Chapter 8

Microbiological Control

Objectives

This chapter provides an overview of microbiological control in the biomanufacturing industry. After completing this chapter the student will be able to:

- Explain why microbiological control is important in a biomanufacturing facility and provide a number of examples as to how it is achieved and maintained.

- Describe the various sources of microbial contamination within a biomanufacturing facility/process and name specific microbial contaminants and their possible sources.

- Explain the different microbiological cleanliness standards required for the manufacture of biopharmaceutical drug substances and drug products.

- Define aseptic processing and provide examples of aseptic processing practices.

- Identify measures taken in controlled and classified environments within cleanrooms to prevent microbial contamination.

- Describe the components of an effective environmental monitoring program along with specific environmental monitoring testing methods.

- Explain the importance of information derived from environmental monitoring and describe how this information is utilized in investigations.

- List the quality control practices that are essential in the Microbiology QC Laboratory.

Terms

Action level: an established microbial or airborne particulate level that, when exceeded, should trigger appropriate investigation and corrective action based on that investigation

Alert level: an established microbial or airborne particle level giving early warning of potential drift from normal operating conditions and triggering appropriate scrutiny and follow-up to address the potential problem. Alert levels are always lower than action levels.

Aseptic: the absence of pathogenic (disease-causing) microorganisms

Aseptic processing: biomanufacturing methods for those axenic products that cannot be subjected to terminal sterilization. Typically utilized for those products that are heat-labile (products that are damaged by heat-sterilization methods)

Aseptic techniques: techniques that prevent contamination by unwanted microorganisms. Used not only in biomanufacturing methods but also with medical procedures

Cleanroom: a room or interconnected rooms maintained and controlled to prevent particle and microbiological contamination of drug products. Cleanrooms are assigned and reproducibly meet an appropriate air cleanliness classification.

Contamination: the presence of any unwanted substance that may affect the purity, safety, identity, or strength of a drug product

Disinfection: the elimination of most recognized disease-causing or harmful microorganisms but not necessarily all microbial forms. It is a less lethal process than sterilization

Pyrogen: A substance which causes fever when present in the blood of an organism.

Sterile: the complete absence of viable (living) microorganisms

Sanitization: the general reduction of the number of microorganisms on a surface

Sterilization: the act or process, either physical or chemical, that destroys, inactivates, or eliminates all forms of life, including bacterial endospores (the most resistant form of microorganism)

Terminal sterilization: the application of a lethal agent to sealed, finished drug products for the purpose of achieving sterility

Foundations of Microbiology

Microorganisms are ubiquitous. They are in the air we breathe, food we eat, water we drink, and surfaces we touch. They range from simple nucleic acid-free entities, termed prions (first recognized approximately 20 years ago), to complex eukaryotic cells such as yeast that have been known since Leeuwenhoek invented the microscope in the 15th Century.

Microbes are only a problem when their presence results in unwanted effects, such as causing infections or contaminating drug products or intermediates. Controlling microbes when manufacturing products that use organic materials is a demanding challenge that has existed for centuries.

In the 19th century French scientist Louis Pasteur helped found the science of microbiology, when he studied the causes behind the spoilage of wine. Pasteur examined properly aged wine under a microscope and noticed the presence of yeast cells. He then discovered that soured wine contained bacterial cells that were producing lactic acid. His findings led him to recommend that vintners (wine producers) heat the wine, which would kill the lactic-acid-producing bacteria. His discovery helped save the French wine industry, which was crucial to the country's economy. This led directly to the heat-treating process named for him, pasteurization, which is used on food products (e.g., dairy products) worldwide today.

There are many other similar instances in which food is adversely affected by inadequately controlled microbes. Many methods traditionally used to preserve foods fundamentally rely on creating an environment that is inhospitable to or kills microbes, such as smoking meats or pasteurization.

As with food production, microbiological control is a key issue in pharmaceutical manufacturing. This is particularly true in the case of biopharmaceuticals and bioprocessing, which use various organic materials to create a range of products. Microbiological control is vital for two main reasons:

1. The majority of biopharmaceutical medicines are designed for parenteral administration (i.e. they are administered to the patient by injections of various types) and must be sterile and free of significant amounts of pyrogens such as endotoxin to prevent infections in the recipient patients

2. Biopharmaceutical drug substances are generally large, complex proteins that are susceptible to degradation, mediated by enzymes produced by contaminating microbes.

This chapter will describe microbial contaminants, how they might enter into the production cycle, and their impacts. It will also cover the controls that are established to prevent microbial contamination of products.

Bacteria, Fungi, and Mycoplasma

Bacteria and fungi are a heterogeneous group of organisms that include both simple non-nucleated prokaryotic cell types (bacteria) and nucleated eukaryotic cells (fungi, including yeasts and molds). All of these are ubiquitous, as they are present in air, water, soil, and even in other living organisms, including humans. In fact our own body cells are outnumbered by the bacteria that we harbor by a factor of a hundredfold!

Life as we know it depends on the activities of bacteria. They help certain animals digest food and convert it to energy. They help produce oxygen by working in symbiotic relationships with plants. They break down dead plants and animals which contributes to the cycle of life. They even help in making foods like bread, yogurt, and cheese, and beverages like wine and beer.

The numbers of bacteria and fungi are almost incomprehensible. It has been estimated that there are 4–6 X 10^{30} total prokaryotic cells on Earth and that every person has approximately 3 X10^8 prokaryotic cells on their skin and 7 X 10^{13} cells in their intestines.

This group of microbes can impact biopharmaceutical processing in various ways and at different levels, from the cell culture to the final dosage form. With cell cultures, the process objective is to maintain an axenic or monoseptic-type culture. This means that only the engineered production cells of interest are present and that extraneous contaminating microbes are excluded. This requires the stringent control of operating conditions and equipment. Despite these controls, however, batches of product are still lost due to contamination. This remains an ongoing problem in the industry. Typically, when a mammalian cell culture becomes contaminated by bacteria or other cells, the contaminating microbes can overgrow the production of the mammalian cells since the former can grow much more quickly than the mammalian cells. This results in the microbes out-competing the mammalian cells for nutrients. This growth is readily apparent through atypical batch parameters like visual appearance (turbidity), pH, and DO (Dissolved Oxygen). Microbial contamination of a microbial cell reactor is more difficult to detect, but no less of an issue.

A common type of contamination at the cell culture level is caused by Mycoplasma, the smallest self-replicating prokaryote. These lack a cell wall, as well as the ability to synthesize one. These organisms depend on their host cells for cholesterol and as such exist as parasites or commensally with their hosts. They are 0.2–0.3 micron in diameter and can be observed as filamentous or coccal forms. And though over 160 species have been identified to date, approximately 90 percent of all cell culture contaminations are caused by only five *Mycoplasma* species: *M. hyorhinis*, *M. arginini*, *M. orale*, *M. fermentans,* and *Acholeplasma laidlawii*.

Mycoplasma can grow to very high concentrations in mammalian cell cultures—to levels near 10^7–10^8 organisms/ml. However, it remains unobservable by regular light microscopy, and generally requires fluorescence staining of the culture to observe. While late-stage *Mycoplasma* contamination can cause cell culture media to become acidic, there are usually no overt signs that cultures are contaminated. *Mycoplasma* can cause changes in growth characteristics, membrane antigenicity, and mammalian cell metabolism. It can also produce chromosomal aberrations, disrupt nucleic acid synthesis, alter transfection rates, and induce virus susceptibility.

Mycoplasma can either directly or indirectly contribute to human disease, representing significant safety and regulatory concerns. Therefore, testing for Mycoplasma in manufacturing cell substrates and the culture media is essential. The main sources of *Mycoplasma* contamination likely arise from the production cell line, the raw materials used in the process, the production personnel, and/or the environment. Specific methodologies are required to examine cell cultures for Mycoplasma infection as defined in the European Pharmacopoeia (Ph.Eur.), section 2.6.7. Traditional culture methods can take up to a month or more to produce results. Recently PCR based techniques have allowed for near real-time detection.

The cell culture process is considered a closed system – production cells are introduced into a closed vessel containing a nutrient medium designed to support the growth of these cells. This culture is incubated for the appropriate time, typically days (for bacteria), weeks, or even months (for mammalian cells), before the cells or the culture medium is harvested. The process of extraction and purification then begins. Typically purification is termed an open process and there are opportunities for environmental microbes to enter the process. In addition, the nature of the equipment and materials used to purify a product, such as filter membranes and chromatography resins, may make sterilization of that equipment impossible. These opportunities for contamination must be understood, monitored, and controlled. The typical microbes of concern at the purification stage are bacteria and molds as the lack of cells means there is nothing to serve as a host for viruses or *Mycoplasma* so these organisms are unable to proliferate in the equipment and systems without them. Bacteria and fungi are typically carried by people and materials and they thrive in moist environments. The concerns about bacteria and molds in open processes are:

1. The simple control of their numbers. While it can be anticipated that they may be present, it is important that the environmental conditions do not promote their proliferation where they can overwhelm the capability of downstream filtration processes designed to remove them.

2. Bacterial products or structures such as endotoxin- these can be dangerous to the patient who ultimately receives that product

Concerning the former, certain bacteria and molds secrete proteolytic enzymes into the growth medium, potentially degrading the therapeutic protein. Regarding the latter, Gram-negative bacteria release a component of their cell wall termed *endotoxin* that can be highly toxic if introduced into patients. It is one example of a **pyrogen**. Pyrogens can cause fever and other immunologic side effects. Consequently, endotoxin levels must be carefully minimized during production.

The majority of biopharmaceuticals are delivered as parenteral medicines. These deliberately bypass the body's external physical barriers against infection (e.g., skin, mucous membranes, digestive system) and, as such, must be delivered in a **sterile state** (free from any viable life forms) to the patient. Microorganisms known to cause infectious diseases in humans are hard to distinguish from those customarily thought to be benign. Once a body's external defense barriers have been breached, virtually any microorganism has the potential to proliferate and cause infection or other unwanted side effects. This is especially true in immunosuppressed or immunodeficient patients. For example, the bacterial species *Citrobacter freundii, Enterobacter*

agglomerans, Enterobacter cloacae, and *Klebsiella aerogenes* were responsible for a spate of fatalities in the 1970s from infusion fluids that were thought to be sterile. These four species, found living in commensal association with healthy humans, were thought to be no more than "opportunistic" pathogens. As recently as 2002, a 77-year old woman died from fungal meningitis after receiving a spinal injection, which subsequently tested positive for a rare fungus species present in the manufacturing plant.

These cases illustrate that achieving sterility in a final product is of vital importance. This effort is complicated, however, by the fact that most biopharmaceutical molecules cannot be subjected to the most common method of generating a sterile product—autoclaving. During the autoclaving process, a finished product in its final container is sterilized using heat. this is referred to as **terminal sterilization**. Biopharmaceuticals, however, are typically very heat-labile molecules, meaning that heat is likely to change them and alter their function. Therefore, they must be sterilized using a sterilizing filtration process applied to the bulk formulation, after which they are placed into pre-sterilized individual containers under aseptic conditions and capped with pre-sterilized closures. Though necessary for completely excluding microbes, the process of aseptic manufacture is highly complex and demanding.

Viruses

Viruses are small, obligate, intracellular infectious agents that can cause a wide variety of diseases in humans, animals, and plants. In their simplest form, viruses consist of genetic information in the form of DNA or RNA packaged within a surrounding protein coat. They can only replicate inside another organism's living cells and therefore cannot be categorized as living.

Viruses were first identified over one hundred years ago by two scientists working independently. Shortly after Pasteur's work with bacteria, the Russian scientist Dmitri Ivanovski was researching the cause of tobacco mosaic disease. A few years later, the Dutch botanist Martinus Beijerinck also studied the same plant disease. Beijerinck named the agent he discovered, which was smaller than bacteria, a virus—based on the Latin word for poison. Beijerinck is now considered the father of virology(a specialization of microbiology).

In the medical field there have been numerous cases over the years in which medicines have been contaminated by viruses—unintentionally infecting patients. The best known example was in the 1980s, when as a result of the development of the AIDS epidemic, the HIV virus that is associated with AIDS began appearing in blood products. Viral infections were devastating to many patients suffering from hemophilia—an inherited disease that impairs the body's ability to control blood clotting (due to the absence of certain clotting proteins) and results in uncontrollable bleeding when blood vessels are damaged (e.g., from a cut, wound, etc.). The treatment of this disease was by the injection of the missing blood clotting proteins, effectively alleviating the disease symptoms and batches of these therapeutic clotting proteins were produced from blood donations. Before the HIV virus had been identified, however, no reliable diagnostic tests were available to detect it. As a result, donated blood could not be screened for the virus. Furthermore, blood donations were often obtained from individuals who were paid to donate and these donors often led unhealthy lifestyles that made them more likely to be

infected. If a single blood donor was infected with the HIV virus, then the entire batch of donated blood samples could be contaminated.

As a result of the above incident, with more than ten thousand hemophiliacs contracting HIV as a result of the tainted blood supply, more stringent controls were initiated. Screening tools for example, are now used to accept or reject blood donors, and diagnostic tests have been developed and are used to detect the virus in donated blood. These tools and tests are now routinely applied to all blood donations in developed countries and the risk of HIV transmission via blood and blood products has been largely eliminated.

In a case from 2010, an important childhood vaccine for the prevention of a deadly infection caused by rotaviruses (which causes severe diarrhea) was demonstrated to be contaminated by an apparently harmless, porcine-derived virus known as a circovirus. Although the circovirus was not known to cause illness in humans, government agencies recommended that medical professionals cease using the vaccine for a period of time until its potential impact to patients could be assessed. The main issue with detecting and controlling viruses is that new viruses are discovered frequently. Thus it is not practical or feasible to test for *all* known viruses. As a result, the approach is to eliminate as many viruses as possible by carefully analyzing all the biological materials used in the production of product. Furthermore, steps are included in the production process that are designed to inactivate and/or remove viruses. Finally, specific tests are performed to check for a range of relatively common viruses known to be of concern in the manufacturing and administration of biologics.

Prions

Prions are a recently-discovered class of infectious agents that simply consist of protein, meaning they do not have any associated nucleic acid but can be replicated by the body. Prions do this because they are misfolded proteins, and once inside an organism can induce further similarly-misfolded proteins to occur by inducing the formation of an amyloid fold. The end result of this is a large number of misfolded proteins of the prion form, causing the disease state. The term *prion* is a linguistic portmanteau of the words *protein infection*. Prions were first proposed in the early 1980s by Stanley Prusiner as the causative agent of two unusual neurological diseases, previously attributed to "slow viruses"- scrapie in sheep and Creutzfeldt-Jakob Disease (vCJD) in humans.

At the time, Prusiner's work was a revolutionary concept that was not widely accepted; however, his work did eventually earn him the Nobel Prize for Medicine in 1997. Since that time, a number of other prion-associated diseases have been identified. Most neurologic prion infections fall under the term Transmissible Spongiform Encephalopathies (TSEs). The most well-known of theses is Bovine Spongiform Encephalopathy (BSE), more popularly known as "mad cow disease." BSE is a disease that affects cattle, causing neurological symptoms in infected animals. Prion diseases are generally transmitted by consumption of nerve tissue from infected animals (i.e., brain, spinal cord). BSE is believed to have been transmitted to large numbers of cattle that were given animal feed containing Meat/Bone Meal (MBM) from other animals (which included ground spinal meat). While MBM has been used for many years in animal feed, it is now thought that a change in the treatment process of this product in the

1970s resulted in the prions no longer becoming properly inactivated. This change started a cycle in which a high proportion of animals became infected with BSE.

What is the relevance of prions to the pharmaceutical manufacturing? The industry generally uses a number of animal-derived materials, such as gelatin and amino acids, which are traditionally harvested from cattle. For biopharmaceuticals, fetal bovine serum is used in the process of growing mammalian cells. If this serum includes material from infected animals, it could potentially infect the producing cell line and ultimately be transmitted to the patient via pharmaceutical product derived from these cells. In the 1960s and 70s, the methods of recombinant DNA and genetic engineering had not yet been developed. All human therapeutic proteins for patients whose bodies did not adequately produce them were derived through the isolation of that protein from human tissues. One of the best and most successful cases was the use of Human Growth Hormone (HGH), used to treat dwarfism in children who made an insufficient amount of this essential hormone. The HGH was extracted from human pituitary glands obtained from cadavers. To make one batch of HGH, a large number of human pituitaries would be gathered and the product extracted. HGH batches created in this manner were effective in reversing the growth deficiency symptoms in the affected patients. Some years later, however, it was discovered that some patients had developed a type of CJD and that donated pituitaries from individuals with CJD could contaminate the manufactured HGH and potentially transmit the infectious prion to all the children who received the product. This event increased the awareness of the dangers posed by this type of infectious agent and expedited the approval of the rDNA-based HGH, which eliminated the need to start with human pituitaries and thus resolved the issue.

When the "mad cow" type of TSE was identified in the United Kingdom in the 1980s, it created a wave of concern, as it was recognized that the prion was extremely difficult to inactivate or remove. Thus if many parts of an infected animal were to enter the production process, the concern was that it might contaminate other previously inert, animal-derived components of pharmaceuticals. This led to concerted global efforts to eliminate the use of animal-derived materials wherever possible. When this was not feasible, the effort was to mitigate the risk by using only certain parts of the animal deemed to be of lower risk or by using only animals from BSE-free areas. To date there have been no reported cases of CJD in humans due to pharmaceuticals contaminated by BSE.

Prions are now well accepted as the causative agent of certain transmissible spongiform diseases and are recognized as being potentially responsible for devastating illness that are transmitted by contaminated pharmaceutical products. However, through careful control of the supply chain inputs (raw materials), this issue has been more successfully controlled.

Microbiological control

The manufacture of biopharmaceutical products begins with the essentially closed, axenic mono-culture process of fermentation, proceeds through the low bioburden process of purification, and ends with the aseptic fill/finish process to produce a sterile dosage form. During this cycle there are various types of microbial agents that can enter these processes in different ways. In some circumstances, extraneous microbes may be tolerated—in other circumstances they are unacceptable. In all instances, however, it is essential that organizations

understand, monitor, and control their products and potential impurities in those products (in this context extraneous microbes can be considered as impurities). Often it is difficult to measure the quantity of these impurities that are present and there are major challenges in proving their complete absence. In order to control them, it is necessary to control the environmental factors that impact their presence. To understand this, a closer examination of the manufacturing process of biopharmaceutical products is necessary.

Typically the manufacturing process can be considered as two independent activities: the manufacture of the drug substance and the manufacture of the drug product. These two activities can occur in separate facilities (at times in separate countries) and can have their own specific, but differing, requirements for microbiological control.

Manufacture of the drug substance

The manufacture of the drug substance consists of a relatively long, discontinuous set of process steps in which the realities of achieving microbiological control are quite different at the various phases. This culminates in the preparation of a single or small number of containers of a substance, typically as a solution that is allowed to have a low bioburden. In other words, it is not sterile and can have a small number of microbes present when tested. During the required steps, the exclusion/control of extraneous microbes is critically important to ensure the required quality substance is achieved. An ongoing analysis and understanding of the microbes (in the air, water, surfaces, and the people relevant to the process) are required. A critical piece of documentation is a bioburden control strategy. This document is used to provide a proactive analysis of the risk that bioburden presents to product quality. It should document how the process reduces the risks posed by microbes to acceptable levels. For example, microbes are eliminated through sterile filtration, toxins may be eliminated through chromatography or viruses may be reduced through an inactivation step.

Manufacture of the drug product

The manufacture of the drug product involves the preparation of individual sterile dosage form units from a biopharmaceutical drug substance or substances. These drug substances are usually not "sterile" but are considered low bioburden solutions. Various other substances (e.g., excipients, preservatives, etc.) and components (e.g., vials, syringes, stoppers, etc.) required for the dosage form are included in this process. Compared to the manufacture of drug substances, the manufacture of the drug product is essentially one relatively short, continuous process.

This process is similar, or in some cases identical, to that used for the aseptic manufacture of different types of parenteral products (aseptic manufacturing is a well-established process that has been used for decades). The process output is a sterile product produced from a series of individual, non-sterile components. There are many controls and conditions related to the facility, people, and process that must be in place to achieve the sterile product. It is important to understand the terms sterile, aseptic, and axenic:

- **Sterile**: the absence of life. All drug products are required to be sterilized once placed in their final container. This is performed to prevent the provision of a product contaminated with microorganisms to patients.

- **Aseptic:** acting in such a way to prevent the introduction of microorganisms. Aseptic processing is used for those drug products that must be sterile but cannot be subject to terminal sterilization due to their heat-labile nature; effectively all biopharmaceutical products are in this category.

- Axenic**:** freedom from foreign organisms. All biopharmaceuticals are intended to be axenic cultures; that is, they only contain the cell line desired, without other foreign organisms

During aseptic processing, previously sterilized components, containers, and closures are assembled within specially designed and controlled environments. These environments are intended to minimize the potential contamination of the product by microbes or particulates. In such processes, aseptic techniques similar to those used in routine microbiology laboratories and in many medical procedures are widely used.

Types of Contamination

Contamination is the presence of any unwanted substance that will affect the safety, integrity, strength, purity, or quality of a drug product. In aseptic biomanufacturing, the goal is to maintain sterility in the final product or to prevent contamination of the sterile components and the end product. Contaminants can be transferred to the sterile components and end product from the environment or by direct contact. Contaminants can be grouped into two categories, particulate (non-viable) or microbial (viable). Neither type may be visible to the naked eye.

Particulate (non-viable) contamination

Particulates are small bits of matter, or particles, usually of microscopic dimensions. Government agencies and industry groups have different means of categorizing particulates based on size and the type of particle. Generally a particle is smaller than a human eye can detect, which is an object of approximately 25 microns in size, about 39 millionths of an inch (0.000039 inch). A micron is one millionth of a meter. Figure 8-1 illustrates the size of one micron.
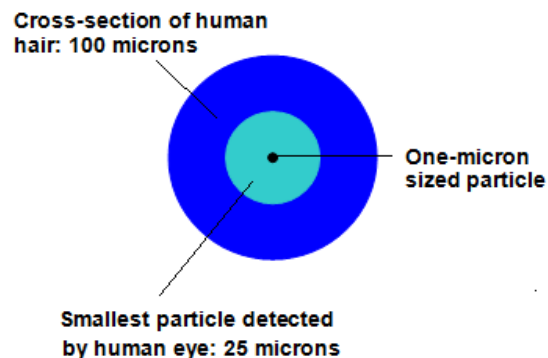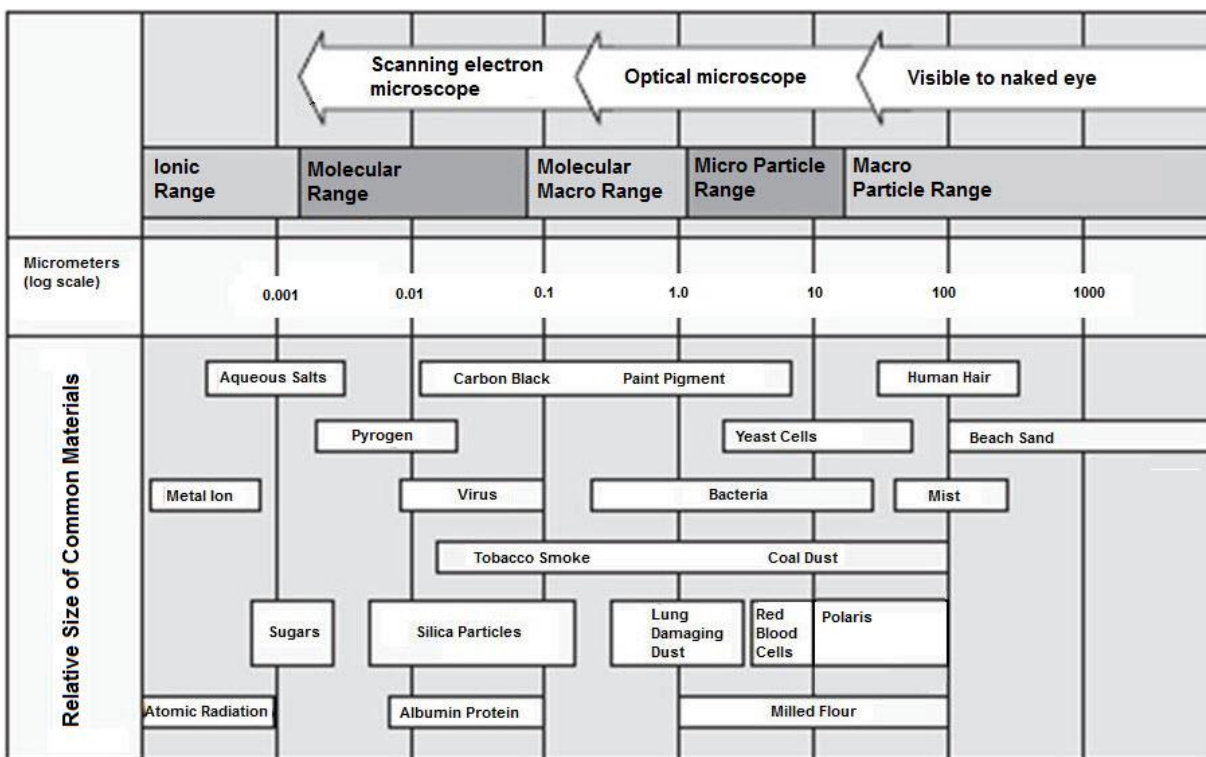

Cross-section of human hair: 100 microns

One-micron sized particle

Smallest particle detected by human eye: 25 microns

Figure 8-1. Comparison of one micron-size particulate to one human hair

Particulates can be composed of any material, either organic or inorganic. They can be found in gases, liquids, or solids as either suspended or settled material:

- gas: the suspended particulate is referred to as an aerosol or airborne contamination (Figure 8-2 illustrates the relative size of common airborne contaminants—a person cannot see up to 97 percent of particulates in the air; dust accounts for approximately three percent of the particulates that flow through an air space).

- liquid: the suspended particulate is referred to as a suspension; when settled at the bottom of a liquid it is referred to as silt.

- solid: the suspended particulate is referred to as included matter.

	Scanning electron microscope		Optical microscope		Visible to naked eye	
Ionic Range	Molecular Range		Molecular Macro Range	Micro Particle Range	Macro Particle Range	

Micrometers (log scale)	0.001	0.01	0.1	1.0	10	100	1000

Relative Size of Common Materials:

- Aqueous Salts
- Carbon Black — Paint Pigment
- Human Hair
- Pyrogen
- Yeast Cells
- Beach Sand
- Metal Ion
- Virus
- Bacteria
- Mist
- Tobacco Smoke — Coal Dust
- Sugars
- Silica Particles
- Lung Damaging Dust
- Red Blood Cells
- Polaris
- Atomic Radiation
- Albumin Protein
- Milled Flour

Figure 8-2. Relative size of common airborne contaminants

Particulates can be harmful to the product or process. In a cleanroom environment, common sources of non-viable particulates include:

- cellulose fiber from paper

- glass particulate from breaking glass vials during filling

- aluminum particles from capping vials

- gown fibers

- hair

- human dead skin cells (one of the most frequently encountered particulates in the cleanroom)

It is important to note that all particulate contaminants on this list, may also present a source of viable (microbial) contamination as well.

Cleanroom classifications are based on the number of particulates allowed in the air. Particulate sizes measured are typically between 0.5 micron and 5.0 microns. These particle sizes are considered to represent sizes of microbes. They are measured using a particle counter.

Microbial (viable) contamination

As previously discussed, microbial contamination is caused by microorganisms such as bacteria, fungi, and viruses. All microorganisms are ultimately excluded to the fullest extent possible from medicinal products. If not controlled, however, they can reproduce. Thus they are referred to as "viable." Bacteria can replicate rapidly—some types as quickly as every twenty minutes. For example, within eight hours one bacterium can multiply to over 16 million bacteria (Figure 8-3 and Table 8-1 illustrates microbial replication). This rapid increase in numbers, along with the associated metabolic products and cell wall components such as bacterial endotoxin, is why microbial contamination is a major concern during aseptic processing. It only takes one bacterium to cause significant contamination of a product.

Table 8-1. Microbial contamination replication

Time of Day	Number of Bacterial Cells
9:00 a.m.	1
9:20 a.m.	2
9:40 a.m.	4
10:00 a.m.	8
10:20 a.m.	16
1:00 p.m.	4,096
1:20 p.m.	8,192
1:40 p.m.	16,384
2:00 p.m.	32,768
2:20 p.m.	65,536
4:40 p.m.	8,388,608
5:00 p.m.	16,777,216
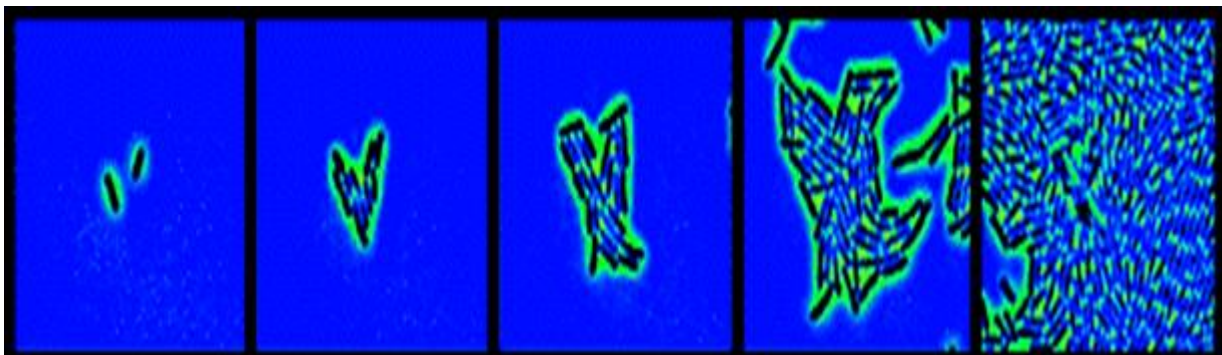
Figure 8-3. Example of bacterial reproduction

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Bacteria are the most common microbial contaminant found in cleanrooms, followed by mold. Bacteria can be divided into Gram-positive and Gram-negative bacteria, based on differences in their cell walls. Table 8-2 is a summary of some of the most frequently encountered microorganisms in a cleanroom setting. Bacteria from skin contribute the most contaminants to cleanrooms. In a well-controlled cleanroom environment, the presence of mold and Gram negative organisms should be minimal.

Table 8-2. Commonly encountered microorganisms in cleanrooms

| Microorganism	Example	Source
Gram-positive cocci	*Staphylococcus* species	humans
Gram-positive cocci	*Micrococcus* species	humans
Gram-positive bacilli	*Bacillus* species	soil

Sources of contamination

Microbial and particulate contamination sources in the cleanroom include humans, air, surfaces, water, and the components used to manufacture the product. Humans are the main source of cleanroom contamination. The very presence of people in a manufacturing process area poses risks to products. Contaminants from humans include shed skin particles and hair, as well as those found under fingernails, on hands, and or on clothes (Table 8-3). People can also expel contaminants by talking, sneezing, and coughing, even while wearing a mask (Figure 8-4). Depending upon the type of mask and other factors, not all particulate contaminants may be blocked.

Table 8-3. Sources of microbial contaminants from humans

Source	Amount
nose secretion	approximately 10 million microbes/gram
Spittle	approximately 100 million microbes/gram
scalp	approximately 1 million microbes/cm^2
forehead	10,000–100,000 microbes/cm^2
Armpit	1–10 million microbes/cm^2
Hands	100–1,000 microbes/cm^2

Note: During the course of a typical day, a person will shed 10 grams of skin particles. This is equivalent to one layer of skin (40 ft) within 3–4 days.
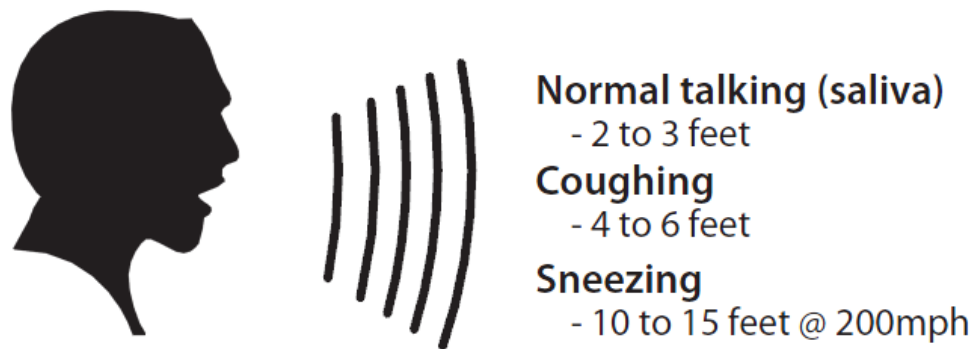
Figure 8-4. Respiratory dispersal of microbes

The process of product contamination is influenced by many factors. Contamination can be introduced by materials, water, the degree of human contact, and the manufacturing environment. People and their specific activities may have direct contact with the product. Contaminated work surfaces, tools, and fixtures can provide a direct transfer of contamination to the product.

Control of Contaminants in the Cleanroom

Several measures must be taken to reduce the likelihood of transferring contaminants from their source to sterile components and the end product. These include properly designing the facility, controlling the air supply for the aseptic environment, sterilizing the manufacturing components, using aseptic gowning, following aseptic techniques, and implementing a cleaning and disinfection program.

Each individual step in the entire biomanufacturing process, from the raw materials at the start to the final finished product, should be examined for potential risks. An approach used to examine the steps in a process is called the Hazard Analysis and Critical Control Points (HACCP). HAACP has been used extensively in industries such as food processing since the 1990s and has recently become more common in biomanufacturing processes. This hazard analysis method leads to the determination of critical control points, which helps establish a preventative monitoring system. This system is used to observe key parameters that have a potential impact on product quality.

Control through facility design

A cleanroom is any room or area where an attempt is made to limit, control, and eliminate the amount of airborne contamination. Many contaminants are continuously generated within a cleanroom by people, processes, equipment, and the facility, which are then transferred

through the flow of air. Eliminating these contaminants is a function of appropriate facility design (see the *Facilities* chapter) and in-process controls described in the following sections.

Control through air supply

It is important to note that there is no such thing as a totally clean room (a room with absolutely no contamination)—there are only degrees of cleanliness. Therefore, it is vital for aseptic processing that a facility's cleanroom be designed properly to maintain the necessary level of cleanliness.

The cleanroom environment is created and maintained by controlling the air that is supplied. Traditional air handling systems, referred to as Heating Ventilation and Air Conditioning (HVAC), use filtration systems that remove large particles and provide air that is adequate for routine manufacturing. Along with controlling particulate emissions, HVAC systems play an important role in regulating a facility's temperature and humidity. This standard HVAC filtration alone is not adequate for aseptic processing though and is not effective in reducing sub-micron airborne contamination.

Cleanrooms used for aseptic processing require High Efficiency Particulate Arrestance (HEPA) filters. HEPA filters are used to supply clean air to controlled areas such as cleanrooms. HEPA filters remove 99.97 percent of the particles suspended in air that are 0.3 microns or larger in size. The number of air changes per hour in a cleanroom is also controlled. The air handling system replaces the air in a cleanroom regularly, effectively purging the particulate matter generated within the room.

Additional ways by which to maintain this environment include specific construction materials, air classifications, pressure differentials, airlocks, restricted entry, and flow of people and materials.

All systems should be properly validated, or demonstrated to be effective in maintaining the proper level of cleanliness, through a rigorous protocol. This protocol will demonstrate that the system can be relied upon with a high level of confidence to provide an environment that is appropriate for its intended use – which is to minimize the risk of contamination.

Control through sterilization of components and equipment

The components and equipment used in aseptic biomanufacturing must be sterilized. Sterilization is defined as the act or process, either physical or chemical, which eliminates or inactivates all forms of life, including bacterial endospores (the most resistant of all microorganisms). An item can only be deemed sterile when there is a complete absence of viable microorganisms. Sterilization methods vary based on the material of the components to be sterilized. Commonly used methods for sterilization of biopharmaceuticals include:

- dry heat
- gamma irradiation
- ethylene oxide
- Vaporized Hydrogen Peroxide (VHP)

- filtration

Sterilizers, their use, and their load patterns need to be initially validated to ensure that each sterilizer is functioning properly and that every item in the load is sterilized by the end of the cycle. Ongoing validation, performed at least annually, ensures that the cycles remain within a validated state. Preventive maintenance ensures the sterilizer is running properly.

The basic principles for validating a sterilizing process are:

- Demonstrate that the equipment used for the sterilization process (autoclave, dry heat oven, and VHP) is capable of operating to achieve the desired end result

- Run cycles to demonstrate actual operational conditions. Ensure that the required parameters of microbial kill (i.e., bioburden reduction) are achieved. Biological indicators are typically used for this purpose. Biological indicators are materials that are inoculated with a known quantity of microorganisms, typically one that is resistant to the sterilization conditions and thus serves as indicators of the worst case scenario for the sterilization cycle.

- Continually monitor the process parameters (e.g., temperature, pressure, etc.) during each sterilization cycle to ensure they are operating within the validated parameters

- Perform continuing validation studies periodically to ensure that the loads are maintained in a validated state

It is important that sterilization processes operate within validated parameters to ensure the sterility of all equipment and components since each and every item used for aseptic processing cannot be tested for sterility. It is also important to check the expiration date and appearance and integrity of sterilized equipment and components before their use. Any equipment that has been compromised or is out of date must not be used.

Steam sterilization involves exposing an item to steam under high temperature (minimum of 121 degrees Celsius) and pressure conditions (15 psi). Steam sterilization can be used for items that are heat and moisture stable and that can be penetrated by steam. An autoclave (Figure 8-5) is a commonly-used method for sterilization.

Figure 8-5. Steam sterilization using an autoclave

To provide a uniform temperature distribution and efficient heat transfer, air must be removed from the sterilization chamber. This is usually accomplished using a vacuum system. Once the correct temperature and pressure conditions are achieved in the autoclave chamber, the sterilization time begins. A steam sterilization cycle is not valid unless the appropriate temperature and pressure conditions are maintained for the required period of time.

Some of the items that can be steam sterilized using an autoclave include:

- culture media

- filters

- glass bottles

- miscellaneous items such as stoppers, caps, tubing and forceps

Prior to sterilization these items are prepared and wrapped using penetrable materials such as Sterilin® bags or parchment paper. All items must be loaded according to validated load patterns to ensure that each item in the load is sterile.

Autoclaving is a consistently effective means of sterilizing most objects, provided the process is done correctly. The principle advantages of steam sterilization are its simplicity, relatively short processing times, and lack of toxic residues. Its main disadvantages are the relatively high temperature and the limited types of materials that can be sterilized in this manner (i.e. those that are not sensitive to moisture, temperature, and/or pressure). Furthermore, there are hazards associated with high temperature and operating pressurized vessels. To prevent burns, operators must wear thermal gloves that allow hot materials to be handled safely.

Steam-In-Place (SIP) procedures are used for large vessels, such as tanks and other types of equipment too large to fit in an autoclave. Clean-In-Place (CIP) procedures must be completed first to prevent the SIP process from baking on any residuals (cross contamination). For the SIP cycle to be effective its validated procedure must be followed exactly.

Dry heat sterilization involves exposing an item to hot air (160–170 degrees Celsius) in an oven-like chamber. To ensure temperature uniformity within the chamber, the air is circulated using a fan-blower system. Dry heat sterilization can be used for items which are heat stable but are sensitive to moisture or cannot be penetrated by moist heat. Glassware can be sterilized by dry heat.

Once the oven correct temperature is achieved in the chamber, the sterilization time begins. A dry heat sterilization cycle is only valid if the appropriate temperature conditions are maintained for the required period of time. As with steam sterilizers, all items must be loaded according to validated load patterns to ensure that each item in the load is properly sterilized.

The main advantages of dry heat sterilization are its simplicity, penetrating power, and lack of toxic residues. Components that might corrode if exposed to moisture are well suited for dry heat sterilization. Another significant advantage is that dry heat has the ability to depyrogenate, or inactivate, all pyrogenic substances (any substance, such as endotoxins, that produce fever when introduced into the body). Steam does not have this ability. Dry heat sterilization's disadvantages are the relatively long processing time and the high temperature. This limits the

types of products and packaging materials that are compatible with this process. Dry heat sterilization takes longer than steam sterilization mainly due to the fact that air is a relatively inefficient heat transfer medium. There is a time delay for the heat to penetrate the load. As with steam sterilization equipment, operators must wear thermal gloves that allow hot materials to be handled safely.

Gamma irradiation provides a simple sterilization alternative for moisture-sensitive and/or heat-sensitive non-liquid items. Some types of liquids which are heat-sensitive can also be sterilized this way. Gamma irradiation involves exposing items to gamma rays produced by isotopes Cobalt-60 or Cesium-137 until the desired dosage is delivered. A gamma ray is an electromagnetic radiation of extremely high energy, emitted by a radioactive atom during decay. It is similar to an X-ray but shorter in wavelength (higher-energy). Gamma rays damage DNA and other cellular structures in living organisms, thus making them effective in killing bacteria and other potential contaminants. Items sterilized by this method are not exposed to any toxic agents and no residues are left behind.

The exposure during gamma irradiation can be quantitatively monitored, making it an easily validated method. The main disadvantage of gamma irradiation, however, is that it is a relatively expensive process. The extremely high capital cost to install gamma irradiators makes it prohibitive for many organizations. However, there are third party organizations that provide gamma-irradiated items to the pharmaceutical industry. There are also safety issues associated with the process since it involves the use of radioactive isotopes. Gamma irradiation is not widely used for aqueous drug products and protein-based pharmaceuticals because it can degrade such products via the electromagnetic radiation.

Vaporized Hydrogen Peroxide (VHP) is an aerosolized, low temperature chemical sterilant that kills microorganisms, including bacterial endospores on environmental surfaces in an enclosed area. It is used to sterilize sealed enclosures such as isolators, workstations, and pass-through rooms. VHP's advantages include its effectiveness against a wide variety of microorganisms, the use of low temperatures, and compatibility with a wide variety of materials and surfaces. Furthermore, once the VHP (31% H_2O_2) is used, it can be sent through a catalytic converter to produce water vapor and oxygen. These by-products are safe and environmentally friendly. The main disadvantage of VHP sterilization is its limitation as a surface sterilant. It is only effective on exposed surfaces. Furthermore, 31% H_2O_2 is toxic and must be used in enclosed or sealed areas. And though it rapidly aerates and converts to harmless by-products, it is important to follow all required safety precautions when working with this substance.

Filtration does not necessarily kill microorganisms but it does remove them from the filtered material. Filtration is used to sterilize heat-sensitive materials. And the process basically consists of passing the liquid or gas product through a porous medium such as cellulose esters or plastic polymers that trap the microorganisms in the filtration matrix or retains them on its surface. Filters are assigned a nominal size rating above which a certain percentage of contaminants will be retained.

Control by aseptic gowning

The human body constantly sheds dead skin cells and hair and excretes oil and moisture—all of which can contain microorganisms that could contaminate critical parts of the process and products. Humans shed approximately 1000 bacteria-carrying cells per minute. Many airborne microorganisms are dispersed in cleanrooms due to human skin cells. A person can shed one outermost layer of epithelial cells every 24 hours which amounts to 10^9 cells per day. A small but significant amount of these skin cells can be associated with microorganisms which can get into the air flow and cause contamination.

Wearing clean, sterilized clothing is necessary to protect the product and critical surfaces from contamination. Aseptic gowns (Figure 8-6), consisting of a gown, mask, gloves, goggles, and boots, cover all or most of a person. Individuals working within cleanrooms must be properly trained on aseptic gowning. The apparel provides a barrier between the individual wearing it and the sterile components and products. However, aseptic gowning does not provide 100 percent protection. Microorganisms have the ability to penetrate woven or non-woven fabrics to emerge into the surrounding air, thus proper gowning reduces but does not eliminate microorganisms shed by people in cleanrooms.

Figure 8-6. Full aseptic gowning

Within a facility, different areas are assigned classifications that determine gowning requirements (Table 8-4).

Table 8-4. Gowning requirements by area classification

Clean Area Classification (0.5 micron particles/ft^3)	ISO Designation[a]	Typical Area	Gowning
Class 100	5	point of fill, sterility testing or other aseptic manipulation	full aseptic gowning, to consist of sterile gown/hood, sterile double gloved, mask, goggles, and boots
Class 10,000	7	background room/area for aseptic manipulation	same as Class 100
Class 100,000	8	personnel and equipment airlocks leading into the cleanroom facility	non-shedding one- or two-piece suit gathered at wrists and ankles; hair, beard, and shoe covers
Controlled Unclassified	NA	access and exit to/from classified areas; packaging areas	employer-issued uniform plus non-shedding smock, hair, beard, and shoe covers

[a] ISO 14644-1 designations provide uniform particle concentration values for cleanrooms in multiple industries.

Individuals working in a cleanroom must follow high standards of personal hygiene and cleanliness to reduce the amount of microorganisms and particulates shed. Anyone with a respiratory or gastrointestinal infection, open skin lesions, or any other condition that causes skin to peel should not work in a cleanroom. For people working in a cleanroom environment, the challenge is being gowned and operating in a slow, controlled manner for long periods of time.

Proper practices for aseptic gowning include:

- wash hands with soap and water prior to entering the gowning room

- keep fingernails trimmed to preserve the integrity of the gloves

- remove watches and jewelry prior to gowning, with the exception of smooth wedding band and medical jewelry; special measures such as additional gloves may be necessary to lower the risk for these exceptions

- follow the organization's established SOP for the gowning process, maintaining the order in which gowning attire is put on to assure that gown cleanliness is maintained as much as possible

- wear face mask for facial hair

- gown, making sure the gowning apparel does not contact walls, floor, bare skin, or equipment; gown again if the apparel contacts a potentially contaminated surface

- wear gloves when entering the cleanroom area; disinfect gloves with sterile 70% isopropyl alcohol; inspect gloves and gowning apparel initially upon entering the cleanroom and periodically to ensure that they have not been compromised; and replace damaged items as necessary

- use gowning materials that fit properly as inadequately-sized gowns or materials may cause problems in performing aseptic work; loose-fitting gowns create the potential for contact between the gown and a critical surface area.

- keep talk to a minimum; avoid sneezing and coughing as particulate matter can be dispersed through such actions

- if necessary, adjust gloves and the sleeves of the gown during aseptic manipulations, as it is possible for the gown and the gloves to separate at the wrist, leaving the wrist exposed

Control through aseptic techniques

Every activity that is done for aseptic processing should be performed with the mindset of reducing the probability of risk to the lowest-acceptable level. Because of its inherent probabilistic nature, risk can never be completely eliminated, but one should understand that his or her behaviors directly and immediately impact the chance for success during aseptic processing. Even though most biosafety cabinets have a rating of Class 100, this rating still allows for the presence, however limited, of particulate matter to circulate.

The most important factor in reducing contamination during aseptic processing is operator technique. This is due to the fact that the most frequent physical interventions in close proximity to the open cell culture are the operator's movements. Given proper equipment and material asepticity, as well as process design (e.g., appropriate filtration steps, SIP following CIPs, etc.), the operators are the major potential sources of contamination for the cell culture.

Extrinsic to aseptic technique, the three major components most important for reducing probability of contamination are: environment, equipment and materials.

Environment

- the environment must be clean with HEPA-filtered air circulating
- the aseptic area should be cleaned before and after use. Cleaning after use reduces the risk of cross-contamination in a multi-product suite and reduces the microbial load on working surfaces

Equipment and materials

- Ensure that equipment and materials are cleanable and fit for purpose, i.e., use equipment that is designed for cleanrooms. Equipment would not be considered cleanable if the presence of air exhaust on the equipment leads to non-aseptic turbulent airflows, surfaces are difficult-to-access or reach for cleaning, or non-suitable materials of construction (e.g., non-slip felt/fabric pads on underside of equipment) are present.

Aseptic technique guidelines when working under a biological safety cabinet

When working with a biological safety cabinet (BSC) as part of the aseptic process, it is important to understand the associated guidelines and limitations of their use.

BSCs and cleanrooms are categorized as Class 100 environments. This means that one cubic foot of air may have no more than 100 particles of 0.5 microns or larger. The critical surfaces in the aseptic processing area in the biological safety cabinet must see the first air from the HEPA filter. No body part, equipment, objects, or contaminated air should come between the HEPA filter and the critical surface. An example is passing a gloved hand over an open bottle of product. Anything on the hand or sleeve could potentially be shed into the product by the flow of air under the hood and subsequently contaminate the product. Techniques for activities as basic as removing or replacing a bottle cap should be well thought out and practiced in order to minimize the chances of contamination. This is called the First Air rule (Figure 8-7).
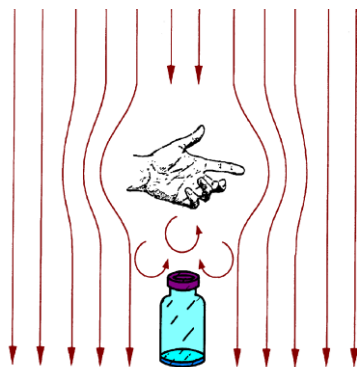
Figure 8-7. Violation of the First Air rule

List of behaviors that should be adhered to in order prevent contamination

The following list of behaviors should be adhered to when working in a biosafety cabinet or cleanroom to prevent contamination.

- verify the proper operation of the BSC - that it has been certified prior to the start of the procedure and that it's indicator lights, alarms, and/or gauges are working properly. The BSC should be on a periodic certification schedule

- plan all activities - arrange materials and plan operations so that there is a minimum of movement and physical contact with materials once aseptic operations begin. Place non-critical materials (e.g., tube racks) in a location where they do not need to be moved or manipulated during the aseptic activity

- movement of personnel into and out of the cleanroom area should be kept to a minimum as fast and unnecessary movements cause turbulence which can disrupt the air flow and contaminate critical surfaces, including the product. A vacuum can be created near the sides of a BSC work area which could pull contaminated air from outside into the BSC and thus it is important that no work be performed near the sides of the BSC work area

- perform any aseptic manipulation above waist level for BSCs and cleanrooms. Based on the concept that the rebound effect of the air may extend approximately one foot above the floor surface, all aseptic manipulations and connections should be performed at least two feet or higher from the floor

- bring only the minimum equipment and materials necessary to perform the activity into the area

- clean all equipment and materials brought into the BSC area with 70% isopropanol

- use materials that are suitably constructed to withstand cleaning without being compromised

- place all materials and equipment in such a fashion as to allow for the activities being performed to be accomplished with a minimum of movements and in a manner so as not to break the first air rule

- Segregate clean and dirty (waste) materials within the BSC

- Prohibit movement of materials and personnel into or out of BSC or cleanroom until all activities being performed in the cabinet are brought to a stage where no product is open to the environment

- Perform all aseptic manipulations at least six inches from the front edge and sides of a biosafety cabinet or as per the manufacturer's recommendations

- Avoid touching critical surfaces. Gloved hands are not considered sterile even after disinfection with alcohol as disinfection reduces the number of microbes but cannot sterilize a surface

- Avoid excess turbulence and particulate shedding by:

- minimizing the number of people and equipment under the hood; only those essential to processing should be present

- limit talking

- working in an unhurried, deliberate manner. Motions should be controlled and methodical (even while sitting or standing still, a person can emit 100,000 particles per minute—the more a person moves the more particulate load is emitted in the area). Perform only those actions that are necessary to accomplish the task being performed. Do not perform unnecessary movements or touch objects when doing so is not needed. Table 8-5 lists the amount of particles that a person can generate.

- Avoid placing equipment on the air intake grate of the BSC as this disrupts air flow.

- Avoid using equipment if its sterility has been compromised—critical surfaces remain sterile if they are handled correctly and the package is intact. Replace any component with a fresh sterile one if contamination is suspected

- refrain from storing materials that generate fibers or particulates (e.g., cardboard, paper, wood, etc.) where aseptic work is performed

- when entering a cleanroom via an airlock adhere to the following behaviors:

  - All equipment must be disinfected in the airlock prior to entering an aseptic area and before an aseptically-gowned person contacts it

  - Opening and closing of airlock doors should be kept to a minimum. They should be opened only to perform necessary activities

  - both doors of an airlock should never be opened simultaneously

Table 8-5. Estimated particles (0.3 micron and larger) generated by various activities

Particles Emitted	Activity
100,000	motionless—sitting/standing
500,000	upper body motion
1 million	upper body and minor leg motion
2.5 million	sitting to standing or vice versa
5–10 million	walking >2.0mph

If specifications require measurement of viable and non-viable particulates during your operations the following experiments can be performed:

- Viable particulates: surface monitoring plates may be used to measure viable contaminants on personnel and equipment surfaces. Air viable devices may be used to measure viable air contaminants.

- Non-viable particulates: a particulate measuring device can also be used to measure the total of both viable and non-viable particles.

Control by cleaning and disinfection

Cleanrooms do not have self-cleanup capabilities to offset any contamination brought into the room or generated by people and/or equipment. Most contaminants introduced in this fashion settle to the floor or other horizontal surfaces and could be introduced into the air by changes in air currents or activity in the room. Thus contamination needs to be removed from these surfaces by frequent cleaning and disinfection. A one-micron anthrax spore can take approximately twenty minutes to float a one meter distance to the floor or horizontal surface.

Cleaning is often confused with disinfection; however, they are not the same. Cleaning is applying a detergent (along with the physical removal of particles and microorganisms from surfaces) by mopping, wiping, or brushing. Disinfection is the elimination of most recognized disease-causing or harmful microorganisms but not necessarily all microbial forms and is a less lethal process than sterilization. Although disinfection might make use of ultraviolet radiation, boiling water, or steam, the term is typically associated with the use of chemicals.

Cleaning

Cleaning is used to remove contaminants and residues that can interfere with the effectiveness of disinfectants. Proper cleaning is important for a successful disinfection. Cleaning does not have to be performed at the same frequency of disinfection. An example of a four-step cleaning process of a surface involves:

1. scrubbing the surface with a mop or wipe and using a detergent solution
2. rinsing the surface before the surface dries
3. collecting any remaining liquid on the surface by wiping or vacuuming
4. allowing the surface to dry then disinfecting

Though vital to the cleaning process, disinfectants can be burdensome. One issue with disinfectants is that they can leave residues. Air pockets can form in the residue when a disinfectant dries and these air pockets can harbor bacterial endospores. If the air pockets break, they can release the endospores. The residues can also be transported on personnel to critical surfaces as well as the product and cause contamination. They can also be extremely corrosive and damage the surfaces on which the disinfectants are applied. Routine removal of these residues through careful cleaning is necessary.

Disinfection

A disinfectant is defined as a chemical agent used on surfaces that destroys disease-causing microorganisms. No single disinfecting agent or procedure is adequate for all disinfection purposes. For example, most disinfectants do not ordinarily destroy bacterial endospores. Thus a variety of disinfectants and disinfectant procedures are used to maintain aseptic processing equipment and facilities.

The effectiveness of a disinfectant depends upon:

- the disinfectant concentration
- the length of exposure to the disinfectant
- the amount of organic matter (e.g. soil, blood) present
- the nature and amount of microorganisms on the surface
- the material to be disinfected

When selecting a disinfectant for an aseptic processing area, these criteria must be considered. All disinfectants must be used at sufficient concentrations and given sufficient time to work. The disinfectant vendor's recommendation for contact time must be followed.

Disinfectants include antimicrobial components. Many different types of antimicrobial components can be used in disinfectants, each with varying degrees of success depending on the type of microorganism. Most disinfectant antimicrobial components require a minimum contact time of five minutes. Disinfectants can also have a detergent component which is used to help remove soil from the area being treated. A disinfectant is more effective once soil has been removed and the surface is clean.

A disinfectant can have an acid or alkaline pH level. The pH of an environment can limit microbial growth. Very few microorganisms can survive in an environment with a pH of less than 4 (acidic) or more than 10 (alkaline). Most microorganisms grow well in an environment close to pH 7. The proper disinfectant can create an environment that is not conducive to the survival of microorganisms.

Chemical agents used as disinfectants in biopharmaceutical manufacturing fall into the following categories based on their antimicrobial component (Table 8-6):

- alcohols
- aldehydes
- halogens
- peroxygens
- phenolics
- surface active agents

Each type of disinfectant is also categorized by its use and the microorganisms against which it is effective- bactericidal agents, fungicidal agents (fungi), virucidal agents (destroy/inactivate

viruses), sporicidal agents (bacterial and fungal spores), and bacteriostatic agents (prevent the growth of bacteria but do not necessarily kill them). Solutions of these disinfectants must be sterile when used in Class 100 and Class 10,000 area classifications. Examples of disinfectants are sodium hypochlorite (halogen), 70% isopropyl alcohol (IPA), and peracetic acid (peroxygen).

Table 8-6. Summary of disinfectant categories and their efficacy

4 = most efficacious in killing

1 = least efficacious in killing

0 = not effective in killing

	Gram-Positive Bacteria	Gram-Negative Bacteria	Endospores	Fungi
Disinfectant Category				
Alcohols	3	3	0	2
Aldehydes	4	4	4	4
Halogens	3	3	1	2
Peroxygens	4	4	4	4
Phenolics	3	2	1	3
Surface Active Agents	3	1	0	2

Many facilities typically rotate the types of disinfectants used in cleanrooms by alternating the use of two different disinfectants. This is usually performed on a monthly basis. The rationale is that one disinfectant would not be effective in killing a particular population of microorganisms, whereas second disinfectant could possibly prove efficacious against a different microorganism or microorganism population.

All equipment and containers should be disinfected in the equipment airlock prior to being transferred into an aseptic processing area. Equipment and containers should be wiped from either top to the bottom or from the cleanest to most contaminated area. Sterile 70% IPA is commonly used to disinfect equipment and components immediately prior to transferring them under a laminar flow hood, as well as immediately prior to use within the laminar flow hood. While working under the laminar flow hood, workers should frequently apply 70% IPA to their gloved hands.

Examples of surface disinfectants for different area classifications are shown in Table 8-7. Table 8-8 shows the frequency of application of various disinfectants.

**Table 8-7. Surface disinfectants usage
on different surfaces in different area classifications**

Area Classification	Ceilings	Walls	Floors	Plastic Curtains	Critical Work Surfaces
Class 100	phenolic and monthly sporicide	phenolic and monthly sporicide	phenolic and monthly sporicide	PA or hypochlorite and monthly sporicide all followed by IPA wipe	PA or hypochlorite and monthly sporicide all followed by IPA wipe
Class 10,000	as above	as above	as above	as above	NA
Class 100,000	phenolic	phenolic	phenolic	NA	NA

PA: Peracetic Acid (peroxygen)
Hypochlorite: Halogen
IPA: Isopropyl Alcohol, NA: Not Applicable

Table 8-8. Frequency of application for disinfectants [a]

Area Classification	Ceilings	Walls	Floors	Plastic Curtains	Critical Work Surfaces
Class 100	daily to weekly mopping	daily to weekly mopping or spraying	daily mopping	daily mopping, wiping, or spraying	daily wiping or spraying
Class 10,000	weekly mopping	weekly mopping or spraying	daily mopping	NA	NA
Class 100,000	monthly mopping	monthly mopping or spraying	daily mopping	NA	NA

[a] The frequency of disinfectant use should be determined by environmental monitoring results.

Environmental Monitoring

Aseptic biomanufacturing environments require strict controls to minimize the potential for microbiological and particulate contamination of the product. A comprehensive environmental monitoring program is required for facilities, personnel, and process utilities to check that the state of control is maintained. Such control is mandated by cGMPs for the consistent production of products that are safe, pure, and effective.

Monitoring alone, however, is not enough. A key disadvantage of environmental monitoring, particularly with microbial monitoring, is that often a potential problem is not identified until several days after the occurrence. Prevention is the preferred means of control over monitoring.

Prerequisites for an environmental monitoring program

To assure a high likelihood of achieving acceptable testing results from the environmental monitoring program, the following vital elements must be in place prior to implementing the program:

HEPA filter certification program: For HEPA filtered equipment supporting classified areas, such as laminar flow hoods and HVAC systems, an ongoing HEPA filter certification program is necessary. Minimal certification includes routine biannual HEPA filter leak testing and air flow testing in Class 100 areas. HEPA filters serving Class 10,000 and Class 100,000 environments should be certified at least annually. Certification must also include the verification of the appropriate pressure differentials between rooms and the room air exchange rate.

Cleanroom cleaning and disinfection program: Procedures must be in place for the cleaning and disinfection of all classified areas, and all individuals performing these tasks must be properly trained.

Cleanroom qualification/requalification program: Prior to use, the cleanroom and its process utility systems must be formally qualified. Performance Qualification, also known as PQ, consists of a series of extensive tests to ensure that the area or process utility is operating as intended and that the environmental requirements are achieved. Cleanrooms and process utilities also need to be re-qualified in response to changes in area integrity (such as power failures affecting operation of laminar flow hoods and HVAC systems).

Airflow pattern visualization program: Smoke studies must be performed to demonstrate the unidirectional flow of HEPA filtered air across product contact surfaces in Class 100 areas and to confirm the appropriate flow of air from the areas of highest criticality outward to less critical areas. These studies should be performed both in static and dynamic modes of operation and recorded on video. Smoke studies are performed prior to PQ testing. They should also be performed on an annual basis to reconfirm the unidirectional nature of the airflow to determine that no routine drift of airflow parameters has occurred.

SOPs on the performance of environmental testing, investigations, documentation of results: SOPs must be in place to provide a written description of the program specifics for the monitoring and testing of classified areas, personnel, and controlled utility systems, along with the alert and action levels. Also, an SOP must document the process for recording, accessing, tracking, and storing of test results.

Personnel training program: Relevant personnel must receive documented training in environmental monitoring and testing procedures. This training must be conducted prior to the performance of any testing. The performance of those conducting the test(s) should be evaluated on an ongoing basis in the field by a trainer or supervisor.

Components of an effective environmental monitoring program

An environmental monitoring program must be established at each biomanufacturing facility to continuously monitor the environmental conditions to which products are exposed during manufacturing. An effective environmental monitoring program should generally include scheduled monitoring of:

- airborne viable microbial and non-viable particulate levels

- microbial contamination on personnel, work surfaces, floors, walls, and equipment

- microbial contamination of clean utilities

- pressure differentials

- direction of air flow

- temperature

- humidity

Awareness of the microorganisms present, as well as the particulate levels, can assist in designing an effective control system. However, no environmental monitoring program, regardless of its design, can provide the level of confidence desired without overall cleanroom systems management. This management is accomplished by adherence to cGMPs, facility design control, effective supervision, sound corrective action steps, and proper employee training.

A critical element to designing an effective environmental monitoring program is developing a well thought-out sampling plan. A sampling plan is a documented plan that:

- describes the procedures and methods for sampling in a cleanroom

- identifies the sampling sites, the sampling frequency, and the number of samples

- describes the method of analysis and how to interpret the results

The specific requirements for environmental monitoring programs are often interpreted differently by different international regulatory agencies. Although there have been efforts among industry groups and regulatory agencies to harmonize guidelines and requirements, significant variations still exist. Therefore, it is important that the quality group within the

organization keep abreast of environmental monitoring guidelines and requirements for the different countries in which the product is to be sold.

Sample site selection

Environmental monitoring programs are designed to provide periodic evaluations of environmental quality and system performance within classified environments where biopharmaceutical manufacturing occurs.

This is accomplished in part by conducting air, surface, personnel, and process utility monitoring at sample sites that are representative of those locations that:

- come in contact with exposed product and/or components

- are in close proximity to exposed product

- are areas of high personnel and equipment flow

- contribute to the particulate and microbial levels within an area (e.g. personnel, equipment, etc.)

- represent the most difficult or inaccessible areas to clean or disinfect

Collectively the resulting test data represent the systems' performance over time and provide the evidence to assess whether the systems are performing as intended for the production of quality products. Each manufacturing process should be carefully evaluated when selecting sample sites for a sampling plan. Representative locations should be identified through the use of a formal mapping process, based on analysis of risk, through smoke studies; and/or through data obtained from the Performance Qualification.

An important consideration is the extent of exposure or contact that each point in the biomanufacturing environment has with the product. Sites determined to have a greater opportunity for contaminating the product should be sampled and monitored. Critical test sites or potential product contact sites can include critical surfaces, manufacturing equipment, gloved hands of personnel, tools, and room air.

It may not always be practical, however, to select a sampling site at the most critical sampling locations. When selecting sample sites, consideration should be given as to whether the sampling location would actually increase the probability of product contamination (e.g. a sampling location that could violate the first air rule). It would also be impractical to have numerous critical site locations as extensive monitoring during aseptic processing alone would add unnecessary bioburden.

Routine test sites are non-product contact sampling locations that include locations such as floors, walls, doors, and ceilings. Sampling locations may not be monitored if there is a low probability of contamination during processing of those sites. Table 8-9 provides an example of recommended critical and routine sampling locations. It is important when developing a sampling plan to have a rationale for selecting each sampling location.

**Table 8-9. Example of critical test site and routine
test site locations and the rationale for selecting each**

Tests	Test Location	Rationale
Critical Sites (Laminar Flow)		
Microbial Air **Particulate Air**	Sample within approximately 1 ft. of the critical process point or product container opening.	Sample is to monitor the air where there is increased activity (mechanical or otherwise) at a location that is close to and representative of the air in contact with the open product.
Surface Contact Plate	Sample within approximately 1 ft. of (or at the nearest obtainable surface to) the product container opening or critical aseptic process step.	Samples are to monitor the critical surfaces where there is increased activity (mechanical or otherwise) at locations that represent surfaces close to or in contact with exposed product or product components.
Personnel Contact Plate	Sample gown in 2 locations (chest, forearm) and gloved fingertips of each hand.	Samples are to reflect the parts of the gowned and gloved person that are in the closest proximity to exposed product.

Table 8-9. Continued

Tests	Test Location	Rationale
Routine Sites (Non-Laminar Flow)		
Microbial Air **Particulate Air**	Sample in a central location in the room or otherwise representative location.	Sample is to reflect general area/room conditions which are representative of room conditions.
Surface Contact Plate	Sample on a representative wall, curtain (outside), door push plate/handle/push button, floor surface, or other frequently-utilized surfaces as determined by the sampling plan.	Sample is to reflect the general condition of the area surfaces.
Personnel Contact Plate	Sample gown in 2 locations (chest, forearm) and gloved fingertips of each hand.	Routine personnel testing is to ensure that gowning is performed properly and that glove disinfection is effective.

Sampling frequency

Environmental testing frequencies in the cleanroom vary depending on the room classification and the nature of operations. The testing frequency typically can be divided into two categories- per process testing and routine testing. For biopharmaceutical manufacturing, microbiological and particulate testing must accompany manufacturing. This testing is referred to as per process or critical site testing. A process is generally defined as a set of interdependent steps or manipulations that are conducted within the same set-up or process. It begins with the area set-up for aseptic processing and concludes at the completion of processing. Recommended monitoring frequencies for critical site testing are shown in Table 8-10. Routine monitoring, or monitoring not performed during processing, is generally performed on a weekly basis (at a minimum). Both per process and routine monitoring provide assurance that operations, cleaning, and HVAC systems are continuing to operate and perform satisfactorily and consistently.

**Table 8-10. Example of critical site test requirements
and frequency of testing for Class 100 areas**

Environmental Test	Class 100 – Critical Site Testing Frequency
Microbial Air (active)	once per process (minimum of 1m³ of air)
Microbial Air (passive)	one settling plate per designated location
Surface Contact Plate	minimum of one site per process
Product Contact Surface Contact Plate (Sterile Filling Only)	minimum of four product contact surface sites to be performed during filling prior to the breakdown of a line at the end of a fill; to include all component bowls and representative fill needles
Personnel Contact Plate	the fingertips of both gloved hands and the chest and forearm of personnel who perform aseptic operations or testing during processing
Particulate Air	minimum of once per hour per process (minimum of 1m³ of air)

Interpreting environmental test results and establishing action and alert levels

Environmental testing results fall into one of three categories (Figure 8-8)- **passing**, **alert level**, or **action level**.

- Passing result indicates that the test result for the sampling site was within acceptable established levels**.** Tests that do not pass are defined as environmental test excursions, which include alert and action levels. Alert and action levels are assigned for each room classification and each type of test performed**.**

- Alert level indicates a test excursion above the environmental norm for the site and is usually set at 10–50 percent of the action level. It is based upon historical statistical data for the sampling site and is typically re-evaluated on a yearly basis. An alert level highlights a potential problem.

- Action level is a test result that reaches or exceeds the area classification as established from industry/regulatory guidelines and/or a statistical analysis of historical data. An action level indicates a possible problem and requires that corrective action be taken. An investigation is conducted to determine the impact on the product and the root cause. Examples of environmental action levels for commonly performed tests are indicated in Table 8-11.
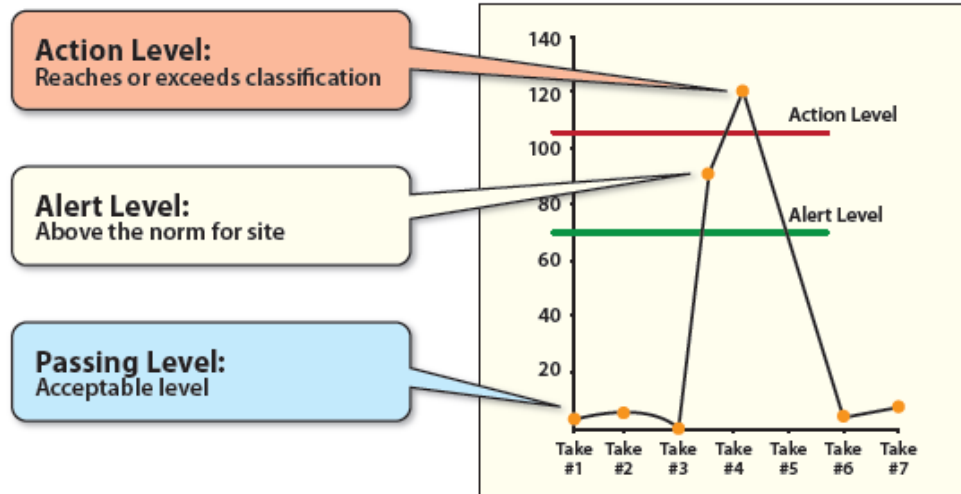
Figure 8-8. The three categories of environmental test results

**Table 8-11: Example of industry/regulatory guidelines
for action levels for commonly performed environmental testing**

Environmental Test	Class 100	Class 10,000	Class 100,000
Particulate Air ($\geq$ 0.5 microns/m³)	> 3,520	> 352,000	> 3,520,000
Particulate Air ($\geq$ 5 microns/m³)	> 29	> 2000	> 20,000
Active Microbial Air (Colony-Forming Unit or CFU/m³)	$\geq$ 1	> 10	> 100
Passive Microbial Air (CFU)	$\geq$ 1	N/A	N/A
Surface Contact Plate (CFU)	$\geq$ 1	> 5	> 25
Floor Contact Plate (CFU)	> 3	> 10	N/A

Table 8-11. Continued

Compressed Gas Particulate (≥ 0.5 microns/m³)	> 3,520	> 352,000	> 3,520,000
Compressed Gas Microbial (CFU/m³)	≥ 1	> 10	> 100

Microbial identification must be performed for all alert and action level results. The identity of the microorganisms found is useful in understanding the potential impact to product safety and the possible source of the contamination. For some categories of non-sterile pharmaceutical products, certain microorganisms are deemed as "objectionable" by the FDA and other agencies. These microorganisms include:

- *Burkholderia cepacia*
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Salmonella* species
- *Shigella* species
- *Staphylococcus aureus*

The organisms specified were chosen because they are either pathogenic (*Salmonella)*, indicator microorganisms for fecal contamination (*Escherichia coli*), or index organisms for poor production hygiene (*Staphylococcus aureus*). Biopharmaceutical parenteral products must all be sterile and thus any microorganism in the product must be considered as objectionable. In the low bioburden processes associated with the manufacture of biopharmaceutical drug substances, however, each identified microorganism must be assessed on its merits in the context of its potential downstream impact on the product and the possible routes by which it entered the production stream rather than by its presence on a list of objectionable microbes.

Environmental excursion investigations

Regulatory agencies require that an investigation be performed whenever an action level or a recurring alert level is identified from a sampling location. Investigations are performed to assess the state of environmental control during manufacturing operations in support of the product release process and the ongoing environmental control requirements for the classified areas. Investigations must identify the time, location, and conditions related to the sampling performed. Additionally, the result in terms of particulate count or microbial count versus the alert and action level criteria for the sampling location must be included in the investigation, along with the representative microorganism identification. This is so that a possible contamination source can be determined. The investigation should also include information

regarding the activities in progress during the environmental sampling, the previous cleaning/disinfection performed, and any potential causal events. When performing an investigation, transient events that occurred during sampling and sampling and testing problems are important points to consider. The investigation should determine a probable cause, unless it is determined that the excursion is a spurious result, where data do not identify a likely cause.

Other elements that should be included in an environmental excursion investigation are:

- review of maintenance or other activity in the area that may have contributed to the excursion

- review of environmental trending results of the sampling site for at least a two-month time period, looking for other environmental excursions or an adverse trend with environmental testing results

- review of environmental data from other sites and personnel tests in the room or system for the week in question. Alert and action levels from these sites should be included in the investigation report.

- identification of a likely source of the microbial isolate(s) found. A review of other sampling locations from which the same microorganism was isolated (to determine if there was any association among the sampling locations) should be performed. This may help to determine how the microorganism got into the cleanroom.

- performance of a physical inspection of the test site to reveal possible causes of the action level. This would include checking other environmental parameters as applicable (e.g., pressure differentials, air flow) to determine what effect any deviations could have had with the excursion.

- pertinent input and findings in the investigation from personnel involved with the process

- possible cause and investigation recommendations to the individual(s) responsible for the affected area

- corrective actions that are being performed at the time the investigation is still being conducted. Interaction with affected area personnel as needed to facilitate improvement of the site/area and to obtain follow-up on recommendations

Examples of some corrective actions that may be included as part of an investigation are:

- restricting activities that may increase the bioburden or the total number of microorganisms detected in or on an article

- increasing the area ventilation, room pressure, or quality of air delivered (e.g. HEPA filtration)

- testing of process HEPA filters and correcting leaks

- increasing or altering cleaning and disinfecting procedures and using a sporicidal agent

- conducting additional personnel training to reduce practices that may add bioburden

- increasing gowning requirements for the area

- restricting improper personnel flow from less clean areas

- improving facility surfaces (e.g., painting, eliminating rough and irregular surfaces, eliminating or covering hard to clean surfaces)

- containing process particulates (via hoods or design modifications)

- improving air lock access (e.g., both air lock doors not opened simultaneously)

- removing extraneous items from the area

Excursions that occur in locations that are critical to the biomanufacturing process could require more rigorous investigation and corrective actions than those occurring in locations that are less critical.

Environmental test results must be reviewed by a quality group to determine the suitability of a particular product lot for release to patients. Alert and action levels results are used to monitor and control aseptic process and should not be treated as product specifications for each manufactured product lot. The focus, therefore, should primarily be on trending environmental data and a thorough investigation rather than individual testing results.

Trending environmental monitoring test results

Although non-viable environmental test results are available in real-time, the results of microbiological environmental monitoring are generally not available until five or more days after sampling. Product is often filled and even packaged by the time microbiological environmental results are available. However, near real-time analytical instrumentation is gaining in use and reducing the time delay in identifying bacteria.

One way in which to combat such delays is to rely on other means for identifying potential problems or issues. Other systems, such as shift and trend analysis of environmental data must be established to enable efficient assessment of control and to allow statements to be made regarding the likely state of cleanliness of locations within the cleanroom. The particulate air, microbial air, and microbial surface data for each monitored cleanroom site should be evaluated regularly for the detection of shifts and trends. This evaluation, which involves plotting the data as graphs, provides for an early warning of a location's potential inability to adhere to previously established environmental conditions.

A shift is defined as the tendency for the most recent six-month period to have either higher or lower results than the previous six-month period. A statistically-significant shift means that there is strong evidence from the data that the shift is real and not due solely to chance variation in the results. A trend analysis, on the other hand, is concerned only with the data from the most recent six-month period. A trend is defined as a tendency for the data to exhibit a continuous movement, either upward or downward, over a six-month period. A statistically-significant trend means there is strong evidence from the data that the trend is real and not due solely to chance variation in the results. Provided there are sufficient data available, trends toward deteriorating conditions can be detected reliably. Operating parameters within the

cleanroom, such as temperature, humidity, air flow, and pressure differential between rooms are typically trended hourly. The data are reviewed on a periodic basis to provide additional assurance that cleanroom operations and maintenance are performing consistently and to assure that the appropriate data are available for investigational purposes.

Test methodology

Environmental monitoring is accomplished using a variety of equipment, such as airborne particle counters for non-viable particles and active air samplers for microbial detection. Any movement of the environmental monitoring equipment between production areas must be controlled to prevent cross contamination. Sample takers must disinfect all equipment prior to use. Ideally environmental monitoring equipment should be dedicated to a specific production area in a cleanroom and not moved to other production areas. Simpler methods of environmental monitoring, which require no sampling equipment, include the use of direct contact plates and settle plates for microbiological sampling.

Environmental testing is normally performed under dynamic conditions. Dynamic, or "in operation," testing confirms that the environment remains under control during processing with personnel and activities in a normal operational mode. Static, or "at-rest," testing is typically only performed as part of the initial Performance Qualification or requalification of a cleanroom. Static testing ensures that the facility environment continues to perform as designed, with no personnel present and no activities in progress.

Microbiological testing requires the use of growth media to demonstrate the recovery of microorganisms from sampling, while non-viable testing does not require the use of growth media. A general growth medium such as soybean casein digest agar (i.e., tryptic soy agar, TSA) is used in Petri plates for microbial air and microbial surface monitoring. Where the media is used for surface contact, the plates should contain a disinfectant neutralizer, such as polysorbate 80 for phenolics or lecithin for quaternary ammonium compounds. The growth promoting quality of the culture media must be demonstrated as part of the normal laboratory quality control. Various guidance documents suggest incubation times and temperatures.

The following example is an acceptable method used in the United States. After sampling, the microbial plates are incubated at 20–25°C for 72 hours, followed by incubating the plates at 30–35°C for 48 hours. At the end of this five-day time period, the plates are examined for microbial growth and compared to established values for sampling locations. Those sampling sites exceeding action and alert levels require identification of each microorganism recovered from testing. Any method used for sampling and testing must be validated. The sample must be reflective of the sampled location. Aseptic techniques must be used in the sample collection. The samples must then be promptly transported to the lab for microbiological testing. Furthermore, sampling must be performed in a way that has no adverse effect on the aseptic process being monitored. It is important to note that the sample taker and the equipment are always potential sources for product contamination.

Air monitoring

Research has shown that airborne contamination (versus surface contamination) has the highest potential for adversely affecting the product quality. It is for this reason that air monitoring is routinely conducted for both microbial and non-viable contamination in cleanrooms.

Microbial air monitoring

There are two general methods by which microbial air testing for viables is performed in the cleanroom environment by active microbial sampling and passive microbial air sampling. Active microbial air sampling requires the use of equipment, typically requiring a vacuum source to draw air over the surface of an agar plate. Passive microbial air sampling does not require any specialized sampling equipment and viables in the air fall on an agar plate through sedimentation. Neither method provides real-time results, however, as incubation of a growth medium is necessary.

The standard unit of measurement for microbiological monitoring is the Colony-Forming Unit (CFU). Active air microbial measurements are stated as CFUs per cubic meter. Passive microbial air sampling measurements are stated as CFUs/4 hours or per unit of time period as dictated by process duration and/or operational factors. Test results for each of these methods of microbial monitoring are available after a minimum five-day incubation of test plates.
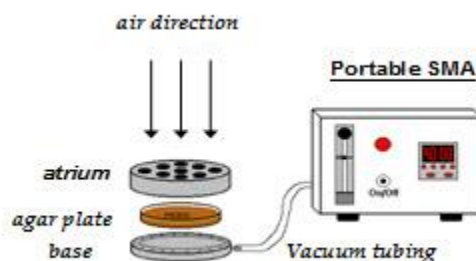
Active microbial air monitoring

Active microbial air monitoring is the preferred approach for classified areas due to the greater likelihood of obtaining actual microbial counts. Active sampling devices draw both air and any microorganisms contained in the air into the instrument. The air is diverted over a culture medium and any microorganisms are deposited onto it. Due to this active mechanism, the sampling duration is less than that of a passive air sampling. The volume of air sampled should be adequate enough to provide counts in most samples. This is normally one cubic meter in Class 100 and 10,000 areas and one cubic foot in Class 100,000 areas. Active airborne microbial air samplers must be calibrated to manufacturer's specifications on an annual basis.

There are a variety of samplers/methodologies used in the biopharmaceutical industry for active microbial air sampling. All the samplers measure known volumes of air, allowing quantification of microbial contaminants by unit volume of air. The selection of a sampler or methodology should address the volume of air that can be sampled, the availability of culture media plates, and the level of technical and vendor support. Following is a brief description of some commercially available active microbial air samplers/methodologies. This list is for informational purposes only and not intended to be all inclusive. It does not constitute endorsement or support of any vendor/product.

- Sterilizable Microbiological Atrium (SMA): The SMA uses a simple stainless steel chamber (Figure 8-9). The unit's cover, or atrium, contains uniformly-spaced orifices. The base accommodates one standard 100mm agar plate. Air is drawn through the unit

by vacuum and allowed to impact the agar surface. Units are available that use a house-vacuum source, while other models use a self-contained vacuum source.

**Figure 8-9. SMA is one method used
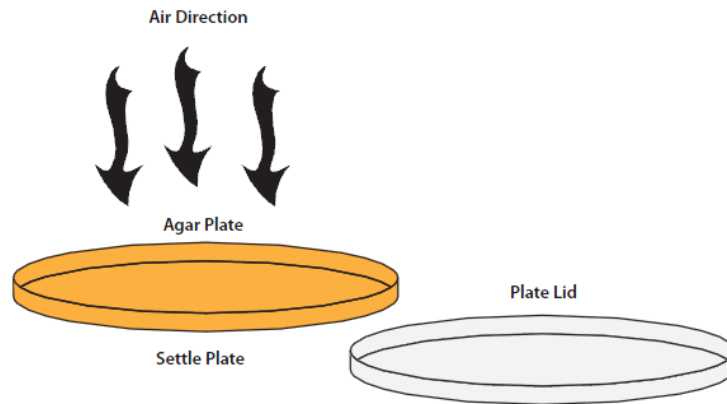for active microbial air sampling**

- Slit-to-Agar (STA): with the STA sampler, a known volume of air is drawn in by a self-contained vacuum pump through a standardized slit. Below the slit is a slowly revolving agar plate. Particles in the air are drawn through the slit and impact the agar surface. The STA machine requires a large 150 mm agar plate.

- Centrifugal Air Sampler: the centrifugal air sampler, such as the Reuter Centrifugal Sampler (RCS), consists of a propeller or turbine that pulls in a known volume of air then propels the air outward to impact on a tangentially-placed nutrient agar strip.

Each of the above active microbial testing methods requires an agar incubation period. The agar plate or nutrient agar strip is incubated for a minimum of five days. Colonies of microbial growth, bacteria, and fungi are then counted and expressed as CFUs.

Passive microbial air monitoring

Passive microbial air monitoring uses settling plates, or exposure plates. The process involves exposing agar-filled Petri dishes to the environment. To perform the test, a 100 mm agar plate is placed in the sampling location (Figure 8-10). The lid of the agar plate is removed and placed in a location to ensure the lid itself does not become a contamination vector (edges down and on a freshly disinfected flat surface). Airborne microorganisms randomly settle by sedimentation on the agar surface during a specified time period. Exposure times of settle plates greater than four hours should be avoided so that the media does not dry out.

Although using settling plates best reflects the number of CFUs that naturally settle on surfaces by gravity, it is qualitative rather than quantitative in nature. This may mean lower count results in classified areas. Therefore, settle plates are used to supplement active air sampling. This method provides more data to support the demonstration of control within an aseptic processing area and can also be used in areas where active air sampling is not feasible. Settle plates can also be useful if continuous monitoring is required (e.g., close to the product's exposure position).

Figure 8-10. A settle plate is used for passive microbial air sampling

Non-viable particulate monitoring

Area classifications within cleanrooms are generally based upon the level of non-viable particulates in the air. Unlike microbial air monitoring, non-viable or particulate air monitoring provides real-time data on the environment and is a useful tool to demonstrate that the environment remains in a state of control with respect to particulate contamination.

It is difficult to draw a direct correlation between particulate monitoring data and microbial contamination—a high particulate count for a specific testing location does not mean that there will be a concurrent high microbial count at the same location. Airborne Particle Counters measure particles in a number of size ranges then display the number of particles in each range as a cumulative count or differential count. Although monitoring non-viable particles in different size ranges may seem practical, particles of 0.5 microns and larger are generally recognized as indicators of environmental contamination. Particulate monitoring for products used outside the United States may also include the need to monitor 5-micron particles.

There are numerous manufacturers for airborne particle counters, however, the sampler/methodology is very similar. A commonly used non-viable particle monitoring method is optical particle counting in which the vacuum pump of the particle counter pulls sample air into the sensor through the inlet nozzle. Particles in the sample pass through the optical detection view where a laser light source is concentrated. Particles scatter the laser light, which is then focused onto a photodiode. The photodiode detects and converts the light signal to electrical pulses. The height of the electrical pulses is directly proportional to the particle size. The electrical pulses are counted and measured by electronics on a circuit board containing counter/threshold circuitry, a microprocessor (CPU), and communications circuitry. The microprocessor displays the count on the front panel as the total particle count in specified size ranges (Figure 8-11). Non-viable particles test results can be expressed as the number of particles ≥ 0.5 microns and ≥ 5.0 microns per cubic meter or cubic foot.
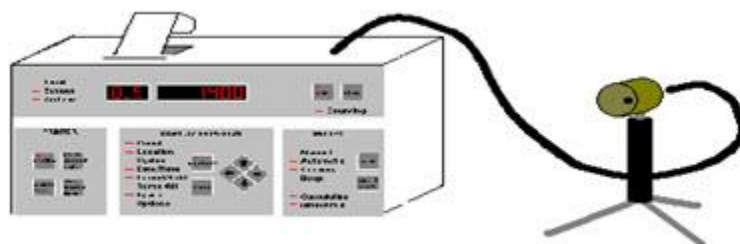
**Figure 8-11. Non-viable portable particle
counter displaying real-time test results**

Particle counters come in two general varieties, portable and permanent. Portable particle counters are easily transportable, allowing an individual to test one location at a time. Permanently-installed particle counters use dedicated sample probes that allow for remote sampling capabilities in biomanufacturing areas, thus minimizing equipment and sampling personnel in these areas. They also allow for automatic continuous particulate monitoring of the biomanufacturing process in multiple locations, provide for centralized data storage, and have alarm capabilities once particulate action or alert levels for a monitoring site are exceeded.

The minimum particle count sample volume is one cubic meter of air for Class 100 and Class 10,000 areas. For Class 100,000 areas, a lesser sample volume may be used, but should not be less than 1 cubic foot. Results are expressed as either particles per cubic meter or particles per cubic foot.

Surface and equipment microbial monitoring

Microbial monitoring of surfaces is performed either per process testing, routinely, or as an investigative tool. All techniques must be qualified using standard laboratory procedures to demonstrate the recovery capability of the method. Contact impression plate and swabbing techniques provide a means for direct microbial monitoring of surfaces.

Contact plates are Petri plates containing solid nutrient agar where the culture medium's convex surface extends above the walls of the base plate. A commonly used contact plate method is called Replicate Organism Detection and Count (RODAC). RODAC impression plates are specifically constructed so that an agar medium can be over-filled, producing a meniscus or dome-shaped surface that can be pressed onto a surface for sampling its microbial burden (Figure 8-12). RODAC plates are 24–30 cm^2 in area. During sampling the raised surface of the agar plate is gently rolled against the test surface. Once sampling is completed, the lid is placed back on the agar plate and the plate is incubated to determine the number of CFUs recovered (Figure 8-13). Contact plate measurements are stated as CFUs/plate.

agar plate

lid

**Figure 8-12. RODAC plate is a contact impression plate
used for microbial monitoring of surfaces**

Figure 8-13. RODAC plate showing colonies of bacterial growth

Swabbing is used for irregularly-shaped cleanroom surfaces, such as equipment surfaces, that are generally difficult to reach with contact plates. The swab should be pre-moistened with sterile culture media prior to sampling. Approximately four square inches of the test surface is swabbed by rolling it until all surfaces are in contact with the swab. Swabs are then submitted to the microbiology laboratory for processing. After collecting the surface sample by either contact plate or swab, the surface sampled must be immediately disinfected by the sample taker.

Microbial monitoring of gowned personnel

Since people are the greatest source of contamination in a cleanroom, proper aseptic gowning and aseptic technique is vital to ensure product quality. Personnel microbial monitoring serves as a tool to demonstrate that workers are adequately trained and following proper gowning procedures and aseptic technique. All individuals working in a cleanroom must undergo initial qualification testing to demonstrate proper gowning techniques prior to being permitted into a cleanroom. Routine periodic gown and fingertip microbial testing is performed on all personnel entering the cleanroom. This testing is performed at a greater frequency (usually based on the process) for those individuals involved with aseptic processing and for those who perform environmental monitoring. At a minimum, microbial sampling using contact plates should be done on the chest, forearm, and the fingertips of both gloved hands (Figure 8-14 and Figure 8-15).

Figure 8-14. Microbial personnel testing:
gloved fingertip RODAC testing

Figure 8-15. Microbial personnel testing: chest RODAC testing

Utility monitoring

During the Initial Qualification (IQ) of a process utility system, it is assured that the actual performance is consistent with the required performance. A system is put in place to assure that these performance criteria are met when used during biomanufacturing processes. Thus, all process utilities that have contact with the product must be monitored on an ongoing basis. Examples of the utilities used in cleanrooms include compressed gases, clean steam, and Water for Injection (WFI). Test data from each process utility system should be evaluated on an annual basis to ensure that required quality is consistently met, to characterize seasonal variations, and to reveal adverse trends. This section of the text will only focus on the methods used to monitor one of these process utilities -high quality water systems. The methods used for monitoring compressed gases for viables and non-viables are the same as those described in the previous sections for air monitoring. Clean steam is tested to the same criteria as high quality water.

High purity water system monitoring

Even though tap water is safe to drink, it should never be used to make sterile medicinal products or to clean equipment used in their manufacture. Although tap water (potable water) must meet the regulations for drinking water of the country in which it is used, it still contains numerous microorganisms and it is not considered high quality water. In the United States up to 500 CFUs of microorganisms per ml of water (with the exception of coliforms) are allowable in tap water.

Since a greater level of control is needed for water used in biomanufacturing, high purity water is used. One type of high quality water is Water for Injection, which is made by filtering and

distilling potable water (Figure 8-16). For more information of WFI, refer to the **_Facilities_** chapter.
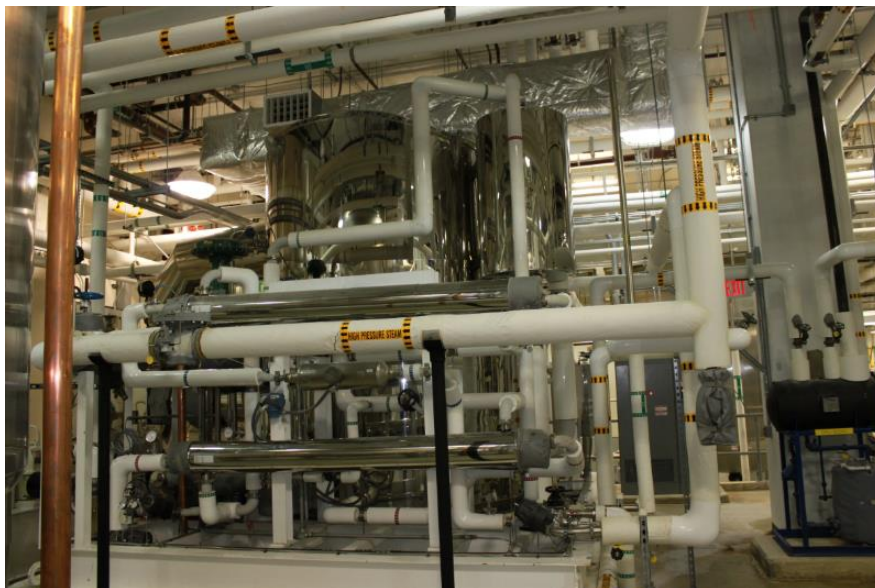
Figure 8-16. WFI system

The United States Pharmacopeia (USP) specifies that water used as an ingredient in the manufacture of sterile pharmaceuticals and for cleaning equipment must meet their WFI standards. Once a WFI system is validated for use in a facility and the WFI system is shown to be in-control, appropriate water samples should be taken periodically from the water system holding tank and distribution system. For water that is used as an ingredient, samples should be taken daily at the points of use. Water used for other purposes can be tested weekly. The tests must assess the microbiological quality (colony count, indicator microorganism, and endotoxin testing).

Regardless of the type of sampling or the frequency, water samples need to be collected in a manner consistent with appropriate biomanufacturing practices. For example, if points of WFI use are routinely flushed prior to use, it is appropriate for water samples to be collected with the same flush cycle. If points of use are not normally flushed prior to use, there should be no flush prior to water sample collection. If biomanufacturing practices require the use of hoses, the sample should be taken from hoses and not directly from the point of use. If the WFI sample cannot be processed immediately for microbial testing, the sample needs to be refrigerated at 2–8°C .

The minimum quantity to be aseptically collected in a sterile container for WFI microbial testing is 200 mL. Multiple containers are required for collecting WFI samples, as samples for other testing, such as endotoxin and chemical testing, are collected concurrently. Water from some sampling sites may be extremely hot, so to prevent the sample taker from getting burned, the appropriate safety equipment (protective gloves, apron, etc.) must be worn.

To perform microbial colony counts on WFI, a plate count is performed by membrane filtration of the water sample with subsequent incubation of the 0.45 micron membrane filter on a low-nutrient agar. Incubation time of the nutrient agar is a minimum of five days at 30–35 degrees Celsius. Representative colonies from all action and alert level growth need to have microbial identification tests performed. Membrane filtration with selective culture media is used to screen WFI for objectionable bacteria such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and coliforms. When a WFI sampling site test fails, it must be investigated to determine if product quality is in jeopardy. A failing WFI site test result can result in product quarantine, at the very least, and could result in product discard.

Media fills

In addition to monitoring the cleanroom environment through an appropriate environmental monitoring program, media fills of each process must be performed. The purpose for performing media fills is to demonstrate the capability of a specific aseptic process to produce sterile drug products, to qualify or certify aseptic processing personnel, and to comply with regulatory requirements. A media fill is a simulation of an aseptic process that uses microbial growth-promoting culture media in the place of actual product. In new cleanroom facilities, or when an aseptic process is developed, it is generally necessary to qualify the microbiological status of the process by running at least three consecutive media fills. For existing facilities or processes, a routine media fill should be conducted on a biannual basis for each type of aseptic process performed. It is important that the conditions that would normally occur during the aseptic process (e.g., full complement of personnel present, all processing steps performed) be included as part of the media fills. The resulting containers of media are incubated for a minimum of 14 days to ensure no microbial growth. Any growth in the containers, along with any microorganisms recovered during the environmental monitoring performed during the media fill, is identified in order to aid in the investigation of the sources of contamination. Failure to pass a media fill requires an investigation and additional satisfactory media fills before processing of product can either begin or continue.

Microbial identification

Identification of microorganisms recovered during microbial monitoring is an important component of the environmental monitoring program. Identification of microorganisms isolated from environmental monitoring should start with a pure culture. Normally, distinct colonies are sub-cultured from the original sampling culture medium to a nutrient agar using the streak plate method. Once a pure culture of a microorganism has been isolated, the microorganism is examined microscopically to determine its morphology and response to the Gram stain.

Following microscopic examination, colonies from the pure culture of the microorganism are identified using either phenotypic methods (i.e., observable physical or metabolic properties) or genotypic methods (i.e., characterization of some portion of a bacterium's genome using molecular techniques for analysis of DNA or RNA). All microorganisms isolated from Class 100 and Class 10,000 areas must be identified at the genus and species level. Microorganisms from Class 100,000 areas should be characterized at least to Gram stain reaction and microscopic

morphology. Identification of mold from all sampling sites at the genus and species level is typically performed by both macroscopic (i.e., visual description of colony) and microscopic methods. Periodically all microbial growth (not only excursions but passing level sampling as well) should be identified to the genus and species level to ascertain the microbial flora of the cleanroom. A change in the microbial flora or the introduction of a previously undetected species should be investigated, as this might signal a breakdown in established controls for the cleanroom.

Utilization of information derived from environmental monitoring

Environmental monitoring programs typically generate ample amounts of data on an ongoing basis. It is not uncommon to have hundreds or thousands of environmental monitoring results in one week. The amount of data will typically be a function of the size of the facility and the level of production activity. These data are gathered in a highly programmed manner, based on environmental monitoring SOP requirements.

Once the data is gathered (generally the data is gathered and interpreted by the specialists in the facility's QC Microbiology lab), the information must be converted into knowledge, which then needs to be actively managed and communicated. It is vital to understand what is happening to the microbiological profile of the facility and this knowledge needs to be communicated to people at various levels in the organization, especially if any subsequent action is required. The data must be monitored to ensure that the area either remains in control or regains control if previously lost.

The pattern of data is an outcome of the actions and activities of all those who work in the facility, whether they are production personnel, warehouse personnel, or various support personnel (e.g., engineers). Part of the Knowledge Management (KM) task is to ensure that the right personnel receive the right information/knowledge at the right level of detail so their actions can be productive. The practice of Knowledge Management was formally introduced into pharmaceutical manufacturing in ICH Q10 and involves acquiring, analyzing, storing, and disseminating information (Figure 8-17). According to ICH Q10, KM and Quality Risk Management are concepts that "will enable a company to implement ICH Q10 effectively and successfully" and "will facilitate achievement of the objectives described in Section 1.5 above by providing the means for science and risk-based decisions related to product quality."
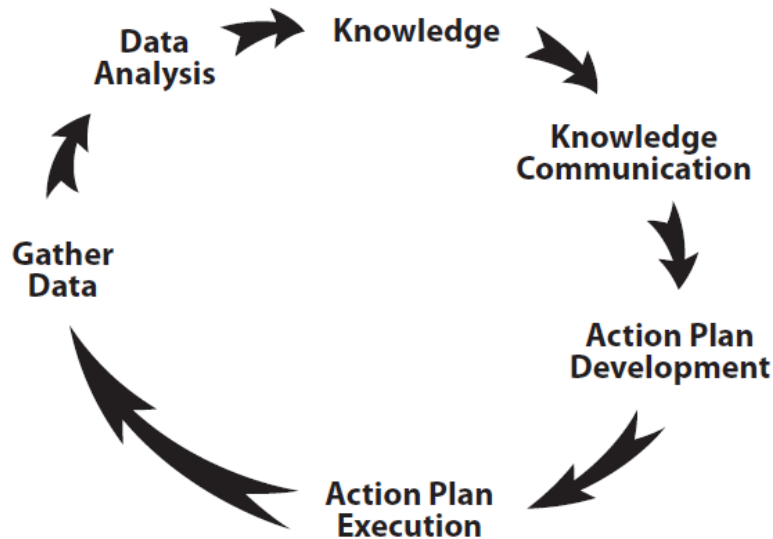
Figure 8-17. Knowledge Management methodology

ICH Q10 describes the expectations regarding Knowledge Management as follows:

"Product and process knowledge should be managed from development through the commercial life of the product up to and including product discontinuation. For example, development activities using scientific approaches provide knowledge for product and process understanding."

It defines KM as:

"...a systematic approach to acquiring, analyzing, storing, and disseminating information related to products, manufacturing processes, and components. Sources of knowledge include, but are not limited to, prior knowledge (public domain or internally documented); pharmaceutical development studies; technology transfer activities; process validation studies over the product lifecycle; manufacturing experience; innovation; continual improvement; and change management activities."

In environmental monitoring, Knowledge Management plays a vital role in the collection and dissemination of information, and in order for the process to be successful, the following questions should be considered:

- What is the data?

- What does the data mean (i.e., what knowledge can we obtain from the data)?

- How is this knowledge owned by the area management?

- How are those who work in the area kept informed of the requisite knowledge?

- How does one monitor the effectiveness of the actions undertaken and have effective investigations in this area?

Quality Control Practices in the Microbiology Laboratory

A quality control program is essential in ensuring reliable, reproducible, and accurate test results in the microbiology laboratory. Quality control practices help to both assure effective laboratory operation and avoid delays in issuing test results. Both governmental agencies and industry professional organizations provide guidelines for developing and maintaining QC programs. And though many expendable supplies procured from commercial sources have been tested for quality control by the manufacturer, in many cases a repeat assessment must be performed in the microbiology laboratory prior to using the supplies. The amount of time allotted to QC will vary with the size of the laboratory and the variety of procedures employed.

While there are a variety of facets to a QC program, many of which are discussed in the ***Quality Assurance*** chapter, the following section will focus on two key aspects that are unique to microbiology laboratories - the quality control of microbiological culture media and the maintenance of microbiological stock cultures.

Quality Control of microbiological culture media

The quality of the work performed in a microbiological laboratory depends in part on the quality of the culture media. The importance of maintaining the quality of the culture media cannot be overstated, as there are few things in the microbiology laboratory that will lead to problems with every aspect of laboratory operations. Culture media are either at or near the top of that short list.

The three primary aspects of the quality control of culture media used for microbiological product sampling and environmental monitoring are:

- the control of the culture media preparation and storage conditions
- the physical and chemical characterization of culture media
- growth promotion testing

Each laboratory must have procedures in place to ensure that culture media that have not undergone quality control testing are not used for microbiological testing procedures. Ideally this would best be accomplished by having a separate storage room for tested and non-tested types. This can also be accomplished through tagging quarantined materials and placing them in a clearly identified area within the same room. All quality control checks on the quarantined culture media should be completed before its documented release for general use. Storage conditions of the quarantined culture media should match those of the released media.

Control of culture media preparation and its storage conditions

The culture media that microbiology laboratories use are obtained in one of two ways - either through producing the culture media in-house or by purchasing the culture media pre-made from a vendor. These preparation methods must be considered separately.

Culture media prepared in-house offer several opportunities for quality control. The raw materials (either the dehydrated complete media or the components) must be stored under appropriate and controlled conditions and used within established expiration dates. The compounding of the media must be controlled to ensure the media is prepared correctly to prevent such errors as incorrect weighing, incorrect water measurement, improper mixing of ingredients, and use of deteriorated stock powders. Agar media, for example, must be pre-warmed to dissolve the agar prior to sterilization but not heated so extensively as to damage any heat-labile components. Also, the sterilization procedure must be under control. This means using a validated autoclave cycle shown to hold the media at 121°C for at least 15 minutes. Each batch of culture media made should be clearly labeled to allow for unambiguous audit of each stage of preparation.

For culture media that is purchased from a vendor there is little opportunity to control the preparation. However all media used should be checked for physical and chemical parameters and growth promotion. As with in-house culture media, commercially-prepared media must be stored under controlled conditions to ensure its quality through its expiration date. Key conditions to be controlled during culture media storage include temperature, humidity, and exposure to light. Although these factors may not be a concern for all culture media, they can be a concern for certain types. For example excesses of heat and cold, in particular, must be avoided for all types of media, while exposure to light will only affect certain ones.

There is variance in the expiration dates of culture media as well. The expiration date for in-house culture media must be established by the laboratory and the expiration date for commercially produced culture media is set by the manufacturer. Furthermore, there are specific regulatory requirements regarding the expiration of the culture media used for certain laboratory tests.

One of the most common problems with culture media storage is the failure to take adequate steps against dehydration of the media. Dehydration should not be an issue with liquid or solid media kept in well-sealed containers but can potentially occur when agar plates prepared in-house are stored, as they are typically not placed in sealed containers after preparation. Media that show obvious signs of dehydration (e.g., separation from edges or cracked surfaces) must be discarded. Dehydration can be significantly reduced by sealing agar plates in plastic bags immediately after preparation. The packaged culture media plates should then be stored in a refrigerator.

Control of physical and chemical characteristics

The initial goal of evaluating the physical and chemical characteristics of culture media is to rapidly screen the culture media for acceptability before investing time in labor intensive tests such as growth promotion testing. If the physical and chemical characteristics do not meet the specified requirements of the culture media, the culture media should be discarded. When a new batch of media is prepared in-house or received from a commercial vendor, it should first be examined visually for clarity and color. Unless the media contain a normally insoluble component, the presence of a precipitate or turbidity indicates that a component of the media has come out of solution. If the precipitate goes back into solution when the culture media is brought to temperature by incubation, the media is satisfactory to use. Otherwise it should not

be used. The color of the media should then be examined and a decision should be made as to its correctness. It should also be checked for any crystal formations or variations in color. The pH of the finished culture media is another critical attribute to confirm. The containers of media should also be thoroughly examined for cracks or defects and all defective units discarded.

Growth promotion testing

It is required that each new batch of culture media either prepared in-house or received from a commercial manufacturer be selectively tested with compendial microorganisms to ensure that the culture media supports their growth. In addition to compendial microorganisms which are required for the growth promotion testing by regulatory agencies, other stock microorganisms, such as those frequently recovered by a specific microbiology laboratory in its testing of a facility, should be included as growth promotion testing as well.

One commonly used method for growth promotion testing is the Miles and Misra method, also known as the surface variable count method. In this method, culture media plates are divided into sectors, and drops of serial dilutions of bacterial suspensions are deposited into the separate sections. The culture media plates are incubated for a minimum of 18 hours at 37°C and observed for bacterial growth expressed as CFUs. If there is either no growth or insufficient growth, the batch of culture media must not be used.

Maintenance of microbiological stock cultures

Along with culture media quality, preserving the stock culture microorganisms is also vital to the success of a microbiology laboratory. Since lab procedures require a pure culture, stock culture microorganisms must be handled carefully at all times to avoid contamination.

The care of the stock culture microorganisms begins upon receipt. A careful stock culture keeper in the laboratory will confirm the identity of the received cultures even those received from a respected source (e.g., a national culture collection), as mistakes can occur with even the most reputable of providers. The use of an incorrect strain in a compendial test has the potential to bring the results of weeks or months of work into question.

Standard strains of microorganisms that conform to the typical morphological, physiological and biochemical characteristics of species represented are needed not only for quality control testing of culture media but also for reagents and various other microbiological procedures. The strains used for stock cultures possess sufficient stability to display these characteristics reproducibly if they are maintained under proper conditions.

There are several methods commonly used for maintaining stock cultures (including simple ones like storing the culture media at the right temperature—either room temperature or in a refrigerator as necessary) and a sufficient stock culture collection can be maintained at little expense and requires relatively little lab personnel time. The culture media used to maintain the stock cultures must be chosen carefully for their ability to maintain the stability and viability of microorganisms over long periods of time without permitting excessive growth or metabolic activity. There are also more complex methods for maintaining stock cultures that require special equipment, such as lyophilization or freeze drying. Lyophilization is particularly effective

for long-term preservation of most microorganisms and most can be preserved indefinitely with this technique.

Barrier Isolation Technology

There is an intrinsic incompatibility between human beings and aseptic processing. This means that there will always be the risk of product contamination from people involved with performing the processing unless the two are completely separated. Barrier isolation technology can accomplish this.

Barrier isolators are airtight enclosures constructed of an impervious material, such as transparent PVC, that protects an internal Class 100 environment from the outside uncontrolled environment. Processing operations within this barrier are conducted either through remotely controlled machinery or from the outside, with individuals wearing half body suits or rubber arm length gloves. Although many organizations in the pharmaceutical industry still rely on aseptic processing in cleanrooms, some are moving toward increased use of barrier isolation technology. It is outside the scope of this chapter, however, to address this technology in more detail.

Check Your Knowledge

1. Contaminants can be introduced into the product by:

   a. equipment
   b. people
   c. work surface
   d. all of the above

2. Microbial contamination is caused by:

   a. gown fibers
   b. dead skin cells
   c. bacteria
   d. dust
   e. all of the above

3. The term *aseptic* means:

   a. sterile
   b. the absence of microorganisms capable of causing disease or contamination
   c. gamma irradiation
   d. all of the above

4. _____ is an example of a non-viable particulate.

   a. yeast
   b. cellulose fiber
   c. bacteria
   d. mold

5. All of the following are effective means of sterilization except:

   a. steam
   b. gamma irradiation
   c. alcohol
   d. dry heat

6. Which of the following is a means of controlling contaminants in the cleanroom?

   a. aseptic gowning and utilization of aseptic technique
   b. sterilization of manufacturing components
   c. controlling the supplied air
   d. all of the above

7. RODAC testing is performed on:

   a. product
   b. fingertips
   c. gowned personnel and critical sites within the cleanroom
   d. b and c

8. Proper cleanroom conduct includes the following:

    a. disinfecting gloves upon using a BSC
    b. never touching a critical surface
    c. limiting talking
    d. all of the above

9. One of the basic principles of aseptic technique is that product and critical surfaces must see "first air" from HEPA filtered air. This means:

    a. only product and components should be under the BSC
    b. nothing must pass between the HEPA filter and the product
    c. all aseptic manipulations should be the first things performed for the day
    d. a new HEPA filter should be installed prior to performing aseptic manipulations

10. Which of the following is not a practice to keep biopharmaceutical products contaminant-free:

    a. careful visual inspection of all final product containers
    b. BSCs
    c. controlling the environment, employees, and equipment
    d. filtering prior to filling the final product containers

Activities

1. Working in groups of three, each person is to prepare three different samples of the environment. Using a sterile swab for each, sample two inanimate objects (e.g., the floor, a table top, a doorknob, a cell phone, etc.). Using a different nutrient agar plate for each sample, roll the swab over the surface of the nutrient agar plate. Next, expose a third nutrient agar plate to the room air by removing the lid from the plate for 30 minutes then replacing the lid. Ensure that you label each agar plate with the sampling location, your initials, and the date sampled. If you have a 37 °C incubator available, incubate the agar plates for 48 hours—otherwise leave the agar plates at room temperature. After 48 hours observe each of the three nutrient agar plates and record the number of microbial colonies, along with their color and size. What surfaces and what area of the room had the most microbial colonies? Write a paragraph explaining the variation in the number of colonies found at the different locations. Compare your group's findings with the rest of the class.

2. Perform microbial sampling of your fingertips by gently pressing four fingertips from your right hand on the surface of a nutrient agar plate. Repeat the sampling of your fingertips using another nutrient agar plate after washing your hands. Ensure that you label each agar plate with the sampling location, your initials, and the date sampled. If you have a 37 °C incubator available, incubate the plates for 48 hours. Otherwise leave the agar plates at room temperature. After 48 hours, observe both nutrient agar plates and compare your results with those of your classmates. What is the primary role of hand washing and or frequent disinfection of gloved hands? Were you able to demonstrate this with the activity?

3. Using the Internet, research the FDA website (*www.fda.gov*) and find two different regulatory inspections involving a deficiency (FDA 483) in the environmental monitoring program. Write a one-page paper summarizing these deficiencies as well as any actions that could have been taken to prevent them.