Microbial Control and Monitoring in Aseptic Processing Cleanrooms

Abstract

Cleanrooms and associated controlled environments provide contamination control (inert particles and microbiological entities) to levels appropriate for accomplishing contamination-sensitive activities. Products and processes that benefit from the control of contamination include those in such industries as aerospace, electronics, food and beverages, cosmetics, general healthcare and medical devices, and pharmaceutical products requiring a variety of clean environments.

Environmental Monitoring (EM), particularly in pharmaceutical manufacturing facilities where the risk of microbial contamination is controlled through aseptic processing, comprises both physical and microbiological test methods. It is a common assumption that if fewer total particulates are present in a cleanroom, it is less likely that airborne microorganisms will be present. This is true only if human operators are the main source of particulate matter in the air. However, it is not possible to clearly distinguish between the total background particulate contamination generated by mechanical operations and the total particulates contributed by personnel. Thus, it is routine for cleanroom environmental monitoring programs to consist of both a total particulate component and a microbiological component.

The data originating from these EM components provides critical information on how well a stable and suitable environment for the aseptic preparation of medicinal products is maintained.

Manufactured Medicinal Products

There are two categories of sterile products (Isaacson, 2009):

- Those that can be sterilized in a final container (terminally sterilized).
- Those that cannot be terminally sterilized and must be aseptically prepared.

Products manufactured in aseptic environments include:

- Pharmaceutical sterile products
- Bulk sterile drug substances
- Sterile intermediates
- Excipients
- Stem cells production and preservation
- Medical devices (some particular cases)

Examples of aseptically-filled sterile products are:

Non-parenteral

- Ophthalmic preparations (e.g., eye drops)
- Topical products (creams, lotions, ointments), gauze
- Irrigation solutions
- Inhalation

Parenteral

- Intrathecal, intracerebral, injections (intravenous, intramuscular, intradermal or subcutaneous)
- Hemodialysis solutions
- Filled in a variety of containers (ampoules, vials, bottles, bags, etc.)

Aseptic processing environments are the most critical in terms of patient risk. As a result, they are heavily regulated and closely inspected.

Microbiologically Controlled Environments in the Pharmaceutical Industry

Nonviable particulate and viable microbiological surveillance are used to evaluate the design and control of a cGMP-manufacturing environment. The nonviable particulate monitoring program plays an important role as it is used on a routine basis to verify the maintenance of air classifications.

In general, a comprehensive environmental monitoring program should include scheduled monitoring of:

1. Airborne viable
2. Total particulates
3. Pressure differentials
4. Direction of air flow
5. Temperature and humidity
6. Surface microbial contaminants on personnel and equipment, work tables, floors, and walls

As a goal, the environmental monitoring program should provide the following pieces of information:

- Information about the state of control in the manufacturing facility
- Data that is required to be evaluated on both a short- and long-term basis
- A detection system for microbial and total particulate ingress into a facility
- Operators behavior and training
- SOP challenge
- Indicator of HVAC, HEPA, and differential pressure issues
- Provides information on the types of organisms recovered in the facility
- Used as a method for evaluating facility change control

It should be noted that the microbial monitoring within an EM program does not provide an exact quantity and quality of the microorganisms present in the manufacturing area. Numerous studies have shown that there is a large proportion of microorganisms that are viable but unable to grow on the traditional agar media. Therefore, these microorganisms, known as viable but not culturable (VBNC) are not detected using the traditional methodology.

In addition, traditional methods are unable to sample everywhere and at every time. Instead, this methodology provides observational windows of time. Consequently, the microbial monitoring program is not a way to guarantee the sterility of a given batch by collecting counts under defined specifications, but rather it contributes to demonstrating the manufacturing process is in a continuous state of control.

Regulatory Guidance, Standards, and Risk Assessment

Significant differences in cleanroom design and EM practices exist between pharmaceutical manufacturers in different countries, and GMP inspectors often have very different interpretations of GMP requirements for cleanrooms and their monitoring. However, during the last ten years, environmental monitoring has become more complex, progressing from random sampling using a grid floor plan over the room and testing each square, to the current focus on risk assessment and the use of risk assessment tools to determine the most appropriate methods for environmental monitoring.

Two events have changed the way cleanrooms are to be designed and monitored. The first was the adoption of the ISO cleanroom definitions by the US and EU GMP organizations. A common standard helps reduce the number of divergent norms that companies serving the international market must conform to (though ISO standards like ISO 14644 and ISO 14698 do not always fit with local regulatory guidance documents because they apply to controlled environments across a range of industries other than pharmaceuticals, where standards can be higher).

The second event was the growing acceptability of a risk-based approach from the International Conference for Harmonization (ICH) Quality by Design (QbD) regulatory project (FDA, 2009). In it, risks inherent to product-specific manufacturing steps are analyzed and specific measures needed to manage or reduce those risks are determined. This approach helps to identify the risks, making it possible to assess whether these are adequately controlled at a particular point or at a later stage in the process (Whyte and Eaton, 2004).

Microbial Limits in Cleanrooms

Tables 1 and 2 show the microbial limits as described by the European Union GMPs (2008) and the USA FDA (2004) GMP guidances currently in place, respectively. These limits are only for reference and the maximum acceptance. For cleanroom monitoring, the alert and action limits should be based on statistical evaluation of a minimum of three months of collected data.

TABLE 1 EU GMPS ANNEX 1 2008

GRADE	AIR SAMPLE cfu/m^3	SETTLE PLATES D = 90 mm cfu/4 hr	CONTACT PLATES D = 55 mm cfu/plate	GLOVE PRINT 5 fingers cfu/glove
A	< 1	< 1	< 1	< 1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

TABLE 2 FDA GUIDELINES 2004			
CLEAN AREA CLASSIFICATION 0.5 µm particles/ft³	**ISO DESIGNATION**	**MICROBIOLOGICAL ACTIVE AIR ACTION LEVELS** cfu/m³	**MICROBIOLOGICAL SETTLING PLATE ACTION LEVEL** cfu/4 hr (D = 90 mm)
A	< 1	< 1	< 1
B	10	5	5
C	100	50	25
D	200	100	50

In order to achieve compliance to regulatory guidance, a microbial monitoring program will include the following components:

- Active air (room or enclosure environment)
- Passive air (settle plates)
- Surfaces (contact plates and swabs)
- Personnel (gloves and garments)
- Compressed gases
- Materials and equipment that could compromise product microbiological quality
- Cleaning and sanitization process understanding

As discussed previously, a microbial monitoring program cannot provide an accurate detection and quantification value of all the microbial contaminants in controlled environments because of the limitations and variability in sampling methods. Samples usually represent a very narrow window of time. Therefore, as discussed in Chapter <1116> of the United States Pharmacopoeia (USP, 2013) both the lack of accuracy and precision of the traditional enumeration methods and the restricted sample volumes that can be effectively analyzed suggest that environmental monitoring is incapable of providing direct quantitative information about sterility assurance. Moreover, no microbiological sampling plan can prove the absence of microbial contamination, even when no viable contamination is recovered.

Contamination Trends and the Case for Contamination Control Rates (CRR)

In order to analyze contamination trends, take into consideration the following issues:

1. The low level of isolated incidents in highly controlled environments
2. The high variability in CFU recovered counts
3. The limited accuracy and precision of microbial counts

Chapter <1116> of the USP recommends that in order to evaluate microbial contamination incidents and the state of control of the manufacturing process, it is better to use the frequency with which contamination is detected, rather than the absolute numbers of CFU detected in any single sample. Table 3 (copied from <1116> of the USP, 2013) summarizes this concept.

TABLE 3 SUGGESTED INITIAL CONTAMINATION RECOVERY RATES IN ASEPTIC ENVIRONMENTS[a]

ROOM CLASSIFICATION	ACTIVE AIR SAMPLE %	SETTLE PLATE D = 90 mm 4 hr exposure %	CONTACT PLATE OR SWAB %	GLOVE OR GARMENT %
Isolator/Closed RABS (ISO 5 or better)	< 0.1	< 0.1	< 0.1	< 0.1
5	< 1	< 1	< 1	< 1
6	< 3	< 3	< 3	< 3
7	< 5	< 5	< 5	< 5
8	< 10	< 10	< 10	< 10

a All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for non-sterile products or other classified environments in which fully aseptic gowns are not donned.

Based on the guidance found in the Table 3 of the USP <1116>, the following points are recommended

1. Consider frequency of contamination instead of absolute numbers (in CFUs) detected in a sample.
2. Determine recovery rates for each cleanroom environment (also per location, building, etc.).
3. Contamination recovery rates are applicable only to environments in which all operators are aseptically gowned.
4. Detection frequency should be based on actual monitoring data and retabulated monthly.

Establishment of Alert and Action Levels

Alert and action levels are initially derived from data obtained during the qualification study and are frequently used to set the initial operating alert and action levels for the routine environmental monitoring program. A good rule of thumb is that the alert level should be at the 95th percentile of observed readings for a given period of time (typically 6 months of data), the action level at the 99th percentile (see the PDA Technical Report #13 and Technical Report #59) for an excellent discussion of setting alert and action levels). While common industry practice is to uncritically accept regulatory recommendations for predefined clean zones, this practice is discouraged in the US (FDA, 2004) and EU (EU GMPs and PICs). There is controversy over the regulatory guidance for highly controlled areas due to concern with control levels set so far below the level of quantification for plate count assays (generally 25 - 30 CFU per plate, compared with regulatory guidance setting alert and action levels as low as single digits). This concern led USP to suggest a frequency distribution approach for these areas (USP, 2013). An interesting discussion of this approach can be found in Caputo and Huffman (2004).

Whichever approach is chosen to the determination of the initial alert and action levels, it should be one of the deliverables from the EM qualification program (Dalmaso, 2012).

Sampling Airborne Microorganisms

Listed below (Table 4) are the most common devices and instruments used to collect samples of airborne microorganisms from cleanroom environments.

TABLE 4 TYPES OF PASSIVE AND ACTIVE MONITORING PRACTICES

Passive Monitoring

- Settling plates

Active Monitoring

- Slit-to-Agar (STA) Air Sampler (Air through narrow slit, rotational agar plate)
- Sieve Impactors (Air through a perforated plate or plates)
- Single-Stage (Contact plates or Petri dishes)
- Multi-Stage Cascade (Stacked perforated plates)
- Sterilizable Atrium (Stainless Steel head collection device)
- Single-Use Sterile Atrium (Disposable)
- Centrifugal Propeller Sampler (Agar coated strip)
- Filtration (Polycarbonate, cellulose acetate, gelatin filters)
- Impinger (Use of liquid medium for particle collection)
- Real Time Laser-Induced Fluorescence Systems

Low shear-force liquid impingers are used for the recovery of stressed organisms and therefore they are claimed to show the best recovery over a wide range of airborne microorganisms. Yet, using an impinger sampler is less portable, more time consuming and requires a greater degree of laboratory sophistication, and therefore is not the first choice for use in routine sampling. Sieve samplers provide particle size distribution of the bioaerosol, whereas slit-to-agar samplers are used to determine the airborne bioburden as a function of time and activity without regard to particle size. Settling plates are suitable to locate point sources of emissions, where larger particles are generated. They rely on gravity and particle dynamics to provide a gradient of contamination, whereas centrifugal samplers and certain sieve samplers provide an easy and rapid means to take numerous samples of the airborne bioburden where viable particle size and temporal considerations are unimportant.

Examples of Instrumentation for Active Air Microbial Monitoring

Based on the list of instrumentation and methods for air microbial monitoring summarized in **Table 4**, this section describes some examples of commonly used active air sampling devices used in the pharmaceutical industry for microbial monitoring (for a full description of these instruments see reference 9).

1. **Slit-to-Agar (STA) Air Sampler:** Air flows through a narrow slit, the agar plate (150 mm) has a rotational base that rotates at a fixed speed. The operator is able to pin-point when the contamination event took place. The airflow is 28.3 liters per minute (LPM). The exhaust air is HEPA filtered to prevent dispersion of any contamination. There is a version for compressed gases.
2. **Single-Stage Sieve Impactors** (Air through a perforated plate(s))
    a. **Single-Stage (contact plates or Petri dishes):** Air is drawn through slits in the sampling head using an internal vacuum pump. The microorganisms are impacted on the agar surface (100 mm) in the pattern designed on the sampling head. Air flow is 25, 50 and 100 LPM. The exhaust air is HEPA filtered. Portable and remote devices, kits for compressed gases and isolator, and connectors for remote use with stainless steel and single use atriums are commercially available.

MiniCapt® Remote (left) and MiniCapt Mobile (right)

b **Sterilizable Atrium (Stainless Steel head collection device):** These impactors are composed of a head with slits (20 slits for the BioCapt), a base with adjustable pins, and connection to a vacuum pump.

BioCapt® Stainless Steel impactors (two designs)

c **Single-Use Sterile Atrium (Disposable polystyrene device combination of impactor and agar plate):**
The incorporated agar plate cannot be accidentally touched by the operator, reducing the risk of contamination by improper handling (false positives)

BioCapt Single-Use

3 **Real Time Laser-Induced Fluorescence Systems:** This type of device continuously monitors viable microorganisms in real time. Extremely sensitive, the limit of detection is down to 1 microbial cell. It provides both total particulate and viable counts.

Sampling Microorganisms on Surfaces

It is important to perform regular sampling of surfaces within the aseptic processing environment, including equipment, walls, floors and counter tops.

Contact Plates

Flat surfaces can be sampled using contact plates. These specialized agar plates are manufactured precisely to ensure a smooth, evenly distributed layer of agar on each plate. Sampling is achieved by gently rolling the domed surface of the agar onto the test area. The plate is then incubated under appropriate conditions to obtain colony counts. Contact plates are usually available for TSA and SDA media, with or without neutralizers (e.g., Lecithin and Polysorbate 80) added to inactivate residual disinfectants or cleaning agents that may be on the test surface. In addition, they can be supplied irradiated and triple-wrapped for use in designated clean areas.

Swabs

In an environmental monitoring program, it is important to include the sampling of surfaces that are not flat or are difficult to access, as these areas may be more difficult to clean and disinfect. Swabs are preferred for this purpose. Early commercially available swabs (cotton or rayon materials) presented a lower microbial recovery (30-50%). However, with the advent of nylon flocked swabs, recoveries of 80% or better can be obtained on a routine basis. Thanks to this development, swabbing is becoming one of the most widely used methods for microbiological examination of surfaces.

Nylon® flocked swabs comprise of a solid molded plastic applicator shaft with a tip that can vary in size and shape. The tip of the applicator is coated with short nylon fibers that are arranged in a perpendicular fashion. This perpendicular arrangement results from a process called flocking, where the fibers are sprayed onto the tip of the swab, while it is held in an electrostatic field. This process creates a highly absorbent thin layer with an open structure. Unlike traditional fiber wound swabs, which resemble a mattress or cushion, Flocked swabs have no internal absorbent core to disperse and entrap the specimen– the entire sample stays close to the surface for fast and complete elution. The perpendicular nylon fibers act like a soft brush and allow improved collection of cell samples. Capillary action between the fiber strands facilitates strong hydraulic uptake of liquid sample, and the sample stays close to the surface further promoting elution.

A typical swabbing kit includes two tubes: one screw cap tube with an attached sterile swab and filled with a small volume of saline solution for moistening the tip of the swab, and a second tube containing a nutrient broth to use as a rinse solution, as a diluent or as recovery medium.

The area to be tested is swabbed, the microorganisms are recovered in a rinse solution and then filtered through a sterile membrane filter. Next, the membrane is placed onto defined agar media. After incubation under the required conditions, a Total Viable Count (TVC) can be deduced and colonies can be identified as necessary. If preferred, instead of the sterile membrane filtration, a dilution series and plate count could be alternatively performed.

Personnel Sampling

Periodic sampling of clothing (gowns and gloves) is used to measure the effectiveness of aseptic precautions. Gloves can be sampled (prior to removing or replacement) by touching all fingers and thumbs onto the surface of an agar plate. Other garments can be sampled using contact plates or swabs.

Conclusions

1. A monitoring program should be able to detect a change from the validated state of control in a facility and to provide information for implementing appropriate countermeasures.
2. Environmental monitoring sampling plans should be flexible with respect to monitoring frequencies, and sample plan location should be adjusted on the basis of the observed rate of contamination and ongoing risk analysis.
3. Oversampling can be as deleterious to contamination control as under-sampling, and careful consideration of risk and reduction of contamination sources can guide the sampling intensity.
4. Studies conclusively show that operators, even when carefully and correctly gowned, continuously slough microorganisms into the environment.
5. In general, fewer personnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from microbial contamination.
6. Periodic excursions are a fact of life in human-scale cleanrooms, but the contamination recovery rate, particularly in ISO 5 environments used for aseptic processing, should be consistently low.

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Gilberto Dalmaso has over 25 years' experience in pharmaceutical microbiology and sterility assurance. His current work is focused on pharmaceutical microbiology and aseptic processes, microbiological contamination control and rapid microbiological methods, Quality by Design (QbD), and PAT in microbiology.

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