are based primarily on the type of dosage form, water activity, and so on. For oral liquids and pharmaceutical products having a high water activity, in general, low microbial contamination limits are given. In this guideline, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida albicans* are shown as specified microorganisms, but it is also necessary to test certain pharmaceutical products for other microorganisms (for example, certain species of *Clostridia, Pseudomonas, Burkholderia, Aspergillus*, and *Enterobacter species*) that may have the potential to present a microbial risk to patients. The selection of the specified microorganisms was based on following criteria; indicator for poor hygienic practices, pathogenic potential for route of administration, and survival profile of the microorganism and recoverability in the product. Due to the inherent precision limitations of the enumeration methods, a value exceeding the target limit by not more than 2 times.

6. Microbial contamination limits for herbal drugs

Target limits of microbial contamination for herbal drugs and herbal drug containing preparations are shown in Table 3. Category 1 indicates herbal drugs and their preparations to which boiling water is added before use, and category 2 indicates other herbal drugs and their preparations. In this guideline, enterobacteria and other gram-negative bacteria, *Escherichia coli, Salmonella*, and *Staphylococcus aureus* are mentioned as specidied microorganisms, but other microorganisms such as certain species of *Bacillus cereus, Clostridia, Pseudomonas, Burkholderia, Asperigillus* and *Enterobacter species* are also necessary to be tested depending on the origin of the herbal drug raw materials or the preparation method of the preparations.

Table 1. Microbial enumeration limits for raw materials

Microorganisms	Target limit (cfu/g or cfu/mL)
Total aerobic microbial count (TAMC)	≦1000
Total combined yeasts/molds count (TYMC)	≦100

Table 2. Microbial enumeration limits for nonsterile finished dosage forms

Route of administration	TAMC (cfu/g or cfu/mL)	TYMC (cfu/g or cfu/mL)	Examples of objectionable microorganisms
Inhalation (liquid)	≦20	≦20	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Inhalation (powder)	≦100	≦50	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Nasal	≦100	≦50	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Varginal	≦100	≦50	*Escherichia coli* *Staphylococcus aureus* *Candida albicans*
Otic or Topical (including transdermal patches)	≦100*	≦50*	*Pseudomonas aeruginosa* *Staphylococcus aureus*
Rectal	≦1000	≦100	*Not specified*
Oral (solid)	≦1000	≦100	*Escherichia coli*
Oral (liquid)	≦100	≦50	*Escherichia coli*

* For transdermal patches, the limits are expressed as cfu per transdermal patch.

Table 3. Microbial enumeration limits for herbal drugs and their preparations

Microorganisms	Category 1 (cfu/g or cfu/mL)	Category 2 (cfu/g or cfu/mL)
Aerobic bacteria	10^7	10^5
Molds and yeasts	10^4	10^3
Enterobacteria and other gram-negative bacteria	*	10^3
Escherichia coli	10^2	not detected
Salmonella	not detected	not detected
Staphylococcus aureus	*	*

* The limits are not specified.

8. Microbiological Evaluation of Processing Areas for Sterile Pharmaceutical Products

This chapter describes the methods for the control and evaluation of microbial contamination in areas used for the processing of sterile pharmaceutical products. Such processing areas are classified into critical areas and clean areas according to the required levels of air-cleanliness. A critical area is a defined space in which the airborne particulate and microorganism levels are controlled to meet grade A. The cleanliness requirements for such a space extend to the surfaces of the facilities and equipment which form or are located within the space, as well as to the supplied raw materials, chemicals, water, etc. Environmental conditions, such as temperature, humidity, and air pressure, are also controlled in this space when required. A clean area is a controlled space such that the levels of contaminants (particulates and microorganisms) in air, gases and liquids are maintained within specified limits, which are less stringent than those of grade A. When sterile pharmaceutical products are manufac-

tured, the environment, facilities/equipment, and personnel should be routinely monitored to ensure appropriate microbiological control in the processing areas. The detection of microorganisms should be performed under normal operational conditions, using an appropriate sampling device, according to an environmental control program established previously. The sampling, cultivation, counting, and evaluation methods for airborne microorganisms, as well as those found on surfaces, should also be chosen appropriately, depending on the monitoring purpose, monitoring items, and microorganisms being detected. Sampling devices, measurement methods, media, culture conditions, frequency of monitoring, and recommended limits for environmental microorganisms shown in this chapter are for information only, and are not requirements.

1. Definitions

For the purposes of this chapter, the following definitions apply.

1) Processing areas: Areas in which actions such as cultivation, extraction/purification, weighing of raw materials, washing and drying of containers and stoppers, preparation of solutions, filling, sealing and packaging are performed, including the gowning area.

2) Action levels: Established microbial levels (and type of microorganisms, if appropriate) that require immediate follow-up and corrective action if they are exceeded.

3) Alert levels: Established microbial levels (and type of microorganisms if appropriate) that give early warning of a potential drift from normal operating conditions, but which are not necessarily grounds for definitive corrective action, though they may require follow-up investigation.

4) Contaminants: Particulates and microorganisms causing contamination by adhering to surfaces or by being incorporated into materials.

5) Cleanliness: A quantity which indicates the condition of cleanliness of a monitored item, expressed as mass or number of contaminants contained in a certain volume or area.

6) Contamination control: The planning, establishment of systems and implementation activities performed in order to maintain the required cleanliness of a specified space or surface.

7) Shift: Scheduled period of work or production, usually less than 12 hours in length, during which operations are conducted by a single defined group of workers.

8) Characterization of contaminants: Procedures for classifying contaminants so that they can be differentiated. In routine control, classification to the genus level is sufficient; as required, identification to the species level is performed.

2. Air-cleanliness of processing areas for sterile pharmaceutical products

Airborne particulates in areas used for the processing of pharmaceutical products may act physically as a source of insoluble particles in the products, and biologically as a carrier of microorganisms. So, it is necessary to control strictly the number of particles in the air. The air-cleanliness criteria are shown in Table 1.

2.1 Terminally sterilized products

Solutions should generally be prepared in a grade C environment. Solution preparation may be permitted in a grade D environment if additional measures are taken to minimize contamination. For parenterals, filling should be done in a grade A workstation in a grade B or C environment. The requirements during the preparation and filling of other sterile products are generally similar to those for parenterals.

2.2 Sterile products prepared aseptically after filtration

The handling of starting materials and the preparation of solutions should be done in a grade C environment. These activities may be permitted in a grade D environment if additional measures are taken to minimize contamination, such as the use of closed vessels prior to filtration. After sterile filtration, the product must be handled and filled into containers under grade A aseptic conditions.

2.3 Sterile products prepared aseptically from sterile starting materials

The handling of starting materials and all further processing should be done under grade A conditions.

3. Microbiological environmental monitoring program

Environmental monitoring is especially important in sterility assurance for sterile pharmaceutical products that are manufactured by aseptic processing. The major purpose of environmental monitoring is to predict potential deterioration of the processing environment before it occurs, and to produce high-quality, sterile pharmaceutical products under appropriate contamination control.

3.1 Monitoring of environmental microorganisms

a) An environmental control program document is prepared for each area used for the processing of sterile pharmaceutical products. The procedures in the document include: 1) items to be monitored, 2) type of microorganisms to be monitored, 3) frequency of monitoring, 4) methods of monitoring, 5) alert and action levels, and 6) actions to be taken when specified levels are exceeded.

b) The aseptic processing areas and other processing areas maintained under controlled conditions are monitored on a routine basis. The critical processing areas where sterile products are in contact with environmental air are monitored during every operational shift. The items to be monitored include the air, floor, walls, equipment surfaces, and the gowns and gloves of the personnel. Table 2 shows suggested frequencies of the environmental monitoring.

c) The sampling devices used for monitoring environmental microorganisms, as well as the methods and culture media, should be suitable to detect microorganisms that may be present (aerobic bacteria, anaerobic bacteria, molds, yeast, etc.). The cultivation conditions, such as incubation temperature and time, are selected to be appropriate for the specific growth requirements of microorganisms to be detected. Table 3 shows the culture media and cultivation conditions that are generally used in testing for environmental microorganisms.

d) The number of microorganisms in the samples is estimated by using the Membrane Filtration, Pour Plating, Spread Plating, or Serial Dilution (Most Probable Number) Methods described in the Microbial Limit Test.

e) Table 4 shows recommended limits for environmental microorganisms. The alert and action levels may be adjusted if necessary after sufficient data have been accumulated. The most important point in environmental monitoring is to confirm that an acceptable value of each monitoring item is maintained consistently.

f) Microorganisms isolated are characterized if necessa-

Table 1. Air-cleanliness requirements for processing of sterile pharmaceutical products

Air cleanliness	Maximum number of airborne particulates per m^3	
Grade*1	at rest	in operation
	0.5 μm	0.5 μm
A (Laminar-airflow zone)	3,530	3,530
B (Non laminar-airflow zone)	3,530	353,000
C	353,000	3,530,000
D	3,530,000	—*2

*1 The maximum permitted number of particles in the "in operation" condition corresponds to the standards described under USP ⟨1116⟩ as follows.
Grade A: Class 100 (M3.5); Grade B: Class 10,000 (M5.5); Grade C: Class 100,000 (M6.5);
Grade D: no corresponding standard.
*2 The limit for this area will depend on the nature of the operation carried out there.

Table 2. Suggested frequency of environmental monitoring

Processing area	Frequency of monitoring
Critical area (Grade A)	Each shift
Clean area adjacent to critical area (Grade B)	Each shift
Other clean areas (Grade C, D)	
Potential product/container contact areas	Twice a week
Non-product/container contact areas	Once a week

Table 3. Media and culture conditions

Microorganisms to be detected	Media*1	Culture conditions
Aerobes	Soybean-casein digest agar (or fluid) medium Brain-heart infusion agar (or fluid) medium Nutrient agar (or fluid) medium	30 – 35°C*2 More than 5 days*3
Yeast and fungi	Soybean-casein digest agar (or fluid) medium Sabouraud dextrose agar (or fluid) medium Potato-dextrose agar (or fluid) medium Glucose peptone agar (or fluid) medium	20 – 25°C*2 More than 5 days*3
Anaerobes*4	Soybean-casein digest agar medium Fluid cooked meat medium Reinforced clostridial agar (or fluid) medium Thioglycolate medium I (or thioglycolate agar medium) for sterility test	30 – 35°C More than 5 days*3

*1 If necessary, antibiotics may be added to media in an appropriate concentration (see Microbial Limit Test). If the existence of disinfectants that may interfere with the test on the surface of the specimen is suspected, add a substance to inactivate them.
*2 When soybean-casein digest agar medium is used for the detection of aerobes, yeast and fungi, incubation at 25 to 30°C for more than 5 days is acceptable.
*3 If a reliable count is obtained in a shorter incubation time than 5 days, this may be adopted.
*4 Generally, anaerobes are not targets for the monitoring. For the detection of anaerobes, agar medium is incubated in an appropriate anaerobic jar.

Table 4. Recommended limits for environmental microorganisms*1

Grade	Airborne microorganisms*2 (CFU/m^3)	Minimum air sample (m^3)	CFU on a surface	
			instruments/facilities	gloves
			(CFU/24–30 cm^2)*3	
A	<1	0.5	<1	<1
B	10	0.5	5	5
C	100	0.2	25	—
D	200	0.2	50	—

*1 Maximum acceptable average numbers of microorganisms under each condition.
*2 These values are by using a slit sampler or equivalent.
*3 Viable microbe cell number per contact plate (5.4–6.2 cm in diameter). When swabbing is used in sampling, the number of microorganisms is calculated per 25 cm^2. For gloves, usually, put their all fingers on the plate.

ry. In addition, analysis of hourly or daily variation of airborne particulate numbers will provide data to assist in the control of the cleanliness of processing areas.

3.2. Evaluation of environmental monitoring data

a) The data from the environmental monitoring are evaluated on a routine basis for each area and location. The source of any discrepancy should be investigated immediately and the investigation should be documented in a report. After corrective action has been taken, follow-up monitoring should be done to demonstrate that the affected area is once again within specification.

b) The report is reviewed and approved by personnel responsible for quality control and distributed to all key personnel associated with the aseptic processing operation.

4. Sampling devices and measuring methodology

Various types of sampling devices and measurement methods are available for the sampling and measurement of microorganisms in the air and on surfaces, and appropriate samplers and measuring methodology are selected according to the purpose of monitoring and the items to be monitored.

4.1 Evaluation of airborne microorganisms

a) Settle plates

Petri dishes of a specified diameter containing a suitable culture medium are placed at the measurement location and the cover is removed there. The plates are exposed for a given time and the microorganisms deposited from the air onto the agar surface are enumerated after incubation. This method is not effective for quantitative monitoring of total airborne microorganisms because it does not detect microorganisms that do not settle onto the surface of the culture media, and the settling velocity of aggregates of microorganisms is affected by air currents and disturbances in airflow. Although the results obtained by the settle plate method are only qualitative or semi-quantitative, this method is suitable for long-term evaluation of possible contamination of products or devices by airborne microorganisms.

b) Active microbial sampling methods

1) Measuring methods

Methods in which a fixed volume of air is aspirated include filtration-type sampling devices and impact-type sampling devices. With the filter-type sampling devices the desired volume of air can be collected by appropriately changing the air intake rate or the filter size. However, care must be taken to ensure that sterility is maintained while the filter is placed in and removed from the holder. When air sampling devices are used in critical areas, care must be taken to avoid disturbance of the airflow around the products. There are two types of filters; wet-type used gelatin filters and dry-type used membrane filters. With the dry-type filters, static electricity effects can make it impossible to collect quantitatively microorganisms on the filter. When an impact-type sampling device is used, the following points are important: 1) The speed at which the collected air strikes the culture medium surface must be sufficient to capture the microorganisms, but must not have an adverse effect on the collected microorganisms. 2) A sufficient volume of air must be sampled so that even extremely low levels of microbiological contaminants are detected, but the procedure must not cause a significant change in the physical or chemical properties of the culture medium. 3) When the device is used in critical areas, care must be exercised to ensure that the processing of the sterile pharmaceutical products is not adversely affected by the air disturbance.

2) Sampling devices

The most commonly used samplers are as follows: Slit sampler, Andersen sampler, pinhole sampler, centrifugal sampler and filtration-type sampler. Each sampler has specific characteristics. The slit sampler is a device to trap microorganisms in a known volume of air passed through a standardized slit. The air is impacted on a slowly revolving Petri dish containing a nutrient agar. The rotation rate of the Petri dish and the distance from the slit to the agar surface are adjustable and it is possible to estimate the number of microorganisms in the air passed through the device for a period of up to 1 hr. The Andersen sampler consists of a perforated cover and several pieces of Petri dishes containing a nutrient agar, and a known volume of air passed through the perforated cover impacts on the agar medium in the Petri dishes. The sampler is suitable for the determination of the distribution of size ranges of microorganism particulates in the air. The pinhole sampler resembles the slit of the slit sampler, but has pinholes in place of the slit. A known volume of air passed through several pinholes impacts on agar medium in a slowly revolving Petri dish. The centrifugal sampler consists of a propeller that pulls a known volume of air into the device and then propels the air outward to impact on a tangentially placed nutrient agar strip. The sampler is portable and can be used anywhere, but the sampling volume of air is limited.

See above 1) on the characteristics of the filtration-type sampler.

4.2 Measurement methods for microorganisms on surfaces

a) Contact plates

Use a contact plate with an appropriate contact surface. The culture medium surface should be brought into contact with the sampling site for several seconds by applying uniform pressure without circular or linear movement. After contact and removal, the plates are covered and, as soon as possible, incubated using appropriate culture conditions. After a contact plate has been used, the site to which the plate was applied must be wiped aseptically to remove any adherent culture medium.

b) Swabs

A piece of sterilized gauze, absorbent cotton, cotton swab, or other suitable material premoistened with an appropriate rinse fluid is stroked in closely parallel sweeps or slowly rotated over the defined sampling area. After sampling, the swab is agitated in a specified amount of an appropriate sterilized rinse fluid, and the rinse fluid is assayed for viable organisms.

5. Test methods for collection performance of a sampling device for airborne microorganisms

The testing of the collection performance of sampling devices for airborne microorganisms is performed in accordance with JIS K 3836 (Testing methods for collection efficiency of airborne microbe samplers) or ISO 14698 - 1 (Cleanrooms and associated controlled environments. Biocontamination control. General principles).

6. Growth-promotion test of media and confirmation of antimicrobial substances

This test and confirmation are performed according to "Effectiveness of culture media and confirmation of antimicrobial substances" in the Microbial Limit Test.

Media

Brain-heart infusion agar medium or Fluid brain-heart infusion medium

Bovine brain extract powder*1	An amount equivalent to 200 g of calf brain
Bovine heart extract powder*2	An amount equivalent to 250 g of the material
Peptone	10.0 g
Glucose	2.0 g
Sodium chloride	5.0 g
Disodium hydrogenphosphate 12-water	2.5 g
Agar	15.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 - 7.6.

Fluid cooked meat medium

Bovine heart extract powder*2	An amount equivalent to 450 g of the material
Peptone	20.0 g
Glucose	2.0 g
Sodium chloride	5.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 - 7.6.

Glucose peptone agar medium or Fluid glucose peptone medium

See Microbial Limit Test. Antibiotic is added if necessary.

Nutrient agar medium or Fluid nutrient medium

Meat extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6 - 7.0.

Potato-dextrose agar medium or Fluid potato-dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

Reinforced clostridial agar medium or Fluid reinforced clostridial medium

Meat extract	10.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Soluble starch	1.0 g
Glucose	5.0 g
L-Cystein hydrochloride monohydrate	0.5 g
Sodium chloride	5.0 g
Sodium acetate trihydrate	3.0 g
Agar	15.0 g
Water	1,000 mL

For fluid medium, add 0.5 g of agar. Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.7 - 6.9.

Sabouraud dextrose agar medium or Fluid sabouraud dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

Soybean-casein digest agar medium or Fluid soybean-casein digest medium

See Microbial Limit Test.

Thioglycolate agar medium or thioglycolate medium I for sterility test

See Sterility Test. The agar concentration of Thioglycolate agar medium is about 1.5%.

*1 **Bovine brain extract powder** Dried extract of bovine fresh brain. A yellow-brown powder having a characteristic odor.

Loss on drying: not more than 5%.

*2 **Bovine heart extract powder** Dried extract of bovine fresh heart. A yellow-brown powder having a characteristic odor.

Loss on drying: not more than 5%.

Rinsing Liquids

Buffered sodium chloride-peptone solution (pH 7.0)

See Microbial Limit Test.

LP liquid

Casein peptone	1.0 g
Soybean lecithin	0.7 g
Polysorbate 80	1.0 - 20.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2.

Phosphate buffered solution (pH 7.2)

See Microbial Limit Test.

Ringer's solution, 1/4 concentration

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride dihydrate	0.16 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.0.

Thiosulfate-Ringer's solution

Sodium thiosulfate pentahydrate	0.8 g
Ringer's solution, 1/4 concentration	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6.

9. Mycoplasma Testing for Cell Substrates used for the Production of Biotechnological/ Biological Products

This document describes the currently available methods of mycoplasma testing that should be performed for cell substrates that are used in the manufacture of biotechnological/biological products.

Methods suggested for detection of mycoplasma are, A. culture method, B. indicator cell culture method, and C.