Environmental Monitoring of Clean Rooms in Vaccine Manufacturing Facilities

Points to consider for manufacturers of human vaccines

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On behalf of the United Nations, the World Health Organization maintains a list of vaccines that are prequalified for procurement by UN agencies. Prequalified vaccines have been assessed by WHO to verify that the product has been manufactured and tested in accordance with the relevant Technical Report Series (TRS) monographs and WHO Good Manufacturing Practices (GMP), that preclinical and clinical evidence supports the quality, safety and efficacy of the vaccine, and that the product meets relevant UN tender specifications. WHO collaborates closely with the national regulatory authorities in the country of manufacture to ensure that the regulatory oversight of the product meets international standards. This Points to consider provides manufacturers with non-binding information concerning the criteria currently used by WHO for the assessment of prequalified human vaccines.

1. Introduction

Medicinal products must be *pure*, that is, free of contaminants that are not part of the product's intended composition. Purity is however a relative term, with its definition varying significantly by pharmaceutical class. For orally-administered chemical pharmaceuticals, the limits set for bacteria or fungi are relatively high. For parenterally-administered vaccines, the possibility of injecting even minimum amounts of unwanted virus, mycoplasma, or bacteria is considered unacceptable.

Quality is built into a product produced by aseptic manufacture when sound process, equipment, and facility design is employed to minimize or eliminate potential contamination hazards. Modern design approaches include systematic evaluation of potential process vulnerabilities and awareness of how daily dynamic operational factors can interact.

A carefully planned and executed environmental monitoring (EM) program provides increased assurance of sterility for aseptically produced products. However, environmental monitoring data is only one of a number of measures used to indicate the state of control in an aseptic manufacturing process. Besides, environmental monitoring is not a direct measure of product batch sterility due to the inherent variability of environmental monitoring methods and more importantly; the lack of a correlation between specific numerical environmental monitoring levels and batch sterility.

EM represents an important means by which the effectiveness of contamination control measures can be assessed and the specific threats to the purity of products being manufactured can be identified. The results of environmental monitoring must be considered when making the decision whether a production batch can be released.

EM describes the microbiological testing undertaken in order to detect changing trends of microbial counts and micro-flora growth within clean rooms or controlled environments. The results obtained provide information about the physical construction of the room, the performance of the Heating, Ventilation, and Air-Conditioning (HVAC) system, personnel cleanliness, gowning practices, the equipment, and cleaning operations.

Over the past decade, environmental monitoring has become more sophisticated in moving from random sampling, using an imaginary grid over the room and testing in each grid, to the current focus on risk assessment and the use of risk assessment tools to determine the most appropriate methods for environmental monitoring.

Significant differences in clean room design and EM practices exist between vaccine manufacturers in different countries, and GMP inspectors often have very different interpretations of GMP requirements for clean rooms and their monitoring.

Two recent events are changing the way clean rooms are to be designed and monitored. The first is the adoption of the ISO clean room definitions by the US, EU, and subsequently, WHO. A common standard should help reduce the number of divergent norms that companies serving the international market must conform to (ISO standards like ISO 14644 and ISO 14698 do not always fit with regulatory guidance documents because they apply to controlled environments across a range of industries other than pharmaceuticals, where standards can be higher). The second event is the emerging acceptance of a risk-based approach. In it, risks inherent to product-specific manufacturing steps are analysed and specific measures needed to manage or reduce those risks are determined. Using risk approaches, GMP requirements that better address the specific problems inherent in the production of vaccines should be possible.

This paper presents how a group of technical and regulatory experts active in assessing prequalification applications interprets current WHO requirements for clean rooms and EM as they are applied to the production of human vaccines. As such, the analysis may be helpful to manufacturers and inspectors of prequalified vaccines in understanding how current WHO requirements are being assessed. Readers are cautioned that views provided here are non-binding and subject to change over time; the official WHO requirements continue to be those approved by the WHO Expert Committee on Biological Standardization and by the WHO Expert Committee on Specifications for Pharmaceutical Products published in the respective WHO Technical Report Series (e.g.: TRS 957, Annex 4).

2. Risk assessment applied to clean room grade recommendations for vaccine production activities

Regardless of how well clean rooms function, potential contaminants can be continuously introduced into production facilities through entry of materials and equipment. Operators are another major source of particulates and microorganisms, shedding particles and microbes from skin, mucous membranes, and through respiratory secretions. Manufacturing procedures such as mixing, concentration, centrifugation, or transfer may also generate spills or aerosols that spread widely through production areas. Where bacteria and fungi are allowed to grow in recesses or when cleaning and sanitation procedures are ineffective, continuous or even resistant environmental strains can be developed.

In a strict interpretation of the WHO GMP for sterile pharmaceutical products annex, for sterile bacterial and viral vaccines where heat sterilization or filtration are impossible due to the nature of the product, the entire manufacturing scheme should be conducted in full aseptic processing in Grade A with a Grade B background. . The selection of which grade or class of clean room to use at each stage of manufacture remains one of the most misunderstood areas in GMP for biological medicinal products. For this reason, a risk-based approach in selecting the clean room grade needed for the various steps in vaccine production is considered an essential component in establishing environmental monitoring practices.

The use of risk assessment approaches is an important current Good Manufacturing Practice (cGMP) tool in microbiological environmental monitoring. However, each suite of clean rooms or isolator will be subtly different. Every aspect of the environment must be considered and what level of monitoring best suits the system decided; and the techniques used and the locations selected must be justified.

As biological medicinal products, vaccines present risks to the patient that must be managed:

a. Some vaccines are not "pure" or well defined / characterized products, but contain complex mixtures of proteins, lipids, and other inherent biological materials. As such, the identification and complete removal of "impurities" can be difficult if not impossible;
b. Some vaccines are produced from highly pathogenic and transmissible microorganisms. These microorganisms are present in high concentrations in the production environment, and cross-contamination of products with viable production microorganisms represent a major GMP risk and risk to the vaccinee;
c. The formulation of some vaccines may be optimized for the survival of microorganisms, making it likely that viable contaminants derived from the production environment (starting materials, operators, and those endemic to the facility) will survive in product substance and be administered to vaccinees;
d. A number of parenterally administered live viral and live bacterial vaccines cannot be sterilized by filtration. Moreover, some viruses and mycoplasma found in the manufacturing environment may potentially pass through sterilizing filters, making the effectiveness of filtration as a method of reducing environmentally-derived microorganisms not completely reliable.

1. Vaccine production methods and GMP, when properly implemented, provide significant safety measures:

    a. Many vaccines are produced in dedicated facilities or under campaign conditions that significantly reduce the possibility of carry-over between products or batches;

b. Many vaccines are produced on highly attenuated strains that have greatly reduced pathogenicity, transmissibility, or survival in the environment;
c. Many vaccine production schemes use antibiotics, antimycotics, specific media, or preservatives that prevent the growth and survival of unwanted microorganisms; final bulk and finished medicinal product may contain traces of these substances (e.g.: antibiotics in viral vaccines).
d. Many vaccines are extensively purified by precipitation, chromatography, or density ultracentrifugation, processes likely to remove most or all environmental contaminants;
e. Many vaccines are subjected to standardized chemical inactivation (e.g., using formaldehyde or β-propiolactone) that kill all bacteria, viruses, mycoplasma, and fungi, as well as detoxifying bacterial toxins.

2.1. Recommended clean room grades for operations during the manufacture of prequalified vaccines

Given the risk assumptions presented above, the clean room grades presented in Table 1 can be viewed as acceptable in most cases for vaccines to be prequalified for procurement by UN agencies. It is stressed that while the grades stated represent the 'normal' situation in vaccine production for different types of products, factors that increase risk in a particular facility may demand a higher clean room grade. In facilities where sterility or contamination failures have occurred at such levels, higher clean room grades for any or all manufacturing steps may be justified.

2. The assumptions upon which the clean room grade recommendations for each activity are based include conformity with WHO GMP standards. Where these standards are not met, the clean room grades provided in Table 1 are not relevant and higher grades may be required.

3. Clean rooms must be designed, qualified, and operated according to international standards, including their layouts, personnel and material flows, air handling systems, utilities, and operator qualifications.

4. Vaccines must be produced according to WHO technical specifications, including those regarding seed and cell bank qualification, adventitious agents' safety, and transmissible spongiform encephalopathy safety.

5. Purification procedures must be effective in removing likely contaminants. Size fractionation columns where the vaccine substance elutes in the void volume may be effective in eliminating molecular contaminants, but inefficient in separating out contaminating microorganisms or macromolecules. The use of sequential chromatography steps (size exclusion, ion exchange, hydrophobic interaction, or affinity columns) may result in highly purified vaccine products. Ultracentrifugation in a density gradient may also yield a highly purified product provided the sample is of limited size.

6. Inactivation with formalin, β-propiolactone, or other chemical inactivating agent or neutralization steps must be conducted by a procedure where the inactivation time, temperature, and concentration of the inactivating agent has been validated to be effective. Testing is conducted to verify that the inactivating chemical was effective in its purpose; and where appropriate, tests for reversion are carried out.

For early steps in vaccine production the common approach of manufacturers has been the use of unidirectional airflow (UDAF) systems in Grade C or even, in some cases, Grade D environments for short time aseptic operations such as aseptic quick connection of tubes or rapid transfers of sterile solutions from one container to another. Normally unidirectional airflow systems should be located in grade B background, but other backgrounds may still be acceptable provided a risk assessment has been done and risks have been eliminated or reduced to an acceptable level. In acknowledgement of this wide-spread practice, definitions of unidirectional airflow systems in Grade C and Grade D environments are provided, and standards by which such units are to be monitored are included. The table below therefore utilizes two important measures for the production of vaccines, closed systems and unidirectional airflow systems in Grade C and D clean rooms.

7. Closed systems are effective in protecting product materials from operator and environmental contamination, and protect employees and facilities against pathogenic vaccine strains. Systems are considered closed when materials are added and removed so that product is not exposed to the room environment at any time. To do so they must be equipped with a barrier technologies allowing the aseptic transfer of solids, liquids, and gasses, such as tube welders, steam-through valves, isolator port assemblies, and other validated transfer systems. Contamination can occur any time materials in a system come into contact with the surrounding room (e.g., opening hatches to add or remove materials, the use of unsealed hoses or stop-cocks for filling sample bottles, the connection of open-ended tubes in the manufacturing environment), these semi closed or intermittently closed systems for the purpose of defining clean room grades are considered open systems. Containers are considered closed when hermetically sealing closures are held in place by a mechanical cap or ring. The closure must prevent contact of material within the container with environmental microorganisms. It is acknowledged that an open activity of short duration and limited exposure poses less risk of contamination than activities where operators must extensively manually manipulate product materials. However, because contamination can even occur instantaneously, maintaining a completely closed system is currently the only situation where a reduction of clean room grade can be unambiguously recommended.

8. Where the scale or nature of production operations restrict the use of aseptic processing, such as in early manufacturing steps where microorganisms are being replicated or manipulated, unidirectional airflow systems in a Grade C or Grade D environment are widely used throughout the industry to protect critical operations. A unidirectional airflow system is defined as a stand-alone work station or biosafety cabinet that effectively flushes the work space with clean, HEPA-filtered air that

corresponds to Grade A regulatory limits. Where the scale or type of operations does not allow the use of an enclosed cabinet, ceiling- or wall-mounted filtration units or mobile carts can offer equivalent conditions, and for purposes of this document "unidirectional airflow" is taken to include any acceptable configuration that supplies HEPA-filtered Grade A unidirectional air to the workspace. For unidirectional airflow systems in Grade B surroundings, all Grade A standards should be met. For unidirectional air flow systems in Grade C or Grade D surroundings, the limits for particulates under "at rest" conditions should be equivalent to those recommended for Grade A, and the limits for microorganisms detected by volumetric samples or settle plates should be equivalent to those recommended for Grade B.

9. When formulations are used that optimize the survival of viruses (such as solutions used during the preparation or storage of live vaccines), contamination of product materials by microorganisms derived from operators, the facility, or adventitious agents may survive until the point of administration to the patient. To reduce this possibility, operators working in the vicinity of open materials in Grade D and Grade C operations should wear gloves and face masks in addition to the gowning requirements specified for the grade by WHO GMP for sterile pharmaceutical preparations.

10. Airlocks are clean room areas to be monitored. The grade of the airlock should correspond to that of the adjoining area with the highest grade. For specialized material airlocks (pass-through boxes), qualification results indicating the number of air changes necessary to reduce particulate and microbial counts to below the regulatory limit (and a strict observance of the time required for such changes during operations) may substitute for routine static and dynamic monitoring. For pass-through boxes too small to admit sampling devices, qualification sampling should be conducted through specially fitted probes. Unqualified, unmonitored material airlocks without HEPA air supply or fumigation capabilities that are connected to grade C or higher clean rooms should not be used.

11. Vaccines that cannot be sterilized represent a class of atypical medicinal products not adequately described in pharmacopoeial or GMP texts, and there is a high degree of variability concerning the clean room classes required for these products. The pharmacopoeial definition of "sterility" is the lack of all viable microorganisms (technically defined as a sterility assurance level of 10^{-6} for all replicating microorganisms). In both pharmacopoeial and GMP definitions sterility cannot be defined by testing, but only by a validated sterilization process (steam, dry heat, ionizing radiation, or filtration through a 0.22 μm bacterial retaining filter). In contrast, a product may be *labelled* as "sterile" simply by passing the pharmacopoeial sterility test. This test cannot detect the viral active ingredients of live vaccines, or viral, mycoplasma, or fastidious species that are common adventitious agents found in tissue or cell cultures. Without GMP or pharmacopoeial recommendations for these atypical products, inspectors predictably vary considerably in their interpretations and approaches. While acknowledging that such products are not sterilized, an

interpretation where some but not all aspects of aseptic processing are required is favoured by WHO.

Table 1. Recommended clean room grades for general activities in the manufacture of prequalified vaccines [1]

General Activities		
Activity	**Open Systems**	**Closed Systems**
Raw materials receipt and storage	▪ UNC (unclassified)	▪ N/A (not applicable)
Raw materials sampling	▪ Non-growth promoting materials: Sampling hoods with dust control/ fume control in UNC (1) ▪ Growth-promoting materials: Sampling hood with HEPA air supply and dust control in D ▪ Sterile materials: in specialized areas (2)	▪ N/A
Preparation of glassware and accessory equipment for sterilization by heat	▪ D	▪ N/A
Storage of glassware and accessory equipment after heat sterilization	▪ D (fully enclosed wrapping, such as autoclave bags) or C (with barrier protection, such as flask openings covered with aluminum foil)	▪ UNC (pharma-sealed containers)
Preparation of media to be sterilized by heat	▪ Component weighing, mixing: D	▪ N/A
Preparation of media to be sterilized by filtration	▪ Component weighing, mixing: C	▪ Media final filtration: UDAF in D (a closed system is normally required)
Storage of media after sterilization	▪ C for sealed but "open" containers	▪ D for closed containers
Preparation of excipients to	▪ Component weighing,	▪ N/A

[1] Recommended clean room grades for general activities in the manufacture of prequalified vaccines are provided as guidance and do not intend to be restrictive.

be sterilized by heat	mixing: D	
Preparation of excipients to be sterilized by filtration	▪ Component weighing, mixing: C ▪ Excipient final filtration: A in B	▪ Excipient final filtration: D
Production of master and working seeds	▪ UDAF or Class II biosafety cabinet in C [3]	▪ Isolator or Class III biosafety cabinet in D
Seed storage	▪ N/A	▪ UNC
Thawing and small-scale expansion of seeds	▪ Open manipulation of seeds / inoculation of flasks, plates, slants:UDAF in D. Alternative use of a Class II biosafety cabinet acceptable.	▪ Manipulation in isolator or Class III biosafety cabinet: D ▪ Incubation: closed containers in D
Inoculation of production media	▪ UDAF in D	▪ D
Large-scale replication	▪ Open systems are discouraged [4]	▪ D
Harvesting	▪ C	▪ D
Pre- inactivation dissociation / purification	▪ C	▪ D
Inactivation	▪ C	▪ D
Purification post-inactivation	▪ C	▪ D
Storage of post-inactivation bulks	▪ Not recommended	▪ D
Formulation of filling bulks prior to sterile filtration	▪ C	▪ D
Final sterile filtration	▪ A in B	▪ D
Formulation after final sterile filtration	▪ A in B	▪ D
Storage of sterile filling bulks	▪ N/A	▪ D [5] or UNC depending on closure
Filling	▪ Filling bulk tank with open connections to be located in A in B ▪ Filling operation in A in B	▪ Closed filling bulk tank: D ▪ Filling in isolator or Class III biosafety cabinet: A in D
Transfer of fully stoppered liquid vaccines prior to capping	▪ Capping areas within aseptic core (A/B) separated from filling zone: A in B.	▪ N/A

	▪ Capping areas outside aseptic core, separated from aseptic filling zone: UDAF for transfer, and UDAF in D for capping / crimping	
Transfer of partially stoppered vials from filling to lyophilization	▪ On a continuous belt: Grade A in Grade B ▪ In a mobile unit: Grade A air with cart in a Grade B surround ▪ Transfer of open ampoules from lyophilizer to sealing: Grade A in Grade B	▪ In closed validated transfer containers: UNC
Loading area of lyophilizer	▪ Grade A in Grade B	▪ N/A
Transfer of fully stoppered vials from lyophilization to capping area	▪ Transfer systems without additional air supply: B ▪ Transfer in a mobile unit providing Grade A air: D [(6)]	▪ In closed validated transfer containers: UNC
Capping of lyophilized vials	▪ Grade A[(7)]	▪ N/A
Visual inspection	▪ UNC	▪ UNC
Labeling	▪ UNC	▪ UNC
Packaging	▪ UNC	▪ UNC
Quality control laboratories	▪ Sterility test: A in B	▪ Sterility test: isolator in D

B. Vaccine-specific production activities

SUBUNIT and CONJUGATE VACCINES		
Activity	**Open Systems**	**Closed Systems**
Cell disruption or dissociation	▪ C	▪ D
Component purification	▪ C	▪ D
Component sterile filtration	▪ Intermediates sterilization: C ▪ Final sterilization: A in C	▪ D
Activation and conjugation reactions	▪ C	▪ D
Conjugate purification	▪ C	▪ D
Conjugate sterilization	▪ N/A	▪ Intermediate sterilization: C Final sterilization: A in B
INACTIVATED VIRAL VACCINES with STERILE FILTRATION		
Activity	**Open Systems**	**Closed Systems**
Viral seed / cell seed storage	▪ N/A	▪ UNC
Tissue collection and disruption (primary cells)	▪ C	▪ N/A
Cell expansion	▪ UDAF in C	▪ D
Thawing and small-scale expansion of seeds	▪ UDAF in C	▪ N/A
Preparation of inoculum	▪ UDAF in D	▪ D
Inoculation of production cells	▪ UDAF in D	▪ D
Viral replication	▪ C	▪ D
Media changes / additions	▪ UDAF in D	▪ D
Harvesting	▪ C	▪ D
Concentration / buffer changes	▪ C	▪ D
Pre-inactivation purification	▪ C	▪ D
Inactivation	▪ C	▪ D
Post-inactivation purification	▪ C	▪ D
Formulation before final sterile filtration	▪ UDAF in C	▪ D
Sterile filtrations	▪ A in B	▪ C

Formulation after final sterile filtration	▪ A in B	▪ C
Filling	▪ Oral or nasal administration: A in B[8] ▪ Parenteral administration: A in B	▪ Filling in isolators requires a grade D background
VACCINES PREPARED WITHOUT STERILE FILTRATION		
Preparation of materials to be heat sterilized	▪ D	▪ N/A
Preparation of materials to be filter sterilized	▪ C	▪ N/A
Preparation of growth cells	▪ UDAF in C	▪ D
Preparation of inoculum	▪ UDAF in C	▪ D
Replication	▪ C with open manipulations in UDAF / C	▪ D
Harvesting, purification	▪ C with open manipulations in UDAF / C	▪ D
Treatment by non-sterilizing temperatures	▪ C with open manipulations in UDAF / C	▪ D
Filling, lyophilization (see general activities), capping	▪ Bulks containing live bacteria for oral administration: A in B[9] ▪ Bulks containing live viruses for oral or nasal administration: A in B[8] ▪ Bulks containing live mycobacteria or viruses, or heat-killed bacteria for SC, ID, or IM administration: A in B[10]	▪ Filling in isolators requires a grade D background
EGG-BASED VACCINES		
Egg incubation and candling	▪ UNC	▪ N/A
Egg inoculation and sealing	▪ UDAF in C	▪ N/A

Inoculated egg incubation	▪ Unsealed eggs: C[(11)]	▪ Sealed eggs: D
Egg harvesting	▪ UDAF in C (in cases where the product is sterile filtered, UDAF in D may be acceptable)	▪ N/A
Pre-inactivation viral purification	▪ C, or UDAF in D	▪ D
Pre-inactivation bulk storage	▪ C	▪ D
Post-inactivation viral purification	▪ C	▪ D
EXPRESSION OF SEQUENCES IN GENETICALLY MODIFIED BACTERIA, YEAST, OR INSECT CELLS		
Storage of production cell	▪ UNC	▪ UNC
Expansion of production cell	▪ D for systems with selective media, C for systems without selective media	▪ D
Harvesting	▪ D for systems with selective media, C for systems without selective media	▪ D
Purification	▪ C	▪ D
Formulation	▪ Pre-sterilization: C ▪ Post-sterilization: A in B	▪ D
CHEMICALLY SYNTHESIZED ANTIGENS		
Chemical synthesis, purification	▪ GMP for active pharmaceutical ingredients	▪ GMP for active pharmaceutical ingredients
Conjugation reactions	▪ D	▪ D
Formulation	▪ D if prior to heat sterilization ▪ C if prior to sterile filtration ▪ A in B if after sterilization	▪ D

Notes:
[(1)] UDAF in C or D or UNC (unclassified) refers to the situation where a unidirectional airflow system may not be classified as Grade A (due to the lack of a Grade B surrounding) but can provide significant additional protection to operations.

(2) Raw materials may not be brought into production areas while under quarantine. Sterile materials that require sampling should be sampled in a testing area if sterility of the raw material is required for sterility of the final product. If no subsequent sterilization procedure is to be used, sampling should be done aseptically, i.e. with grade A in B, or a grade A isolator in grade D background. However, testing of raw materials should not result in contamination of the finished product sterility testing area or any alteration in sterility test sensitivity, as could be case with the sampling of antibiotics, growth-promoting materials, preservatives, and so on. In such cases, the manufacturer should employ a separate sampling area, or conduct effective cleaning and sanitization of the area prior to product testing. Where material sterility is required for a production step, but the product material so manipulated will be subsequently sterilized, the manufacturer should itself decide which grade is needed; at the minimum, a grade C is required when product material is to be final sterilized by filtration. Following sampling, if the container closure seal can be re-established, materials may be stored under the same conditions as non-sampled containers. Where the container closure seal cannot be re-established, the material can be aseptically transferred to a sterile container, aseptically re-wrapped, or stored under grade A/B conditions. The testing of separate samples furnished by the material supplier can in many cases avoid the problem of sampling sterile materials.

(3) Due to the increased risk of contamination of seeds by adventitious agents derived from operators or the surrounding environment, in addition to the use of a UDAF or biosafety cabinet, increased personal protection equipment and procedures should be employed. The use of surrounding areas of Grade C or higher is recommended for open manipulation of seeds.

(4) The use of open systems for replication of production microorganisms is discouraged due to the lack of control it offers. However, with the use of highly selective media or other growth conditions that limit the growth of contaminating microorganisms, some manufacturers may choose to continue this practice. Where microorganisms not assayed by sterility testing are a likely contaminant (e.g., mycoplasma or viruses), effective measures must be taken to avoid this. Where quick connections are be made between containers (such as in the use of cell factories), these connections should be made either aseptically or, exceptionally, in a UDAF in Grade D or higher. The degree of product protection afforded by the UDAF (such as the uniformity and velocity of air flow at product level and the effectiveness in excluding operators from the working area during operations) is a critical factor in assessing the acceptability of such practices.

(5) Final filling bulks are often closed but not fully sealed, such as during the use of bottles equipped with screw-tops. Where such closures are used, additional protection of the bulk container (such as double wrapping) may be necessary during transport through uncontrolled areas. Fully sealed unbreakable pharma-quality containers corresponding to closed systems do not require additional protection during transport and can be stored in UNC areas..

(6) The carts utilized for this transfer may not be the same as those used to transfer from filling to lyophilization within the Grade B surround.

(7) Vial capping can be undertaken as an aseptic process using sterilized caps or as a clean process outside the aseptic core. Where this latter approach is adopted, vials should be protected by grade A conditions up to the point of leaving the aseptic processing area,

and thereafter stoppered vials should be protected with a grade A air supply until the cap has been crimped.
(8) Due to the pharmacopoeial sterility test requirement, at least grade B dynamic (in operation) limits should be met in both static and dynamic conditions.
(9) Due to the presence of formulations promoting the stability of viable microorganisms, at least grade B dynamic (in operation) limits should be met in both static and dynamic conditions.
(10) Depending on the design and historical state of control of the surrounding area, grade B (dynamic) limits may be accepted for area qualification during both static and dynamic conditions.
(11) National regulatory agencies may accept specialized procedures for seasonal or pandemic influenza vaccines.

3. Classification and environmental monitoring (EM) of clean rooms and laminar flow workstations

3.1. Clean room classification schemes

A number of different schemes have existed to define clean rooms. In the past WHO has harmonized its classification and EM requirements to those of the European Union (EU) and more recently with ISO standard 14644-1. Some countries have established their own norms for clean rooms, and others have harmonized to norms established by WHO, the US FDA, the EU, or adopted those set by non-governmental organizations such as ISO or PIC/S. As such, manufacturers are often confronted with a large number of conflicting norms to which their facility must conform.

Due to this heterogeneity in national requirements, WHO employs its own GMP code as the basis for assessments of vaccines to be procured for global use. However, it is recognized that certain national standards are similar to those of WHO, and when the manufacturer can demonstrate that such standards provide essentially the same clean room and EM procedures as WHO, this is acceptable as a basis for prequalification.

3.2. Clean room classification based on airborne particulates

3.2.1. WHO requirements

Table 2. Maximum permitted airborne particulate concentration per air grade[2]

Grade	At rest		In operation	
	Max. permitted particles / m^3		Max. permitted particles / m^3	
	≥ 0.5 μm	≥ 5.0 μm	≥ 0.5 μm	≥ 5.0 μm
A	3,520	20	3,520	20
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

3.2.2. Use of other standards for prequalification purposes

12. Companies considering prequalification of vaccines using limits presented in a different format or units of measurement than those of WHO should submit a clear chart or table where the correlation between their own classification scheme and WHO's scheme is presented. This should include evidence that:

    a. Distinct clean room grades are defined that correspond to the WHO Grades A, B, C, and D;

    b. Limits for particulates and microorganisms are numerically similar to those recommended by WHO. As an example, the minor numerical differences that exist between EU, WHO, and ISO limits are not considered statistically significant;

    c. Particle sizes at both ≥ 0.5 μm and ≥ 5 μm are measured and the limit for each size range is met;

    d. Viable microorganisms are measured both by active and passive air sampling and by sampling of surfaces and personnel;

    e. Measurements in both static (*at rest*) and dynamic (*in operation*) conditions are made for particulates and microorganisms; and

[2] Table 2 shows the maximum permitted airborne particle concentration as established in Annex 4 WHO Good Manufacturing Practices for sterile pharmaceutical products in TRS 957, 2010

f. Only one standard is used for the purpose of prequalification, even when a manufacturer conforms to multiple standards simultaneously.

13. Discontinued standards, unless specifically authorized in the medicines law of the country of manufacture, are not acceptable for prequalification purposes.

3.2.3 Particulate sampling methods[3]

14. Sampling procedures may be conducted by quality control, quality assurance, production personnel, or other designated personnel or contractors with specialized training and skills to conduct the activity.

15. Particles should be measured by a light-scattering instrument designed to detect airborne particles of defined sizes in a clean room environment. The instrument should have a valid calibration certificate, with the frequency of calibration dependent on the type of instrument and its use; the manufacturer's instructions for calibration and set-up provide valuable information in this regard. Particles of the two size ranges stated in the WHO requirements must be analysed. Isokinetic sample heads should be used in unidirectional airflow systems.

16. Samples should be taken at approximately working levels (guidance value: within 30 cm from operations); where HEPA filters are located distant from operations, or where objects are likely to generate turbulent flow, qualification of HEPA filters may not be representative of the grade of the clean room area. In such cases, additional sampling must be conducted.

17. The connection of the sampling probe to the particle counters should not result in loss of larger particles on tubing surfaces. Where long (> 2 m) connection hoses or hoses with bends are needed, specific sampling devices validated for both particle sizes to be measured should be used.

18. When portable counters are transported between areas, companies must demonstrate the effectiveness of measures taken to avoid cross-contamination. Specially segregated areas, such as for spore-forming microorganisms or microorganisms handled in biosafety facilities, must have dedicated particle counters.

3.2.4. Clean room classification

Two types of particulate sampling may be distinguished, clean room classification / qualification and clean room routine environmental monitoring according ISO 14644-1. Classification studies measure several parameters of the clean room, including the ability of the HVAC system to attain and maintain the clean room limits for airborne particulates

[3] Particulate sampling methods described are based on ISO standard 14644-1

both in *at rest* and *in operation* conditions. This differentiation is important because the regulatory limits for *at rest* and *in operation* differ, and a single air system must be capable of meeting both the lower and upper grade limits depending on the activities present. Qualification results should be no older than 12 months to be valid. Filter integrity testing at a frequency of every 6 months for Grades A and B is recommended. If continuous monitoring results indicate an out of trends (OOT) in Grade A and B areas, an investigation should be conducted and appropriate actions should be taken, and a full requalification should be considered. .

19. For a room with *x* square meters of surface area (rounded up to the whole number), $\sqrt{x}$ equally spaced points should be sampled. Where an area of a differing grade (such as a unidirectional air flow area) is contained in a room, this area should be considered as separate for the area of the room. Where the shape of the room does not allow for equally spaced grid points to be selected, points in equal areas should be selected. If room is small and only one location needs to be probed, at least three replicates should be made and values may be averaged.

20. The volume sampled at each location should normally be at least two litres of air, with a minimum sampling time at each location of one minute. The ISO recommendation of $Vs = (1000)\ (20/Cn,m)$, where *Vs* is the volume to sample and *Cn,m* is the regulatory limit for the largest particle size is acceptable for purposes of prequalification. If a test fails, it can be repeated only once with an increased number of points. The number, location, and size of dynamic sampling points for qualification are the same as for static monitoring.

    - Both size distributions must be measured and limits for both sizes met.

    - For all forms of environmental monitoring, the assumption should be made that contaminants are introduced into the clean room from finite points, and their subsequent distribution may be limited or sporadic. For this reason, averaging of values across sampling points is not appropriate for *in-operation* monitoring and for *at rest* monitoring.

    - In small areas such as within isolators or cabinets where only one sampling site is possible, three replicates must be taken. Results of these tests should not be averaged.

3.2.5. Routine monitoring for particulates

For each clean room, companies should conduct an analysis of the layout of the room, the materials, equipment, and personnel present, the types of activities conducted, and the potential risk to the product. From this analysis, a risk-based routine sampling plan detailing sampling sites, volumes, and frequencies can be devised; this plan, a schematic drawing of the room showing sampling locations, and a justification of the choice of sampling locations should be clearly documented. Risk assessments should be kept up to date. Modifications to the area risk assessment and sampling plan should reflect EM

results that indicate unsuspected areas of contamination or dispersion. Additionally, in new, renovated facilities or after plant shutdowns, additional sampling is recommended to find possible locations where contamination is recurrent.

21. Locations should be representative of all areas in the clean room, but locations where product is put at high risk of contamination should be included during routine monitoring. As an example, in rooms where open operations are carried out in a unidirectional airflow hood, the hood should be sampled routinely; the surrounding area may be sampled at a lower frequency, or in multiple sites sampled on a rotating basis. Areas of low risk (such as those distant from product, materials, or air flows) should be sampled occasionally to provide confidence that low levels of contamination are maintained in such areas. Sampling plans where a central point in a room is chosen and samples exclusively taken at this point are not an optimal use of EM.

22. For Grade A unidirectional airflow systems in which multiple operators perform different activities on exposed product (where multiple sources of particles or aerosols are present), multiple sampling sites and times may be necessary. For biosafety cabinets where operations obstruct airflows or turbulent airflow is created where product is exposed, additional sampling may be necessary. The regulatory limits set for biosafety cabinets are equivalent to those for unidirectional airflow systems.

23. Sampling probes should be positioned at work height and pointed in a direction such that the probability of detecting particles is maximized. Where possible, probes should point into the airflow that has just passed the product; where this is not possible; probes should be directed towards the area surrounding the product and not towards clean air flowing directly out of the HEPA filter.

24. Aseptic filling of final containers must be monitored for the full duration of operation. Probes should be mounted close to the point(s) of filling where product is exposed to the clean room environment. Probes may be mounted vertically or horizontally with the inlet at an approximate distance of 30 cm from the most critical area of the filling operation. Where operations involving exposed product are spread over large surface areas, separate probes may be needed for filling, transport, and stoppering activities.

25. Enclosed work spaces (isolators and Class III biosafety cabinets) should be monitored by means of probes. The connection between the sampling probe and the particulate detection machine should be kept short enough so that loss of particles does not occur.

Static (at rest) routine monitoring of particulates

26. Static monitoring is not normally done for routine environmental monitoring. However, when conducted, the number of samples is determined by the manufacturer's risk assessment. WHO recommends sampling of at least one point where product is put at greatest risk, and at least one point in the area that surrounds it.

Dynamic (in-operation) particulate measurements

Because operations and personnel generate particles, the limit is the same as the "static" limit for the next higher grade (except for Grade A).

27. In-operation samples should be taken under both routine and "worst-case" conditions for contamination. This would include when products are in open containers in direct contact with the environment, or where personnel are in proximity to open product materials. The maximum number of personnel normally involved in operations should be present; having reduced personnel present will lower particulate and microorganism shedding and invalidate the sampling data.

28. Production areas often have maximum and minimum operational schedules depending on the number of batches or doses manufactured per unit time. Peak operations affect utilities, material and personnel flows, and environmental control systems. Where a facility operates under differing throughput levels, environmental monitoring should be conducted according a risk assessment e.g. at peak levels rather than minimum operating conditions. The environmental monitoring sampling plan should detail these conditions.

29. Sampling points should be close enough to the product to sample the immediate environment that could contain particulates or microorganisms without unduly obstructing operations. Where sampling points are not in "worst-case" areas due to obstruction of operations or where sampling activities could themselves put products at increased risk of contamination, the decisions justifying the acceptable sampling procedure should be documented.

30. Isolators or other closed working areas must be monitored according the risks involved in the process. Where working areas are not sealed during operations, e.g., operators can open panels to adjust equipment or materials, monitoring during these incursions should be part of the sampling period.

3.2.6. Sampling frequencies for routine monitoring of particulates

Environmental monitoring should be conducted based on a schedule determined by a documented risk assessment conducted by the manufacturer. Operations where products are likely to be contaminated and affect the health of the vaccinee require more frequent EM sampling. Areas where values exceeding the regulatory limit have been detected require increased EM sites and frequency compared to areas where monitoring results consistently fall within set specifications over time. The recommended minimum monitoring frequencies for volumetric particulates (in operation) are showed in table 3:

Table 3. Monitoring frequencies for *in operation* routine particulate sampling

Classification	In operation (dynamic) routine particulate sampling
Grade A (filling operation)	For the full duration of operation
Grade B	Daily[1]
Grade C	Weekly
Grade D	Not required
UDAF work stations in B	Daily [(1)]
UDAF work stations in C	Weekly
UDAF work stations in D	Monthly
UDAF in UNC areas	Routine re-qualification of UDAF is sufficient

[(1)] Working days. Monitoring can be omitted on e.g., weekends if no production activities are taking place.

3.2.7. Particulate routine monitoring data analysis

31. For discrete or defined samples, each sample should be analysed separately, and averaging should not be conducted for results of multiple sites, or a single site sampled at multiple time points. Any value above the regulatory limit should be viewed as an excursion requiring investigation.

32. Particle-generating events of limited duration, such as brief procedures, equipment failures, or spills, will affect some products in a batch but not all. EM samples close to the workspace may thus be more important than those distal, and samples taken during certain activities may be more relevant than those conducted when these activities are not under way. Results should be analysed taking such factors into account, and samples that reflect the greatest risk to product purity, potency, safety, and efficacy should be weighted more than others.

3.3. Environmental monitoring of microorganisms

3.3.1. WHO requirements

Table 4. WHO recommended limits for microorganisms during operation[4]

Grade	Air sample (CFU/m^3)	90 mm diameter settle plates (CFU/4hours)	55 mm diameter contact plates (CFU/plate)	Glove print (5 fingers) (CFU/glove)
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

3.3.2. Monitoring of microorganisms

33. WHO interprets the listing of four independent tests as meaning that *all four* tests (volumetric sampling, settle plates, contact plates, and finger dabs) must be performed during clean room monitoring, although not every test must be conducted during every monitoring activity in every type of clean room and operation. For Grade A, volumetric sampling, settle plates, and finger dabs must be performed during each shift of operations. At the discretion of the manufacturer, multiple samples may be taken during each run, e.g., at the beginning, middle, and end of the operations to assist in investigations when EM values exceeding the regulatory limit occur or when the product fails sterility testing.

34. Environmental monitoring should be conducted based on a schedule determined by a documented risk assessment performed by the manufacturer. Sample sites can be chosen where product is exposed to the clean room environment, where operators manipulate or otherwise come into proximity to products, and where materials and surfaces that will later come in contact with product are manipulated. For each clean room, an analysis of sampling sites and sampling frequencies should be made and documented. A system where highly used areas (such as unidirectional air flow workspaces) are sampled routinely but less-trafficked areas are sampled on a rotating basis is acceptable.

[4] Table 4 shows the WHO recommended limits for microbial contamination during operation as established in Annex 4 WHO Good Manufacturing Practices for sterile pharmaceutical products in TRS 957, 2010

35. The accessibility to high risk zones for monitoring equipment and procedures is governed by a risk-benefit relationship. The manufacturer should develop ways to take samples that minimize risks to the product, such as the use of sampling ports during sterile operations rather than bringing sampling devices into an unidirectional airflow area. Where EM operations are impossible or where risks to product are considered unacceptable, this decision must be evidence-based, formally approved, and documented.

3.3.4. Growth promotion testing

Environmental bacteria and fungi are impacted or settle onto growth-promoting surfaces, but unless they survive capture and grow to visible colonies, they will not be detected. Particularly in clean room grades where the regulatory limits are low, the use of insensitive detection methods that recover only a small percentage of contaminating microorganisms present in the clean room defeats the intent and the utility of environmental monitoring. Growth promotion testing is therefore of particular importance for environmental monitoring of biological products.

There should be a formal program governing media growth promotion testing. This program should include the evaluation of media used for QC compendial/release testing as well as for environmental monitoring. Each aseptic manufacturer should consistently evaluate the growth promotion properties of media for a predefined list of organisms and must be able to prove that their microbial media are suitable to consistently recover enviromental contaminats (assuming they would be present). This standardized list should include compendial organisms and/or environmental isolates and should represent a reasonable range of "representative" microorganisms that could be encountered in manufacturing environments (e.g., Gram positive rods; Gram positive coccus; filamentous mold or yeast; Gram negative rods). It is suggested that each standardized list contains a minimum of five unique microbial strains.

36. Agar plates used for environmental monitoring should be tested for their growth-promoting capacity. Growth promoting capacity should be performed in every lot of agar plates prepared and sterilized in one cycle.

37. Plates should be tested for their ability to cultivate low numbers of standard bacteria and fungi (e.g., < 100 CFU of *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger*) when incubated at the standard times and temperatures used for analysing EM samples. The reliable recovery of small numbers microorganisms is a prerequisite for successfully monitoring clean rooms. When > 50% of microorganisms fail to be detected for each species tested, the procedures utilized should be carefully investigated and revised.

38. In addition to standard bacterial and fungal species used for growth promotion and sterility test procedures, validation studies should demonstrate that bacterial or fungal species found in the production environment (such as environmental isolates that may have become resistant to disinfection procedures and production strains) are detectable by the method used.

39. Expiry dates should be assigned to agar plates based on real-time growth promotion testing. Agar plates used for surface monitoring should contain disinfectant neutralizers as necessary.

40. Settle plates placed in areas of high airflow, turbulence, or where high temperature or low humidity conditions exist may dry out or otherwise change their properties (e.g., changes in dissolved gasses, pH, or deterioration of certain components of the media) so that bacteria or fungi that previously settled on the plates die, as do newly captured ones. Validation studies should be conducted to determine how long a settle plate can be left under the specific conditions of use and still retain full growth promotion for the microorganisms of interest. Care should be taken during the validation studies that dried plates are not rehydrated by the volume of the inoculum ($\leq$ 0.1 ml is a recommended maximum).

3.3.5. Volumetric air sampling for microorganisms

Volumetric air samples can quantify bacteria and fungi suspended in the air space surrounding open product. Active sampling can detect homogeneous suspensions of microorganisms in air, but it is not a reliable measure of sporadic contamination that occurs during operations.

41. There are several types of air samplers for microorganisms that are commercially available. WHO does not specify which type of device to be used, but the device chosen should be shown to correspond to current standards of sensitivity and detection.

42. Sample locations must be chosen by the manufacturer based on a risk analysis. Environmental monitoring should be conducted based on a schedule determined by a documented risk assessment conducted by the manufacturer. Although sampling procedures can themselves pose a threat to sterility, the closer to the process that samples are taken, and the longer the length of sampling, the more representative will be the sample of the production environment.

43. Sample duration (volume) requires a balance between the need for a sample representative of the process (where large samples should be taken over extended periods of time) and sensitivity (large volumes or high impaction forces can dry out capture plates, disrupt bacterial or fungal clumps that promote stability, or decrease

viability of microorganisms already captured), and the threat to the aseptic process itself. In general, the manufacturer of the sampling instrument will recommend sample sizes, and this recommendation must be taken into account in the design of sampling strategies. Sample sizes of ≥ 1 m^3 should be taken for each measurement. In the case where this sample size results in an unreadable number of colonies, reduced volumes may be employed to monitor Class C and D areas if properly justified.

44. The design (including among other factors sampling size and intake velocity) and the validation of volumetric air sampling, should include the efficiency of the instrument to capture standard test microorganisms as well as indigenous microbial flora of interest, such as production strains, EM isolates, or endemically circulating microorganisms likely to infect operators.

45. The effect of capture plate drying during sampling and transport to the microbiological laboratory should be determined by a validation study. Time limits should be set to ensure that microorganisms remain viable up to the point when transferred to a growth-promoting detection environment.

46. Risks involved in removing potentially pathogenic environmental or production microorganisms from the manufacturing area, transporting them through other areas, and their growth and analysis in the microbiology laboratory should be assessed. Where instruments that can be sterilized or sanitized are required, they should be used.

Settle plates

Settle plates can detect bacteria and fungi that descend in the column of air over the plate. While sensitivity depends both on the size of the plate, the settling rate of the individual microorganisms, and the growth promotion properties of the plate chosen, settle plates are the only method that provides continuous monitoring of microorganisms.

47. Settle plates should be placed in areas of high risk of product contamination. They should be placed as close to activities as possible without causing obstruction of activities or contamination by the plates themselves.

48. Settle plate measurements should be made during periods of high activity or when aerosolizing of materials may occur.

49. Where settle plate drying occurs, exposed plates may be replaced by fresh ones so that the total time of exposure is reached.

50. Settle plates should be exposed for the duration of the production process. Individual settle plates exposure times should be determined by own validation data. Where sporadic or short-time operations are carried out (such as combining sterile solutions

or connecting equipment in an unidirectional airflow system), the total exposure time may be reduced to correspond to the period of time when operations take place.

3.3.6. Surface sampling for microorganisms

Contact plates

Contact plates and glove prints both detect microorganism contamination in the immediate vicinity of the work area. When manual operations are being carried out contamination can often be detected by sampling the gowns and hands of staff. Where sterile technique results in frequently sanitization of work surfaces and gloves (or where double-gloving with frequent glove changes is used), the utility of these glove monitoring techniques is reduced.

51. Contact plates should be used to detect microorganisms on surfaces that could lead to product contamination. The manufacturer should devise a sampling scheme based on a risk assessment considering the type of activity performed to monitor relevant surfaces where contaminating microorganisms could be found. These surfaces may include working surfaces, equipment surfaces, and walls and ceilings of unidirectional air flow systems. When spills dropped materials are likely to contaminate floors, these should be sampled. When operators work in close proximity to exposed product, such as in an open flow hood, gown fronts, sleeves, masks, or other representative areas should be sampled. Every sampling site need not be sampled every time; the manufacturer may devise a sampling system whereby multiple points can be sampled in a random or rotating order.

52. Contact samples should be taken after completion of production activities or in such a way that contamination of sterile areas by monitoring does not occur. Samples should be taken before sanitization of the area. Where frequent sanitization (e.g., through spraying with alcohol solutions) occur, samples should be taken prior to the sanitation procedure to maximize the likelihood that microorganisms are detected. Where surfaces are still wet with sanitization solutions, contact plate measurements are invalid.

53. The recovery of microorganisms from contact plates should be validated. Greater than 50% of microorganisms should be recoverable during validation studies.

Swabs

54. Swabs or other adsorbent materials wetted with sterile water or other diluents can be used to sample irregular or constrained surfaces such as equipment, filling nozzles, tubing, or corners. They are also useful for sampling large areas, such as after cleaning or sanitization procedures. The recovery of microorganisms from swabs should be validated, including the chosen sampling method, the suitability of the

swab moisturizing liquid, and the transfer of microorganisms to growth media. Normally > 50% of microorganisms should be recoverable during validation studies. If the area to be sampled is large but not standardized, no regulatory limits are applicable to swabs; however, the detection of microorganisms using this method should be investigated as part of batch release. Broth or other liquids used to recover microorganisms from swabs should contain disinfectant neutralizers if necessary.

Glove prints (finger dabs)

55. Fingertips are the most likely area to come into contact with microbial contamination on work surfaces, on materials, or arising from the operator and then be transferred to products. Glove prints including all five fingers should be taken to monitor this possibility. Sampling should be conducted before routine sanitization of gloves with alcohol, or before changing of outer gloves in cases where double-gloving is used.

3.3.7. Microbiological classification of clean rooms

56. Microorganisms detected during environmental monitoring need to be considered as part of clean room classification. Microorganism monitoring for this purpose should be conducted in connection with particulate monitoring at intervals of no greater than 12 months (6 month intervals may be necessary for requalification of Grades A and B when frequent detection of environmental microorganisms occurs).

57. Microorganism monitoring should be performed both at *at-rest* (static) and *in-operation* (dynamic) conditions during classification studies. These conditions should be described by the manufacturer and documented.

3.3.8. Routine monitoring of microorganisms

58. If an area is not in use for a period of time (over weekends, or during shut-downs), environmental monitoring may be suspended during the period. Before re-starting work in the area after long shut-down (weeks or months), intensive sampling should be conducted to ensure that the area corresponds fully to the set clean room classification.

59. After shut-down, maintenance work on ventilation, or major changes in equipment function or procedures, a short series of repeated sampling should be conducted to ensure that the area corresponds fully to the set clean room classification.

60. Recommended frequencies for in-operation routine monitoring (Table 5) for microorganisms are shown. Companies may use higher or lower frequencies when this is justified by monitoring results, except in Grade A and in Grade B. Static routine monitoring is recommended to assure that the cleanliness of the clean room is

maintained when area is not in use for short periods of time or to verify cleaning efficacy prior to operations.

Table 5. Microorganism *in-operation (dynamic)* routine monitoring frequencies

Classification	Volumetric[(2)]	Settle plate[(2)]	Contact plate	Glove print
Grade A (filling operations)[1]	Once per shift	Once per shift	Once per shift	Once per shift
Grade B	Daily	Daily	Daily	Daily
Grade C	Weekly	Weekly	Weekly	N/A
Grade D	Monthly	Monthly	N/A	N/A
UDAF in B	Once per shift	Once per shift	Once per shift	Once per shift
UDAF in C	Weekly	Weekly	Weekly	Weekly
UDAF in D	Monthly	Monthly	Monthly	N/A

[(1)] Where Grade A or B areas are in constant use, dynamic sampling is conducted on a per shift basis, and the area operates consistently in a state of control, at the discretion of the manufacturer static monitoring frequencies may be reduced to monthly sampling or eliminated.
[(2)] The practice of air sampling at the start, middle, and end of filling operations provides better environmental monitoring and facilitates investigations related to filling batch release. This approach should be part of a general environmental monitoring strategy based on risk analysis and considering the types of activities performed.

3.3.9. Laboratory testing of environmental samples

61. Microorganisms of interest (e.g., production strains; strains previously identified during EM; resistant or sensitive strains; or potential infections of operators predicted by local endemic conditions) may require special conditions for capture and growth.

62. 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^{o}C followed by incubation 30 to 35^{o}C for an additional 2-3 days has been shown to be sufficient to detect most bacteria and fungi. The method chosen by each manufacturer should be carefully validated and standardized. Alternative methods are acceptable when high recoveries (>90%) of microorganisms of interest can be consistently demonstrated.

63. Where mycobacteria, mycoplasma, anaerobic, thermophilic, micro-aerophilic or nutritionally deficient or otherwise fastidious bacteria or fungi or other microorganisms of concern are probable, methods should be developed to detect such microorganisms during the environmental monitoring program. The sampling and detection strategy should take into account the need for specific methods as necessary

(e.g., specific nutrients, growth temperatures, gas balances, humidity, anaerobic conditions, longer incubation times, etc.).

64. When bacteria or fungi are detected in critical areas above the action level or limit, their identity should be ascertained at the species level. When this is impossible, the rationale should be documented.

65. Repetitive detection of the same microorganism indicates that a constant source of contamination is present. Where the detection of house flora becomes recurrent due to a population of strains resistant to antibiotics or cleaning agents, corrective actions must be taken. Sporicidal agents should be utilized to kill spore-forming species.

66. Unexpected or exotic (non-indigenous to the local country) species may result from contaminated raw materials (indicating a change in supplier or supplies) or operators recently exposed to a disease not endemic to the site of the manufacturer. In such cases corrective actions may be more urgent.

67. Averaging EM results across multiple locations and times should be avoided. When the procedure is not identical over time and events occur that could produce aerosolized particulates of microorganisms (vortexing or shaking liquids, opening pressurized containers, transfers of liquids, machine failures, spills, breaking of glass vials or syringes, intrusions of operators into clean areas, operator coughing or sneezing, etc.), then statistically "diluting" the true value with other values is unacceptable. If multiple sites within a work area are chosen but only one is likely to be representative of the risk of contamination (e.g., close to the operator's hands, in the airflow around activities, etc.), "diluting" out the relevant site with irrelevant sites gives no information on the potential risk to the product.

3.3.10. Future trends

EM requirements often refer to bacterial and fungal sterility testing, which through its specification of growth media limit the types of microorganisms that can be detected. This represents one of the major inconsistencies that exist between the GMP for chemical sterile pharmaceuticals and biological vaccines. EM GMP was written in an era when bacteria and fungi were the only microorganisms that could be readily identified, and septicaemia due to intravenously administered solutions was a major problem. However, the efficiency and capacity of filters used for sterilization has improved markedly over the intervening decades, and in the rare case where a localized infection may occur following intramuscular or subcutaneous injections, antibiotics are normally available for treatment.

The situation is very different when one considers the viruses and other microorganisms that potentially contaminate vaccine production facilities. To use inactivated polio vaccine (Salk-IPV) as an example, thousands of litres of highly pathogenic poliovirus must be produced and purified in the manufacturing facility prior to inactivation. During concentration and purification procedures, often in pressurized equipment, viral titers can approach millions of human infective doses per millilitre. In the event of a minute spill or leak, large surface areas could be contaminated with infective virus, and once dispersed, polioviruses are hardy survivors in the environment with virtual immortality in the cold. As a small picornavirus, poliovirus cannot be removed by filtration, making sterilization ineffective in controlling the major threat to the patient; the inclusion of even one viable virus in the final vaccine could have catastrophic effects. Adventitious agents such as the oncogenic SV40 virus, commonly found in primate primary cells, are similarly not controlled by sterilization. Any risk-assessment approach to environmental control would immediately identify cross-contamination of vaccine materials with viable production strains as the major risk to the vaccinee, and emphasize that neither sterile filtration nor sterility testing are of any real utility in detecting or avoiding this. Similarly, potential contamination of production facilities with mycoplasma (another potential contaminant in the cell cultures used for viral replication) is not addressed adequately by current EM requirements.

Recognizing these concerns, some of the more scientifically advanced vaccine manufacturers have begun to develop sampling, recovery, and automated analytical methods capable of detecting a wide range of viruses or other microorganisms likely to be the major environmental contaminants found in biologics production sites. They measure the survival of viruses and other microorganisms within the production environment and take this into account when designing clean room maintenance and monitoring. They actively validate cleaning and fumigation methods under the conditions actually used, rather than relying on immersion studies provided by detergent producers that have little relevance to the cleaning procedures actually performed in manufacturing facilities. With the initiation of a risk-based approach to GMP replacing strict conformity to legal requirements, vaccine manufacturers that opt to follow EM requirements as currently formulated run an ever-increasing risk of scrutiny of their cleaning, disinfection, changeover, and personal health and hygiene validation activities.

4. Investigations and Corrective and Preventive Actions (CAPA)

A prequalified manufacturer should have a system for implementing corrective actions and preventive actions resulting from the investigations of deviations, non-conformances, product rejections, complaints and recalls, audits and regulatory inspections findings and product quality monitoring.

The correct functioning of investigations and a CAPA system also for environmental monitoring is a critical component of the operation of a prequalified vaccine manufacturer.

Particulate and viable count limits (see tables 2 and 6) for clean room grades represent the recommended limits of acceptability for environments in which vaccines are manufactured. Values exceeding these limits are variously known as excursions, peaks, deviations, or out-of-specification (OOS) results. When values exceeding the regulatory limit (recommended limit of acceptability) occur, the impact of the manufacturing environment on batch release should be considered, especially for critical locations located in Grade A/B.

Handling environmental excursions is one of the most difficult tasks for a vaccine manufacturer. The conduct of thorough and unbiased investigations to rule out any possible impact on product quality, purity, or safety is an essential indicator of the functioning of a manufacturer's quality system. Under poor systems, the considerable pressures on personnel and management to release valuable batches of vaccine can override any reasonable scientific concern that a batch has been contaminated. The detection of non-functional quality system will normally result in rejection of applications for prequalification, or in the case of previously prequalified products, revocation of acceptability for procurement to UN agencies.

4.1. Action and alert limits for environmental monitoring

While particulate and viable count limits for clean rooms are recommendations set by WHO, alert and action limits are set by the manufacturer. Alert and action limits should be set to trigger activities to return the system to normal operations before regulatory limits are exceeded. Any clean room has variability in particulate and microorganism values, with the degree of variation over time the best indicator of the level of control of the clean room. For well functioning clean rooms, variability in values will be low, and values outside the norm are often indicative of a new problem in the clean room or a problem in the environmental sampling method.

68. Alert limits should be set by the manufacturer for their benefit. Alert values for particulates or microorganisms are a designated value within the maximum specification for the clean room grade, but sufficiently above normal variation in results so that increased attention should be paid whether this was a unique event, or part of an upward (and undesirable) trend. The response to a value above the alert limit is often just a notation of the event on a trend analysis, and a check that the event is not part of a cluster of abnormally high values.

69. Action limits should be set by the manufacturer for their benefit. Action limits for particulates or microorganisms are a designated value normally above the alert limit,

but normally below the regulatory limit for the clean room grade. Alert and action limits may, at the discretion of the manufacturer, be set at the same value. Where the sensitivity of methods used by the manufacturer to detect non-viable and viable contaminants is high and an area has been shown to operate consistently in a state of control, action limits may be set at the regulatory limit. Action limits should trigger the necessary investigations and corrective actions. Values over the action limit should trigger staff activities; as such, values should be carefully chosen so that it protects against batch failure, but the number of actions taken is not excessive. Depending on the particular situation, actions could include:

a. Investigation of possible alternations in procedures or equipment (including water systems and other utilities) that might be responsible for high EM levels;

b. Review of personnel operations and behaviours in the affected area that might be responsible for high EM levels;

c. Analysis (trending of viable or particulate counts as a function of time, seasonality in water or raw material supplies, endemic diseases that may infect operators);

d. Repeating monitoring, or increasing the frequency of monitoring;

e. Increasing monitoring locations;

f. Checks of equipment function or maintenance, including analysis of whether processes are operating within design limits and upper operating limits, as defined by valid validation studies;

g. Identification of microorganisms detected, with follow-up investigation as to their possible source (e.g., emergence of resistant strains, or infection of operators with a transmissible microorganism);

h. Alerting personnel to the problem, with re-training as needed;

i. Revalidation of equipment or processes using relevant process parameters.

4.2. Investigations

There should be a detailed SOP on how to investigate and react to an environmental excursion. This should be recorded and documented; the event should be investigated and the results of the investigation recorded; and an unbiased, scientific decision should be made whether the excursion could negatively affect the products purity, potency, safety, or efficacy.

70. Batch release by QA should be delayed until investigations have been successfully completed and the result of the investigation indicates that no unacceptable risk to the product or patient exists as a result of the environmental excursion.

71. A thorough investigation into the cause of the environmental excursion should be carried out. The results of the investigation should be documented and reviewed as part of batch release.

72. The impact of the environmental excursion on all batches produced in the area while the condition existed must be considered. Companies often make the mistake of sampling too infrequently, and then ignoring the fact that the area could have been continuously out of compliance for weeks or even months. Practically, if a malfunctioning filter or area contamination with a pathogenic microorganism has been discovered, all batches produced in the area since the last successful result was obtained are considered suspect. Batch failures and recall procedures for all such released batches must be specifically considered in the investigation, and failure to do so represents a severe failure in the manufacturer's quality assurance system.

73. When a process generates particles or microorganisms (such as a process where an aerosol of a live bacterial vaccine is generated), it may be difficult or even impossible to demonstrate compliance with EM requirements. In such cases a detailed validation study should be conducted that demonstrates that the nature of the product alone is responsible for these results. This may take the form of repetitive simulation studies (e.g., using an innocuous replacement of product such as growth media) where all EM results are found to be acceptable.

74. Where EM excursions have been obtained and clean room design and function do not meet specifications, this should generally result in batch failure. If a piece of equipment does not meet specifications (e.g., HVAC systems, autoclaves, fermenters, or lyophilizers cannot be qualified, or operating parameters cannot be validated) and they are possible causes of the EM excursions, this should generally result in batch failure. Where the clean room grade of the area in which the excursion occurred does not comply with GMP requirements, this should be reported in each investigation summary.

75. In the absence of other circumstances, "trending" of results is a major tool available to the manufacturer to determine whether an event is indicative of a serious problem or not. When the area has been in continuous control over a long period of time and a single, unexpected, low-level episode of contamination occurs, this could be used to support the decision to release the batch. Conversely, where environmental excursions have occurred consistently over time, or where a cluster of events is under way, the concern over batch acceptability is more serious. For microbiological

contaminants, identification of the microorganism can similarly be used to determine whether the event is the result of a continuing contamination of the facility by one or more species, or represents the introduction of a new, potentially more dangerous species via unknown mechanisms. Trends as a function of time should be analysed and displayed in such a way that "normal" and "abnormal" values are readily apparent to the viewer.

4.3. Corrective and Preventive Actions

Corrective actions and preventive actions (CAPA) are the logical result of investigations that take place following EM values above the action limit and the recommended limit. Production, engineering, quality control, and quality assurance all have an interest that clean rooms function according to specifications, and each can contribute to the design and conduct of CAPA.

If high amounts of particles are produced by a certain operation, the operation can be modified or segregated to minimize the problem. If microorganisms are detected in the work space of a particular operator, the hygiene, work practices, and training of the operator can be examined and then improved.

When excursions occur but the investigation fails to identify the cause, greater efforts must be made to improve clean room function and prevent the occurrence of further events. Environmental monitoring can be increased to cover more areas with more frequency to try to pin-point the source of contaminants, and gain assurance that the clean room area is actually operates according to specifications. Independent analyses of the operations, material and personnel flows, and personnel behaviour can be conducted. The procedures and effectiveness of cleaning the area can be reviewed and re-validated. Fortunately, with effective CAPA programs excursions diminish, resulting in increased production efficiency and employee satisfaction; companies that fail to implement effective investigations and CAPA programs inevitably waste precious resources repeating investigations that, ultimately, will result in batch failures.

76. Following investigations, if corrective or preventive actions are needed to ensure the acceptability clean room design and function and EM values, these should be analysed, defined and documented.

77. Where significant manufacturer resources are involved in implementing CAPA measures, formal approval from management should be obtained and documented. Where this approval is not obtainable this should be documented.

78. Following approval of CAPA activities, the time frame for completion of needed improvements should be defined, and persons or departments responsible for the corrective action determined. Where delays occur or unduly long periods of time are encountered in implementing needed improvements, the reasons for this and manufacturer response should be documented.

79. Following completion of CAPA activities, the effectiveness of the measures in eliminating the triggering problem should be analysed and documented.

5. Acknowledgements

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A second draft prepared from input of the expert group was discussed with representatives of the Developing Country Vaccine Manufacturers Network (DCVMN) in a workshop taking place in Chiang Mai, Thailand on February 18, 2010, including Mr.Prashant Akut, Serum Institute of India Ltd., Pune, India; Michael Begg, The Biovac Institute, Pinelands, South Africa; Dr Antoine Diatta, Institut Pasteur, Dakar, Sénégal; Mrs. Rosane Cuber Guimarães, Bio-Manguinhos /FIOCRUZ, Rio de Janeiro, Brazil; Dra. Verena L. Muzio Gonzalez, Centro de Ingenieria Genetica y Biotecnologia, Habana, Cuba; Ms. Nguyen Vu Thu Huong, Vabiotech, Company for Vaccine and Biological Production No.1, Hanoi, Vietnam; Dr. Alexander Kiktenko, Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russian Federation; Dr. Anchalee La-Ard, BioNet-Asia Company, Ltd., Ayutthaya, Thailand; Maria da Luz Fernandes Leal, Bio-Manguinhos/FIOCRUZ, Rio de Janeiro, Brazil; Mr. Zhang Lei, Chengdu Institute of Biological Products, Chengdu, P.R. China; Dr. Weining Meng, Sinovac Biotech Ltd., Beijing, P.R. China; Dr Duangporn Pornmuttakul, Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand; Dr. Rayasam Prasad, Biological E. Limited, Hyderabad, India; Dr. Susarla Sai, Krishna Indian Immunologicals Ltd., Rakshapuram, India; Mr. Kirtikumar Satpute, Serum Institute of India Ltd., Pune, India; Mr. Liu Shaoxiang, Chengdu Institute of Biological Products (CDIBP), Chengdu, P.R. China; Dr. Novilia Sjafri Bachtiar, P.T. Bio Farma Ltd., Bandung, Indonesia; Mr. Kurada Sreekanth, Panacea Biotec Limited, New Delhi, India; Mr. Trinh Tuan Viet, Vabiotech, Company for Vaccine and Biological Production No.1, Hanoi, Vietnam; Dr. Sompon

Virojanadara, Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand; and the members of the expert group. Based on comments received a third draft was prepared and distributed to the Developing Country Vaccine Manufacturers Network (DCVMN) and the International Federation of Pharmaceutical Manufacturers & Association (IFPMA) for input and comments. Based on the input received, the fourth draft was prepared for comment and was presented to the Informal consultation with the ad hoc committee on vaccines prequalification for the revision of the procedure for assessing the acceptability , in principle, of vaccines for purchase by UN agencies, May 12-14, 2010 in WHO, Geneva. Following this meeting the fifth draft was prepared incorporating suggestions received and was submitted to the Quality Assurance and Safety of: Medicines (QSM) team. Comments and suggestions received were discussed with QSM and incorporated in the sixth (current) draft.

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