

monograph and note whether the contractions produced by the preparation with the added histamine correspond to the amount of histamine added. If this is not the case, or if the contractions caused by the substance to be examined are not reproducible or if subsequent responses to "high" and "low" doses of histamine are diminished, the results of the tests are invalid and the test for depressor substances (2.6.11) must be carried out.

Solution A

Sodium chloride	160.0 g
Potassium chloride	4.0 g
Calcium chloride, anhydrous	2.0 g
Magnesium chloride, anhydrous	1.0 g
Disodium hydrogen phosphate dodecahydrate	0.10 g
Water for injections R sufficient to produce	1000 ml

Solution B

Solution A	50.0 ml
Atropine sulphate	0.5 mg
Sodium hydrogen carbonate	1.0 g
Glucose monohydrate	0.5 g
Water for injections R sufficient to produce	1000 ml

Solution B should be freshly prepared and used within 24 h.

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2.6.11. DEPRESSOR SUBSTANCES

Carry out the test on a cat weighing not less than 2 kg and anaesthetised with chloralose or with a barbiturate that allows the maintenance of uniform blood pressure. Protect the animal from loss of body heat and maintain it so that the rectal temperature remains within physiological limits. Introduce a cannula into the trachea. Insert a cannula filled with a heparinised 9 g/l solution of sodium chloride into the common carotid artery and connect it to a device capable of giving a continuous record of the blood pressure. Insert into the femoral vein another cannula, filled with a heparinised 9 g/l solution of sodium chloride, through which can be injected the solutions of histamine and of the substance to be examined. Determine the sensitivity of the animal to histamine by injecting intravenously at regular intervals, doses of *histamine solution R* corresponding to 0.1 µg and 0.15 µg of histamine base per kilogram of body mass. Repeat the lower dose at least 3 times. Administer the second and subsequent injections not less than 1 min after the blood pressure has returned to the level it was at immediately before the previous injection. The animal is used for the test only if a readily discernible decrease in blood pressure that is constant for the lower dose is obtained and if the higher dose causes greater responses. Dissolve the substance to be examined in sufficient of a 9 g/l solution of sodium chloride or other prescribed solvent, to give the prescribed concentration. Inject intravenously per kilogram of body mass 1.0 ml of *histamine solution R*, followed by 2 successive injections of the prescribed amount of the solution to be examined and, finally, 1.0 ml of *histamine solution R*. The second, third and fourth injections are given not less than 1 min after the blood pressure has returned to the level it was at immediately before the preceding injection. Repeat this series of injections twice and conclude the test by giving 1.5 ml of *histamine solution R* per kilogram of body mass.

If the response to 1.5 ml of *histamine solution R* per kilogram of body mass is not greater than that to 1.0 ml the test is invalid. The substance to be examined fails the test if the mean of the series of responses to the substance is greater than the mean of the responses to 1.0 ml of *histamine solution R* per kilogram of body mass or if any one dose of the substance causes a greater depressor response than the concluding dose of the histamine solution. The test animal must not be used in another test for depressor substances if the second criterion applies or if the response to the high dose of histamine given after the administration of the substance to be examined is less than the mean response to the low doses of histamine previously injected.

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2.6.12. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: TOTAL VIABLE AEROBIC COUNT

This general chapter presents 2 sets of tests. The 1st set gives the reference methods for determining compliance with monographs. Reference to this chapter in a monograph therefore implies compliance with the 1st set of tests, unless use of the 2nd set of tests has been authorised. The tests in the 2nd set also constitute official methods of the European Pharmacopoeia and may be referred to as such, notably in applications for marketing authorisation. It is intended to replace the 1st set by the 2nd set once the monographs concerned have been revised. The 2nd set presents tests developed in co-operation with the Japanese Pharmacopoeia and the United States Pharmacopoeia to achieve harmonised requirements.

A. METHOD OF THE EUROPEAN PHARMACOPOEIA

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

The tests are designed primarily to determine whether or not a substance that is the subject of a monograph in the Pharmacopoeia complies with the microbiological requirements specified in the monograph in question. 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 tests may also be used for the test for *Efficacy of antimicrobial preservation (5.1.3)* as described in the Pharmacopoeia. They may furthermore be used for monitoring raw material quality and may be used in association with guidelines on microbiological quality (5.1.4). When used for such purposes, for example by a manufacturer for raw materials and/or finished product monitoring or for process validation, the conduct of the tests including the number of samples to be taken and the interpretation of the results are matters for agreement between the manufacturer and the competent authority.

Carry out the determination under conditions designed to avoid accidental contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any micro-organisms that are revealed in the test. If the product to be examined has antimicrobial activity, this must be adequately neutralised. If inactivators are used for this purpose, their efficacy and non-toxicity versus micro-organisms are demonstrated.

Determine the total viable aerobic count by the membrane filtration method or the plate-count method, as prescribed in the monograph.

The Most Probable Number (MPN) method is reserved for bacterial counts when no other method is available. The choice of a method may be based on factors such as the nature of the product and the expected number of micro-organisms. Any method that is chosen must be properly validated.

When used in conjunction with chapter 5.1.3 or 5.1.4, the pour-plate method, the surface-spread method and the membrane filtration method may be used.

PREPARATION OF THE SAMPLE

Sampling plan. Sampling of the product must follow a well-defined sampling plan. The sampling plan will be dependent on factors such as batch size, health hazard associated with unacceptably highly contaminated products, the characteristics of the product and the expected level of contamination. Unless otherwise prescribed, use sample(s) of 10 g or 10 ml of the substance or preparation to be examined, taken with the precautions referred to above. Select the sample(s) at random from the bulk material or from the available containers of the preparation. If necessary, to obtain the required quantity, mix the contents of a sufficient number of containers to provide each sample, depending on the nature of the substance or preparation to be examined.

An example of a sampling plan applicable to products where homogeneity with respect to the distribution of micro-organisms may be a problem, is the three-class sampling plan. In this case five samples from each batch are drawn and investigated separately. The three recognised classes are:

- (i) acceptable samples, i.e. samples containing less than m CFU (colony-forming units) per gram or millilitre, where m is the limit specified in the relevant monograph;
- (ii) marginal samples, i.e. with more than m CFU but less than $10m$ CFU per gram or millilitre;
- (iii) defective samples, i.e. containing more than $10m$ CFU per gram or millilitre.

Water-soluble products. Dissolve or dilute 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten dilution is prepared. However, the characteristics of the product or the required sensitivity may necessitate the use of other ratios. If the product is known to have antimicrobial activity, an inactivating agent may be added to the diluent. If necessary, adjust the pH to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Non-fatty products insoluble in water. Suspend 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten suspension is prepared, but the characteristics of some products may necessitate the use of larger volumes. A suitable surface-active agent such as 1 g/l of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If the product is known to have antimicrobial activity, an inactivating agent may be added to the diluent. If necessary, adjust to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Fatty products. Homogenise 10 g or 10 ml of the product to be examined with not more than half its weight of sterile polysorbate 80 or another suitable sterile surface-active agent, 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 or in an incubator. Add sufficient pre-warmed buffered sodium chloride-peptone solution pH 7.0 to make a one in ten dilution of the original product. Mix carefully whilst

maintaining the temperature for the shortest time necessary for the formation of an emulsion and in any case for not more than 30 min. Further serial tenfold dilutions may be prepared using buffered sodium chloride-peptone solution pH 7.0 containing a suitable concentration of sterile polysorbate 80 or another sterile surface-active agent.

Transdermal patches. Remove the protective cover sheets ('release liners') of ten patches of the transdermal preparation using sterile forceps, and place them, the adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile gauze (or a woven-filter type monofilament polymer grid), if necessary, and transfer the ten patches to a minimum volume of 500 ml of buffered sodium chloride-peptone solution pH 7.0 containing suitable inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 min (preparation A). Prepare another ten patches in the same way, place them in a minimum volume of 500 ml of broth medium D and shake vigorously for at least 30 min (preparation B).

EXAMINATION OF THE SAMPLE

Membrane filtration. Use membrane filters having a nominal pore size not greater than 0.45 µm and whose effectiveness to retain bacteria has been established. The type of filter material is chosen such that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. Cellulose nitrate filters, for example, may be used for aqueous, oily and weakly alcoholic solutions, and cellulose acetate filters, for example, for strongly alcoholic solutions. The filtration apparatus is designed to allow the transfer of the filter to the culture medium.

Transfer a suitable amount of the sample prepared as described in the section Preparation of the sample (preferably representing 1 g of the product, or less if large numbers of colony-forming units are expected) to each of 2 membrane filters and filter immediately. Wash each filter with 3 quantities, each of about 100 ml of a suitable liquid such as buffered sodium chloride-peptone solution pH 7.0. To this solution, surface-active agents such as polysorbate 80, or inactivators of antimicrobial agents may be added. If validated, less than 3 washes may be applied. Transfer one of the membrane filters, intended primarily for the enumeration of bacteria, to the surface of a suitable agar medium, such as medium B and the other, intended primarily for the enumeration of fungi, to the surface of a suitable agar medium, such as medium C. Incubate the plate of agar medium B at 30-35 °C, and the plate of agar medium C at 20-25 °C, for 5 days, unless a reliable count is obtained in a shorter time. Select plates with the highest number below 100 colonies and calculate the number of colony-forming units per gram or millilitre of product.

When examining transdermal patches, filter 50 ml of preparation A separately through each of 2 sterile filter membranes. Transfer one membrane to agar medium B for total aerobic microbial count, and the other membrane to agar medium C for the count of fungi.

Plate-count methods

a. Pour-plate method. Using Petri dishes 9 cm in diameter, add to each dish 1 ml of the sample prepared as described in the section Preparation of the sample and 15-20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B), or 15-20 ml of a liquefied agar medium suitable for the cultivation of fungi (such as medium C) at not more than 45 °C. If larger Petri dishes are used the amount of agar is increased accordingly. Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates at 30-35 °C (20-25 °C for fungi) for 5 days, unless a reliable count is obtained in a shorter time.

Select the plates corresponding to 1 dilution and showing the highest number of colonies less than 300 (100 for fungi). Take the arithmetic average of the counts and calculate the number of colony-forming units per gram or millilitre.

b. Surface-spread method. Using Petri dishes 9 cm in diameter, add 15-20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B) or a liquefied agar medium suitable for the cultivation of fungi (such as medium C) 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 an incubator. Spread a measured volume of not less than 0.1 ml of the sample prepared as described in the section Preparation of the sample over the surface of the medium. Use at least 2 Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of colony-forming units proceed as described for the pour-plate method.

Most-probable-number method

Table 2.6.12.-1. – *Most-probable-number values of bacteria*

3 tubes at each level of dilution							
Number of positive tubes			MPN per gram	Category*		95 per cent confidence limits	
0.1 g	0.01 g	0.001 g		1	2		
0	0	0	< 3			–	–
0	1	0	3		x	< 1	17
1	0	0	3	x		1	21
1	0	1	7		x	2	27
1	1	0	7	x		2	28
1	2	0	11		x	4	35
2	0	0	9	x		2	38
2	0	1	14		x	5	48
2	1	0	15	x		5	50
2	1	1	20		x	8	61
2	2	0	21	x		8	63
3	0	0	23	x		7	129
3	0	1	38	x		10	180
3	1	0	43	x		20	210
3	1	1	75	x		20	280
3	2	0	93	x		30	390
3	2	1	150	x		50	510
3	2	2	210		x	80	640
3	3	0	240	x		100	1400
3	3	1	460	x		200	2400
3	3	2	1100	x		300	4800
3	3	3	> 1100			–	–

* Category 1: Normal results, obtained in 95 per cent of the cases.
 * Category 2: Less likely results, obtained in only 4 per cent of cases. These are not to be used for important decisions. Results that are even less likely than those of category 2 are not mentioned and are always unacceptable.

The precision and accuracy of the most-probable-number method (MPN) is less than that of the membrane filtration method or the plate-count methods. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the

enumeration of bacteria 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 subsequent tenfold dilutions of the product as described in the section Preparation of the sample. From each level of dilution, 3 aliquots of 1 g or 1 ml are used to inoculate 3 tubes with 9-10 ml of a suitable liquid medium (such as broth medium A). 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 for 5 days at 30-35 °C. Record for each level of dilution the number of tubes showing microbial growth. If the reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or on a suitable agar medium (such as agar medium B), for 18-24 h at the same temperature and use these results. Determine the most probable number of bacteria per gram or millilitre of the product to be examined from Table 2.6.12.-1.

EFFECTIVENESS OF CULTURE MEDIA AND VALIDITY OF THE COUNTING METHOD

Grow the bacterial test strains separately in containers containing a suitable liquid medium (such as broth medium A) at 30-35 °C for 18-24 h. Grow the fungal test strains separately on a suitable agar medium (such as medium C without antibiotics) at 20-25 °C for 48 h for *Candida albicans* and at 20-25 °C for 7 days for *Aspergillus niger*.

<i>Staphylococcus aureus</i>	such as ATCC 6538 (NCIMB 9518, CIP 4.83)
<i>Escherichia coli</i>	such as ATCC 8739 (NCIMB 8545, CIP 53.126)
<i>Bacillus subtilis</i>	such as ATCC 6633 (NCIMB 8054, CIP 52.62)
<i>Candida albicans</i>	such as ATCC 10231 (NCPF 3179, IP 48.72)
<i>Aspergillus niger</i>	such as ATCC 16404 (IMI 149007, IP 1431.83)

Use buffered sodium chloride-peptone solution pH 7.0 to make reference suspensions containing about 100 colony-forming units per millilitre. Use the suspension of each of the micro-organisms separately as a control of the counting methods, in the presence and absence of the product to be examined. When testing the membrane filtration method or the plate-count method, a count of any of the test organisms differing by not more than a factor of 5 from the calculated value from the inoculum is to be obtained. When testing the most-probable-number method the calculated value from the inoculum is to be within the 95 per cent confidence limits of the results obtained. To test the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the method using sterile sodium chloride-peptone solution pH 7.0 as the test preparation. There must be no growth of micro-organisms.

INTERPRETATION OF THE RESULTS

The bacterial count will be considered to be equal to the average number of colony-forming units found on agar medium B. The fungal count will be considered to be equal to the average number of colony-forming units on agar medium C. The total viable aerobic count is the sum of the bacterial count and the fungal count as described above. If there is evidence that the same types of micro-organisms grow on both media this may be corrected. If the count is carried out by the most-probable-number method the calculated value is the bacterial count.

When a limit is prescribed in a monograph it is interpreted as follows:

10² micro-organisms: maximum acceptable limit: 5 × 10²;

10^3 micro-organisms: maximum acceptable limit: 5×10^3 ; and so forth.

If a sampling plan such as the three-class sampling plan, for example, is used, proceed as follows.

Calculate the total viable aerobic count separately for each of the five samples. The substance or preparation passes the test if the following conditions are fulfilled:

- (i) none of the individual total viable aerobic counts exceeds the prescribed limit by a factor of 10 or more (i.e. no 'unacceptable samples');
- (ii) not more than 2 of the individual total viable aerobic counts are between the prescribed limit and 10 times this limit (i.e. no more than 2 'marginal samples').

The solutions and culture mediums recommended are described in the general chapter 2.6.13.

B. HARMONISED METHOD: MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

1. INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that 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 Pharmacopoeia 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 that 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 a very low bioburden, it may be the most appropriate method.

The choice of method is based on factors such as the nature of the product and the required limit of micro-organisms. The chosen method must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the method chosen 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 them 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 2.6.12.-2.

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 2.6.12.-2, 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 2.6.12.-2, using a separate plate of medium for each. Incubate in the conditions described in Table 2.6.12.-2.

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 upon 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.

Table 2.6.12.-2. – 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 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 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 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 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 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

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 agent, 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 agent.

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 liners') of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays.

Cover the adhesive surface with a 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 neutralisation, dilution or filtration.

4-5-3. Neutralisation/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 specific or general neutralising agents into the diluent, (3) membrane filtration, or (4) a combination of the above measures.

Neutralising agents. Neutralising agents may be used to neutralise the activity of antimicrobial agents (Table 2.6.12.-3). They may be added to the chosen diluent or the medium preferably before sterilisation. If used, their efficacy and their absence of toxicity for micro-organisms must be demonstrated by carrying out a blank with neutraliser and without product.

Table 2.6.12.-3. – *Common neutralising agents for interfering substances*

Interfering substance	Potential neutralising method
Glutaraldehyde, mercurials	Sodium hydrogensulphite (sodium bisulphite)
Phenolics, alcohol, aldehydes, sorbate	Dilution
Aldehydes	Glycine
Quaternary Ammonium Compounds (QACs), parahydroxybenzoates (parabens), bis-biguanides	Lecithin
QACs, iodine, parabens	Polysorbate
Mercurials	Thioglycollate
Mercurials, halogens, aldehydes	Thiosulphate
EDTA (edetate)	Mg ²⁺ or Ca ²⁺ ions

If no suitable neutralising 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 product 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 such 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 2.6.12.-2. 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 2.6.12.-2, at least 2 Petri dishes are used. Incubate the plates as indicated in Table 2.6.12.-2. 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 an incubator. For each of the micro-organisms listed in Table 2.6.12.-2, 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 in 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 2.6.12.-4.

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 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.

Table 2.6.12-4. – *Most-probable-number values of micro-organisms*

Observed combinations of numbers of tubes showing growth in each set			MPN per grams or per millilitre of product	95 per cent confidence limits
Number of grams or millilitres of product per tube				
0.1	0.01	0.001		
0	0	0	< 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	> 1100	

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 at 30-35 °C for 3-5 days. 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 2.6.12-4.

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 the 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 the 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:

- 10¹ CFU: maximum acceptable count = 20;
- 10² CFU: maximum acceptable count = 200;
- 10³ CFU: maximum acceptable count = 2000, and so forth.

The recommended solutions and media are described in general chapter 2.6.13 (under section B, Harmonised method).

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2.6.13. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: TEST FOR SPECIFIED MICRO-ORGANISMS

This general chapter presents 2 sets of tests. The 1st set gives the reference methods for determining compliance with monographs. Reference to this chapter in a monograph therefore implies compliance with the 1st set of tests, unless use of the 2nd set of tests has been authorised. The tests in the 2nd set also constitute official methods of the European Pharmacopoeia and may be referred to as such, notably in applications for marketing authorisation. It is intended to replace the 1st set by the 2nd set once the monographs concerned have been revised. The 2nd set presents tests developed in co-operation with the Japanese Pharmacopoeia and the United States Pharmacopoeia to achieve harmonised requirements.

A. METHOD OF THE EUROPEAN PHARMACOPOEIA

In this general method the use of certain selective media is proposed. A feature common to all selective media is that sub-lethally injured organisms are not detected. As

sub-lethally injured organisms are relevant for the quality of the product, a resuscitation must be included in examination procedures that rely on selective media.

If the product to be examined has antimicrobial activity, this must be adequately neutralised.

Enterobacteria and certain other gram-negative bacteria

Although the test has been designed to detect bacteria belonging to the family of Enterobacteriaceae, it is recognised that other types of organisms (e.g. *Aeromonas*, *Pseudomonas*) may be recovered.

Detection of bacteria. Prepare the product to be examined as described in the general method 2.6.12, but using broth medium D in place of buffered sodium chloride-peptone solution pH 7.0, homogenise and incubate at 35-37 °C for a time sufficient to revive the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 h but not more than 5 h). Shake the container, transfer the quantity of the contents (homogenate A) corresponding to 1 g or 1 ml of the product to 100 ml of enrichment medium E and incubate at 35-37 °C for 18-48 h. Subculture on plates of agar medium F. Incubate at 35-37 °C for 18-24 h. The product passes the test if there is no growth of colonies of gram-negative bacteria on any plate.

Quantitative evaluation. Inoculate suitable quantities of enrichment broth medium E with homogenate A 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 35-37 °C for 24-48 h. Subculture each of the cultures on a plate of agar medium F to obtain selective isolation. Incubate at 35-37 °C for 18-24 h. Growth of well-developed colonies, generally red or reddish, of gram-negative bacteria 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 2.6.13-1 the probable number of bacteria.

Table 2.6.13-1

Results for each quantity of product			Probable number of bacteria per gram 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

When testing transdermal patches, filter 50 ml of preparation B as described in the general method 2.6.12 through a sterile filter membrane, place the membrane in 100 ml of enrichment broth medium E and incubate at 35-37 °C for 18-24 h. After incubation, spread on agar medium F for the detection of Enterobacteria and other gram-negative micro-organisms.

Escherichia coli

Prepare the product to be examined as described in the general method 2.6.12 and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate 100 ml of broth medium A, homogenise and incubate at 35-37 °C for 18-48 h. Shake the container, transfer 1 ml to 100 ml of broth medium G and incubate at 43-45 °C for 18-24 h. Subculture on plates of agar medium H at 35-37 °C for 18-72 h. Growth of red, non-mucoid colonies of gram-negative rods indicates the possible presence of *E. coli*. This is confirmed by suitable biochemical tests, such as indole production. The