4.05 Microbiological Examination of Non-sterile Products

Change to read as follows:

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 neutralized. 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, Suitability of the Counting Method and Negative Controls

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-I-1.

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.

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
Staphylococcus aureus 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	
Pseudomonas aeruginosa 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	
Bacillus subtilis 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	
Candida albicans 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
Aspergillus niger 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

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. A negative control is also performed when testing the products as described under 5. A failed negative control requires an investigation.

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 standardized 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 wettable substances. If necessary adjust to pH 6 – 8. Further dilutions, where necessary, are prepared with the same diluent.

Fatty products—Dissolve in isopropyl myristate, sterilized 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 toxicity for micro-organisms must be demonstrated by carrying out a blank with neutralizer and without product.

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.

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

Interfering substance	Potential neutralizing agents/method
Glutaraldehyde, Mercurials	Sodium hydrogen sulfite (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

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

For each of the micro-organisms listed in Table 4.05-I-1, 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 mm. 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 in Table 4.05-I-1, 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-airflow 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 platecount 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 milliliter 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 milliliter (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.

5-2-3 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.

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*.

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	

II. Microbiological Examination of Non-sterile Products: Tests for Specified Micro-organisms

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

1 Introduction

The tests described hereafter will allow determination of the absence of, or limited occurrence of specified microorganisms 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 neutralized 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, Suitability of the Test and Negative Controls

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 microorganisms 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. A negative control is also performed when testing the products as described under 4. A failed negative control requires an investigation.

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.

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 microorganism. 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 1 g 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.

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 – 359C 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 a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the product to be examined as described in *Microbial enumeration tests*.

Divide the sample into two portions of at least 10 mL. Heat 1 portion at 80°C for 10 min and cool rapidly. Do not heat the other portion.

4-6-2 Selection and subculture

Use 10 mL or the quantity corresponding to 1 g or 1 mL of the product to be examined of both portions to inoculate suitable amounts (determined as described under 3-4) of *Reinforced clostridium medium*. Incubate under anaerobic conditions at 30 – 35°C for 48 hours. After incubation, make subcultures from each container on *Columbia agar* and incubate under anaerobic conditions at 30 – 35°C for 48 – 72 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*. This is confirmed by identification tests.

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

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 provided that their suitability can be demonstrated.

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 dehydrate	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 dehydrate	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 1009C 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 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°C; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

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

Medium	Property	Test strains
Test for bile-tolerant gram-negative bacteria		
Enterobacteria enrichment broth-Mossel	Growth promoting	*E. coli* *P. aeruginosa*
	Inhibitory	*S. aureus*
Violet red bile glucose agar	Growth promoting + Indicative	*E. coli* *P. aeruginosa*
Test for *Escherichia coli*		
MacConkey broth	Growth promoting	*E. coli*
	Inhibitory	*S. aureus*
MacConkey agar	Growth promoting + Indicative	*E. coli*
Test for *Salmonella*		
Rappaport Vassiliadis Salmonella enrichment broth	Growth promoting	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony
	Inhibitory	*S. aureus*
Xylose, lysine, deoxycholate agar	Growth promoting + Indicative	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony
Test for *Pseudomonas aeruginosa*		
Cetrimide agar	Growth promoting	*P. aeruginosa*
	Inhibitory	*E. coli*
Test for *Staphylococcus aureus*		
Mannitol salt agar	Growth promoting + Indicative	*S. aureus*
	Inhibitory	*E. coli*
Test for Clostridia		
Reinforced medium for Clostridia	Growth promoting	*Cl. sporogenes*
Columbia agar	Growth promoting	*Cl. sporogenes*
Test for *Candida albicans*		
Sabouraud dextrose broth	Growth promoting	*C. albicans*
Sabouraud dextrose agar	Growth promoting + Indicative	*C. albicans*

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

4.06 Sterility Test

Change to read as follows:

This test is harmonized with the European Pharmacopoeia and the U. S. Pharmacopeia.

The test is applied to substances, preparations or articles which, according to the Pharmacopoeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating micro-organism has been found in the sample examined in the conditions of the test.

1. Precautions against microbial contamination

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any micro-organisms which are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

2. Culture media and incubation temperatures

2.1. Introduction

Media for the test may be prepared as described below, or equivalent commercial media may be used provided that they comply with the growth promotion test.

The following culture media have been found to be suitable for the test for sterility. Fluid thioglycollate medium is primarily intended for the culture of anaerobic bacteria; however, it will also detect aerobic bacteria. Soya-bean casein digest medium is suitable for the culture of both fungi and aerobic bacteria.

2.2. Fluid thioglycollate medium

Fluid thioglycollate medium

L-Cystine	0.5 g
Agar	0.75 g
Sodium chloride	2.5 g
Glucose monohydrate/anhydrous	5.5 / 5.0 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate or	0.5 g
Thioglycollic acid	0.3 mL
Resazurin sodium solution (1 in 1000), freshly prepared	1.0 mL
Water	1 000 mL
(pH after sterilization 7.1 ± 0.2)	

Mix the L-cystine, agar, sodium chloride, glucose, water-soluble yeast extract and pancreatic digest of casein with water, and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycollic acid in the solution and, if necessary, add sodium hydroxide TS so that, after sterilization, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling and filter while hot through moistened filter paper. Add the resazurin sodium solution (1 in 1000), mix and place the medium in suitable vessels which provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. If the medium is stored, store at a temperature between 2 °C and 25 °C in a sterile, tight container. If more than the upper one-third of the medium has acquired a pink colour, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink colour disappears and cooling quickly, taking care to prevent the introduction of non-sterile air into the container. Do not use the medium for a longer storage period than has been validated.

Fluid thioglycollate medium is to be incubated at 30-35 °C.

For products containing a mercurial preservative that cannot be tested by the membrane-filtration method, fluid thioglycollate medium incubated at 20-25 °C may be used instead of soya-bean casein digest medium provided that it has been validated as described in growth promotion test.

Where prescribed or justified and authorized, the following alternative thioglycollate medium might be used. Prepare a mixture having the same composition as that of the fluid thioglycollate medium, but omitting the agar and the resazurin sodium solution (1 in 1000), sterilize as directed above. The pH after sterilization is 7.1 ± 0.2. Heat in a water bath prior to use and incubate at 30-35 °C under anaerobic conditions.

2.3. Soya-bean casein digest medium

Soya-bean casein digest medium	
Pancreatic digest of casein	17.0 g
Papaic digest of soya-bean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate/anhydrous	2.5 / 2.3 g
Water	1000 mL
(pH after sterilization 7.3 ± 0.2)	

Dissolve the solids in water, warming slightly to effect solution. Cool the solution to room temperature. Add sodium hydroxide TS, if necessary, so that after sterilization the solution will have a pH of 7.3 ± 0.2. Filter, if necessary, to clarify, distribute into suitable vessels and sterilize using a validated process. Store at a temperature between 2 °C and 25 °C in a sterile tight container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

Soya-bean casein digest medium is to be incubated at 20-25 °C.

3. Suitability of the culture medium

The media used comply with the following tests, carried out before or in parallel with the test on the product to be examined.

Sterility

Incubate portions of the media for 14 days. No growth of micro-organisms occurs.

Growth promotion test of aerobes, anaerobes and fungi

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of micro-organisms are indicated in Table 4.06 -1.

Inoculate portions of fluid thioglycollate medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Clostridium sporogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*.

Inoculate portions of soya-bean casein digest medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*.

Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi.

Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than five passages removed from the original master seed-lot.

The media are suitable if a clearly visible growth of the micro-organisms occurs

Table 4.06 -1 — *Strains of the test micro-organisms suitable for use in the Growth Promotion Test and the Method suitability Test*

Aerobic bacteria	
Staphylococcus aureus	ATCC 6538, NBRC 13276, CIP 4.83, NCTC 10788, NCIMB 9518
Bacillus subtilis	ATCC 6633, NBRC 3134, CIP 52.62, NCIMB 8054
Pseudomonas aeruginosa	ATCC 9027, NBRC 13275, NCIMB 8626, CIP 82.118
Anaerobic bacterium	
Clostridium sporogenes	ATCC 19404, NBRC 14293, CIP 79.3, NCTC 532 , ATCC 11437
Fungi	
Candida albicans	ATCC 10231, NBRC 1594, IP 48.72, NCPF 3179
Aspergillus niger	ATCC 16404, NBRC 9455, IP 1431.83, IMI 149007

4. Method suitability test

Carry out a test as described below under Test for sterility of the product to be examined using exactly the same methods except for the following modifications.

Membrane filtration

After transferring the content of the container or containers to be tested to the membrane add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the final portion of sterile diluent used to rinse the filter.

Direct inoculation

After transferring the contents of the container or containers to be tested to the culture medium add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the medium.

In both cases use the same micro-organisms as those described above under Growth promotion test of aerobes, anaerobes and fungi. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of micro-organisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity and repeat the method suitability test.

This method suitability is performed:
a) when the test for sterility has to be carried out on a new product;
b) whenever there is a change in the experimental conditions of the test.

The method suitability may be performed simultaneously with the Test for sterility of the product to be examined.

5. Test for sterility of the product to be examined

5.1. Introduction

The test may be carried out using the technique of membrane filtration or by direct inoculation of the culture media with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits, that is, for filterable aqueous preparations, for alcoholic or oily preparations and for preparations miscible with or soluble in aqueous or oily solvents provided these solvents do not have an antimicrobial effect in the conditions of the test.

5. 2. Membrane filtration

Use membrane filters having a nominal pore size not greater than 0.45 µm whose effectiveness to retain micro-organisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. Specially adapted filters may be needed for certain products, e.g. for antibiotics.

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilized by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions; it permits the aseptic removal of the membrane for transfer to the medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

Aqueous solutions

If appropriate, transfer a small quantity of a suitable, sterile diluent such as a 1 g / L neutral solution of meat or casein peptone pH 7.1 ± 0.2 onto the membrane in the apparatus and filter. The diluent may contain suitable neutralizing substances and/or appropriate inactivating substances for example in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary after diluting to the volume used in the method suitability test with the chosen sterile diluent but in any case using not less than the quantities of the product to be examined prescribed in Table 4.06-2. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than three times by filtering through it each time the volume of the chosen sterile diluent used in the method suitability test. Do not exceed a washing cycle of 5 times 100 mL per filter, even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into two equal parts and transfer one half to each of two suitable media. Use the same volume of each medium as in the method suitability test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

Table 4.06-2 — Minimum quantity to be used for each medium

Quantity per container	Minimum quantity to be used for each medium unless otherwise justified and authorised
Liquids	
– less than 1 mL:	The whole contents of each container
– 1 – 40 mL:	Half the contents of each container but not less than 1 mL
– greater than 40 mL and not greater than 100 mL	20 mL
– greater than 100 mL :	10 per cent of the contents of the container but not less than 20 mL
Antibiotic liquids	1 mL
Insoluble preparations, creams and ointments to be suspended or emulsified	Use the contents of each container to provide not less than 200 mg
Solids	
– less than 50 mg	The whole contents of each container
– 50 mg or more but less than 300 mg	Half the contents of each container but not less than 50 mg
– 300 mg – 5 g	150 mg
– greater than 5 g	500 mg

Soluble solids

Use for each medium not less than the quantity prescribed in Table 4.06-2 of the product dissolved in a suitable solvent such as the solvent provided with the preparation, water for injection, saline or a 1 g / L neutral solution of meat or casein peptone and proceed with the test as described above for aqueous solutions using a membrane appropriate to the chosen solvent.

Oils and oily solutions

Use for each medium not less than the quantity of the product prescribed in Table 4.06-2. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight then filter, applying the pressure or suction gradually. Wash the membrane at least three times by filtering through it each time about 100 mL of a suitable sterile solution such as 1 g / L neutral meat or casein peptone containing a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability of the test, for example polysorbate 80 at a concentration of 10 g / L. Transfer the membrane or membranes to the culture medium or media or vice versa as described above for aqueous solutions, and incubate at the same temperatures and for the same times.

Ointments and creams

Use for each medium not less than the quantities of the product prescribed in Table 4.06-2. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1 per cent in isopropyl myristate as described above, by heating, if necessary, to not more than 40 °C. In exceptional cases it may be necessary to heat to not more than 44 °C. Filter as rapidly as possible and proceed as described above for oils and oily solutions.

5.3. Direct inoculation of the culture medium

Transfer the quantity of the preparation to be examined prescribed in Table 4.06-2 directly into the culture medium so that the volume of the product is not more than 10 per cent of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralising this with a suitable neutralising substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. Where appropriate the concentrated medium may be added directly to the product in its container.

Oily liquids

Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability of the test, for example polysorbate 80 at a concentration of 10 g / L.

Ointments and creams

Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as a 1 g / L neutral solution of meat or casein peptone. Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However when fluid thioglycollate medium is used for the detection of anaerobic micro-organisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

6. Observation and interpretation of results

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by

visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined.

The test may be considered invalid only if one or more of the following conditions are fulfilled:

a) the data of the microbiological monitoring of the sterility testing facility show a fault;
b) a review of the testing procedure used during the test in question reveals a fault;
c) microbial growth is found in the negative controls;
d) after determination of the identity of the micro-organisms isolated from the test, the growth of this species or these species may be ascribed unequivocally to faults with respect to the material and/or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid it is repeated with the same number of units as in the original test.

If no evidence of microbial growth is found in the repeat test the product examined complies with the test for sterility. If microbial growth is found in the repeat test the product examined does not comply with the test for sterility.

7. Application of the test to parenteral preparations, ophthalmic and other non-injectable preparations required to comply with the test for sterility

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in Table 4.06-2, diluting where necessary to about 100 mL with a suitable sterile solution, such as 1 g / L neutral meat or casein peptone.

When using the technique of direct inoculation of media, use the quantities shown in Table 4.06-2, unless otherwise justified and authorised. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of two or more containers are used to inoculate the different media.

8. Minimum number of items to be tested

The minimum number of items to be tested in relation to the size of the batch is given in Table 4.06-3.

Table 4.06-3. Minimum number of items to be tested

Number of items in the batch*	Minimum number of items to be tested for each medium, unless otherwise justified and authorised**
Parenteral preparations	
–Not more than 100 containers	10 per cent or 4 containers whichever is the greater
–More than 100 but not more than 500 containers	10 containers
–More than 500 containers_	2 per cent or 20 containers (10 containers for large-volume parenterals) whichever is the less
Ophthalmic and other non-injectable preparations	
–Not more than 200 containers	5 per cent or 2 containers whichever is the greater
–More than 200 containers	10 containers
–If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral use	
Bulk solid products	
–Up to 4 containers	Each container
–More than 4 containers but not more than 50 containers	20 per cent or 4 containers whichever is the greater
–More than 50 containers	2 per cent or 10 containers whichever is the greater

* If the batch size is not known, use the maximum number of items prescribed
**If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

6.09 Disintegration Test

Change to read following part under Apparatus:

Disks-The use of disks is permitted only where specified or allowed. Each tube is provided with a cylindrical disk 9.5±0.15 mm thick and 20.7±0.15 mm in diameter. The disk is made of a suitable, transparent plastic material having a specific gravity of between 1.18 and 1.20. Five parallel 2±0.1 mm holes extend between the ends of the cylinder. One of the holes is centered on the cylindrical axis. The other holes are centered 6±0.2 mm from the axis on imaginary lines perpendicular to the axis and parallel to each other. Four identical trapezoidal-shaped planes are cut the wall of the cylinder, nearly perpendicular to the ends of the cylinder. The trapezoidal shape is symmetrical; its parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centers of two adjacent holes 6 mm from the cylindrical axis. The parallel side of the trapezoid on the bottom of the cylinder has a length of 1.6±0.1 mm, and its bottom edges lie at a depth of 1.5-1.8 mm from the cylinder's circumference. The parallel side of the trapezoid on the top of the cylinder has a length of 9.4±0.2 mm, and its center lies at a depth of 2.6±0.1 mm from the cylinder's circumference. All surfaces of the disk are smooth. If the use of disks is specified, and a desk to each tube, and operate the apparatus as directed under Procedure. The disks conform to dimensions found in Fig.6.09-1. The use of automatic detection employing modified disks is permitted where the use of disks is specified or allowed. Such disks must comply with the requirements for density and dimension given in this chapter.

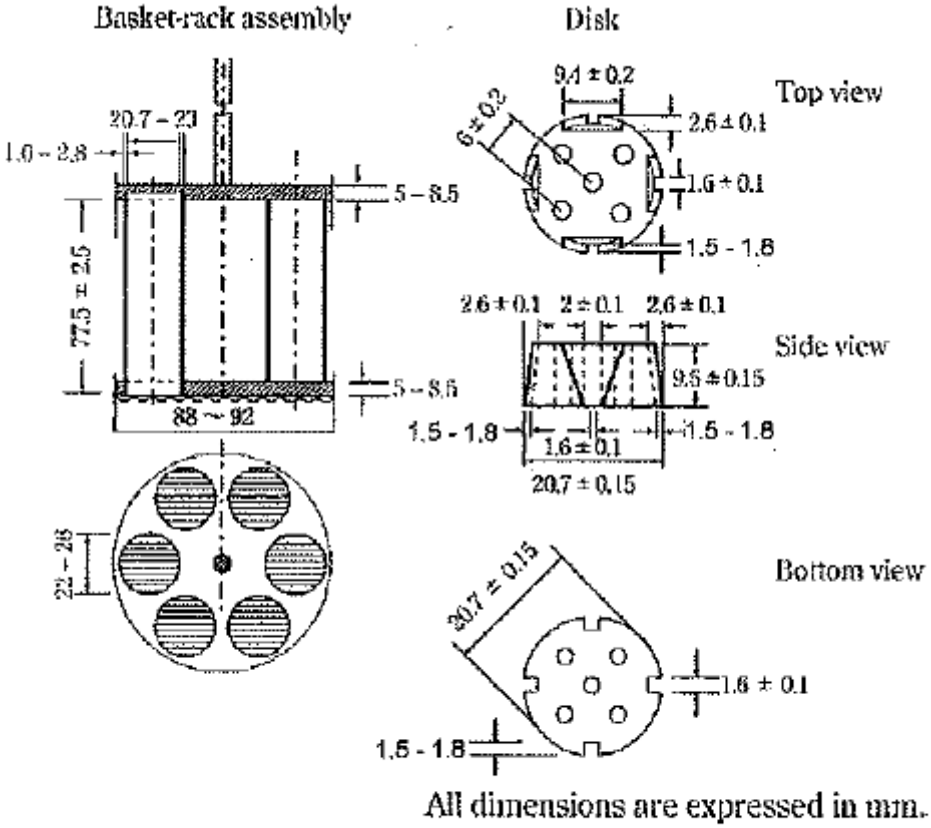

Fig. 6.09-1 Disintegration apparatus

6.10 Dissolution Test

Change to read following part under Procedure for Basket or Paddle Methods:

IMMEDIATE-RELEASE DOSAGE FORMS

Procedure—Place the stated volume of the dissolution medium (±1%) in the vessel of the specified apparatus, assemble the apparatus, equilibrate the dissolution medium to 37±0.5℃, and remove the thermometer. Place 1 dosage unit in the apparatus, taking care to exclude air bubbles from the surface of the dosage unit, and immediately operate the apparatus at the specified rate. Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the Dissolution Medium and the top of the rotating basket or blade, not less than 10 mm from the vessel wall. [NOTE—Where multiple sampling times are specified, replace the aliquots withdrawn for analysis with equal volumes of fresh Dissolution Medium at 37℃ or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.] Perform the analysis using an indicated assay method.[*3] Repeat the test with additional dosage units.

If automated equipment is used for sampling or the apparatus is otherwise modified, verification that the modified apparatus will produce results equivalent to those obtained with the standard apparatus described in this chapter, is necessary.

Dissolution Medium-A specified dissolution medium is used. The volume specified refers to measurements made between 20℃ and 25℃. If the dissolution medium is a buffered solution, adjust the solution so that its pH is within 0.05 unit of the specified pH. [NOTE-Dissolved gases can cause bubbles to form, which may change the results of the test. If dissolved gases influence the dissolution results, remove dissolved gases prior testing.[*4]]

Time—Where a single time specification is given, the test may be concluded in a shorter period if the requirement for minimum amount dissolved is met. Specimens are to be withdrawn only at the stated times, within a tolerance of ±2%.

Change to read following part under Apparatus for Flow-Through Cell Method:

Apparatus for Flow-Through Cell Method (Apparatus 3)—The assembly consists of a reservoir and a pump for the dissolution medium; a flow-through cell; a water bath that maintains the dissolution medium at 37±0.5℃. Use the cell size specified in the individual monograph.

The pump forces the dissolution medium upwards through the flow-through cell. The pump has a delivery range between 4 and 16 mL per minute, with standard flow rates of 4, 8, and 16 mL per minute. It must deliver a constant flow (±5 percent of the nominal flow rate); the flow profile should be sinusoidal with a pulsation of 120±10 pulses per minute. A pump without the pulsation may also be used. Dissolution test procedures using the flow-through cell must be characterized with respect to rate and any pulsation.

The flow-through cell (see Figures 6.10-3 and 6.10-4), of transparent and inert material, is mounted vertically with a filter system (specified in the individual monograph) that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1-mm diameter with one bead of about 5 mm positioned at the apex to protect the fluid entry tube; a tablet holder (see Figures 6.10-3 and 6.10-4) is available for positioning of special dosage forms. The

cell is immersed in a water bath, and the temperature is maintained at 37±0.5℃.

The apparatus uses a clamp mechanism of two O-rings to assemble the cell. The pump is separated from the dissolution unit in order to shield the latter against any vibrations originating from the pump. The position of the pump should not be on a level higher than the reservoir flasks. Tube connections are as short as possible. Use suitably inert tubing, such as polytef, with about 1.6-mm inner diameter and inert flanged-end connections.

Apparatus Suitability—The determination of suitability of a test assembly to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as given above. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the dissolution medium, rotation speed (Basket Method and Paddle Method), and flow rate of medium (Flow-Through Cell Method).

Determine the acceptable performance of the dissolution test assembly periodically.