

2.6. BIOLOGICAL TESTS

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2.6.1. STERILITY

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. Guidance for using the test for sterility is given at the end of this text.

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 (such as those indicated in the appropriate European Community Directives and associated guidance documents on GMP).

CULTURE MEDIA AND INCUBATION TEMPERATURES

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.

Other media may be used provided that they pass the growth promotion and the validation tests.

Fluid thioglycollate medium

L-Cystine	0.5 g
Agar, granulated (moisture content not in excess of 15 per cent)	0.75 g
Sodium chloride	2.5 g
Glucose monohydrate/anhydrous	5.5 g/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 g/1 of resazurin sodium), freshly prepared	1.0 ml
Water R	1000 ml

pH of the medium after sterilisation 7.1 ± 0.2

Mix the L-cystine, agar, sodium chloride, glucose, water-soluble yeast extract and pancreatic digest of casein with the *water R* and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycollic acid in the solution and, if necessary, add *1 M sodium hydroxide* so that, after sterilisation, 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, 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. Sterilise using a validated process. If the medium is stored, store at $2-25\text{ }^{\circ}\text{C}$ in a sterile, airtight container. If more than the upper 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 $30-35\text{ }^{\circ}\text{C}$.

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 g/2.3 g
Water R	1000 ml

pH of the medium after sterilisation 7.3 ± 0.2

Dissolve the solids in *water R*, warming slightly to effect solution. Cool the solution to room temperature. Add *1 M sodium hydroxide*, if necessary, so that after sterilisation the medium will have a pH of 7.3 ± 0.2 . Filter, if necessary, to clarify, distribute into suitable vessels and sterilise using a validated process. Store at $2-25\text{ }^{\circ}\text{C}$ in a sterile well-closed 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 $20-25\text{ }^{\circ}\text{C}$.

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 the ingredients. Suitable strains of micro-organisms are indicated in Table 2.6.1-1.

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 5 passages removed from the original master seed-lot.

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

VALIDATION 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.

Table 2.6.1-1 – *Strains of the test micro-organisms suitable for use in the Growth Promotion Test and the Validation Test*

Aerobic bacteria	
<i>Staphylococcus aureus</i>	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518
<i>Bacillus subtilis</i>	ATCC 6633, CIP 52.62, NCIMB 8054
<i>Pseudomonas aeruginosa</i>	ATCC 9027, NCIMB 8626, CIP 82.118
Anaerobic bacterium	
<i>Clostridium sporogenes</i>	ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437
Fungi	
<i>Candida albicans</i>	ATCC 10231, IP 48.72, NCPF 3179
<i>Aspergillus niger</i>	ATCC 16404, IP 1431.83, IMI 149007

Membrane filtration. After transferring the contents 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 (for catgut and other surgical sutures for veterinary use: strands) 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 validation test.

This validation is performed:

- when the test for sterility has to be carried out on a new product,
- whenever there is a change in the experimental conditions of the test.

The validation may be performed simultaneously with the test for sterility of the product to be examined.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

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.

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 sterilised 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 neutralising 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 validation 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 2.6.1-2. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than 3 times by filtering through it each time the volume of the chosen sterile diluent used in the validation test. Do not exceed a washing cycle of 5 times 200 ml, even if during validation 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 2 equal parts and transfer one half to each of 2 suitable media. Use the same volume of each medium as in the validation test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

Soluble solids. Use for each medium not less than the quantity prescribed in Table 2.6.1-2 of the product dissolved in a suitable solvent such as 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 2.6.1-2. 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

Table 2.6.1.-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
<i>Liquids</i>	
– 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
<i>Antibiotic liquids</i>	1 ml
<i>Other preparations soluble in water or in isopropyl myristate</i>	The whole contents of each container to provide not less than 200 mg
<i>Insoluble preparations, creams and ointments to be suspended or emulsified</i>	The whole contents of each container to provide not less than 200 mg
<i>Solids</i>	
– 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 to 5 g	150 mg
– greater than 5 g	500 mg
<i>Catgut and other surgical sutures for veterinary use</i>	3 sections of a strand (each 30 cm long)

3 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 validation 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 2.6.1.-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.

Direct inoculation of the culture medium. Transfer the quantity of the preparation to be examined prescribed in Table 2.6.1.-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 validation 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 thioglycollate medium or other

similar medium is used for the detection of anaerobic micro-organisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

Catgut and other surgical sutures for veterinary use. Use for each medium not less than the quantities of the product prescribed in Table 2.6.1.-2. Open the sealed package using aseptic precautions and remove 3 sections of the strand for each culture medium. Carry out the test on 3 sections, each 30 cm long, cut off from the beginning, the centre and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 ml to 150 ml).

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:

- the data of the microbiological monitoring of the sterility testing facility show a fault,
- a review of the testing procedure used during the test in question reveals a fault,
- microbial growth is found in the negative controls,
- 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.

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 2.6.1.-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 2.6.1.-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 2 or more containers are used to inoculate the different media.

GUIDELINES FOR USING THE TEST FOR STERILITY

The purpose of the test for sterility, as that of all pharmacopoeial tests, is to provide an independent control analyst with the means of verifying that a particular material meets the requirements of the European Pharmacopoeia. A manufacturer is neither obliged to carry out such tests nor precluded from using modifications of, or alternatives to, the stated method, provided he is satisfied that, if tested by the official method, the material in question would comply with the requirements of the European Pharmacopoeia.

Precautions against microbial contamination. Aseptic conditions for performance of the test can be achieved using, for example, a class A laminar-air-flow cabinet located within a class B clean-room, or an isolator.

Guidance to manufacturers. The level of assurance provided by a satisfactory result of a test for sterility (the absence of contaminated units in the sample) as applied to the quality of

the batch is a function of the homogeneity of the batch, the conditions of manufacture and the efficiency of the adopted sampling plan. Hence for the purpose of this text a batch is defined as a homogeneous collection of sealed containers prepared in such a manner that the risk of contamination is the same for each of the units contained therein.

In the case of terminally sterilised products, physical proofs, biologically based and automatically documented, showing correct treatment throughout the batch during sterilisation are of greater assurance than the sterility test. The circumstances in which parametric release may be considered appropriate are described under Methods of preparation of sterile products (5.1.1). The method of media-fill runs may be used to evaluate the process of aseptic production. Apart from that the sterility test is the only analytical method available for products prepared under aseptic conditions and furthermore it is, in all cases, the only analytical method available to the authorities who have to examine a specimen of a product for sterility.

The probability of detecting micro-organisms by the test for sterility increases with their number present in the sample tested and varies according to the readiness of growth of micro-organism present. The probability of detecting very low levels of contamination even when it is homogenous throughout the batch is very low. The interpretation of the results of the test for sterility rests on the assumption that the contents of every container in the batch, had they been tested, would have given the same result. Since it is manifest that every container cannot be tested, an appropriate sampling plan should be adopted. In the case of aseptic production, it is recommended to include samples filled at the beginning and at the end of the batch and after significant intervention.

Guidance on the minimum number of items recommended to be tested in relation to the size of the batch is given in Table 2.6.1.-3. The application of the recommendations must have regard to the volume of preparation per container, to the validation of the sterilisation method and to any other special considerations concerning the intended sterility of the product.

Table 2.6.1.-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*
<i>Parenteral preparations</i>	
– 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, whichever is less
<i>Ophthalmic and other non-injectable preparations</i>	
– 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	
<i>Catgut and other surgical sutures for veterinary use</i>	2 per cent or 5 packages whichever is the greater, up to a maximum total of 20 packages
<i>Bulk solid products</i>	
– 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
<i>Pharmacy bulk packages of antibiotics (greater than 5 g)</i>	6 containers
*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.	

Observation and interpretation of results. Conventional microbiological/biochemical techniques are generally satisfactory for identification of micro-organisms recovered from a sterility test. However, if a manufacturer wishes to use condition (d) as the sole criterion for invalidating a sterility test, it may be necessary to employ sensitive typing techniques to demonstrate that a micro-organism isolated from the product test is identical to a micro-organism isolated from the test materials and/or the testing environment. While routine microbiological/biochemical identification techniques can demonstrate that 2 isolates are not identical, these methods may not be sufficiently sensitive or reliable enough to provide unequivocal evidence that two isolates are from the same source. More sensitive tests, for example, molecular typing with RNA/DNA homology, may be necessary to determine that micro-organisms are clonally related and have a common origin.

01/2005:20602

2.6.2. MYCOBACTERIA

If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurilsulfate solution.

Inoculate 0.2 ml of the sample in triplicate onto each of 2 suitable solid media (Löwenstein-Jensen medium and Middlebrook 7H10 medium are considered suitable). Inoculate 0.5 ml in triplicate into a suitable liquid medium. Incubate all media at 37 °C for 56 days.

Establish the fertility of the media in the presence of the preparation to be examined by inoculation of a suitable strain of a *Mycobacterium* sp. such as BCG and if necessary use a suitable neutralising substance.

If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test.

If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

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2.6.7. MYCOPLASMAS

Where the test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot or for control cells, both the culture method and the indicator cell culture method are used. Where the test for mycoplasmas is prescribed for a virus harvest, for a bulk vaccine or for the final lot (batch), the culture method is used. The indicator cell culture method may also be used, where necessary, for screening of media.

CULTURE METHOD

CHOICE OF CULTURE MEDIA

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers of mycoplasmas that may be present in the product to be examined. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the organisms shown below. The nutritive properties of each new batch of medium are verified for the appropriate organisms in the list.

Acholeplasma laidlawii (vaccines for human and veterinary use where an antibiotic has been used during production)

Mycoplasma gallisepticum (where avian material has been used during production or where the vaccine is intended for use in poultry)

Mycoplasma hyorhinis (non-avian veterinary vaccines)

Mycoplasma orale (vaccines for human and veterinary use)

Mycoplasma pneumoniae (vaccines for human use) or other suitable species of D-glucose fermenter

Mycoplasma synoviae (where avian material has been used during production or where the vaccine is intended for use in poultry).

The test strains are field isolates having undergone not more than fifteen subcultures and are stored frozen or freeze-dried. After cloning the strains are identified as being of the required species by a suitable method, by comparison with type cultures, for example:

<i>A. laidlawii</i>	NCTC 10116	CIP 75.27	ATCC 23206
<i>M. gallisepticum</i>	NCTC 10115	CIP 104967	ATCC 19610
<i>M. hyorhinis</i>	NCTC 10130	CIP 104968	ATCC 17981
<i>M. orale</i>	NCTC 10112	CIP 104969	ATCC 23714
<i>M. pneumoniae</i>	NCTC 10119	CIP 103766	ATCC 15531
<i>M. synoviae</i>	NCTC 10124	CIP 104970	ATCC 25204

INCUBATION CONDITIONS

Divide inoculated media into two equal parts and incubate one in aerobic conditions and the other in microaerophilic conditions; for solid media maintain an atmosphere of adequate humidity to prevent desiccation of the surface. For aerobic conditions, incubate in an atmosphere of air containing, for solid media, 5 to 10 per cent of carbon dioxide. For microaerophilic conditions, incubate in an atmosphere of nitrogen containing, for solid media, 5 to 10 per cent of carbon dioxide.

NUTRITIVE PROPERTIES

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test organisms; use not more than 100 CFU (colony-forming units) per 60 mm plate containing 9 ml of solid medium and not more than 40 CFU per 100 ml container of the corresponding liquid medium; use a separate plate and container for each species of organism. Incubate the media in the conditions that will be used for the test of the product to be examined (aerobically, microaerophilically or both, depending on the requirements of the test organism). The media comply with the test for nutritive properties if there is adequate growth of the test organisms accompanied by an appropriate colour change in liquid media.

INHIBITORY SUBSTANCES

Carry out the test for nutritive properties in the presence of the product to be examined. If growth of the test organisms is notably less than that found in the absence of the product to be examined, the latter contains inhibitory substances that must be neutralised (or their effect otherwise countered, for example, by dilution) before the test for mycoplasmas is carried out. The effectiveness of the neutralisation or other process is checked by repeating the test for inhibitory substances after neutralisation.

TEST FOR MYCOPLASMAS IN THE PRODUCT TO BE EXAMINED

For solid media, use plates 60 mm in diameter and containing 9 ml of medium. Inoculate each of not fewer than two plates of each solid medium with 0.2 ml of the product