

MINISTRY OF HEALTH OF THE RUSSIAN FEDERATION

GENERAL PHARMACOPOEIA MONOGRAPH

Sterility

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The present General Pharmacopoeia Monograph applies to sterility test methods used for various medicinal products: injectable preparations, infusions, eye drops, films, drug substances, and excipients, including biological medicinal products and their solvents, which should be sterile according to the Normative Documents or Pharmacopoeia Monographs.

Test conditions

Testing for sterility should be performed in an aseptic environment, in laminar flow cabinets, clean areas or Purity Class A isolators. Measures preventing contamination should not be harmful to microorganisms that may be contained in tested samples of immunobiological medicinal products. The test conditions should be regularly controlled for compliance with Good Manufacturing Practice and Good Laboratory Practice.

Sterility test methods

Testing for sterility may be done using two methods: by direct inoculation or by membrane filtration. The membrane filtration method is used in all cases where the nature of the medicinal product and its physicochemical properties permit filtration through membrane filters.

The direct inoculation method is employed for sterility testing of medicinal products without antimicrobial activity or whose antimicrobial activity may be eliminated by dilution or inactivation, as well as of medicinal products that cannot be tested with the membrane filtration method.

Appropriate negative control experiments should be simultaneously carried out during sterility testing.

1. Test method suitability validation (determination of antimicrobial activity)

A sterility test method should be validated for suitability in the following cases:

- a) when a new medicinal product is tested for sterility;
- b) if any changes are made in the experimental test conditions;
- c) upon a change in the composition of the medicinal product or a change in the manufacturing technology.

The antimicrobial activity test should utilize the same test strains as for the assessment of the growth-promoting properties of culture media (Table 3).

The antimicrobial activity test should use the same methods and the same test conditions as those of the sterility test.

Membrane filtration. The suitability check (determination of antimicrobial activity) may be performed simultaneously with the sterility test for the tested medicinal product (Paragraph 2.2.). After the required amount of the tested medicinal product is transferred to the filter, not more than 100 colony-forming units (CFU) of the microbial test strains should be introduced into the last portion of the washing liquid (Paragraph 2.2.8.).

Direct inoculation. When checking for suitability (determining antimicrobial activity), suspensions of test strains should be prepared with a final concentration of not more than 100 CFU per mL. The test should be carried out twice for every particular microorganism.

Use 4 test tubes for each of the test strains, containing 10 or 20 mL (for immunobiological medicinal products) of the respective culture medium. Transfer

a 1 mL portion of the tested sample into each of the first two test tubes with the microbial culture and a 1 mL portion of the solvent (positive control) into the other two test tubes. Introduce a 1 mL portion of the respective test strain into each of the four test tubes.

When inoculating thioglycollate medium, incubate at temperature 32.5 ± 2.5 °C for 3 days. When inoculating fluid soybean casein digest medium or Sabouraud fluid medium, incubate at temperature 22.5 ± 2.5 °C for 5 days.

Results are assessed by visual examination in passing light, comparing the growth of the microbial test strains in the experimental and control inoculums. If the growth observed in the experimental test tubes is comparable on visual examination with the growth in the control inoculums that do not contain the tested medicinal product, a conclusion should be made that the medicinal product exhibits no antimicrobial activity under the particular test conditions. In this case, the sterility test should be conducted using standard methods.

If a growth of the test strain is observed in the control inoculums and no growth is seen in the test inoculums, it should be concluded that the tested medicinal product possesses an antimicrobial activity that should be eliminated.

1.1. Elimination of the antimicrobial activity of the tested medicinal product

The following measures should be taken to eliminate the antimicrobial activity of the tested medicinal product:

A) Increase the dilution of the tested product taking a higher volume of the solvent / diluent / culture medium (but not more than 200 mL). Dilution with the culture medium is only acceptable for immunobiological medicinal products.

The ratio of the volumes of the culture medium and the inoculated material shown in experiment to neutralize the antimicrobial activity of the medicinal product should be maintained when testing this medicinal product for sterility.

B) The membrane filtration method may be used, with subsequent washing of the filters, if the medicinal product is soluble in aqueous diluents or in isopropyl myristate (IPM).

C) Instead of the standard diluent, a sterile neutralizing liquid (commercial or laboratory-prepared) may be used that should have the following composition:

- Tween 80 -30.0 g
 - Egg lecithin -3.0 g
 - L-histidine hydrochloride -1.0 g
 - Peptone (meat or casein) -1.0 g
 - Sodium chloride -4.3 g
 - Potassium phosphate monobasic -3.6 g
 - Sodium phosphate dibasic -7.2 g
 - Purified water -1000 mL
- pH 7.0 ± 0.2

D) Use non-specific inactivators. To inactivate preservatives included in the formulation of some medicinal products, the following non-specific inactivators may be added to the diluent and / or culture media before sterilization: 3 % Tween-80 or 0.3% lecithin (egg or soy) (for the medium volume). If the product contains more than two preservatives of different chemical structures, the following should be added to the medium simultaneously: 3 % Tween-80, 0.3 % lecithin, 0.1 % L-histidine, and 0.5 % sodium thiosulfate. If the dilution in the aforementioned solution does not inactivate the antimicrobial properties of the medicinal product, the concentration of Tween-80 or lecithin should be increased.

Some inactivators of the antimicrobial activity of medicinal products are specified in Table 4 of the General Pharmacopoeia Monograph «Microbial contamination».

As the thioglycollate medium contains sodium thioglycollate, which is an inactivator of mercury-containing compounds, the neutralizing properties of this medium should be demonstrated before testing immunobiological medicinal products containing mercurial preservatives by direct inoculation in order to confirm the inactivation.

Inactivators should not be used to neutralize the effects of other preservatives included in the formulation of immunobiological medicinal products; dilution by the culture medium is the main method to eliminate their activity. The tested medicinal product should be inoculated into the culture medium at the 1:20 ratio, with consideration given to the drug antimicrobial activity test results.

E) Specific inactivators may be used that neutralize the antimicrobial activity of the medicinal product but do not inhibit microbial growth.

To inactivate penicillins and cephalosporins, regardless of their pharmaceutical form, a sterile beta-lactamase solution in the quantity specified in the Pharmacopoeia Monograph or in the Normative Document should be added under aseptic conditions to the buffer solution used for dissolution, suspension, or emulsification of the sample, as well as to the culture media before they are used.

The inhibitory influence of β -lactamase on penicillins and cephalosporins should be determined by adding 50 to 100 CFU's of *S. aureus* to media containing the enzyme and the antibiotic. A typical growth of the test strain in the culture medium demonstrates that the concentration of the β -lactamase enzyme is sufficient.

To inactivate sulfanilamide preparations, independently of their pharmaceutical form, para-aminobenzoic acid (PABA) at a concentration of 0.05 to 0.1 g/L of medium should be introduced into the buffer solution used for dissolution, suspension, or emulsification of the tested sample, as well as into the culture media, prior to sterilization if necessary.

When developing new medicinal products, the Pharmacopoeia Monograph and the Normative Document should include information concerning the presence or absence of antimicrobial activity of the product, along with recommendations for its elimination and information on the sterility test method. The absence of antimicrobial activity should be demonstrated after any change in the manufacturing process or the composition of the medicinal product.

2. STERILITY TESTING

2.1. The sampling procedure

When performing a sterility test, the amount of primary packs to be controlled should be determined on the basis of the total number of units in the batch. Product samples should be collected as recommended in Table 1.

Sterility testing within the manufacturing procedure for an immunobiological medicinal product should be carried out in accordance with the manufacture regulations.

If necessary, special requirements may be instituted with regard to the necessary quantity of controlled containers that should ensure reliability of the sterility control for the medicinal product.

The size of the sample that should be used for inoculation into the respective culture medium can be found in Table 2.

Table 1 – Quantity of product units required for sterility testing by batch size

Number of units (ampoules, vials, etc.) per batch*	Minimum quantity of units (ampoules, vials, etc.) required for inoculation into each culture medium**
Medicinal products	
1. Parenteral medicinal products:	
• Not more than 100	10 % or 4
• From 100 to 500	10
• More than 500	2 % or 20
• Large-volume parenteral medicinal products (more than 100)	2 % or 10
• Antibiotics, solid forms, Angro (more than 5 g)	6
2. Non-injectable medicinal products (including ophthalmic products):	
• Not more than 200	5 % or 2
• More than 200	10
• Preparations in single-dose containers	See the “Parenteral medicinal products” graph
3. Solid forms, Angro:	
• Not more than 4 packs	Every pack
• More than 4 and less than 50	20 % or 4
• More than 50	2 % or 10

* if the quantity of units per batch is unknown, the maximum quantity indicated in the column should be used.

** if the contents of one drug container (except for immunobiological medicinal products) are sufficient for inoculation into two culture media, this column should specify the quantity of samples required for sterility testing on two culture media.

Table 2 - Minimum quantity of the tested medicinal product to be inoculated into culture media

Quantity of the medicinal product in the primary	Minimum quantity of the medicinal product
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pack	to be inoculated into each culture medium
Liquid	
• Less than 1 mL	entire volume of primary packs pooled to 1 mL
• 1 – 40 mL	half the contents, but not less than 1 mL
• 40 – 100 mL	20 mL
• More than 100 mL	10 % of the contents, but not less than 20 mL
• Antibiotics (liquids)	1 mL
• Other medicinal products soluble in water or IPM	pack contents, but not less than 200 mg
Insoluble emulsifiable or suspendable preparations, ointments, and creams	pack contents, but not less than 200 mg
Solid	
• Less than 50 mg	entire contents
• 50 - 300 mg	half the contents, but not less than 50 mg
• 300 mg – 5 g	150 mg
• More than 5 g	500 mg

2.2. The membrane filtration method

The membrane filtration method should be preferred to test for sterility medicinal products with pronounced antimicrobial activity and medicinal products presented in containers with a capacity exceeding 100 mL. The only exception is medicinal products with antimicrobial activity that are insoluble in aqueous diluents or isopropyl myristate.

The membrane filtration procedure of the sterility test consists of the following main stages: membrane drenching, sample preparation and filtration of the contents of all containers through membrane filters, washing of the membrane filters with an appropriate sterile solution, addition of a culture medium, and incubation of the inoculums.

The test is carried out using open- or closed-type filtration systems that should allow aseptic transfer and filtration of the tested medicinal product through membrane filters (external diameter 47 mm; pore diameter 0.45 µm) capable of entrapping microorganisms. An open-type filtration system should be assembled so that aseptic transfer and filtration of the tested sample can be ensured. After the end of filtration, the membrane should be aseptically transferred into the culture medium. When using a closed-type sterile system with a membrane built in a can, the culture medium should be transferred after filtration directly onto the

membrane in the can. Filters made of nitrate cellulose nitrate should be used for aqueous, oily, and weakly alcoholic solutions, while filters made of acetate cellulose are utilized for concentrated alcoholic solutions and acids. The hydrophobic edge of the filter and its low sorptive capacity ensure effective washing of the membrane and minimize adsorption of the product with antimicrobial activity.

Filters with no hydrophobic edge moistened prior to filtration with the diluent used may be utilized for medicinal products without antimicrobial activity.

If the tested medicinal product exhibits no antimicrobial activity, the filter washing procedure may be excluded from the course of the test.

2.2.1. Testing of aqueous solutions of medicinal products

Collect the required quantity of the medicinal product from all samples using sterile technique, stir, and transfer it aseptically onto one or several pre-moistened filters. Remove the filters aseptically from the filter holder and place in the media or immerse them in the filter holder container. If a closed-type system is used, fill the cans with equal volumes of the media. Make sure to avoid aeration of the thioglycollate medium.

2.2.2. Testing of liquid medicinal products non-miscible with water

The test should be performed in the same manner as for aqueous solutions of medicinal products. When testing viscous liquids, add a sufficient quantity of an appropriate sterile solvent to the general sample aseptically before filtration to increase the filtration rate.

If the tested medicinal product contains lecithin, an oil, or a preservative and an antimicrobial activity has been demonstrated for the product, use liquid No. 2 to wash the filters.

2.2.3. Sample preparation for ointments and creams soluble in isopropyl myristate and for oily solutions

Ointments in a fatty base and emulsions of the water-in-oil type are dissolved in isopropyl myristate, which is sterilized beforehand by membrane filtration (using membranes with a pore size of 0.22 μm). Heat the sterile diluent /

solvent and, if necessary, the tested medicinal product to not more than 44 °C immediately prior to filtration. First let 5.0 mL of sterile isopropyl myristate through the membrane. After that, filter the isopropyl myristate solution of the product. A layer of solution should remain over the filter throughout the filtration procedure to ensure maximum efficacy of the process. After the filtration, wash the membrane with three portions of liquid No. 2 (100 mL each). The test should be performed on culture media with 1 g/L of Tween 80 added.

If vaseline is included in the composition of the medicinal product, use liquid No. 3 to wash the filters. Let 5.0 mL of sterile isopropyl myristate through the filter before initiating filtration. A small amount of warm solution should remain over the filter throughout the filtration procedure to ensure maximum efficacy of the process. After filtering the sample, wash the filter with three portions of liquid No. 3 (100 mL each). Place the filters in the culture media as specified above.

If the medicinal product is an oily solution, the filter and the system should be thoroughly dried prior to use.

2.2.4. Testing of medicinal products presented in syrettes

Transfer the contents of each syrette into the membrane filtration system, or collect a general sample into a sterile test tube for subsequent transfer onto the filter.

2.2.5. Testing of solid pharmaceutical forms for injection (except for antibiotics)

The medicinal product is diluted as required by the Prescribing Information, and the test is carried out in accordance with the technique described in Sections 2.2.1. and 2.2.2.

2.2.6. Testing of sterile aerosolized products

Transfer the required amount of the medicinal product from the spraying container under aseptic conditions into a sterile volumetric flask by pressing on the plunger of the spraying valve. If possible, remove the propellant by evaporation.

Add liquid No. 2 into the flask and mix carefully. This test should be carried out as described in Sections 2.2.1. and 2.2.2.

2.2.7. Membrane filter washing liquids used for testing of medicinal products with antimicrobial activity

Any solvent shown not to suppress microbial growth may be used to wash the filters:

- 0.9 % sodium chloride solution, pH 7.0 ± 0.2 (after sterilization).
- Liquid No. 1: dissolve 1 g of enzymatic peptone in 1,000 mL of water, filter or centrifuge for defecation, pour into receptacles, and sterilize; pH 7.0 ± 0.2 .

When filtering samples of penicillins or cephalosporins (if necessary), add to liquid No. 1 the validated beta-lactamase quantity specified in the Pharmacopoeia Monograph and the Normative Document and sufficient for inactivation of the residual antimicrobial activity of the antibiotic on the filter.

- Liquid No. 2: add 1 mL of Tween 80 to 1,000 mL of liquid No. 1, pour into receptacles, and sterilize; pH 7.0 ± 0.2 .
- Liquid No. 3: dissolve 5 g of enzymatic peptone, 3 g of meat extract, and 10 g of Tween 80 in 1,000 mL of water, pour into vials, and sterilize; pH 7.0 ± 0.2 .

When testing immunobiological medicinal products, the membrane filters may be washed with any sterile solution shown not to suppress microbial growth and used for the antimicrobial activity test for the medicinal product, for instance: 0.9 % sodium chloride solution (pH 7.0 ± 0.2) or liquid No. 1.

2.2.8. Validation of the membrane filtration method for testing of medicinal products with antimicrobial activity

Filter a volume of the tested sample using the same number of units (ampoules, vials, etc.) for one filter as in the sterility test (Table 2). Wash the filter with at least three portions of the respective liquid (100 mL each). Inoculate the last portion of the washing liquid with 1 mL of the prepared microbial test strain suspensions (each separately) with a concentration of 100 CFU/mL (Table 3).

Place the filter in a receptacle containing 100 mL of the respective culture medium or add the culture medium into the can of the closed-type system. Incubate the inoculums at appropriate temperature for not more than 3 days for bacteria and not more than 5 days for fungi.

Estimate the growth of the microbial test strains by visual examination in passing light for result interpretation. If a growth is observed, this means that the antimicrobial activity has been completely inactivated, and a test for sterility should follow using the same quantity of the medicinal product, the same volume of the washing liquid, and the same culture media.

If no growth of the microbial test strain is observed, this means that the antimicrobial activity has not been inactivated, and the test should be repeated increasing the volume of the filter washing liquid (not exceeding 500 mL) or with different neutralization methods (Paragraph 1.1).

2.3. The direct inoculation method

The direct inoculation method is used for sterility testing of medicinal products exhibiting no antimicrobial activity or medicinal products that cannot be tested with the membrane filtration method.

If the medicinal product exhibits no antimicrobial activity in the test conditions, it should be neutralized by adding appropriate inactivators or increasing the volume of the culture medium (Paragraph 1.1). The added inactivator, at the given concentration, should not suppress the growth of the test strains. If necessary, the inactivator may be added to the culture medium as well.

The tested samples should be inoculated immediately into the culture media at the 1:10 or 1:20 ratio. The ratio of the tested material and the culture medium utilized should be determined during the antimicrobial activity test conducted for the medicinal product.

For immunobiological medicinal products increasing the turbidity of the culture medium (drugs containing a sorbent, microbial cells, etc.), when the presence or absence of microbial growth cannot be determined by visual examination or the interpretation of obtained results is doubtful, inoculation

should be done according to the directions above and re-inoculation into a fresh culture medium should be performed in 5 to 7 days. All inoculums should be kept at adequate temperature until the end of incubation (14 days after the primary inoculation).

2.3.1. Testing of non-filterable liquids

Collect the medicinal product volume sufficient for inoculation in the culture media in the 1:10 ratio from a certain amount of vials, ampoules, etc. (Table 1) using aseptic technique. Mix the culture medium carefully after inoculation, avoiding aeration.

2.3.2. Testing of ointments, creams, and oily solutions

Collect the required amount of units from each tested batch (Table 1).

Oily solutions. Prepare an emulsion of the medicinal product in the 1:10 dilution by placing glass beads with a diameter of 5 to 6 mm and, if necessary, a certain amount of Tween 80 in a sterile volumetric flask containing an appropriate sterile diluent.

Inoculums of oily solutions should be carefully mixed every day.

Ointments and creams. Disinfect the tubes (vials) before the test, open them using aseptic technique, and discard the initial portion of the medicinal product without testing it.

Ointments and creams easily emulsifiable in water. Prepare a 1:10 dilution of the medicinal product by placing the sample in a sterile volumetric flask containing an appropriate sterile diluent (such as 0.9 % sodium chloride solution or liquid No. 1) and glass beads with a diameter of 5 to 6 mm. Heat the mixture on a water bath to 40 °C and shake vigorously for 5 to 15 minutes to obtain a homogeneous emulsion, which should be inoculated into liquid culture media: thioglycollate medium, soybean casein digest medium, or Sabouraud fluid medium.

Ointments and creams poorly miscible with water. Prepare a 1:10 dilution of the medicinal product by placing the sample in a sterile volumetric flask containing an appropriate sterile diluent (such as 0.9 % sodium chloride solution or liquid No.

3), Tween 80 (50 % of the sample's weight), and glass beads with a diameter of 5 to 6 mm. Heat the mixture on a water bath to 40 °C (to 45 °C in exceptional cases) and shake vigorously for 5 to 15 minutes (maximum, 30 minutes) to obtain a homogeneous emulsion, which should then be inoculated into liquid culture media: thioglycollate medium, soybean casein digest medium, or Sabouraud fluid medium.

2.3.3. Testing of solid pharmaceutical forms

Take the medicinal product presented as a powder and transfer the quantity specified in Table 2 into liquid culture media: thioglycollate medium, soybean casein digest medium, or Sabouraud fluid medium, then stir carefully. If a sterile solvent was added to the sample, the obtained suspension should be tested for sterility.

2.4. Inoculum incubation conditions

Incubate the inoculums for at least 14 days at temperature 32.5 ± 2.5 °C in fluid thioglycollate medium and at temperature 22.5 ± 2.5 °C in fluid soybean casein digest medium or Sabouraud fluid medium (regardless of the inoculation method).

When testing immunobiological medicinal products, only the thioglycollate medium may be used and inoculums may be incubated only in two temperature regimens: 32.5 ± 2.5 °C and 22.5 ± 2.5 °C.

2.5. Reporting and interpretation of test results

Inoculums should be examined from time to time during incubation. A microbial growth should be assessed by visual examination in passing light. If the tested medicinal product makes the culture medium turbid and the presence or absence of microbial growth cannot be determined by visual examination, not less than 1 mL of the turbid medium should be transferred into test tubes with the equivalent sterile medium in 14 days after the start of the test. The original inoculums and re-inoculums should be incubated. The total incubation time should be not less than 14 + 4 days from the start of the test.

For immunobiological medicinal products that make the culture medium turbid, re-inoculation into the equivalent culture medium should be done in 5 to 7 days, with subsequent incubation for 14 days from the day of primary inoculation.

The tested medicinal product is regarded as complying with the requirements of the sterility test if no growth of microorganisms occurs.

If a microbial growth is detected by visual examination (on the basis of turbidity, sedimentation, flocculation, or other changes in the medium) and confirmed by microscopy, the tested sample is regarded as not complying with the requirements of the test for sterility, and the causes of this non-compliance should be investigated in such cases.

Sterility test results may be considered unreliable if one or more of the following conditions are fulfilled:

- 1) unsatisfactory results of the microbiological monitoring of the environment (air environment, surfaces, personnel's hands, etc.) are obtained during the sterility test;
- 2) faults made in the course of the testing procedure are revealed;
- 3) a microbial growth is identified in the negative controls (control of the sterile solvent / diluent or the culture medium);
- 4) the culture medium is non-sterile and / or its growth-promoting properties are unsatisfactory;
- 5) faults are revealed in the conduct of the materials sterilization procedure.

If the test results are deemed unreliable (mistakes in the course of the analysis are revealed), the test should be repeated on the same number of samples as in the original test, with the exception of immunobiological medicinal products, which should be re-tested on a double number of samples.

If no microbial growth is identified in the repeat test, the medicinal product has passed the test for sterility. If a microbial growth is identified in the repeat test, the medicinal product has not passed the test for sterility.

If the correctness of the sterility test has been demonstrated in the course of the investigation, the medicinal product is considered to have failed the sterility test.

3. Culture media

Liquid media: thioglycollate medium, soybean casein digest medium, or Sabouraud fluid medium should be used for this test. Thioglycollate medium should be used to detect aerobic and anaerobic bacteria. Fluid soybean casein digest medium is used to detect fungi and aerobic bacteria. Sabouraud fluid medium should be used to detect fungi.

Sabouraud fluid medium is not recommended for sterility testing of immunobiological medicinal products.

When testing for sterility immunobiological medicinal products, including those containing mercurial preservatives, use of thioglycollate medium only may be sufficient as it is a universal tool to detect both aerobic and anaerobic bacteria and fungi (provided that its growth-promoting and neutralizing properties are determined beforehand using the test microorganisms specified in Table 3). Inoculums should be incubated in two temperature regimens.

3.1. Preparation of culture media

Culture media should be prepared in a laboratory using commercial dry culture media or individual components. Ready-for-use culture media may be used, provided that they are accompanied by the manufacturer's Certificate. Culture media prepared in a laboratory should be tested for sterility and growth-promoting properties.

Unless otherwise required by the Pharmacopoeia Monograph or the Normative Document, culture media should be sterilized in an autoclave over 15 minutes at 121 °C.

Thioglycollate medium

L-cystine	- 0.5 g
Sodium chloride	- 2.5 g
Glucose monohydrate	- 5.5 g

Agar, microbiological (moisture content not exceeding 15 %)	- 0.75 g
Yeast extract (water-soluble)	- 5.0 g
Pancreatic digest of casein	- 15.0 g
Sodium thioglycollate or thioglycollic acid	- 0.5 g - 0.3 g
Resazurin sodium solution (1:1000), freshly prepared	- 1.0 mL
Purified water	- 1000.0 mL

pH of the medium after sterilization: 7.1 ± 0.2 .

Add the L-cystine, microbiological agar, sodium chloride, glucose, water-soluble yeast extract, and pancreatic digest of casein into the purified water and heat until complete dissolution of the substance. After the, add the sodium thioglycollate or thioglycollic acid and, if necessary, adjust the pH value of the culture medium to the required level with 1 M sodium hydroxide solution. Add the resazurin sodium solution, mix, pour into test tubes of appropriate volume, and sterilize.

Fluid soybean casein digest medium:

Pancreatic digest of casein	- 17.0 g
Papaic digest of soybean meal	- 3.0 g
Sodium chloride	- 5.0 g
Potassium phosphate dibasic	- 2.5 g
Glucose	- 2.5 g
Purified water	- 1000.0 mL

pH of the medium after sterilization: 7.3 ± 0.2 .

Dissolve the components in the water (heating simultaneously if necessary). Cool down at room temperature. If necessary, add 1 M sodium hydroxide solution so that the pH value of the medium after sterilization is 7.3 ± 0.2 . Filter to obtain a clear medium, pour the medium into test tubes, and sterilize.

Sabouraud fluid medium:

Enzymatic peptone	- 10.0 g
Glucose monohydrate	- 40.0 g
Purified water	- 1000.0 mL
pH of the medium after sterilization: 5.6 ± 0.2 .	

Add the peptone and glucose into the purified water and dissolve completely while slowly heating. Cool down to room temperature and bring the pH value to the required level. Filter, if necessary, then pour the medium into test tubes, and sterilize.

The composition of dry and ready-for-use commercial culture media may be different, provided that their growth-promoting properties meet the requirements.

3.2. Sterility of culture media

Following sterilization, place at least 5 % of the containers in each lot of the culture medium in a thermostat and incubate for at least 14 days for sterility monitoring, simultaneously with inoculating the tested sample for the sterility test. No microbial growth should be observed.

3.3. Testing culture media for growth-promoting properties

Growth-promoting properties of culture media are determined for every culture medium batch that is manufactured commercially and assigned a batch number, and for every culture medium lot prepared in laboratory settings.

Introduce every microbial species (10 to 100 CFU/mL) into an individual portion of the tested medium (into two test tubes). Incubate in accordance with the conditions described in Table 3. If a microbial growth is observed in the inoculated media upon visual examination during the required incubation period, such medium is considered suitable for use.

3.3.1. Preparation of microbial test strains

Bacterial and fungal test strains taken from specialized collections should be used, which should have typical cultural-morphological and biochemical properties.

The number of working culture passages should not exceed five.

Prior to the test, cultures of aerobic bacteria should be inoculated into a soybean casein digest slant, medium No. 1, or another adequate dense culture medium; *C. albicans* and *A. brasiliensis* fungal cultures into a Sabouraud agar slant (or medium No. 2); and *Clostridium novyi* and *C. sporogenes** anaerobic cultures into media for anaerobic microorganisms (such as fluid thioglycollate medium); the cultures should be incubated at the appropriate temperature.

* A culture may be inoculated into an aerobic medium, provided that it is incubated in an anaerobic culture apparatus.

3.3.2. Inoculate preparation

The grown bacterial test strain cultures (including *C. sporogenes* grown in an anaerobic environment) and *C. albicans* should be washed off the surface of the agar slant with sterile 0.9 % sodium chloride solution. A suspension of each test strain should be prepared that should correspond to 10 units of the optical turbidity standard sample.

Table 3 – Microbial test strains used for the determination of the growth-promoting properties of culture media and for testing medicinal products for antimicrobial activity *

Culture media	Microbial test strains	Incubation conditions	
		Temperature	Time
Fluid thioglycollate medium	Aerobic bacteria:	32.5 ± 2.5°C	3 days
	<i>Bacillus subtilis</i> SCPM 010011, ATCC 6633 or <i>Bacillus cereus</i> SCPM 010014, ATCC 10702		
	<i>Staphylococcus aureus</i> SCPM 201108, ATCC 6538		
	<i>Pseudomonas aeruginosa</i> SCPM 190155, ATCC 9027		
	<i>Alcaligenes faecalis</i> 415** SCPM 300205		2 days
	Anaerobic bacteria:		
	<i>Clostridium sporogenes</i> 272 SCPM 300524, ATCC 19404		3 days
	<i>Clostridium novyi</i> 198** SCPM 242484		2 days
	Fungi**:		
<i>Candida albicans</i> NCTC885-653, ATCC 10231	22.5 ± 2.5°C	5 days	

Fluid soybean casein digest medium	Aerobic bacteria:	32.5 ± 2.5°C	3 days
	<i>Bacillus subtilis</i> SCPM 010011, ATCC 6633 or <i>Bacillus cereus</i> SCPM 010014, ATCC 10702		
	Fungi:		
	<i>Candida albicans</i> NCTC 885-653, ATCC 10231	22.5 ± 2.5°C	5 days
	<i>Aspergillus brasiliensis</i> ATCC 9642, ATCC 16404		
Sabouraud fluid medium	Fungi:	22.5 ± 2.5°C	5 days
	<i>Candida albicans</i> NCTC 885-653, ATCC 10231		
	<i>Aspergillus brasiliensis</i> ATCC 9642, ATCC 16404		

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* other test strains from different collections may be used, they should have typical cultural-morphological, tinctorial, and biochemical properties. The range of test strains may be changed depending on the method of administration or composition of the tested medicinal product.

** the test strains specified are for cases where thioglycollate medium is used as a universal medium to test immunobiological medicinal products. Culturing should be performed in two temperature regimens: $-32.5 \pm 2.5^\circ\text{C}$ and $22.5 \pm 2.5^\circ\text{C}$.

Cell concentrations should be brought to 1×10^7 CFU/mL for *B. subtilis*, *C. albicans*, *A. brasiliensis* and to 1×10^9 CFU/mL for *S. aureus*, *P. aeruginosa*, *C. sporogenes*, *A. faecalis*. A *C. novyi* culture obtained in a liquid culture medium for anaerobic microorganisms (after 2 re-inoculations) should be diluted, after being centrifuged at 3000 rpm for 20 minutes, with the sterile liquid of the following composition:

- sodium chloride - 8.5 g,
- thioglycollic acid - 0.3 mL,
- purified water - 1000 mL,

Post-sterilization pH value: 7.2 ± 0.2 .

Sterile 0.9 % sodium chloride solution containing 0.05 % Tween 80 should be used to wash off *A. brasiliensis* conidia. The quantity of conidia per millilitre of the wash fluid should be determined using a Goryaev chamber or by inoculating an appropriate dilution into Sabouraud agar or medium No. 2.

Standardized bacterial and fungal suspensions should be brought to concentrations 10 – 100 CFU/mL with sterile 0.9 % sodium chloride solution by means of ten-fold serial dilutions for subsequent inoculation into liquid and semi-liquid culture media – to permit determination of their growth-promoting properties.

To verify the obtained concentration, inoculates of bacteria, including *C. sporogenes* (if the latter is incubated in an anaerobic culture apparatus), should be transferred into soybean casein digest medium (medium No. 1 or a specialized Clostridia medium, respectively) – 0.1 mL from a 10³ CFU/mL suspension, and *C. novyi* inoculates should be transferred into a specialized Clostridia medium. Fungal inoculates should be transferred to Sabouraud agar (or medium No. 2).

3.4. Determination of the neutralizing properties of thioglycollate medium

When testing immunobiological medicinal products containing merthiolate (thiomersal), the *Alcaligenes faecalis* 415 test strain should be used for the determination of the neutralizing properties of the thioglycollate medium (see Paragraph 3.3.2. for the inoculate preparation). In advance, 0.5 mL of recently prepared 0.01% thiomersal solution pre-diluted with sterile 0.9 % sodium chloride solution should be introduced into each test tube, into the middle of the thioglycollate medium column, before inoculating the culture.

The thioglycollate medium is considered to have appropriate neutralizing properties if a growth of the *A. faecalis* 415 test strain is observed on visual examination within 5 days of inoculate incubation at 32.5 ± 2.5° C.

3.5. Storage of culture media

Culture media prepared in laboratory settings should be stored in the temperature range from 2 to 25 °C, in a place protected from light, over a period of not more than 1 month or within a different period of time yielded during validation testing.

If the upper layer of a resazurin-containing thioglycollate medium (more than one-third of its volume) acquires a pink colour during storage, such medium may be regenerated by heating on a boiling water-bath over 10 to 15 minutes until the pink colour disappears, with subsequent rapid cooling down. If the colour does not disappear upon heating, such medium is considered unsuitable for use. The medium may be regenerated only once.

Ready-for-use commercial culture media should be stored in tightly closed containers, and their sterility and growth-promoting properties should be maintained throughout the shelf-life.

Commercial dry culture media should be stored in accordance with their Instructions for Use and disposed of after the expiry date specified by the manufacturer.