

事務連絡
平成 20 年 1 月 9 日

各都道府県衛生主管部（局）
薬務主管課 御中

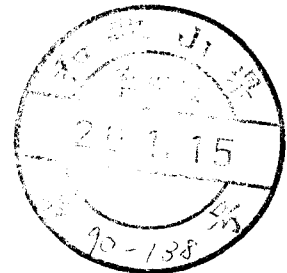
厚生労働省医薬食品局審査管理課

日本薬局方における国際調和について

近年、優れた新医薬品の地球規模での研究開発の促進と患者への迅速な提供を図るため、承認申請資料等の国際的ハーモナイゼーション推進の必要性から薬局方の試験法については、薬局方検討会議（PDG）において、日本薬局方、欧州薬局方及び米国薬局方（以下、「三薬局方」という。）間の国際調和の推進が図られているところです。また、日米 EU 医薬品規制調和国際会議（以下、「ICH」という。）が組織され、品質、安全性及び有効性の各分野でのハーモナイゼーションの促進を図るための活動も行われています。

今般、第十五改正日本薬局方第一追補一般試験法 4.05 微生物限度試験法及び参考情報 23.非無菌医薬品の微生物学的品質特性について、三薬局方間で調和合意がなされましたので、別添のとおり当該英語文を御連絡いたします。

なお、日米 EU の規制当局間における相互受け入れについては、ICHQ4B 専門家会合において検討が進められているところです。



4.05 Microbiological Examination of Non-Sterile Products

This chapter includes microbial enumeration tests and tests for specified micro-organisms. For the test, use a mixture of several portions selected at random from the bulk or from the contents of a sufficient number of containers. If test specimens are diluted with fluid medium, the test should be performed quickly. In performing the test, precautions must be taken to prevent biohazard.

I. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

These tests are harmonized with the European Pharmacopoeia and the U.S. Pharmacopeia.

1 INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi which may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken and interpret the results as stated below.

The methods are not applicable to products containing viable micro-organisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeial method has been demonstrated.

2 GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any micro-organisms which are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised. If inactivators are used for this purpose their efficacy and their absence of toxicity for micro-organisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated.

3 ENUMERATION METHODS

Use the membrane filtration method, or the plate-count methods, as prescribed. The most probable number (MPN) method is generally the least accurate method for microbial counts, however, for certain product groups with very low bioburden, it may be the most appropriate method.

The choice of a method is based on factors such as the nature of the product and the required limit of micro-organisms. The method chosen must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the chosen method must be established.

4 GROWTH PROMOTION TEST AND SUITABILITY OF THE COUNTING METHOD

4-1 GENERAL CONSIDERATIONS

The ability of the test to detect micro-organisms in the presence of product to be tested must be established.

Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

4-2 PREPARATION OF TEST STRAINS

Use standardised stable suspensions of test strains or prepare as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 4.05-1-1.

Table 4.05-I-1 Preparation and Use of Test Micro-organisms

Micro-organism	Preparation of test strain	Growth promotion		Suitability of counting method in the presence of the product	
		Total aerobic microbial count	Total yeasts and moulds count	Total aerobic microbial count	Total yeasts and moulds count
<i>Staphylococcus aureus</i> such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276	Casein soya bean digest agar or casein soya bean digest broth 30 – 35°C 18 – 24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30 – 35°C ≤ 3 days		Casein soya bean digest agar/ MPN casein soya bean digest broth ≤ 100 CFU 30 – 35°C ≤ 3 days	
<i>Pseudomonas aeruginosa</i> such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275	Casein soya bean digest agar or casein soya bean digest broth 30 – 35°C 18 – 24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30 – 35°C ≤ 3 days		Casein soya bean digest agar/MPN casein soya bean digest broth ≤ 100 CFU 30 – 35°C ≤ 3 days	
<i>Bacillus subtilis</i> such as ATCC 6633, NCIMB 8054, CIP 52.62 or NBRC 3134	Casein soya bean digest agar or casein soya bean digest broth 30 – 35°C 18 – 24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30 – 35°C ≤ 3 days		Casein soya bean digest agar/MPN casein soya bean digest broth ≤ 100 CFU 30 – 35°C ≤ 3 days	
<i>Candida albicans</i> such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594	Sabouraud–dextrose agar or Sabouraud–dextrose broth 20 – 25°C 2 – 3 days	Casein soya bean digest agar ≤ 100 CFU 30 – 35°C ≤ 5 days	Sabouraud–dextrose agar ≤ 100 CFU 20 – 25°C ≤ 5 days	Casein soya bean digest agar ≤ 100 CFU 30 – 35°C ≤ 5 days MPN: not applicable	Sabouraud–dextrose agar ≤ 100 CFU 20 – 25°C ≤ 5 days,
<i>Aspergillus niger</i> such as ATCC 16404, IMI 149007, IP 1431.83 or NBRC 9455	Sabouraud–dextrose agar or potato–dextrose agar 20 – 25°C 5 – 7 days, or until good sporulation is achieved	Casein soya bean digest agar ≤ 100 CFU 30 – 35°C ≤ 5 days	Sabouraud–dextrose agar ≤ 100 CFU 20 – 25°C ≤ 5 days	Casein soya bean digest agar ≤ 100 CFU 30 – 35°C ≤ 5 days MPN: not applicable	Sabouraud–dextrose agar ≤ 100 CFU 20 – 25°C ≤ 5 days,

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions; to suspend *A. niger* spores, 0.05 per cent of polysorbate 80 may be added to the buffer. Use the suspensions within 2 h or within 24 h if stored at 2 – 8°C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. niger* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2 – 8°C for a validated period of time.

4-3 NEGATIVE CONTROL

To verify testing conditions a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms.

4-4 GROWTH PROMOTION OF THE MEDIA

Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described

Inoculate portions/plates of *casein soya bean digest broth* and *casein soya bean digest agar* with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 4.05-I-1, using a separate portion/plate of medium for each. Inoculate plates of *Sabouraud-dextrose agar* with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 4.05-I-1, using a separate plate of medium for each. Incubate in the conditions described in Table 4.05-I-1.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

4-5 SUITABILITY OF THE COUNTING METHOD IN THE PRESENCE OF PRODUCT

4-5-1 Preparation of the sample

The method for sample preparation depends on the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed.

Water-soluble products—Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in *buffered sodium chloride-peptone solution pH 7.0*, *phosphate buffer solution pH 7.2* or *casein soya bean digest broth*. If necessary adjust to pH 6–8. Further dilutions, where necessary, are prepared with the same diluent.

Non-fatty products insoluble in water—Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in *buffered sodium chloride-peptone solution pH 7.0*, *phosphate buffer solution pH 7.2* or *casein soya bean digest broth*. A surface-active agent such as 1 g/L of polysorbate 80 may be added to assist the suspension of poorly wetttable substances. If necessary adjust to pH 6–8. Further dilutions, where necessary, are prepared with the same diluent.

Fatty products—Dissolve in isopropyl myristate, sterilised by filtration or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another non-inhibitory sterile surface-active reagent, heated if necessary to not more than 40°C, or in exceptional cases to not more than 45°C. Mix carefully and if necessary maintain the temperature in a water-bath. Add sufficient of the pre-warmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully whilst maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial tenfold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another non-inhibitory sterile surface-active reagent.

Fluids or solids in aerosol form—Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

Transdermal patches—Remove the protective cover sheets ("release liner") of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile porous material, for example sterile gauze, to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 min.

4-5-2 Inoculation and dilution

Add to the sample prepared as described above (4-5-1) and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 CFU. The volume of the suspension of the inoculum should not exceed 1 per cent of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralization, dilution or filtration.

4-5-3 Neutralization/removal of antimicrobial activity

The number of micro-organisms recovered from the prepared sample diluted as described in 4-5-2 and incubated following the procedure described in 4-5-4, is compared to the number of micro-organisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example, (1) an increase in the volume of the diluent or culture medium, (2) incorporation of a specific or general neutralizing agents into the diluent, (3) membrane filtration or (4) a combination of the above measures.

Neutralizing agents—Neutralizing agents may be used to neutralize the activity of antimicrobial agents (Table 4.05-I-2). They may be added to the chosen diluent or the medium preferably before sterilization. If used, their efficacy and their absence of tox-

icity for micro-organisms must be demonstrated by carrying out a blank with neutralizer and without product.

Table 4.05-I-2 *Common neutralizing agents/method for interfering substances*

Interfering substance	Potential neutralizing agents/method
Glutaraldehyde, Mercurials	Sodium hydrogensulfite (Sodium bisulfite)
Phenolics, Alcohol, Aldehydes, Sorbate	Dilution
Aldehydes	Glycine
Quaternary Ammonium Compounds (QACs), Parahydroxybenzoates (Parabens), Bis-biguanides	Lecithin
QAC, Parabens, Iodine	Polysorbate
Mercurials	Thioglycollate
Mercurials, Halogens, Aldehydes	Thiosulfate
EDTA (edetate)	Mg or Ca ions

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the article is not likely to be contaminated with the given species of the micro-organism. However, it is possible that the product only inhibits some of the micro-organisms specified herein, but does not inhibit others not included amongst the test strains or for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

4-5-4 Recovery of micro-organism in the presence of product

For each of the micro-organisms listed, separate tests are performed. Only micro-organisms of the added test strain are counted.

4-5-4-1 Membrane filtration

Use membrane filters having a nominal pore size not greater than 0.45 μm . The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the micro-organisms listed, one membrane filter is used.

Transfer a suitable amount of the sample prepared as described under 4-5-1 to 4-5-3 (preferably representing 1 g of the product, or less if large numbers of CFU are expected) to the membrane filter, filter immediately and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of *casein soya bean digest agar*. For the determination of total combined yeasts/moulds count (TYMC) transfer the membrane to the surface of *Sabouraud-dextrose agar*. Incubate the plates as indicated in Table 4.05-I-1. Perform the counting.

4-5-4-2 Plate-count methods

Perform plate-count methods at least in duplicate for each medium and use the mean count of the result.

4-5-4-2-1 Pour-plate method

For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under 4-5-1 to 4-5-3 and 15 - 20 mL of *casein soya bean digest agar* or *Sabouraud-dextrose agar*, both media being at not more than 45°C. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the micro-organisms listed in Table 4.05-I-1, at least 2 Petri dishes are used.

Incubate the plates as indicated in Table 4.05-I-1. Take the arithmetic mean of the counts per medium and calculate the number of CFU in the original inoculum.

4-5-4-2-2 Surface-spread method

For Petri dishes 9 cm in diameter, add 15 - 20 mL of *casein soya bean digest agar* or *Sabouraud-dextrose agar* at about 45°C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a laminar-air-flow cabinet or in an incubator. For each of the micro-organisms listed in Table 4.05-I-1, at least 2 Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample prepared as described under 4-5-1 to 4-5-3 over the surface of the medium. Incubate and count as prescribed under 4-5-4-2-1.

4-5-4-3 Most-probable-number (MPN) method

The precision and accuracy of the MPN method is less than that of the membrane filtration method or the plate-count method. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least 3 serial tenfold dilutions of the product as described under 4-5-1 to 4-5-3. From each level of dilution, 3 aliquots of 1 g or 1 mL are used to inoculate 3 tubes with 9 – 10 mL of *casein soya bean digest broth*. If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if 3 levels of dilution are prepared 9 tubes are inoculated.

Incubate all tubes at 30 – 35°C for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or *casein soya bean digest agar*, for 1 – 2 days at the same temperature and use these results. Determine the most probable number of micro-organisms per gram or millilitre of the product to be examined from Table 4.05-I-3.

4-6 RESULTS AND INTERPRETATION

When verifying the suitability of the membrane filtration method or the plate-count method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in 4-5-2 in the absence of the product must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95 per cent confidence limits of the results obtained with the control.

If the above criteria cannot be met for one or more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

5 TESTING OF PRODUCTS

5-1 AMOUNT USED FOR THE TEST

Unless otherwise prescribed, use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g. tablet, capsule, injection) is less than or equal to 1 mg or the amount per gram or millilitre (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount of sample to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

For materials used as active substances where sample quantity is limited or batch size is extremely small (i.e. less than 1000 mL or 1000 g), the amount tested shall be 1 per cent of the batch unless a lesser amount is prescribed or justified and authorised.

For products where the total number of entities in a batch is less than 200 (e.g. samples used in clinical trials), the sample size may be reduced to 2 units, or 1 unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

5-2 EXAMINATION OF THE PRODUCT

5-2-1 Membrane filtration

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown suitable as described in section 4 and transfer the appropriate amount to each of 2 membrane filters and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of *casein soya bean digest agar*. For the determination of TYMC, transfer the other membrane to the surface of *Sabouraud-dextrose agar*. Incubate the plate of *casein soya bean digest agar* at 30 – 35°C for 3 – 5 days and the plate of *Sabouraud-dextrose agar* at 20 – 25°C for 5 – 7 days. Calculate the number of CFU per gram or per millilitre of product.

When examining transdermal patches, filter 10 per cent of the volume of the preparation described under 4-5-1 separately through each of 2 sterile filter membranes. Transfer one membrane to *casein soya bean digest agar* for TAMC and the other membrane to *Sabouraud-dextrose agar* for TYMC.

5-2-2 Plate-count methods

5-2-2-1 Pour-plate method

Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates of *casein soya bean digest agar* at 30 – 35°C for 3 – 5 days and the plates of *Sabouraud-dextrose agar* at 20 – 25°C for 5 – 7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per gram or per millilitre of product.

5-2-2-2 Surface-spread method

Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare at least 2 Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of CFU proceed as described for the pour-plate method.

5-2-2-3 Most-probable-number method

Prepare and dilute the sample using a method that has been shown to be suitable as described in section 4. Incubate all tubes for 3 – 5 days at 30 – 35°C. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of micro-organisms per gram or millilitre of the product to be examined from Table 4.05-I-3.

Table 4.05-I-3 *Most-probable-number values of micro-organisms*

Observed combinations of numbers of tubes showing growth in each set			MPN per g or per mL of product	95 per cent confidence limits
Number of g or mL of product per tube				
0.1	0.01	0.001		
0	0	0	Less than 3	0 - 9.4
0	0	1	3	0.1 - 9.5
0	1	0	3	0.1 - 10
0	1	1	6.1	1.2 - 17
0	2	0	6.2	1.2 - 17
0	3	0	9.4	3.5 - 35
1	0	0	3.6	0.2 - 17
1	0	1	7.2	1.2 - 17
1	0	2	11	4 - 35
1	1	0	7.4	1.3 - 20
1	1	1	11	4 - 35
1	2	0	11	4 - 35
1	2	1	15	5 - 38
1	3	0	16	5 - 38
2	0	0	9.2	1.5 - 35
2	0	1	14	4 - 35
2	0	2	20	5 - 38
2	1	0	15	4 - 38
2	1	1	20	5 - 38
2	1	2	27	9 - 94
2	2	0	21	5 - 40
2	2	1	28	9 - 94
2	2	2	35	9 - 94
2	3	0	29	9 - 94
2	3	1	36	9 - 94
3	0	0	23	5 - 94
3	0	1	38	9 - 104
3	0	2	64	16 - 181
3	1	0	43	9 - 181
3	1	1	75	17 - 199
3	1	2	120	30 - 360
3	1	3	160	30 - 380
3	2	0	93	18 - 360
3	2	1	150	30 - 380
3	2	2	210	30 - 400

3	2	3	290	90 - 990
3	3	0	240	40 - 990
3	3	1	460	90 - 1980
3	3	2	1100	200 - 4000
3	3	3	More than 1100	

5-3 INTERPRETATION OF THE RESULTS

The total aerobic microbial count (TAMC) is considered to be equal to the number of CFU found using *casein soya bean digest agar*; if colonies of fungi are detected on this medium, they are counted as part of TAMC. The total combined yeasts/mould count (TYMC) is considered to be equal to the number of CFU found using *Sabouraud-dextrose agar*; if colonies of bacteria are detected on this medium, they are counted as part of TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, *Sabouraud-dextrose agar* containing antibiotics may be used. If the count is carried out by the MPN method the calculated value is the TAMC.

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- 10^1 CFU: maximum acceptable count = 20,
- 10^2 CFU: maximum acceptable count = 200,
- 10^3 CFU: maximum acceptable count = 2000, and so forth.

The recommended solutions and media are described in *Tests for specified micro-organisms*.

II. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: TESTS FOR SPECIFIED-MICRO-ORGANISMS

These tests are harmonized with the European Pharmacopocia and the U.S. Pharmacopeia. The parts of the text that are not harmonized are marked with symbols (♦ ♦).

1 INTRODUCTION

The tests described hereafter will allow determination of the absence of, or limited occurrence of specified micro-organisms which may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken and interpret the results as stated below.

Alternative microbiological procedures, including automated methods may be used, provided that their equivalence to the Pharmacopoeial method has been demonstrated.

2 GENERAL PROCEDURES

The preparation of samples is carried out as described in *Microbial enumeration tests*.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised as described in *Microbial enumeration tests*.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated as described in *Microbial enumeration tests*.

3 GROWTH PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA AND SUITABILITY OF THE TEST

The ability of the test to detect micro-organisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

3-1 PREPARATION OF TEST STRAINS

Use standardised stable suspensions of test strains or prepare as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

3-1-1 Aerobic micro-organisms

Grow each of the bacterial test strains separately in containers containing *casein soya bean digest broth* or on *casein soya bean digest agar* at 30 – 35°C for 18 – 24 hours. Grow the test strain for *Candida albicans* separately on *Sabouraud-dextrose agar* or in *Sabouraud-dextrose broth* at 20 – 25°C for 2-3 days.

Staphylococcus aureus such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276,
Pseudomonas aeruginosa such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275,
Escherichia coli such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972,
Salmonella enterica subsp. *enterica* serovar Typhimurium such as ATCC 14028

or, as an alternative,

Salmonella enterica subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39,
Candida albicans such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 hours or within 24 hours if stored at 2 – 8°C.

3-1-2 Clostridia

Use *Clostridium sporogenes* such as ATCC 11437 (NBRC 14293, +NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.3). Grow the clostridial test strain under anaerobic conditions in reinforced medium for Clostridia at 30 – 35°C for 24 – 48 hours. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *Cl. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2 – 8°C for a validated period.

3-2 NEGATIVE CONTROL

To verify testing conditions a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms.

3-3 GROWTH PROMOTION AND INHIBITORY PROPERTIES OF THE MEDIA

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients.

Verify suitable properties of relevant media as described in Table 4.05- II -1.

Table 4.05- II -1 Growth promoting, inhibitory and indicative properties of media

Medium	Property	Test strains
Test for bile-tolerant gram-negative bacteria		
<i>Enterobacteria enrichment broth-Mossel</i>	Growth promoting	<i>E. coli</i> <i>P. aeruginosa</i>
	Inhibitory	<i>S. aureus</i>
<i>Violet red bile glucose agar</i>	Growth promoting + Indicative	<i>E. coli</i> <i>P. aeruginosa</i>
Test for Escherichia coli		
<i>MacConkey broth</i>	Growth promoting	<i>E. coli</i>
	Inhibitory	<i>S. aureus</i>
<i>MacConkey agar</i>	Growth promoting + Indicative	<i>E. coli</i>
Test for Salmonella		
<i>Rappaport Vassiliadis Salmonella enrichment broth</i>	Growth promoting	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium or <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Abony
	Inhibitory	<i>S. aureus</i>
<i>Xylose, lysine, deoxycholate agar</i>	Growth promoting + Indicative	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium or <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Abony
	Indicative	<i>E. coli</i>
Test for Pseudomonas aeruginosa		
<i>Cetrimide agar</i>	Growth promoting	<i>P. aeruginosa</i>
	Inhibitory	<i>E. coli</i>

Test for <i>Staphylococcus aureus</i>		
<i>Mannitol salt agar</i>	Growth promoting+ Indicative	<i>S. aureus</i>
	Inhibitory	<i>E. coli</i>
Test for <i>Clostridia</i>		
<i>Reinforced medium for Clostridia</i>	Growth promoting	<i>Cl. sporogenes</i>
<i>Columbia agar</i>	Growth promoting	<i>Cl. sporogenes</i>
Test for <i>Candida albicans</i>		
<i>Sabouraud dextrose broth</i>	Growth promoting	<i>C. albicans</i>
<i>Sabouraud dextrose agar</i>	Growth promoting + Indicative	<i>C. albicans</i>

Test for growth promoting properties, liquid media: inoculate a portion of the appropriate medium with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for growth promoting properties, solid media: perform surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for inhibitory properties, liquid or solid media: inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

Test for indicative properties : perform surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

3-4 SUITABILITY OF THE TEST METHOD

For each product to be tested perform sample preparation as described in the relevant paragraph in section 4. Add each test strain at the time of mixing, in the prescribed growth medium. Inoculate the test strains individually. Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation.

Perform the test as described in the relevant paragraph in section 4 using the shortest incubation period prescribed.

The specified micro-organisms must be detected with the indication reactions as described in section 4.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see 4-5-3 of *Microbial Enumeration Tests*).

If for a given product the antimicrobial activity with respect to a micro-organism for which testing is prescribed cannot be neutralised, then it is to be assumed that the inhibited micro-organism will not be present in the product.

4 TESTING OF PRODUCTS

4-1 Bile-tolerant gram-negative bacteria

4-1-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbial enumeration tests*, but using *casein soya bean digest broth* as the chosen diluent, mix and incubate at 20 – 25°C for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 hours but not more than 5 hours).

4-1-2 Test for absence

Unless otherwise prescribed use the volume corresponding to 1g of the product, as prepared in 4-1-1 to inoculate *enterobacteria enrichment broth-Mossel*. Incubate at 30 – 35°C for 24 – 48 hours. Subculture on plates of *violet red bile glucose agar*. Incubate at 30 – 35°C for 18 – 24 hours.

The product complies with the test if there is no growth of colonies.

4-1-3 Quantitative test

4-1-3-1 Selection and subculture

Inoculate suitable quantities of *enterobacteria enrichment broth-Mossel* with the preparation as described under 4-1-1 and/or dilutions of it containing respectively 0.1 g, 0.01 g and 0.001 g (or 0.1 mL, 0.01 mL and 0.001 mL) of the product to be examined. Incubate at 30 – 35°C for 24 – 48 hours. Subculture each of the cultures on a plate of *violet red bile glucose agar*. Incubate at 30 – 35°C for 18 – 24 hours.

4-1-3-2 Interpretation

Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from Table 4.05- II-2 the probable number of bacteria.

Table 4.05- II-2 Interpretation of results

Results for each quantity of product			Probable number of bacteria per gram or mL of product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	+	+	more than 10 ³
+	+	–	less than 10 ³ and more than 10 ²
+	–	–	less than 10 ² and more than 10
–	–	–	less than 10

4-2 *Escherichia coli*

4-2-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbial enumeration tests* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of *casein soya bean digest broth*, mix and incubate at 30 – 35°C for 18 – 24 hours.

4-2-2 Selection and subculture

Shake the container, transfer 1 mL of *casein soya bean digest broth* to 100 mL of *MacConkey broth* and incubate at 42 – 44°C for 24 – 48 hours. Subculture on a plate of *MacConkey agar* at 30 – 35°C for 18 – 72 hours.

4-2-3 Interpretation

Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

4-3 *Salmonella*

4-3-1 Sample preparation and pre-incubation

Prepare the product to be examined as described in *Microbial enumeration tests* and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under 3-4) of *casein soya bean digest broth*, mix and incubate at 30 – 35°C for 18 – 24 hours.

4-3-2 Selection and subculture

Transfer 0.1 mL of *casein soya bean digest broth* to 10 mL of *Rappaport Vassiliadis Salmonella enrichment broth* and incubate at 30 – 35°C for 18 – 24 hours. Subculture on plates of *xylose, lysine, deoxycholate agar*. Incubate at 30 – 35°C for 18 – 48 hours.

4-3-3 Interpretation

The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centres. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-4 *Pseudomonas aeruginosa*

4-4-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbial enumeration tests* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of *casein soya bean digest broth* and mix. When testing transdermal patches, filter the volume of sample corresponding to 1 patch of the preparation described in *Microbial enumeration tests* (4-5-1) through a sterile filter membrane and place in 100 mL of *casein soya bean digest broth*. Incubate at 30 – 35°C for 18 – 24 hours.

4-4-2 Selection and subculture

Subculture on a plate of *cetrimide agar* and incubate at 30 – 35°C for 18 – 72 hours.

4-4-3 Interpretation

Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

4-5 *Staphylococcus aureus*

4-5-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbial enumeration tests* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of *casein soya bean digest broth* and homogenise. When testing transdermal patches, filter the volume of sample corresponding to 1 patch of the preparation described in *Microbial enumeration tests* (4-5-1) through a sterile filter membrane and place in 100 mL of *casein soya bean digest broth*. Incubate at 30 – 35°C for 18 – 24 hours.

4-5-2 Selection and subculture

Subculture on a plate of *mannitol salt agar* and incubate at 30 – 35°C for 18 – 72 hours.

4-5-3 Interpretation

The possible presence of *S. aureus* is indicated by the growth of yellow/white colonies surrounded by a yellow zone. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-6 *Clostridia*

4-6-1 Sample preparation and heat treatment

Prepare the product to be examined as described in *Microbial enumeration tests*.

Take 2 equal portions corresponding to not less than 1 g or 1 mL of the product to be examined. Heat 1 portion at 80°C for 10 min and cool rapidly. Do not heat the other portion.

4-6-2 Selection and subculture

Transfer the quantity corresponding to 1 g or 1 mL of the product to be examined from each of the mixed portions to 2 containers (38 mm x 200 mm) or other containers containing 100 mL of *reinforced medium for Clostridia*. Incubate under anaerobic conditions at 30 – 35°C for 48 hours. After incubation, make subcultures from each tube on *Columbia agar* and incubate under anaerobic conditions at 30 – 35°C for 48 hours.

4-6-3 Interpretation

The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of *Clostridia*.

If no anaerobic growth of micro-organisms is detected on *Columbia agar* or the catalase test is positive, the product complies with the test.

4-7 *Candida albicans*

4-7-1 Sample preparation and pre-incubation

Prepare the product to be examined as described in *Microbial enumeration tests* and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL to inoculate 100 mL of *Sabouraud-dextrose broth* and mix. Incubate at 30 – 35°C for 3-5 days.

4-7-2 Selection and subculture

Subculture on a plate of *Sabouraud-dextrose agar* and incubate at 30 – 35°C for 24 – 48 hours.

4-7-3 Interpretation

Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

The following section is given for information.

5 RECOMMENDED SOLUTIONS AND CULTURE MEDIA

The following solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopoeia. Other media may be used if they have similar growth promoting and inhibitory properties.

Stock buffer solution. Transfer 34 g of potassium dihydrogen phosphate to a 1000 mL volumetric flask, dissolve in 500 mL of purified water, adjust to pH 7.2 ± 0.2 with sodium hydroxide, add purified water to volume and mix. Dispense in containers and sterilize. Store at a temperature of 2 – 8°C.

Phosphate buffer solution pH 7.2

Prepare a mixture of purified water and stock buffer solution (800:1 V/V) and sterilize.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g equivalent to 0.067 mol phosphate
Sodium chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified water	1000 mL

Sterilize in an autoclave using a validated cycle.

Casein soya bean digest broth

Pancreatic digest of casein	17.0 g
Papaic digest of soya bean	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Purified water	1000 mL

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Casein soya bean digest agar

Pancreatic digest of casein	15.0 g
Papaic digest of soya bean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1000 mL

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Sabouraud-dextrose agar

Glucose	40.0 g
Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1)	10.0 g
Agar	15.0 g
Purified water	1000 mL

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Potato dextrose agar

Infusion from potatoes	200 g
Glucose	20.0 g
Agar	15.0 g
Purified water	1000 mL

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Sabouraud- dextrose broth

Glucose	20.0 g
Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1)	10.0 g
Purified water	1000 mL

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Enterobacteria enrichment broth-Mossel

Pancreatic digest of gelatin	10.0 g
Glucose monohydrate	5.0 g
Dehydrated ox bile	20.0 g
Potassium dihydrogen phosphate	2.0 g
Disodium hydrogen phosphate dihydrate	8.0 g
Brilliant green	15 mg
Purified water	1000 mL

Adjust the pH so that after heating it is 7.2 ± 0.2 at 25°C . Heat at 100°C for 30 min and cool immediately.

Violet red bile glucose agar

Yeast extract	3.0 g
Pancreatic digest of gelatin	7.0 g
Bile salts	1.5 g
Sodium chloride	5.0 g
Glucose monohydrate	10.0 g
Agar	15.0 g
Neutral red	30 mg
Crystal violet	2 mg
Purified water	1000 mL

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°C . Heat to boiling; do not heat in an autoclave.

MacConkey broth

Pancreatic digest of gelatin	20.0 g
Lactose monohydrate	10.0 g
Dehydrated ox bile	5.0 g
Bromocresol purple	10 mg
Purified water	1000 mL

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

MacConkey agar

Pancreatic digest of gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose monohydrate	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	30.0 mg
Crystal violet	1 mg
Purified water	1000 mL

Adjust the pH so that after sterilization it is 7.1 ± 0.2 at 25°C . Boil for 1 min with constant shaking then sterilize in an autoclave using a validated cycle.

Rappaport Vassiliadis Salmonella Enrichment Broth

Soya peptone	4.5 g
Magnesium chloride hexahydrate	29.0 g
Sodium chloride	8.0 g
Dipotassium hydrogen phosphate	0.4 g
Potassium dihydrogen phosphate	0.6 g
Malachite green	36 mg
Purified water	1000 mL

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°C . The pH is to be 5.2 ± 0.2 at 25°C after heating and autoclaving.

Xylose, lysine, deoxycholate agar

Xylose	3.5 g
L-Lysine	5.0 g
Lactose monohydrate	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	80 mg
Agar	13.5 g
Sodium deoxycholate	2.5 g
Sodium thiosulfate	6.8 g
Ammonium iron (III) citrate	0.8 g
Purified water	1000 mL

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25°C . Heat to boiling, cool to 50°C and pour into Petri dishes. Do not heat in an autoclave.

Cetrimide agar

Pancreatic digest of gelatin	20.0 g
Magnesium chloride	1.4 g
Dipotassium sulfate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Purified water	1000 mL
Glycerol	10.0 mL

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilization it is 7.2 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Mannitol salt agar

Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0 g
Beef extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Agar	15.0 g
Phenol red	25 mg
Purified water	1000 mL

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilization it is 7.4 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Reinforced medium for Clostridia

Beef extract	10.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Soluble starch	1.0 g
Glucose monohydrate	5.0 g
Cysteine hydrochloride	0.5 g
Sodium chloride	5.0 g
Sodium acetate	3.0 g
Agar	0.5 g
Purified water	1000 mL

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is about 6.8 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle.

Columbia agar

Pancreatic digest of casein	10.0 g
Meat peptic digest	5.0 g
Heart pancreatic digest	3.0 g
Yeast extract	5.0 g
Corn starch	1.0 g
Sodium chloride	5.0 g
Agar, according to gelling power	10.0 g to 15.0 g
Purified water	1000 mL

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25°C . Sterilize in an autoclave using a validated cycle. Allow to cool to $45 - 50^{\circ}\text{C}$; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

12. Microbial Attributes of Nonsterile Pharmaceutical Products

This test is harmonized with the European Pharmacopoeia and the U.S. Pharmacopoeia. The parts of the text that are not harmonized are marked with symbols (♦ ◆).

The presence of certain micro-organisms in non-sterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage and distribution of pharmaceutical preparations.♦ This chapter provides guidelines for acceptable limits of viable micro-organisms (bacteria and fungi) existing in raw materials and nonsterile pharmaceutical products.◆ Microbial examination of non-sterile products is performed according to the methods given in Microbiological Examination of Non-sterile Products <4.05> on *Microbial Enumeration Tests* and *Tests for Specified Micro-organisms*. ♦When these tests are carried out, a microbial control program must be established as an important part of the quality management system of the product. Personnel responsible for conducting the tests should have specialized training in microbiology, biosafety measures and in the interpretation of the testing results. ◆

♦1. Definitions

- 1.1 Non-sterile pharmaceutical products: Non-sterile drugs shown in monographs of the JP and non-sterile finished dosage forms.
- 1.2 Raw materials: All materials, including raw ingredients and excipients, used for the preparation of drugs, except for water and gases.
- 1.3 Bioburden: Number and type of viable micro-organisms existing in non-sterile pharmaceutical products.
- 1.4 Action levels: Established bioburden levels that require immediate follow-up and corrective action if they are exceeded.
- 1.5 Alert levels: Established bioburden levels that give early warning of a potential drift from normal bioburden level, but which are not necessary grounds for definitive corrective action, though they may require follow-up investigation.
- 1.6 Quality management system: The procedures, operation methods and organizational structure of a manufacturer (including responsibilities, authorities and relationships between these) needed to implement quality management.

2. Scope

In general, the test for total viable aerobic count is not applied to drugs containing viable micro-organisms as an active ingredient.

3. Sampling plan and frequency of testing

3.1 Sampling methods

Microbial contaminants are usually not uniformly distributed throughout the batches of non-sterile pharmaceutical products or raw materials. A biased sampling plan, therefore, cannot be used to estimate the real bioburden in the product. A sampling plan which can properly reflect the status of the product batch should be established on the basis of the bioburden data obtained by retrospective validation and/or concurrent validation. In general, a mixture of samples randomly taken from at least different three portions, almost the same amount for each portion, is used for the tests of the product. When the sampling is difficult in a clean area, special care is required during sampling to avoid introducing microbial contamination into the product or affecting the nature of the product bioburden. If it is confirmed that the product bioburden is stable for a certain period, as in the case of non-aqueous or dried products, it is not necessary to do the tests, immediately after the sampling.

3.2 Testing frequency

The frequency of the tests should be established on the basis of a variety of factors unless otherwise specified. These factors include:

- a) Dosage forms of non-sterile pharmaceutical products (usage);
- b) Manufacturing processes;
- c) Manufacturing frequency;
- d) Characteristics of raw materials (natural raw material, synthetic compound, etc.);
- e) Batch sizes;

- f) Variations in bioburden estimates (changes in batches, seasonal variations, etc.);
- g) Changes affecting the product bioburden (changes in manufacturing process, supplier of raw materials, batch number of raw materials, etc.);
- h) Others.

In general, the tests may be performed at a high frequency during the initial production of a drug to get information on the microbiological attributes of the product or raw materials used for the production. However, this frequency may be reduced as bioburden data are accumulated through retrospective validation and/or concurrent validation. For example, the tests may be performed at a frequency based on time (e.g., weekly, monthly or seasonally), or on alternate batches.

4. Microbial control program

When the “Microbiological Examination of Non-Sterile Products <4.05>” is applied to a non-sterile pharmaceutical product, the methods for the recovery, cultivation and estimation of the bioburden from the product must be validated and a “Microbial control program” covering the items listed below must be prepared.

- a) Subject pharmaceutical name (product name);
- b) Frequency of sampling and testing;
- c) Sampling methods (including responsible person, quantity, environment, etc. for sampling);
- d) Transfer methods of the samples to the testing area (including storage condition until the tests);
- e) Treatment of the samples (recovery methods of microbial contaminants);
- f) Enumeration of viable micro-organisms (including testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);
- g) Detection of specified micro-organisms (including testing quantity, culture media, growth-supporting test of the media, culturing methods, etc.);
- h) Estimation of the number of and characterization of microbial contaminants;
- i) Establishment of “Microbial acceptance criteria” (including alert level and action level);
- j) Actions to be taken when the levels exceed “Microbial acceptance criteria”;
- k) Persons responsible for the testing and evaluation, etc.;
- l) Other necessary items.

5. Microbial acceptance criteria for non-sterile pharmaceutical products

By establishing “Microbial acceptance criteria” for non-sterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC), it is possible to evaluate at the initial processing stage of the product whether the microbiological quality of the raw materials is adequate or not. Furthermore, it is then possible to implement appropriate corrective action as needed to maintain or improve the microbiological quality of the product. The target limits of microbial levels for raw materials (synthetic compounds and minerals) are shown in Table 1.

In general, synthetic compounds have low bioburden levels due to the high temperatures, organic solvents, etc., used in their manufacturing processes. Raw materials originated from plants and animals in general have higher bioburdens than synthetic compounds.

The microbial quality of the city water or purified water used in the processing of active ingredients or non-sterile pharmaceuticals may have a direct effect on the quality of the finished dosage form. This means it is necessary to keep the level of microbial contaminants in the water as low as possible.

Acceptance criteria for microbiological quality for non-sterile finished dosage forms are shown in Table 2. These microbial limits 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 acceptance criteria are given.

Table 1. Acceptance criteria for Microbiological Quality of Non-Sterile Substances for Pharmaceutical use

	Total Aerobic Microbial Count (CFU/g or CFU/ mL)	Total Combined Yeasts/Moulds Count (CFU/g or CFU/ mL)
Substances for pharmaceutical use	10 ³	10 ²

Table 2 includes a list of specified micro-organisms for which acceptance criteria are set. The list is not necessarily exhaustive and for a given preparation it may be necessary to test for other micro-organisms depending on the nature of the starting materials and the manufacturing process.

Table 2. – *Acceptance criteria for microbiological quality of non-sterile dosage forms*

Route of administration	Total Aerobic Microbial Count (CFU/g or CFU/ mL)	Total Combined Yeasts/Moulds Count (CFU/g or CFU/ mL)	Specified Micro-organism
Non-aqueous preparations for oral use	10 ³	10 ²	Absence of <i>Escherichia coli</i> (1 g or 1 mL)
Aqueous preparations for oral use	10 ²	10 ¹	Absence of <i>Escherichia coli</i> (1 g or 1 mL)
Rectal use	10 ³	10 ²	–
Oromucosal use	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL)
Gingival use			Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL)
Cutaneous use			
Nasal use			
Auricular use			
Vaginal use	10 ²	10 ¹	Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL) Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Candida albicans</i> (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> (1 patch) Absence of <i>Pseudomonas aeruginosa</i> (1 patch)
Inhalation use (special requirements apply to liquid preparations for nebulization)	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL) Absence of bile-tolerant gram-negative bacteria (1g or 1 mL)

If it has been shown that none of the prescribed tests will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the micro-organisms listed in Table 2, the significance of other micro-organisms recovered should be evaluated in terms of:

- the use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract);
- the nature of the product: does the product support growth, does it have adequate antimicrobial preservation?
- the method of application;
- the intended recipient: risk may differ for neonates, infants, the debilitated
- use of immunosuppressive agents, corticosteroids;
- presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and the interpretation of microbiological data. For raw materials, the assessment takes account of processing to which the product is subjected, the current technology of testing and the availability of materials of the

desired quality. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g. direct plating methods).

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- 10^1 CFU: maximum acceptable count = 20,
- 10^2 CFU: maximum acceptable count = 200,
- 10^3 CFU: maximum acceptable count = 2000, and so forth.

◆6. Acceptance criteria 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 specified micro-organisms, but other micro-organisms 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 3. Acceptance criteria for herbal drugs and their preparations

Micro-organisms	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
<i>Escherichia coli</i>	10^2	Not detected
<i>Salmonella</i>	Not detected	Not detected
<i>Staphylococcus aureus</i>	※	※

※ : The limits are not specified.◆