been leak tested.

B) Sample Heads

Ideally, the stainless steel or aluminum sample heads should be autoclaved. The CDC recommends steam sterilization as the *process of choice* because it is efficient, fast, and inexpensive. [74]

Ultrasonic energy is an effective technology routinely used by healthcare facilities to clean surgical and dental instruments prior to terminal sterilization. Alconox detergent is an excellent choice to remove any surface debris that could shield pathogens from being properly autoclaved or otherwise sterilized. Many

[74] CDC, Biosafety in Microbiological and Biomedical Laboratories (BMBL), 6th Edition (2020).

smaller life science organizations do not have an autoclave. In these scenarios, we would recommend a sonic bath using **Cidex OPA** or **Rapicide** (or similar) after the **Alconox** soaking. Soak times will vary depending on what you are trying to kill, from 1 hour to 48 hours.

Treatment with gases is also a sterilization alternative. Such gases include ethylene oxide, formaldehyde, glutaraldehyde, propylene oxide, hydrogen peroxide (VHP) and chlorine dioxide (GCD). Note, both chlorine dioxide and hydrogen peroxide are oxidizing agents and registered sterilants, which means both are capable of eliminating all viruses, bacteria, fungi and spores. Gaseous Chlorine Dioxide (GCD) has been shown effective against pinworm eggs, beta lactams, and amplicons, all of which hydrogen peroxide is unproven against. Gaseous Chlorine Dioxide (GCD) is less oxidizing than VHP, and subsequently less corrosive. [75] [76]

All Climet microbial air samplers and particle counters have been tested, and are 100% compatible with VHP and GCD.[77] Use with these gases will not void or reduce the warranty.

Comparison of major advantages and limitations of fumigation with formaldehyde, hydrogen peroxide, and chlorine dioxide.

	Formaldehyde	**Hydrogen Peroxide**	**Chlorine Dioxide**
Ability to handle soil loads	Does not penetrate soil loads, pre-cleaning required	Soil loads greatly decrease efficacy, pre-cleaning required	Soil loads do not decrease efficacy, although pre-cleaning is recommended
Carcinogen	Yes	No	No
Residue or toxic by-products	Substantial residue left behind due to neutralization, extensive clean-up required	No residues or toxic by-product	Minimal NaCl residue, may require minimal clean-up, no toxic by-products
Average concentration and exposure length*	8,000-10,000 ppm for average of 12 hours	750-1,500 ppm for 1-4 hours	360-1,800 ppm for 0.5-2 hours
Broad spectrum activity	Yes	Yes	Yes
Relative humidity required	70%-90%	VHP: 10%-40% HPV: ambient RH	70%-90%
Stable fumigant	Yes	No	No
Simple apparatus	Yes	No	No
Expensive equipment	No	Yes	Yes

*Large rooms or whole facilities may require much longer exposure times.

[75] https://orf.od.nih.gov/TechnicalResources/Documents/Technical%20Bulletins/13TB/Vaporous%20%20Gaseous%20Decontamination%20June%202013%20Bulletin_508.pdf

[76] Gordon, et al. "Gaseous Decontamination Methods in High-containment Laboratories, Public Health Agency of Canada, Applied Biosafety, vol. 17, No. 1 (2012). https://www.liebertpub.com/doi/pdf/10.1177/153567601201700107

[77] https://www.climet.com/library/app_notes/VHP/2023-Curis-Climet-VHP/Curis-VHP-Testing-2023.pdf and https://www.climet.com/library/app_notes/VHP/VHP%20Application%20Note%20190328-3.pdf

Gaseous Carbon Dioxide (GCD) may leave a minimal residue of Sodium Chloride (NaCl), commonly known as table salt. This residue may require minimal clean-up. When sanitizing a particle counter with GCD, Climet recommends capping the inlet to prevent possible residue contamination of the sensor, blower, and HEPA filter. Similarly, for microbial air samplers, it is also recommended to cap the sample head to mitigate the possible residue contamination of the blower and HEPA filter.

OBJECTIONABLE SPECIES

The practice of routing species identification, as per the "Objectionable Species" section below, is a best practice mentioned in FDA cGMP 2004. This holds true regardless of whether the action or alert levels are exceeded or not.

Per EU GMP, Annex 1:2022, §9.31*, Microorganisms detected in the grade A and grade B areas should be identified to species level and the potential impact of such microorganisms on product quality (for each batch implicated) and overall state of control should be evaluated. Consideration should also be given to the identification of microorganisms detected in grade C and D areas (for example where action limits or alert levels are exceeded) or following the isolation of organisms that may indicate a loss of control, deterioration in cleanliness or that may be difficult to control such as spore-forming microorganisms and molds and at a sufficient frequency to maintain a current understanding of the typical flora of these areas*.

Characterization of recovered microorganisms provides vital information for the environmental monitoring program. Environmental isolates often correlate with the contaminants found in a media fill or product sterility testing failure, and the overall environmental picture provides valuable information for an investigation. Monitoring critical and immediately surrounding clean areas as well as personnel should include **routine identification of microorganisms to the species** (or, where appropriate, genus) level. In some cases, environmental trending data have revealed migration of microorganisms into the aseptic processing room from either uncontrolled or lesser controlled areas. **Establishing an adequate program for differentiating microorganisms in the lesser-controlled environments, such as Class 100,000 (ISO 8), can often be instrumental in detecting such trends**. At minimum, the program should require species (or, where appropriate, genus) identification of microorganisms in these ancillary environments at frequent intervals to establish a valid, current database of contaminants present in the facility during processing (and to demonstrate that cleaning and sanitization procedures continue to be effective).

Genotypic methods have been shown to be more accurate and precise than traditional biochemical and phenotypic techniques. These methods are especially valuable for investigations into failures (e.g., sterility test; media fill contamination). However, appropriate biochemical and phenotypic methods can be used for the routine identification of isolates.

As stated in the first chapter of this document, the goal of microbiological monitoring is to reproducibly detect microorganisms for purposes of monitoring the state of environmental control. Consistent methods will yield a database that allows for sound data comparisons and interpretations. The microbiological culture media used in environmental monitoring should be validated as capable of detecting fungi (i.e., yeasts and molds) as well as bacteria and incubated at appropriate conditions of time and temperature. Total aerobic bacterial count can be obtained by incubating at 30°C to 35°C for 48 hours to 72 hours. Total combined yeast and mold count can generally be obtained by incubating at 20°C to 25°C for 5 days to 7 days. It is an industry practice to identify all CFUs enumerated to determine if it is an objectionable species. The growth of a highly pathogenic microorganism must be immediately remedied with the assistance of a competent microbiologist, infection control professional or industrial hygienist. [FDA Bad Bug Book]

STERILITY HOLD PERIOD

Sterility Testing is defined as a test that confirms products are free from the presence of viable microorganisms. This type of testing is crucial for medical devices, pharmaceuticals, preparations, tissue materials and other materials that claim to be sterile or free from viable microorganisms.

According to USP<71>, sterility testing and the validation of sterility testing, also known as Bacteriostasis and Fungistasis Testing (B&F), must be conducted. This test is necessary to demonstrate that the product is free from inhibiting factors, thereby eliminating the occurrence of false negative results in sterility testing.

This sterility hold period will vary depending on the risk assessment and product manufactured.

TREND ANALYSIS

Sample data should be uploaded into a database to allow for trend analysis. The primary objective of trend analysis is to identify deteriorating trends before they escalate into deviations, which would necessitate a substantial amount of paperwork and cost. By identifying an early trend, you can start your investigation and take early corrective actions before a problem occurs. An increase in colony-forming unit (CFU) counts may occur gradually over the course of months. Those responsible for monitoring should be familiar with the cleanroom's normal bioburden and be attentive to any emerging trends. There are Laboratory Information Management System (LIMS) solutions that large pharmaceutical companies utilize.

TESTING COMPRESSED GASES

Microbial monitoring of manufactured cleanrooms, RABS, and isolators should include compressed gases, surfaces, room or enclosure air, and any other materials and equipment that might produce a risk of contamination.[78]

Depending on the pharmaceutical producer, typically you see monitoring of gases for microbial contamination, particle contamination, and in many cases, both. This is largely dependent on the risk management report, validation studies, and product or substance being manufactured.

In either case, a **high pressure diffuser** would be required to ensure the measurement instrument (particle counter or microbial sampler) is not damaged by excessive pressure, and should always be used. [79]

[78] USP <1116> page 787
[79] https://www.climet.com/products/ci302.html

FACTORS TO CONSIDER WHEN SELECTING AN AIR SAMPLER

According to ISO 14698-1, Annex A.3.2:

> The sampling rate, duration of sampling and type of sampling device can strongly influence the viability of the microorganisms that are collected. Impingement devices may not be suitable for sampling airborne viable particles because of their low sampling volume and low rate of sampling, and their tendency to disrupt clumps of viable particles.

For this reason, the majority of pharmaceutical manufacturers opt for a Multi-Jet impaction sampler, which is characterized by having between 200 to 400 holes. Given the wide variety of microbial air sampling systems commercially available, the selection for a particular application should, *at a minimum*, consider the following factors (ISO 14698-1, Section A.3.2):

a) Type and size of viable particles to be sampled;

b) Sensitivity of the viable particles to the sampling procedure;

c) This is where the d50 is considered, in which case the experimental d50 should be less than 2 µm to comply with BS EN 17141 and ISO 14698.

d) Expected concentration of viable particles;

e) Ability to detect high or low levels of biocontamination;

> *Again, the d50 is important, and the experimental d50 should be less than 2 µm in biopharmaceutical clean areas.*

f) Appropriate culture media;

> *Use of two media for air sampling:* ***Soybean Casein Digest Medium (SCDM)*** *or* ***Trypticase Soy Agar (TSA)***[80] *are used to culture bacteria, and* ***malt extract agar*** *(or other suitable media that supports the growth of fungi) for fungi in high-risk compounding areas. (Ref. page 35; <USP 797>, page 26; and FDA 2004, page 35).* The most ubiquitous propagation of agars are

[80] Pharmaceutical Microbiology Manual (PMM): 2014, version 1.2, Chapter 9: Environmental Monitoring, Section A(2)(u), page 57. https://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM397228.pdf

tryptone soya agar (TSA) for aerobic bacteria and **Sabouraud dextrose agar (SDA)/Sabouraud maltose agar (SMA)** for Fungi (yeasts and molds).[81]

Also, refer to 'AGAR MEDIA & INCUBATION' section on page 17 of this document, which discusses the possibility of using TSA with 1% glucose as a multi-purpose agar.

g) Time and duration of sampling;

(Ref. 'SAMPLE VOLUME & DURATION,' on page 4)

h) Ambient conditions in the environment being sampled;

i) Disturbance of unidirectional airflow by the sampling apparatus;

j) Sampler properties such as:

1) Appropriate suction flow rate for low levels of viable airborne particles;

Usually 100 LPM. Grade A critical processing may wish to use a 25 LPM flow rate. Flow rate of the sampler will typically be addressed in the risk assessment or sampling plan.

2) Appropriate impact/airflow velocity;

Can be determined by experimental method (side-by-side). Otherwise, you may be able to determine appropriateness of sampler by obtaining and understanding computational and experimental d50 characteristics.

3) Collection accuracy and efficacy;

Can be determined by experimental method (side-by-side).

4) Ease of handling (weight, size) and operation (ease of use, auxiliary equipment, dependence on vacuum pumps, water, electricity, etc.);

5) Ease of cleaning and disinfection or sterilization;

[81] European Journal of Parenteral & Pharmaceutical Sciences 2016: 21(2): 50-55. Pharmaceutical and Healthcare Sciences Society. http://www.climet.com/library/app_notes/Best_Practices_Viable/The_use_of_a_single_growth.pdf

*Stainless steel enclosures are recognized by Quality Managers in the pharmaceutical industry as the simplest **to clean and sanitize**.* Stainless steel is one of the few materials that are static neutral, which mitigates contamination from airborne particles. Aluminum is a secondary alternative. However, plastics can carry a very high negative static charge, attracting particles of all sizes, including microbe-carrying particles.[82] Plastics can also serve as a nutrient source for certain bacteria, such as *Ideonella sakainesis*. Consequently, plastic enclosures increase the risk of bioburden in a cleanroom.

Plastic biodegradation occurs from bacteria, enzymes, UV Light, and exposure to moisture and harsh chemicals, which cause the plastic to crack and develop micro-fractures. In areas where bacteria and fungi are allowed to grow, or when cleaning and sanitation procedures are ineffective, continuous or even resistant environmental strains can develop. [83]

Plastics are easily scuffed and cracked. This seemingly harmless damage can create perfect hiding places for bacteria and other viable microorganisms.

Given the aforementioned, it is an industry best practice that all cleanroom laboratory equipment is constructed of stainless steel. Alternatively, aluminum is the second-best choice.

6) Possible intrinsic addition of viable particles to the biocontamination to be measured.

Air samplers with plastic enclosures, or air samplers that do not have a HEPA filtered exhaust.

Other factors:

1. HEPA Filtered Exhaust

Per ISO 14698-1:2003, Section A.3.2: The exhaust air from the sampling apparatus should not contaminate the environment being sampled or be reaspirated by the sampling device. In summary, the exhaust air must be HEPA filtered or must be removed from the clean area.

[82] USP <1116>
[83] World Health Organization. "Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities." Page 4.

According to a Pharmaceutical Microbiology Form (PFM) Newsletter, one of their members received a regulatory observation as their particle counter did not have a HEPA filtered exhaust. The auditor expressed concern over the quality of the air being exhausted from the instrument.

If you plan to use a microbial air sampler ***without a HEPA filtered exhaust***, it is highly recommended to conduct a validation study. This study should prove that the bioburden and foreign particulate matter exhausted from the instrument do not affect product quality. With a new instrument, you can expect to see thousands of particles being exhausted. On an older instrument, the number of particles exhausted could potentially reach hundreds of thousands. Additionally, any bioburden not impacted onto the collection media will remain in the airflow, and could be *reaspirated* into the clean area by the air sampler. In most cases, it is extremely difficult to justify that a healthcare product is not adversely affected by bioburden and particle burden. For these reasons, a HEPA filtered exhaust should be considered a requirement.

2. Automatic Flow Control

Avoiding deviation reports and investigations, product recalls, batch rejection, and similar actions is extremely important. Most Quality Assurance Managers would prohibit the use of a particle counter in the cleanroom if it does not have automatic flow control and regulation. Microbial air samplers, which are predominantly used in critical aseptic processing areas, should also incorporate automatic flow control. This is highly recommended to mitigate or eliminate deviation reports and failure investigations due to out-of-tolerance conditions. However, if the application is an Indoor Air Quality (IAQ) investigation of a commercial office space, hotel, or other hospitality business, automatic flow control is less critical.

3. VHP Compatible

Both particle counters and microbial air samplers should have their interiors sanitized at least quarterly, and preferably monthly while the instrument is turned ON. During sanitation, the instrument should be 'in operation' to decontaminate the interior of the device, which is ultimately the purpose of this exercise (to mitigate cross-contamination). In biopharmaceutical applications, having a HEPA filtered exhaust, which prevents or substantially mitigates cross-contamination, is an important feature. An instrument that is truly VHP compatible will not have a reduced or voided warranty, or an expectation of a limited life cycle. Beware of terms such as 'VHP resistant'. The manufacturer should have an independent test laboratory certify VHP Compatibility.

According to the Pharmaceutical Microbiology Form (PFM) Newsletter, they report that one of their members received a regulatory citation for not cleaning the interior of a particle counter. The reason for concern was cross-contamination. In addition to cross contamination concerns, it is equally important to consider the safety of the individual conducting the calibration of the instrument. It is an industry best practice to ensure all particle counters and microbial air samplers are fully VHP compatible, with VHP testing/certification performed by an independent laboratory.

4. **Programmable Delay**

When monitoring a cleanroom, it is good practice to let the air settle before sampling. Many of our customers program a delay between 30 seconds to two minutes. This gives the user a chance to step back 6 - 10 feet, letting the air settle (keep in mind, people are a source of particles). Users must also monitor the process *in operation*, and therefore evacuating an entire cleanroom to do monitoring is not recommended.

5. **Remote Sampling Capabilities (BSC, isolator, RABS)**

This is important in biopharmaceutical applications where a biological safety cabinet, isolator, or RABS are employed. The CDC advises against introducing a particle counter or microbial air sampler inside these enclosed environments. For BSCs, a fragile air curtain can be significantly disrupted when these instruments are introduced or removed from the environment, which increases the risk of biocontamination and introduces cross-contamination. Additionally, the exhaust from these instruments disrupts the laminar air flow, further increasing the risk of biocontamination. [84]

6. **Ability to sample gases** (Air, DC02, Argon, , etc.)

In biopharmaceutical applications, microbial air sampling of high pressure gases is required. It is crucial to avoid directly connecting the high pressure gases to a microbial air sampler, as the extremely high impaction velocity will kill microorganisms. Instead, it is recommended and considered best practice to use a high pressure diffuser.

7. **Data Integrity Reporting** (Per ISO 14698-1, 5.3.2.2; and 6)
    - 7.1. Location ID
    - 7.2. User ID
    - 7.3. Unit ID
    - 7.4. Date and Time (start and stop times, flow rate, and volume)
    - 7.5. Room Activity (i.e., occupancy state)
    - 7.6. Culture Medium
    - 7.7. Any alarms/deviations during sampling.

[84] *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition, CDC, HHS Publication No. (CDC) 21-1112: Dec. 2009

8. Internal Memory Buffer Size

Creating sample metadata by hand can lead to inefficiencies and inaccuracies, thereby violating ALCOA principles. The metadata outlines in Section 7 above must all be recorded by hand. This process can introduce inaccuracies during transcription and data entry into the LIMS systems. Furthermore, handwritten entries that are input into a computer must be audited to ensure compliance with Good Documentation Practices (GDP).

Climet's CI-97 microbial air sampler automatically generates required metadata and can be automatically downloaded into the LIMS at the end of each shift, thus eliminating the need for a manual audit. The benefit to the employer includes enhanced accuracy and increased labor efficiency.

9. **Barcode Scanner**

A barcode scanner is often used to scan location IDs or print labels, providing traceability of the petri dish number (issued by LIMS) to the specific sampling site/location.

Evaluating Air Samplers Collection Efficiency

When evaluating a microbial air sampler, the end-user should ask each manufacturer the following questions:

Sampler Under Test:

a. What is the theoretical d50?
b. What is the experimental d50?
c. Explain your test methodology for measuring physical collection efficiency?
d. Do you have an independent laboratory report evidencing your biological collection efficiency? Can you provide this report? What was the *average percent recovery* of the sampler under test compared to the reference sampler? The biological efficiency testing must include at least ten separate samples to comply with ISO 14698.
e. Do you have any ***internal studies***? Internal studies should be taken more skeptically than independent laboratory reports. These internal studies should support the independent laboratory conclusions, and caution should be exercised when taken on their own merit.

Reference Sampler:

f. What is the manufacturer and model of the air sampler you compared your instrument against?
g. Why did you choose this instrument to compare against your sampler? Here, we want to ensure the reference sampler is a high efficiency sampler.
h. What is the reference sampler's theoretical d50?
i. What is the reference sampler's experimental d50?

j. What articles or other documentation are available to evidence this is a high efficiency sampler?

Test for Repeatability. Basic metrology is equally interested in both: (i) accuracy of measurement, and (ii) the stability (or precision) of measurement. If the 'sampler under test' is a high efficiency sampler, and is compared against another high efficiency sampler, one would expect a very strong positive linear relationship in which both sets of data points reflect similar high or low results. This is referred to as 'stability of measurement' (or repeatability). The microbial air sampler must have an independent laboratory conduct a comparative biological efficiency test with at least ten separate samples. Next, create a table with two columns: one consisting of raw CFU (Colony Forming Unit) counts from the *sampler under test*, and the other for the *reference sampler*. Then, calculate the Correlation Coefficient (*r*) between these two data sets. Ideally, you want to see a near perfect linear relationship (***Correlation Coefficient*** (*r*) > 0.9). This is also a sanity test.

If there is *not* a strong positive linear relationship, it might suggest that the manufacturer tested their instrument against a low efficiency sampler, or it could indicate other test or performance irregularities. Most manufacturers do not conduct this test, which means you might need to calculate the Correlation Coefficient yourself.

About Climet

As a point of reference, Climet's computational d50 is 1.05 µm, and our experimental d50 is less than or equal to 0.3 µm. We conducted tests on our microbial air samplers at an independent lab, comparing them against the SKC BioSampler, which is a labor-intensive glass impingement sampler with a low flow rate of 12 LPM (83 minutes to complete a full cubic meter sample). During Climet's study, the independent lab collected ten samples.

The reason Climet tested its microbial air sampler against the SKC BioSampler is because of an article in *Aerosol Science and Technology, 32:184-196 (2000)*. This article studied a number of microbial air samplers, and identified the SKC BioSampler as having the highest physical collection efficiency on the market, along with outstanding biological collection efficiencies. Climet tested our microbial air sampler against what we believed to be the metaphorical 'gold standard.'

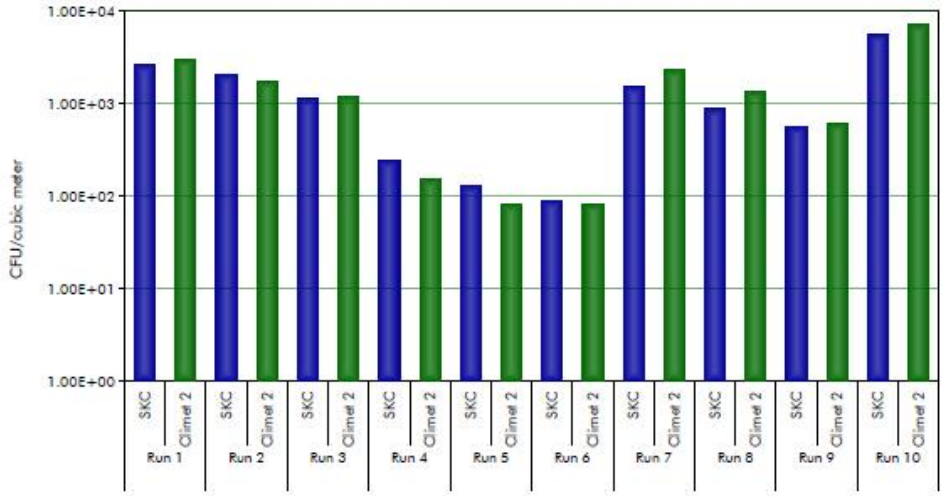
Regarding Climet's biological efficiency testing, Climet's average percent recovery was *comparable* to the SKC BioSampler (1.4% variance), and the two datasets had a correlation coefficient (*r* = 0.98). This is defined statistically as a "*near perfect linear relationship*."

Therefore, end-users can rest assured that Climet microbial air samplers are high efficiency samplers that provide both exceptional physical and biological collection efficiency. (See image below)

ANTIMICROBIAL TEST LABORATORIES

Results of the Study

The following is a graphical representation of the charts above:



Correlation Coefficient (r) = 0.98

The results of this study apply to the tested substances(s) only. Extrapolation of findings to related materials is the responsibility of the Sponsor.

PREVENTATIVE MAINTENANCE

- **Enclosure inspection**: On May 16, 2023, the FDA issued a warning letter to a compounding site in Woburn, MA (USA). One of the issues mentioned was "scratched decks [work surfaces] on several ISO 5 clean hoods," for which there was no "documented justification/risk evaluation." What's important here is that there is at least one FDA Auditor who is looking for damaged laboratory equipment that could potentially present a viable contamination risk. Climet technicians inspect enclosures and, where appropriate, will recommend a replacement. Given the current situation, Climet recommends at least an annual inspection of laboratory **instrumentation enclosures,** including but not limited to particle counters and microbial air samplers, as well as **laboratory equipment work surfaces.** This is to ensure there are no scuffs, scratches, or other external damage that potentially represents a possible viable contamination risk. Where such damage is found, appropriate corrective action should be taken.

- **O-Ring Inspection/Replacement**: Climet recommends an annual inspection of the microbial air sampler's O-Ring, which serves a critical function by ensuring a secure seal between the sample plate and sample head. This inspection should occur each time the instrument is calibrated, but should nonetheless also be performed by the user at least once a year. If there is noticeable flattening, or degradation (such as cracking), the O-Ring should be replaced immediately.

- **HEPA Filter Testing**: As discussed in this document, Climet particle counters and microbial air samplers are equipped with an internal HEPA filter. Customers may optionally consider having their Calibration Technician re-test and certify the HEPA filter to ISO Class 3 when the instrument reaches five (5) years old. Subsequent testing every 2-3 years would be more than satisfactory. Please note, this testing is optional.

- **VHP sanitation**: As discussed in this document, Climet particle counters and microbial air samplers are ***VHP compatible***, and exposure to VHP, GCD, or other oxidizing agents will not void or reduce the warranty. Climet recommends that instrumentation be exposed to VHP at least quarterly, particularly if the instrument is used in a critical area.

- **Battery**: Per the battery section of this document, Climet recommends the implementation of a **Preventative Maintenance Program.** This program should include changing batteries on all portable instruments at regular intervals, as detailed in the chart below.

  Climet counters and air samplers are equipped with a low battery alarm. This alarm is triggered when the remaining battery life is insufficient to *guarantee the completion of a sample*. As a result, this feature enables the user to replace the battery with a fresh one.

  Several microbial air sampler manufacturers require a factory service when replacing the battery, which also requires a calibration. This can result in a relatively high-cost service event exceeding over

$800. Therefore, it is highly recommended to select a high efficiency microbial air sampler that features a **user-replaceable battery**.

	Nickel-Metal Hydride Battery (NiMH)	**Lithium Ion Battery (Li-Ion)**
Climet Models	CI-x5x Series, CI-90, CI-95	CI-x70 Series, CI-97
Storage Temperature	10°C and 28°C (50°F and 82°F).	**5°C and 20°C (41°F and 68°F)**
Storage Charge Rate	Should be charged at around 80% of capacity when placed into storage	Should be charged between 40%-50% of capacity when placed into storage.
Extended Storage	Recommend checking the battery every month or two months, and if necessary, recharge to 80% of capacity. Annually, the battery should be reconditioned (see below). If a NiMH battery becomes over-discharged, for example, running the battery to 0% and then storing it, the battery will be irreversibly damaged and should be immediately replaced. In all cases, dispose of the battery if it cannot be recharged.	Do not leave batteries unused for an extended period. Will slowly discharge (self-discharge) when not in use or in storage, plus the battery is susceptible to aging (see below). Remove the battery and store it separately from the counter or sampler. Self-discharge loss is about 0.5%-3% of their charge per month. The rate increases with temperature and state of charge. Check unused batteries every 6 months (at least) for charge status and recharge the battery to between 40%-50% of capacity if needed. **HAZARD**: If a Li-Ion battery is fully discharged (or a deep discharge) and the Climet battery charge LED indicates 0%, consider the battery damaged and replace it immediately. Do NOT attempt to recharge or use the battery.
Reconditioning	Reconditioning restores a battery to its original capacity. This consists of charging and discharging a battery several times. Climet can recondition NiMH batteries or perform a capacitive test as an additional calibration service.	Climet can *recalibrate* the battery, which will frequently improve performance. Climet can recalibrate a Li-Ion battery as an additional service during the interval calibration.
Aging Loss of capacity over time, which will reduce run time.	A battery may require reconditioning if stored for prolonged periods.	Li-Ion batteries will gradually lose their capacity to hold a charge (aging), which is irreversible.
Consider Battery Replacement	When run times drop noticeably. In these cases, you probably have one or more bad cells. **Replace at 3 years (recommended) and every 5 years (required)**	Replace the battery if the run-time falls below 80% of the original run-time; or if the recharge time is taking significantly longer. **Replace at 2 years (recommended) and every 3 years (required)**

REAL TIME MICROBIAL DETECTION (RTMD) USING LIF

Climet is frequently contacted by regulated life science organizations intent on using a Real Time Microbial Detection (RTMD) system that employs Laser Induce Fluorescence (LIF) technology. After conducting less than two days of research and development on this technology, Climet abjectly disqualified RTMD/LIF as being "*not suitable for commercial purposes intended*."

According to the WHO, "Alternative methods are acceptable when high recoveries (>90%) of the microorganisms of interest can be consistently demonstrated.":[85] Per EU GMP, Annex 1:2022, §9.28, "The adoption of suitable alternative monitoring systems such as rapid methods should be considered by manufacturers in order to expedite the detection of microbiological contamination issues and to reduce the risk to product. **These rapid and automated microbial monitoring methods may be adopted *after validation* has demonstrated their equivalency or superiority to the established methods**."

There are, however, several problems with LIF technology that make validation near impossible.

Laser Induced Fluorescence (LIF) is not a new technology, and has been around since at least 1995.[86] LIF detection was originally conceived to be used on the battlefield or, for example, in an airport or subway to help detect biological attacks. In February 2009, the U.S. Army Research Lab confirmed that *bioaerosols are a mere subset of organic carbon aerosols that fluoresce.* [87]

A joint academic and industry study was conducted in 2012 by the University of Glasgow and AstraZeneca, which confirmed that viable microorganisms fluoresce, as do dead microorganisms; sterile skin flakes; and other organic carbon particles such as some Active Pharmaceutical Ingredients (APIs); particles from cleanroom garments, chemicals in biocides, pollens and chlorophyll, some minerals and compounds, IPA spray, white paper particles, dyes, and certain polymers, vitamin B2, *et cetera*.[88] The study confirmed that the false-positive rate was significant and varied between 700% and 9,400%. In fact, the University of Glasgow and AstraZeneca merely confirmed what the U.S. Army Research Lab had earlier concluded - *bioaerosols are a*

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[85] World Health Organization: Environmental Monitoring of Cleanrooms in Vaccine Manufacturing Facilities (Nov 2012), no. 62 https://www.climet.com/library/app_notes/Best_Practices_Viable/WHO_env-monitoring-cleanrooms.pdf

[86] Pinnick, et al., (Ronald G. Pinnick, Steven C. Hill , Paul Nachman , J. David Pendleton, Gilbert L. Fernandez , Michael W. Mayo & John G. Bruno). Fluorescence Particle Counter for Detecting Airborne Bacteria and Other Biological Particles, Aerosol Science and Technology, 23:4, (1995)ONLINE: https://www.climet.com/rtmd/docs/02786829508965345.pdf
653-664, DOI: 10.1080/02786829508965345

[87] U.S. Army Research Lab, Fluorescence of Bacteria, Pollens, and Naturally Occurring Airborne Particles: Excitation/Emission Spectra. ARL-TR-4722 (February 2009).
https://www.climet.com/library/app_notes/Best_Practices_Viable/ADA494347.pdf

[88] Eaton, T., Davenport, C., and Whyte, W. (2012) Airborne microbial monitoring in an operational cleanroom using an instantaneous detection system and high efficiency microbial samplers. European Journal of Parenteral and Pharmaceutical Sciences, 10 (4). pp. 339-351. ISSN 0964-4679 [Online] https://www.climet.com/rtmd/docs/Eatib-microbial-recovery.pdf

mere subset of organic carbon aerosols that fluoresce. [89] It is probably not surprising that the joint academic and industry study concluded that the use of RTMD using LIF technology in pharmaceutical clean areas is "unfeasible".[90] Moreover, although orginally considered for the battlefield to identify possible biological attacks, the seasonal variances in, for example, a common organic particle such as pollen, make the use of LIF technology impracticle.

Climet is also aware of a pharmaceutical producer who purchased a Real Time Microbial Detection system for $60,000. Immediately after installing the instrument, the Quality Manager realized that their API fluoresces. The seller refused to return and refund the instrument.

In a discussion with a former employee of a particle counter manufacturer, his prior employer purchased a company that manufactured an RTMD/LIF instrument. During validation, considerable shortcomings were discovered with this technology, particularly associated with extremely high false-positive rates. As told to Climet, due to his former employer's investment in the acquisition, the company nonetheless introduced the product to market and failed to fully disclose said technological or operational concerns and shortcomings.

Many RTMD manufacturers have in recent years taken their product off the market. For example, BioVigilant, one of the pioneers in RTMD/LIF instruments discontinued their IMD product line without advanced warning or a *last time buy* notification. Moreover, BioVigilant immediately halted calibrations of the instrument. Why they did this is uncertain. What we know today, is that several years later the company has since permanently closed. Moreover, all, or virtually all Climet competitors offered a RTMD/LIF instrument at one time. Yet again, today, all but one has discontinued their RTMD/LIF product. It is Climet's opinion, that this is due to the dissatisfaction and subsequent gross lack of demand caused by, let's say, imaginative advertising – that being, **1 florescence = 1 viable particle = 1 bio-count**, which since 2009 we've known is nonsense. A far more appropriate name for a "Real Time Microbial Detection" would be "Real Time Organic-Carbon Particle Detection."

Font-Scatter Optical Sensor: All, or virtually all RTMD/LIF instruments use a *front-scatter optical sensor*. This type of sensor is used in handheld particle counters, and are notorious for their inability to provide accurate counts, and their inability to maintain ISO 21504-4 compliant measurements due to significant short-term calibration drift. Indeed, Glasgow University and AstraZeneca confirmed significant variances in inert particle counts compared to a remote particle sensor.[91]

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[89] U.S. Army Research Lab, Fluorescence of Bacteria, Pollens, and Naturally Occurring Airborne Particles: Excitation/Emission Spectra. ARL-TR-4722 (February 2009).
https://www.climet.com/library/app_notes/Best_Practices_Viable/ADA494347.pdf

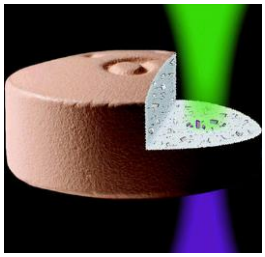
[90] Eaton, T., Davenport, C., and Whyte, W. (2012) Airborne microbial monitoring in an operational cleanroom using an instantaneous detection system and high efficiency microbial samplers. European Journal of Parenteral and Pharmaceutical Sciences, 10 (4). pp. 339-351. ISSN 0964-4679 [Online] https://www.climet.com/rtmd/docs/Eatib-microbial-recovery.pdf

[91] Eaton, T., Davenport, C., and Whyte, W. (2012) Airborne microbial monitoring in an operational cleanroom using an instantaneous detection system and high efficiency microbial samplers. European Journal of Parenteral and Pharmaceutical Sciences, 10 (4). pp. 339-351. ISSN 0964-4679 [Online] https://www.climet.com/rtmd/docs/Eatib-microbial-recovery.pdf

Tubing Loss – The system typically requires the use of an Isokinetic probe and 3/8" ID transport tubing. Climet testing has shown that 5 µm particle loss, even after 1 meter, is approximately 50%. According to USP <1116>, the average size of airborne microbe-carrying particles (MCPs) in cleanrooms is between 10 µm to 20 µm. However, as of the date of this writing, no RTMD manufacturer has conducted a 20 µm tubing loss study. As discussed in this document, isokinetic sampling is not recommended for viable air sampling due to significant tubing loss over relatively short distances.

Imaginative Marketing - The technology incorporates a particle counter (typically front scatter sensor) that uses a laser of a given frequency, i.e., Laser Induced Florescence (LIF). The instrument also includes a fluorescence detector. Manufacturers of these RTMD LIF instruments claim that when a *viable particle is struck by the laser, it will fluoresce; and* *non-viable particles do not fluoresce*. To the contrary, it has been known for decades that non-viable organic carbon particles fluoresce.[92] These particles include sterile skin cells and flakes, dead microorganisms, virtually anything chlorophyll or plant-based; fibers from cleanroom garments; some APIs; chemicals in biocides, some minerals and compounds, IPA spray, white paper particles, dyes, certain polymers, vitamin B2, *and many more*.[93] In fact, **viable microorganisms are only a subset of organic carbon-based particles that fluoresce** according to the U.S. Army Research Lab. A 2012 academic article written by the University of Glasgow and AstraZeneca confirms that RTMD/LIF use in a regulated life science cleanroom is "unfeasible".[94]

False-Positive Rates – As discussed above, viable microorganisms are only a subset of carbon-based particles that fluoresce. Climet has spoken to several drug producers who confirmed that their API (Active Pharmaceutical Ingredient) fluoresces. One study conducted the University of Glasgow and AstraZeneca confirmed that a false-positive rate could potentially range from 700% to 9,400%. (Eaton, 2012)



Poor Validation Method - Validation of RTMD technologies is described by their manufacturers to include testing in an isolation chamber with a reference air sampler used as a comparison. However, this method fails to confirm the large number of false-positives that will result from organic carbon particles that will fluoresce in a "real world" cleanroom environment.

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[92] U.S. Army Research Lab, Fluorescence of Bacteria, Pollens, and Naturally Occurring Airborne Particles: Excitation/Emission Spectra. ARL-TR-4722 (February 2009).
https://www.climet.com/library/app_notes/Best_Practices_Viable/ADA494347.pdf

[93] Eaton, T., Davenport, C., and Whyte, W. (2012) Airborne microbial monitoring in an operational cleanroom using an instantaneous detection system and high efficiency microbial samplers. European Journal of Parenteral and Pharmaceutical Sciences, 10 (4). pp. 339-351. ISSN 0964-4679 [Online] https://www.climet.com/rtmd/docs/Eatib-microbial-recovery.pdf

[94] Eaton, T., Davenport, C., and Whyte, W. (2012) Airborne microbial monitoring in an operational cleanroom using an instantaneous detection system and high efficiency microbial samplers. European Journal of Parenteral and Pharmaceutical Sciences, 10 (4). pp. 339-351. ISSN 0964-4679 [Online] https://www.climet.com/rtmd/docs/Eatib-microbial-recovery.pdf

FDA Citations & Increased Scrap – FDA citations have been issued to drug producers who have validated RTMD instrumentation and have it deployed in an isolator or RABS. Specifically, these producers were cited for not scrapping certain lots where the particle counter and air sampler showed no excursions, yet "bio-counts" have been identified by the RTMD device. Indeed, RTMD false-positives can be expected to average between 700% and 9,400%.[95] Subsequently, one can expect higher scrap due to false-positive measurements.

Unable to Identify Viable Microorganisms – RTMD counters are unable to identify microorganisms, which is a compendial requirement. EU GMP, Annex 1, §9.31 states, "*Microorganisms detected in the grade A and grade B areas should be identified to species level and the potential impact of such microorganisms on product quality (for each batch implicated) and overall state of control should be evaluated. Consideration should also be given to the identification of microorganisms detected in grade C and D areas (for example where action limits or alert levels are exceeded) or following the isolation of organisms that may indicate a loss of control*."

In summary, real time microbial detection methods using Laser Induced Fluorescence (LIF) are generally known to provide inaccurate particle counts and sizing as they use a front scatter optical sensor commonly found in handheld particle counters. Furthermore, LIF instrument bio-counts are grossly overcounted and susceptible to an extremely high false-positive rate. Indeed, the University of Glasgow and AstraZeneca confirmed ***the 5.0 µm channel on a particle counter provides a more accurate measurement of microbial contamination compared to RTMD LIF instruments employing fluorescence technologies.***[96]

It is perhaps safe to assume that any surviving RTMD LIF manufacturers will remain silent on these system limitations.

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[95] Eaton, T., Davenport, C., and Whyte, W. (2012) Airborne microbial monitoring in an operational cleanroom using an instantaneous detection system and high efficiency microbial samplers. European Journal of Parenteral and Pharmaceutical Sciences, 10 (4). pp. 339-351. ISSN 0964-4679 [Online] https://www.climet.com/rtmd/docs/Eatib-microbial-recovery.pdf

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