3.2 Test for sterility

This text is based on the internationally-harmonized texts developed by the Pharmacopoeial Discussion Group (PDG). Some editorial modifications have been made in order to be in line with the style used in The International Pharmacopoeia.

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

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 microorganisms 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.

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.

- ***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 g/l of resazurin sodium), freshly prepared	1.0 mL
Water R	1000 mL

pH after sterilization 6.9–7.3.

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 sodium hydroxide (1 mol/l) VS so that, after sterilization, the solution will have a pH of 9–7.3. 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. Sterilize using a validated process. If the medium is stored store at a temperature between 2 °C and 25 °C in a sterile, airtight 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.

- ***Alternative thioglycollate medium***

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,

sterilize as directed above. The pH after sterilization is 6.9–7.3. Heat in a water-bath prior to use and incubate at 30–35 °C under anaerobic conditions.

-*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	2.5g
Glucose monohydrate/anhydrous	2.5/2.3 g
Water R	1 000 mL
pH after sterilization 7.1–7.5.	

Dissolve the solids in water R, warming slightly to effect solution. Cool the solution to room temperature. Add sodium hydroxide (1 mol/l) VS, if necessary, so that after sterilization the solution will have a pH of 7.1–7.5. 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, 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 °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 microorganisms 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 microorganisms are indicated in Table 1.

Inoculate portions of fluid thioglycollate medium with a small number (not more than 100 CFU) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *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 microorganisms, using a separate portion of medium for each of the following species of microorganism: *Aspergillus brasiliensis*, *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 microorganisms 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 microorganisms occurs.

Table 1. Strains of the test microorganisms suitable for use in the Growth promotion test and the Method suitability test

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

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 microorganisms (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 microorganisms (not more than 100 CFU) to the medium.

In both cases use the same microorganisms 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 microorganisms 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.

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 microorganisms 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 6.9–7.3 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 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 five 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 2. Minimum quantity to be used for each medium

Quantity per container	**Minimum quantity to be used for each medium unless otherwise justified and authorized**
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% 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 2 of the product dissolved in a suitable solvent such as the solvent provided with the preparation, water for injections R, sodium chloride (9 g/l) TS or peptone (1 g/l) TS1 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. 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 R 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 peptone (1 g/l) TS1 containing a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability 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. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1% in isopropyl myristate R 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 directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity carry out the test after neutralizing this with a suitable neutralizing 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 peptone (1 g/l) TS1. 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 microorganisms, keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

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 microorganisms 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, diluting where necessary to about 100 mL with a suitable sterile solution, such as peptone (1 g/l) TS1.

When using the technique of direct inoculation of media use the quantities shown in Table 2, unless otherwise justified and authorized. 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.

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 3.

Table 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 authorized****
Parenteral preparations	
Not more than 100 containers	10% or 4 containers whichever is the greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2% or 20 containers (10 containers for large-volume parenterals) whichever is the less
Ophthalmic and other non-injectable	
Not more than 200 containers	5% 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% or 4 containers whichever is the greater
More than 50 containers	2% 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.