CELL THERAPY
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White Paper

Environmental Monitoring Considerations for Cellular Therapy Products

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Disclaimer:

I am an independent consultant. The opinions expressed in this paper are mine alone and should not be interpreted as the policies or positions of any other organization.

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INTRODUCTION

This paper provides considerations for environmental monitoring (EM) of controlled environments used for the manufacturing of cell therapy products with emphasis on products where microbiological control and risk of contamination is controlled by aseptic processing. Although not exclusively, emphasis is also placed on cell therapy products that are regulated and manufactured under cGMPs, or section 351 of the Public Health Service (PHS) Act. Nonetheless, some of the EM concepts herein may also apply to human cells, tissues, and cellular and tissue-based products HCT/P's regulated under section 361 of the PHS Act. [4] While several EM guidelines for aseptically processed products are available including from the FDA, USP, and EU Annex 1, these guidelines are predominantly written for pharmaceutical filling processes and do not specifically address some of the unique aspects present in manufacturing of cell therapy products.[1] [5] [7]

The Parenteral Drug Association (PDA) Technical Report No. 13 has defined that EM programs should meet three basic criteria. Namely, that EM programs should be meaningful, manageable, and defendable. [3] This paper attempts to address some of the unique considerations for setting up an EM program for cell therapy product manufacturing while maintaining these three basic criteria.

SETTING UP A SAMPLING PLAN

Controlled environments for cellular therapy processing areas will often contain equipment and or materials that are not found in pharmaceutical aseptic processing environments, such as biological safety cabinets (BSCs), cell culture incubators, centrifuges, and other materials that support this type of process manufacturing. Moreover, the cell therapy manufacturing process may contain several differences in terms personnel operations from a traditional vial filling campaign. Furthermore, due to critical timing and other constraints, cell therapy manufacturing areas may more resemble and operate like a laboratory rather than an a filling operation by requiring test equipment such as microscopes, flow cytometers or cell counters, ELISA and/or endotoxin plate readers, and hand-held pipettes in the same controlled area. These additional kinds of

equipment and manufacturing operations add further considerations in establishing an appropriate risk-based EM sampling program for cell therapy products.

The new revision to USP general chapter ⟨1116⟩ *Microbiological Control and Monitoring of Aseptic Processing Environments* advances the concept of performing a risk-based approach to setting up an EM sampling program. It states "*The user should conduct a prospective risk analysis and develop a rationale for the sampling locations and frequencies for each controlled environment*." Risk analysis tools such as HACCP or FMEA can be used for developing this sampling plan rationale. For example, human activity, entry doors and transfer pass-through boxes, proximity of operations to the product, and material/personnel traffic and flow patterns are some of the important control points to consider.

Sampling locations, specifically airborne samples, should initially be determined based on a grid profile of the controlled area depending on area size in square feet or square meters. There are different means to establishing the grid plan or layout, however, a common approach is to utilize the particulate air monitoring calculations from ISO-14644-1 for controlled area classification.[(2)] Further considerations may be given to the location of HEPA filter units, exhaust air returns, and location of equipment and resulting air flow patterns.

Air sampling site locations should be evenly distributed throughout the environment except as limited by equipment. According to ISO 14644-1 the minimum number of sampling sites is determined by calculating the $\sqrt{A}$ where A is the area of the clean room in square meters. In cell therapy processing the critical area, due to the nature of the product, is typically the BSC not a dedicated laminar flow filling area or zone. For an ISO 5 (Grade A) BSC that has a clean zone area of less than 1 m^2 (e.g. 4' wide BSC), one site meets the ISO classification requirements, however, one may choose to add sample sites during equipment qualification activities. A 6' wide BSC may require up to 2 air sample sites to meet the ISO requirements depending on the whether the hood model has a calculated work area of >1 m^2. Table 1 provides ISO 14644 -1 calculations for an example cell processing facility shown below in Figure 1.

Table 1. Number of Airborne Particulate Monitoring Sites

Area	Class	Area, m^2 (A)	$\sqrt{A}$	Minimum no. of sites[1]
Gowning Area	ISO 8	8	2.8	3
Cell Processing Area	ISO 7	30	5.5	6
Biological Safety Cabinets (~4' wide BSC)	ISO 5	0.8	0.9	1

[1] Rounded up to the nearest whole number.

The ISO 8 gowning area and ISO 7 cell processing areas are divided into grids based on the ISO 14644-1 calculations. In Figure 1 there are three sampling grids for the ISO 8 gowning area and six grids for the ISO 7 cell processing area based on the minimum number of sites required.

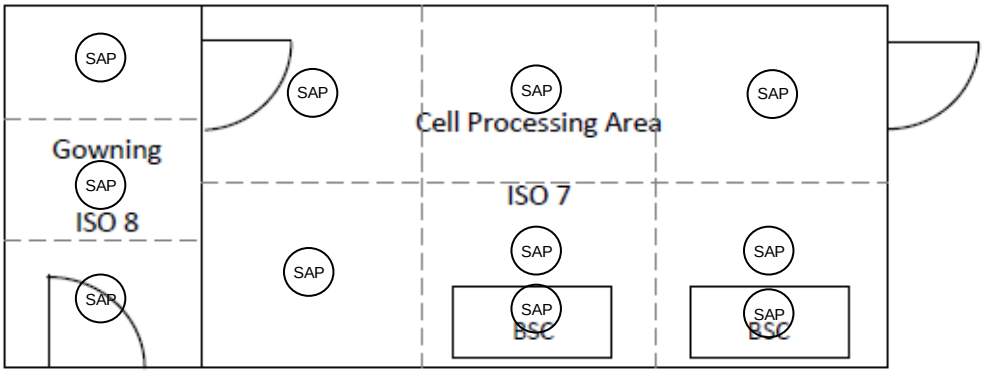

Figure 1. Cell Processing Facility Floor Plan

To begin with EM sampling locations for airborne non-viable particulates (P) are assigned in the approximate center of each grid except as limited by the location of equipment. For convenience with sampling plans and SOPs, air viable samples (A) are assigned at the same locations as non-viable particulates. Furthermore, surface viable samples (S) are assigned on the facility floors to be coincident with the air sampling locations to evaluate surface sanitization effectiveness. Depending on location of the BSCs with respect to the grids, ISO 7 sample locations in the BSC grid may be located near or in front of the BSC opening as the most appropriate risk-based location.

Other surface sample locations are also assigned utilizing risk-based assessments. A series of questions can be asked to help aid in making these assessments. For example: *What locations are in close proximity to processing activity? What sites or equipment are contacted by personnel or gloves? What sites represent the most difficult areas to clean and disinfect? Where is the greatest amount of activity? What are the material and personnel flows? Where are the entry points where materials transfer from lower to higher classification?* With these questions asked, several additional sampling sites in a cellular therapy processing area can be considered. In this facility example from Figure 1, additional surface sampling sites have been added and are shown in Figure 2.

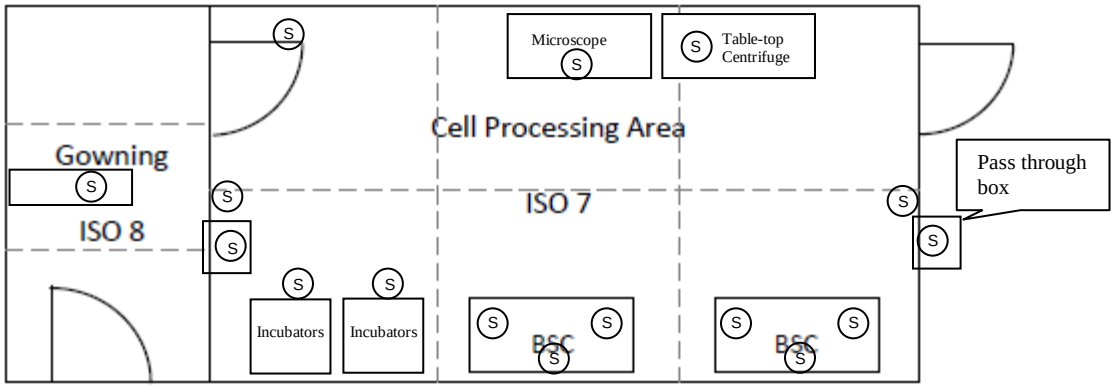

Figure 2. Cell Processing Facility with Additional Surface Sites

The additional surface sampling sites depicted show typical equipment and furniture such as a gowning bench, work benches with an inverted microscope, and a table-top centrifuge. Other sites depict clean room walls, an entry door handle and a wall phone that are contacted by personnel gloves, cell culture incubators, and pass-through boxes to transfer materials or samples in or out of the area.

For the BSCs which are the most critical area where cell product is exposed, open manipulations and/or aseptic connections occur, surface sampling sites are performed in close proximity to operations and include sampling of BSC workbench surfaces and walls.

Although not shown in the drawing, surface monitoring of personnel gloves and other gown locations such as arms that enter the BSC are also a part of an EM sampling plan. Personnel monitoring is typically performed at the conclusion of process operations.

SAMPLING FREQUENCIES

USP ⟨1116⟩ provides guidance on sampling frequencies for aseptic processing areas. Using this as a general reference, an example for sampling frequency for aseptic cell processing is shown in Table 2.

Table 2. Sampling Frequencies for Aseptic Processing Areas

ISO 5 critical area (BSCs)	**Frequency and Timing**
Air Sampling	Continuous during processing
Surface Sampling	At end of processing
Personnel Monitoring (e.g. gloves, arms)	At end of processing
ISO 7 Cell Processing Area	**Frequency**
Air sampling	During processing [a]
Surface sampling	At end of processing [a]
Personnel Monitoring (e.g. facemask, chest)	At end of processing
Adjacent ISO 8 Support Area	**Frequency**
All Sampling (except personnel)	Once/week

[a] Sampling may be isolated to those locations in close proximity to BSC and possibly reduced based on historical data.

Not all sites need to be sampled during each process. One may devise a sampling system whereby multiple points can be sampled in a random or rotating order such as the interior versus exterior of incubators, or the work tables versus equipment that personnel contact with their gloves.

MEDIA AND INCUBATION CONDITIONS

Typically, general purpose microbiological growth media such as soybean-casein digest medium (SCDM), also referred to as tryptic soy agar (TSA), is used for cultivation of a wide range of bacteria and fungi. Industry guidelines including the FDA aseptic guidance, USP informational chapter ⟨1116⟩, and the World

Health Organization (WHO) [9] offer similar media and incubation conditions but are not completely harmonized. For example, the FDA guidance for sterile products produced by aseptic processing states the following:

> "*The microbiological culture media used in environmental monitoring should be validated as capable of detecting fungi (i.e., yeasts and molds) as well as bacteria and incubated at appropriate conditions of time and temperature. Total aerobic bacterial count can be obtained by incubating at 30 to 35°C for 48 to 72 hours. Total combined yeast and mold count can generally be obtained by incubating at 20 to 25°C for 5 to 7 days."*

USP chapter ⟨1116⟩ states the following:

> *"Time and incubation temperatures are set once the appropriate media have been selected. Typically, for general microbiological growth media such as SCDM, incubation temperatures in the ranges of approximately 20°–35° have been used with an incubation time of not less than 72 hours."*

The WHO draft document "Points to Consider for Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities" states the following:

> *"Environmental monitoring samples should be incubated at a minimum of two temperatures to detect both bacteria and fungi. In practice, the use of 3 to 5 days of incubation at 20 to 25 °C followed by incubation 30 to 35 °C for an additional 2-3 days has been shown to be sufficient to detect most bacteria and fungi."*

So with these differences, what is the best incubation scheme for cell therapy manufacturing EM samples? An important point in answering this question is to consider the cell product shelf-life. The guidance documents were written for traditional aseptically manufactured pharmaceuticals and not specifically for cell therapy products that may have a very short shelf-life. Therefore one may consider an incubation scheme that will provide valid EM results in the least amount of time in order to promptly respond and perform corrective measures if excursions were to occur. One strategy is to first incubate at the higher temperature range of 30-35°C for a minimum of 48 hours per FDA guidance, perform CFU enumeration, then return plates to incubation at 20-25°C for another 3-5 days for recovery of slower-growers or yeast and molds. If short product shelf-life is not a factor, the two temperature incubation approach per the WHO guidance is commonly

used. Regardless of what approach is used, each facility should validate their incubation scheme by performing growth promotion studies using a low-level CFU challenge (e.g., < 100 CFU of *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus niger* QC strains). Furthermore, a range of expected or "representative" microorganisms that could be encountered in the manufacturing environment (e.g., Gram positive rods; Gram positive cocci; filamentous molds; and Gram negative rods) should be considered for qualifying and standardizing media and incubation conditions.

One may choose to add periodic surveillance (e.g. quarterly) for fungi using selective mycological media such as Sabouraud Dextrose Agar (SDA), or perhaps sampling for fungi when certain conditions warrant. Conditions that are warranted may be a repeated recovery of molds in the EM program; or where damp and dark conditions, or when potentially trapped or lingering moisture have been observed.

Monitoring for strict anaerobes or microaerophilic organisms in general is not routinely performed. However there are some scenarios where anaerobic monitoring is justified. For example, where the process creates anaerobic conditions or the product is packaged under anaerobic conditions such as a nitrogen overlay or purge (this is unlikely for cell therapy manufacturing processes), and when anaerobic organisms are found in product sterility testing. Some firms have performed monitoring for anaerobes during initial facility qualification start-up activities. Regardless, one must consider if monitoring for anaerobic or microaerophilic organisms, to incubate in appropriate conditions. For example, the skin contaminant *Propionibacterium acnes* is a slow-growing microaerophilic organism that grows best anaerobically. Therefore it is important to ensure samples are incubated for a sufficient duration (at least 5-7 days or more) to allow this slow-growing organism to grow and be visually observed.

MONITORING LEVELS

Environmental monitoring facility action levels vary in industry depending on local and regulatory guidance. Pharmaceuticals manufacturers where the product can be shipped to multiple regions throughout the world will typically require that EM levels be equal to the most strict regulatory guidance where the product is

distributed (i.e. the lowest alert/action levels). However, with cell therapy products that contain live cells, distribution is often limited to the local region due to the short product shelf-life. Therefore EM action levels, at least initially, should be established with respect to the guidance or regulations where the cell product is administered.

Aseptic processing facility cleanliness levels for the United States (FDA) and for the EU (Annex 1) are displayed in Tables 3 – 5. Although the guidance list criteria for all four classifications from ISO 5 to 8 (Grade A-D), a cell therapy manufacturing area commonly has three classifications consisting of an ISO 5 BSC, a surrounding ISO 7 clean room, and often an adjacent ISO 8 support areas such as gowning room or transfer airlock. An area classified as ISO 6 (Grade B) may not exist in cell therapy manufacturing. The concept of ISO 5 BSC surrounded by an ISO 7 zone is outlined in the USP informational chapter <1046> *Cellular and Tissue-Based Products*.[(5)]

Table 3. Airborne Cleanliness Levels (FDA Aseptic Processing Guidance)

Environment Classification	Particulate Levels (for particle size ≥ 0.5 µm)		Microbiological Levels	
			Active Air	Settle Plates
ISO Class	particles / m^3	particles / ft^3	(CFU / m^3)	CFU / 4 hrs
5	3,520	100	1 [a]	1 [a]
6	35,200	1,000	7	3
7	352,000	10,000	10	5
8	3,520,000	100,000	100	50

[a] Samples from critical environments should normally yield no microbiological contaminants.

Table 4. EU Annex 1 Airborne Particulate Classifications

Grade	At rest[a]		In Operation[a]	
	Maximum permitted number of particles/m^3 equal to or above			
	0.5 µm	5.0 µm	0.5 µm	5.0 µm
A	3,520	20	3,520	20
B[b]	3,520	29	352,000	2,900
C	352,000	2,900	3,520,000	29,000
D	3,520,000	29,000	Not defined	Not defined

[a]The particulate conditions given in the table for the "at rest" state should be achieved after a short "clean up" period of 15-20 minutes (guidance value) in an unmanned state after completion of operations.

Table 5. EU Annex 1 Microbial Contamination Recommended Limits

	Recommended limits for microbial contamination [a]			
Grade	**Active Air sample cfu/m^3**	**Settle Plates (diam. 90 mm) cfu/4 hours[b]**	**Contact Plates**	**glove print 5 fingers cfu/glove**
A	<1	<1	<1	<1
B[c]	10	5	5	5
C	100	50	25	-
D	200	100	50	-

Notes
[a] These are average values.
[b] Individual settle plates may be exposed for less than 4 hours.

USP chapter ⟨1116⟩ has redefined levels for microbiological EM samples using the concept of trending by contamination "incidence" rates rather than comparing results against low numerical CFU values. The contamination rate is a percentage defined as the number of samples that have any growth regardless of CFU count divided by the total number of samples, multiplied by 100. The main rationale behind this is to use a more scientifically sound means for trending microbiological EM data which is by nature is more variable. As a result of this inherent variability along with variability in sampling methods themselves, the differences between, for example, an alert level of 1 CFU and an action level of 3 CFU are not analytically significant. Moreover, these CFU ranges are below the limit of quantification for microbiological plate count assays therefore treating these values as numerically different is not scientifically valid. The suggested contamination rates for aseptic processing areas are shown in Table 6.

Table 6. Suggested Microbiological Recovery Contamination Rates

Environment Classification	**Active Air Sample**	**Settle Plate 4 h exposure**	**Surface Viable Contact Plate**	**Personnel Monitoring**	
				Gloves	**Gown**
ISO Class	**(%)**	**(%)**	**(%)**	**(%)**	**(%)**
5	< 1	< 1	< 1	< 1	< 1
6	< 3	< 3	< 3	< 3	< 3
7	< 5	< 5	< 5	< 5	< 5
8	< 10	< 10	< 10	< 10	< 10

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

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As stated previously, USP ⟨1116⟩ was not really written with the nuances of cell process manufacturing in mind. Cell process manufacturing can be very labor intensive with personnel moving about from the BSC to other work stations and back, and often with multiple entries in and out of the critical area (the BSC). These activities, coupled with the knowledge that personnel are the highest source of contamination in controlled environments, the contamination rates shown, especially for personnel gloves and gown areas may not be reasonable for all cell therapy applications. Nevertheless, contamination rates are a good tool to trend and track for each individual facility to determine whether changes over the normal baseline are occurring or unfavorable conditions are approaching.

Since USP ⟨1116⟩ eliminated the concept of trending against numerical alert and action levels, nonetheless it still addresses the possibility of recovering abnormally high or significant CFU counts. Therefore, based on the concept of microbiological variability, excursions beyond approximately 15 CFU, especially from a single ISO 5 BSC sample, although infrequent may be indicative of a significant loss of control and require a prompt and thorough investigation.

A final word regarding EM numerical action level numbers. These have been purposefully stated as *Levels* only, and not referred to as *Limits* or *Specifications*. EM data should not to be treated as limits or specifications because failure to meet these criteria does not necessarily imply an environmental, process, or a product failure. Rather these levels are best understood as process control tools designed to evaluate the ongoing state of control of the aseptic processing area.

TYPES OF SAMPLES

Airborne Particulates

Non-viable particulate sampling is accomplished using discreet laser particle counters. In all regions controlled area particulate measurements in healthcare industry require sampling for particle sizes of 0.5 micron or larger. The EU Annex 1 guidance also requires sampling for particle sizes of 5.0 microns or larger as well which actually qualifies the critical zone (Grade A) as ISO 4.8 instead of ISO 5.

In order to collect a meaningful sample EU Annex 1 requires a minimum sample volume of $1m^3$ at each sample location in the critical zone. Since particulate counters are typically standardized at a sampling flow rate of 1.0 cubic foot per minute, this would require a total sampling time of about 35 minutes to collect the required 1 m^3 sample. For pharmaceutical filling operations, at least for critical Grade A zone, it is not expected that one would sample for 35 minutes in a single sample volume, but rather 1 m^3 would be captured in 35 one minute sample frequencies to try and capture immediate alarms or alerts for *"all interventions, transient events, and any system deterioration..."*. Furthermore, it is expected that continuous particulate sampling be performed throughout the aseptic process in the critical zone.

So how do these guidelines, written for traditional drug filling operations, translate to cell therapy processing where "vial filling" might not occur? This again is where a sound risk-based assessment of the individual process is needed. For example, one should list what are the critical process steps such as open manipulations and/or aseptic connections, versus which process steps utilize "closed systems". If practical, one may consider performing sampling in order to bracket just the open process steps. However, it might not be practical or feasible to capture ALL individual interventions and transient events like a filling campaign, therefore continuous particulate sampling such as a 1 m^3 single sample volume that spans the process from beginning to end can be acceptable.

For ISO 7 and 8 areas where product is not exposed to the environment, a particulate sampling volume of at least one cubic foot (one minute sample time) will meet the ISO 14644-1 volume requirements and can be acceptable.

To minimize particle counting loss, especially if performing particle counts for ≥ 5.0 micron size particles, it is necessary to keep the particle count tubing at a minimum length such as no more than 3 meters (approximately 10 feet) or less. Additionally, bends or arcs in the particle count tubing should have a sufficient radius to prevent larger particle size loss.

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Airborne Viables

Viable air sampling is divided into two sample types *Active* versus *Passive*. Active sampling is accomplished with a volumetric sampling device measuring the CFU count per unit volume, while passive sampling is accomplished with the means of settling plates. Active sampling is required in all zones and is the method of choice for its ability to capture airborne microorganisms for recovery and make quantitative measurements. Active sampling in the ISO 5 critical zone where the level of contamination is expected to be very low, a larger sample size of at least 1 m^3 is needed to increase sensitivity. For ISO 7 or 8 areas, sample volume may be decreased.

Although not necessarily a requirement in the U.S., settle plates can have several advantages for monitoring during cell therapy process manufacturing. First, it is a simple and inexpensive method for qualitative assessment of airborne contamination. Secondly, as opposed to most active sampling devices and portable devices, settle plates may be placed over prolonged periods of time such as 4 hours for continuous sampling. Thirdly, the plates can be positioned in strategic locations without being invasive to the manufacturing process. Finally, settle plates are not disruptive to the airflow as opposed to active sampling devices. In fact, in some cases the use of a portable active sampling device may be disruptive and intrusive to the aseptic process in a small BSC if attempting to perform active sampling during processing. Therefore the use of settle plates over active sampling may be acceptable where justified. In these cases, active sampling of a BSC should be performed on a routine basis or perhaps at the end of processing where process disruption or intrusion are no longer a concern.

Surface Viables

Surface viables are typically accomplished by use contact (RODAC) plates as means for assessing facility cleanliness on regular flat surfaces, and for evaluating the effectiveness of facility sanitizers and disinfectants. Facility floors, walls, workbenches, equipment, and personnel gown and even glove sampling can be easily accomplished with contact plates. The swabbing method may be for irregular surfaces such as process

equipment. Cell therapy manufacturing in most cases may use equipment that do not have direct product contact (e.g. cell culture incubators or centrifuges) and therefore the use of the swabbing method may not be necessary.

Proper sampling technique with contact plates is very important to ensure the best chances for recovery and consistent results for trending. This is especially important since recovery efficiency from surfaces is not very high and in one study has been reported to be in < 20% [(8)].

Contact plate media formulations often contain added neutralizers to aid in recovery from surfaces where residual disinfectants may be present. Common neutralizers are Lecithin and Polysorbate (Tween) 80. Lecithin can act to neutralize quaternary ammonium compounds and Tween 80 can act to neutralize phenolics. It is important to consider using contact plates with appropriate neutralizers for facility surfaces that may contain residual sanitizing agents.

CONCLUSIONS

In this paper I have to put forth several considerations for environmental monitoring of cellular therapy products, and specifically products that are aseptically processed in controlled environments. These considerations were offered to help manufacturers of cell therapy products to set-up and implement an appropriate risk-based EM program that meets the criteria of being meaningful, manageable, and defendable.

REFERENCES

1. European Commission. Volume 4 EU Guidelines to Good Manufacturing Practice. Medicinal Products for Human and Veterinary Use. Annex 1. 2008. "Manufacture of Sterile Medicinal Products (corrected version)". Brussels, Belgium.

2. International Organization for Standardization (ISO). 14644-1. 1999. "Clean rooms and associated environments, part 1: classification of air cleanliness". Geneva, Switzerland.

3. Parenteral Drug Association. Technical Report No. 13 (corrected version). 2000. "Fundamentals of an Environmental Monitoring Program. PDA J of Pharmaceutical Science and Technology". Vol. 54. No. 3.

4. U.S. Department of Health and Human Services, Food and Drug Administration. 21 CFR Part 1271, Human Cells, Tissues, and Cell and Tissue-Based Products.

5. U.S. Department of Health and Human Services, Food and Drug Administration. 2004. "Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing —Current Good Manufacturing Practice". Rockville, MD.

6. United States Pharmacopeia. General Informational Chapter ⟨1046⟩, "Cellular and Tissue-Based Products". Rockville, MD.

7. United States Pharmacopeia. General Informational Chapter ⟨1116⟩, "Microbiological Control and Monitoring of Aseptic Processing Environments". Rockville, MD.

8. Whyte, W. 1989. "Methods for Calculating the Efficiency of Bacterial Surface Sampling Techniques". *J Hosp Infect*. 13:33-41

9. World Health Organization. 2011. Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities. "Draft Document: Points to consider for manufacturers of human vaccines". Geneva, Switzerland.

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