Sterility	**GM.1.2.4.0003.15** **Instead of State Pharmacopeia (SP) X** **Instead of SP XI, ver. 2** **Instead of GM 42-0028-05,** **SP XII, Part 1, GM 42-0066-07**

This General Monograph applies to test methods for sterility of pharmaceutical products (PPs): injections, infusions preparations, eye drops, films, pharmaceutical substances and excipients, including biological medicinal preparations and their solvents, which should be sterile in accordance with the regulatory documentations or pharmacopoeial monographs.

Test conditions

Sterility test should be conducted under aseptical conditions in laminar flow hoods, clean rooms or grade A cleanliness isolators. The measures to prevent contamination should not exert damaging effects on microorganisms that may be present in test samples of immunobiological drugs (IBDs). The test conditions are regularly monitored in accordance with appropriate production rules and laboratory practices.

Methods of sterility tests

The sterility tests are carried out in two ways: by a method of direct inoculation or by a membrane filtration method. The membrane filtration method is used in all cases if the nature of the drug and its physical and chemical properties allow filtering it through the membrane filters.

The method of direct inoculation is used for the sterility test of PPs that have no antimicrobial effect or which antimicrobial effect can be eliminated by dilution or

inactivation, as well as of the preparations which test cannot be performed by the method of membrane filtration.

When testing for sterility, one carries out simultaneously the respective negative controls.

1. Check of the test method suitability (determination of antimicrobial effect)

The suitability check of the test method for sterility should be carried out in the cases as follows:

a) During test for sterility of a new preparation;

b) When changing experimental conditions of the test;

c) In case of changing the drug formulation or its production technology.

To test the antimicrobial effect, one uses the same test-strains as when evaluating growth properties of culture media (see Table 3).

One determines the antimicrobial effect by the same methods and under the same conditions as the sterility test.

Membrane filtration method. Suitability test (determination of antimicrobial effect) can be held simultaneously with the test for sterility of a drug (see para. 2.2.). After transferring the required amount of a test drug on a filter, the last portion of liquid is added with not more than 100 colony forming units (CFU) of test-strains of microorganisms (see para. 2.2.8.).

Direct inoculation method. When checking the suitability (determination of antimicrobial effect), one prepares suspensions of test-strains with a final concentration of not more than 1 CFU/mL. The test is performed with each type of microorganisms.

Four test tubes are used for each test-strain containing 10 or 20 mL (for an IBD) of the corresponding culture medium. The first two test tubes with microorganism culture are filled with 1 mL of a test sample, and the other two: with 1 mL of solvent (positive control). All four test tubes are added with 1 mL of the appropriate test-strain.

The inoculations in the thioglycolic medium are incubated at a 32.5±2.5 °C temperature for 3 days. The inoculations in the liquid casein soya bean digest medium or liquid Sabouraud medium are incubated at a 22.5±25 °C temperature for 5 days.

The results are analyzed visually in regredient light, by comparing the growth of the test-strains of microorganisms in experimental and control inoculations. If the detected growth in test tubes is visually comparable to the growth in the control inoculations that do not contain any test drug, one makes a conclusion the preparation under test has no antimicrobial effect. In this case, the sterility test is carried out by standard methods.

If in the control, one observes the growth of the test-strain, and in the experience, the growth is absent, one believes the test preparation has an antimicrobial effect to be eliminated.

1.1. Elimination of an antimicrobial effect

To eliminate the antimicrobial effect of the preparation, one does the following:

A) One increases the degree of the preparation dilution by using the greater volume of solvent/diluent/culture medium (but not more than 200 mL). For IBDs, it is allowed only dilution by culture medium.

The ratio experimentally found of the volume of culture medium and the inoculation material that ensures the neutralization of the antimicrobial effect of the preparation should be observed when testing the drug for sterility.

B) One applies the method of membrane filtration, followed by washing the filters, if the drug is soluble in aqueous diluents or isopropyl myristate (IPM).

C) Instead of a standard diluent, one can use sterile neutralizing liquid of industrial production or prepared in the laboratory, of the composition as follows:

- Tween-80 -30.0 g
- Egg lecithin -3.0 g
- L-histidine hydrochloride -1.0 g
- Peptone (meat and casein) -1.0 g
- Sodium chloride -4.3 g

- Potassium Phosphate monobasic -3.6 g
- Disodium hydrogen phosphate -7,2 g
- Purified water -1,000 mL

  pH 7.6±0.2

D) One uses non-specific inactivators. To inactivate preservatives included in some preparations, in a diluent and/or culture media prior to sterilization, one places nonspecific inactivators as follows: 3% Tween-80 and 0.3% lecithin (egg or soya bean) of the medium volume. If the preparation has more than two preservatives of a different chemical structure, one adds to the medium 3% Tween-80, 0.3% lecithin, 0,1% L-histidine and 0.5% sodium thiosulfate simultaneously. If the dilution in the above solution does not inactivate the antimicrobial properties of PPs, one increases the concentration of Tween-80 or lecithin.

Some inactivators of antimicrobial effect of a PP are listed in Table 4 of a "Microbiological purity" general monograph.

Given that the composition of thioglycollate medium includes sodium thioglycolate, i.e. inactivator of mercurials, prior to testing IBDs containing mercury preservatives, by direct inoculation method; one determines neutralizing properties of this medium confirming inactivation.

To neutralize the effect of other preservatives included in an IBD, inactivators are not used, and the main way to eliminate their action is to dissolve them with culture medium. The inoculation of a test preparation in culture medium is carried out at a ratio of 1:20, based on detection results of antimicrobial effect of the drug.

E) One uses specific activators to neutralize the antimicrobial effect of a PP but not suppressing the growth of microorganisms.

To inactivate penicillins and cephalosporins regardless of their dosage form, the buffer solution used for dissolving, suspending and emulsification of a sample as well as in culture media prior to their use, one places aseptically sterile solution of β-lactamase in the amount that was specified in a pharmacopoeial monograph or regulatory documentation.

The inhibiting effect of β-lactamase to penicillins and cephalosporins should be determined by adding the medium with an enzyme and an antibiotic with 50-100 CFU of *S. aureus*. Typical growth of a test-strain in culture medium confirms the concentration of the β-lactamase enzyme is sufficient.

To inactivate sulfonamides, regardless of their dosage forms, the buffer solution used for dissolving, suspending and emulsification of a sample as well as in culture media if necessary, prior to sterilization, one pours paraaminobenzoic acid (PABA) at a 0.05-0.1 g/L ratio of the medium.

When developing new preparations, in a pharmacopoeial monograph and regulatory documentation, one should include information on the presence/absence of the antimicrobial effect of a preparation, with the recommendations on its eliminations and the information about the method of a sterility test. If one changes the production process or the composition of the drug, it is necessary to confirm the absence of an antimicrobial effect.

2. STERILITY TEST

2.1. Sampling for testing

When testing for sterility, the number of primary packages to be controlled is determined by considering the total number of units in series. The preparation samples are taken as indicated in Table 1.

The sterility test during the production of an IBD is performed according to production rules.

If necessary, specific requirements can be regulated regarding the required number of vials to be controlled, that provide the reliable control of the drug sterility.

For inoculation in the appropriate culture medium, one uses a sample in the amount given in Table 2.

Table 1: The preparation quantity to fulfill a sterility test depending on series volume

Quantity of units (ampoules, vials, etc.) in series*	Minimum quantity of units (ampoules, vials, etc.) for inoculation in each culture medium**
Pharmaceutical products	
1.Parenteral pharmaceutical products:	
• Not more than 100	10% or 4
• From 100 to 500	10

• More than 500	2% or 20
• Parenteral pharmaceutical products of large volume (more than 100)	2% or 10
• Antibiotics, solid forms, in bulk (more than 5 g)	6
2. Non-injectable pharmaceutical products (including eye ones):	
• Not more than 200	5% or 2
• More than 200	10
• Preparations in unit-dose package	See line “Parenteral Pharmaceutical products”
3.Solid forms, in bulk:	
• Not more than 4 packages	Each
• More than 4, but not greater than 50	20% or 4
• More than 50	2% or 10

* If the quantity of units in the series is unknown, one uses the maximum amount specified in the column.

** If the content of one PP container (except for an IBD) is sufficient to inoculate two culture media, this column contains the quantity of samples needed for the sterility test in two culture media.

Table 2: Minimum quantity of a test product to be inoculated in culture media

Preparation quantity in primary package	Minimum preparation quantity to be inoculated in each culture medium
Liquid dosage forms	
• More than 1 mL	The entire volume of primary packages, combined to 1 mL
• 1–40 mL	½ of the contents, but not less than 1 mL
• 40–100 mL	20 mL
• More than 100 mL	10% of the contents, but not less but not less than 20 mL
• Antibiotics (liquids)	1 mL
• Other products, water-soluble ones or in IPMs	Package contents, but not less than 200 mg
Insoluble preparations, ointments and creams, emulsifiable or suspendable	Package contents, but not less than 200 mg
Solid dosage forms	
• Less than 50 mg	All the contents
• 50–300 mg	½ of the contents, but not less than 50 mg
• 300 mg – 5 g	150 mg
• More than 5 g	500 mg

2.2. Method of membrane filtration

When determining the sterility of PPs with a strong antimicrobial effect, and the PPs in containers of more than 100 mL volume, the preferred method is the membrane filtration method. Exceptions are drugs with antimicrobial effect, insoluble in water diluents or IPMs.

Test procedure for sterility by the membrane filtration method consists of the stages as follows: wetting of the membranes, preparation of samples and filtration of the contents of all vessels through the membrane filters, washing out of the membrane filters with corresponding sterile solution, the addition of the culture medium and incubation of inoculations.

The test is performed using open or closed filtration plants that allow transferring and filtering the test preparations through membrane filters (of a 47 mm outer diameter, 0.45 mcm pore diameter) under aseptic conditions, capable to capture microorganisms. An open-type filtration unit should be mounted so the test sample can be introduced and filtered under aseptic conditions. The filtration finished, the membrane is aseptically transferred to culture medium. When using a closed sterile system with a membrane mounted in a canister, after filtration, culture medium is directly introduced into the canister on the membrane. Filters made of cellulose nitrate are used for water, oil and weak alcoholic solutions, filters of acetyl cellulose – for concentrated alcoholic solutions and acids. The hydrophobic filter edge and low sorption capacity provide the efficient washing of the membrane and minimize adsorption of the drug, which has an antimicrobial effect.

For products that have no antimicrobial effect, one can use filters without hydrophobic edge, by soaking them prior to the filtration with a diluents used.

If the test drug has no antimicrobial effect, during the test, filters washing may be excluded.

2.2.1. Test of PP water solutions

A certain amount of the drug that was selected under sterile conditions from all samples is stirred and aseptically transferred to one or more pre-wetted filters. The filters are aseptically removed from the filter holder and placed in media or poured in the containers with filter holders. When using a closed system, the containers are filled with the equal volume of media. At the same time one should avoid aeration of thioglycolic medium.

2.2.2. Test of liquid preparations that are water-immiscible

The test is performed in the same manner as for the PP aqueous solutions. When testing viscous liquids, to a common sample before filtration, one aseptically adds a sufficient amount of a suitable sterile solvent to increase the filtration rate.

If the composition of a test product includes lecithin, oil or a preservative, and the preparation itself has antimicrobial effect, the Liquid No. 2 is used for washing the filters.

2.2.3. The preparation of sample of ointments, creams that are soluble in IPM, and solutions in oils

Fat-based ointments and emulsion of “water in oil” type are dissolved in IPM with presterilization by filtration method (a membrane with a 0.22 mcm pore diameter). A sterile diluent/solvent, and if necessary, a test preparation immediately before filtration is heated to a temperature not exceeding 44 °C. Initially, through a membrane one passes a sterile IPM in the amount of 5 mL. Then the drug solution is filtered in IPM. For maximum efficiency of the process, during the filtration a solution layer should always be above the filter. After filtration, the membrane is washed with three portions Liquid No. 2 by 100 mL each. The test is performed in culture media with the addition of 1 g/L Tween-80.

If the composition of the test drug includes petrolate, to wash filters, one uses Liquid No. 3. Before filtration, through a filter, one passes 5.0 mL of sterile IPM. For maximum efficiency of the process, during the filtration, a small amount of warm solution should always be above the filter. After the sample filtration, the filter is washed with three portions of Liquid No. 3 by 100 mL each. The filters are placed in culture media as described previously.

If the drug is the oil solution, the filter and the plant should be thoroughly dried prior to use.

2.2.4. Test of the preparations in syringe - tubes

The contents of each syringe-tube are transferred to plants for membrane filtration or collected in one sample to a sterile test tube for the subsequent transfer to the filter.

2.2.5. Test of solid dosage forms for injections (except for antibiotics)

The drug is diluted, as specified in the instructions for use, and the test is carried out according to the procedure set out in Sections 2.2.1. and 2.2.2.

2.2.6. Test of sterile aerosols

The required amount of a drug in the aerosol package is aseptically transferred to a sterile flask by pressing the dispenser valve pin. If possible, the propellant is removed by evaporation. Liquid No. 2 is added to the flask and cautiously stirred. The test is performed as specified in Sections 2.2.1. and 2.2.2.

2.2.7. Liquids to wash membrane filters when testing pharmaceutical products with antimicrobial effect

To wash filters, one can use any sterile liquid that do not inhibit the growth of microorganisms:

• 0.9% sodium chloride solution with pH 7.0±0.2 (after sterilization).

• Liquid No. 1: 1 g of enzymic peptone is dissolved in 1,000 mL of water, filtered or centrifuged for clarification, filled into containers, and sterilized; pH 7.0±0.2.

When filtering samples of penicillins or cephalosporins (if necessary), to Liquid No. 1, one adds validated amount of β-lactamase specified in a pharmacopoeial monograph and regulatory documentation, sufficient to inactivate the residual antimicrobial effect of an antibiotic on the filter.

• Liquid No 2: 1 mL of Tween-80 is added to 1,000 mL of Liquid No. 1, poured into vials and sterilized; pH 7.0±0.2

• Liquid No. 3: 5 g are dissolved in enzymic peptone, 3 g of meat extract and 10 g of Tween-80 in 1,000 mL of water, bottled and sterilized; pH 7.0±0.2.

When testing the IBD, the washing of membrane filters can be carried out by any sterile solution that do not inhibit the growth of microorganisms, used when determining the antimicrobial effect of the drug, for example: 0.9% sodium chloride solution (pH 7.0±0.2) or liquid No 1.

2.2.8. Check of the suitability of a membrane filtration method when testing PPs with antimicrobial effect

The amount of a test sample is filtered using the same quantity of units (ampoules, vials, etc.) as when test for sterility (see Table 2). The filter is washed at least by three portions of the respective liquid, by 100 mL each. The last portion of liquid for washing is added with 1 mL of prepared suspensions of the test-strains of microorganisms test strains (individually) with a concentration of 102 CFU/mL (see Table 3).

The filter is placed in a container with 100 mL of the appropriate culture medium or medium is added into the closed system canister. The inoculations are incubated at the appropriate temperature for no longer than 3 days for bacteria and for 5 days for fungi.

During the results analysis, one visually determines the presence of growth of the test-strains of microorganisms in regredient light. If the detected growth, one believes the antimicrobial effect is totally inactivated, and the test for sterility is fulfilled by using the same amount of preparation, the similar volume of liquid and the same culture media.

If the growth of test-strains is absent, one makes a conclusion the antimicrobial effect of the preparation is inactivated. The test is repeated, by increasing the volume of the liquid to wash the filter (but not more than 500 mL) or other ways of neutralization are used (see para. 1.1).

2.3. Direct inoculation method

Direct inoculation method is used to test the sterility of PPs that have no antimicrobial effect or the drugs which test cannot be done by the membrane filtration method.

If the drug has an antimicrobial effect under test conditions, it is neutralized by the addition of suitable inactivators or increasing the volume of the culture medium (see para. 1.1). The inactivator added at a predetermined concentration should not inhibit the growth of the test-strains. If necessary, the inactivator can be added in the culture medium.

The test samples are inoculated directly into the culture medium in 1:10 or 1:20 ratios. The ratio of the quantity of a test material and the culture medium used should be determined when checking the antimicrobial effect of the drug.

For IBDs that cause clouding of the culture medium (preparations containing sorbent, microbial cells, etc.), if the presence/absence of the growth of microorganisms cannot be visually determined or one doubts about the results, one makes inoculations by a scheme above, and on the 5th-7th day, one subcultures in fresh medium. All the inoculations are maintained at an adequate temperature until the end of the incubation (14 days from the date of the primary inoculation).

2.3.1. Test of nonfiltering liquids

From a certain quantity of vials, ampoules, etc. (see Table 1), one aseptically collects the amount of the drug sufficient for inoculation in culture medium in a 1:10 ratio. After inoculation, one gently stirs medium, excluding aeration.

2.3.2. Test of ointments, creams, and oil solutions

From each test series, one selects a required number of units (see Table 1).

Oil solutions. One prepares a preparation emulsion in a 1:10 dilution rate, by placing glass beads of a 5-6 mm diameter in a sterile flask, with an appropriate sterile diluent, and if necessary, a certain amount of Tween-80.

Daily, the inoculations in oil solutions are gently stirred.

Ointments and creams. Prior to testing, tubes (vials) are disinfected, aseptically opened, and the first portion of the drug is removed, without examination

Ointments and creams that are easily emulsified in water. One prepares a 1:10 dilution ratio of a PP by placing the sample into a sterile flask with an appropriate sterile diluent (e.g. 0.9% sodium chloride solution or Liquid No. 1) and glass beads of a 5-6 mm diameter. The mixture is heated in water bath to a 40 °C temperature and shaken vigorously for 5-15 minutes until a homogeneous emulsion which is plated in liquid media: thioglycolic, casein soya bean digest or Sabouraud.

Ointments and creams that are hardly water-miscible. One prepares a 1:10 dilution ratio of a drug by putting it into a sterile flask with an appropriate sterile diluent

(e.g., 0.9% sodium chloride or Liquid No. 3), Tween-80 in the amount of 50% of the sample weight and the glass beads of a 5-6 mm diameter. The mixture is heated in water bath to a 40 °C temperature (exceptionally up to a 45 °C temperature), shaken vigorously for 5-15 minutes (maximum for 30 minutes) until a homogeneous emulsion, which is then inoculated in liquid media: thioglycolic, casein soya bean digest or Sabouraud.

2.3.3. Test of solid dosage forms

A PP as powder is transferred in the amount shown in Table 2, in the liquid media: thioglycolic, casein soya bean digest or Sabouraud and agitated gently. If the sample is added with a sterile diluent, the suspension received is subject to testing for sterility.

2.4. Inoculations incubation conditions

Inoculations are incubated for at least 14 days at a 32.5±2.5 °C temperature in a liquid thioglycolic medium and a 22.5±25 °C temperature in liquid casein soya bean medium or Sabouraud medium (regardless of method of inoculation).

When testing an IBD, one can use only thioglycolic medium and incubation of the inoculations at two temperature conditions of 32.5±2.5 °C and 22.5±25 °C.

2.5. Accounting and interpretation of the test results

During incubation, one periodically examines the inoculations. The presence of microbial growth is determined visually in regredient light. If the test PP causes clouding of the culture medium and one cannot visually determine the presence or absence of microbial growth, 14 days after the start of the test, one transfers at least 1 mL of darkened medium in sterile test tubes with the same sterile medium. One incubates the original and repeated inoculations. The total incubation time should be not less than 14+4 days from the start of the test.

For IBDs that cause the clouding of the culture medium, one makes the reinoculation in the same culture on the 5^{th}-7^{th} day with the subsequent incubation for 14 days from the date of the primary inoculation.

If the absence of the microbial growth, one believes the test preparation complies with the test requirements on sterility.

Upon detection of the microbial growth to be defined visually by the presence of turbidity, sediment, flakes and other changes in the medium to be confirmed by microscopic examination, it is believed the test preparation does not meet the test requirements on sterility. In this case, one investigates the causes of non-compliance.

Sterility test results may be considered unreliable if one or more of the conditions listed below is met:

1) The negative results of the microbiological control of the environment (air, surfaces and hands of personnel, etc.) are obtained when testing for sterility;
2) The mistakes are revealed that are made during the test;
3) The growth of microorganisms is detected in the negative control (control of the sterile solvent/diluent or culture medium);
4) The culture medium is non-sterile and/or its growth properties are unsatisfactory;
5) The errors are detected during the process of materials sterilization.

If the test results are considered unreliable (in the case of the error detection during the analysis), the test is repeated on the same number of samples as the original, except for IBDs products which re-test is carried out on a twice number of samples.

If the re-test does not detect the growth of microorganisms, it is believed the drug meets the test requirements on sterility. If the result of a repeated test reveals the growth of microorganisms, it is believed the drug does not meet the test requirements on sterility.

If the examination proved the correctness of the test on sterility, one considers the drug does not meet the test requirements on sterility.

3. Culture media

For the sterility testing, one uses liquid media: thioglycolic, casein soya bean digest or Sabouraud. Thioglycolic medium is used to detect aerobic and anaerobic

bacteria. Liquid casein soya bean digest is used for detecting fungi and aerobic bacteria. Liquid Sabouraud medium is used to identify fungi.

When testing the IBD on sterility, it is not recommended to use a liquid Saburo medium.

When testing the IBD on sterility, including those containing mercury preservatives, it is permissible to use only thioglycolic medium as universal for the detection of aerobic and anaerobic bacteria and fungi (provided preliminary determination of its growth and neutralizing properties using test-microorganisms in accordance with Table 3). The incubation of inoculations is carried out at two temperature modes.

3.1. Culture media preparation

Culture media are prepared in the laboratory using dry culture media of industrial production or separate components. It is allowed to use media, ready for use, with the manufacturer's certificate. The culture media prepared in the laboratory are tested for sterility and their growth properties are determined.

The culture medium is sterilized in an autoclave at a 121 °C temperature for 15 minutes unless otherwise stated in the pharmacopeia monograph or regulatory documentation.

Thioglycolic medium

- L-Cysteine - 0.5g
- Sodium chloride - 2.5 g
- Glucose monohydrate - 5.5 g
- Microbiological agar (moisture of not more than 15 %) - 0.75 g
- Yeast extract (water soluble) - 5.0 g
- Pancreatic digest of casein - 15.0 g
- Sodium thioglycollate - 0.5 g
  or thioglycol acid - 0.3 g
- Resazurin sodium solution (1:1,000 ratio), fresh - 1.0 mL
- Purified water - 1,000.0 mL

pH after sterilization 7.1±0.2.

To purified water, one adds L-Cysteine, microbiological agar, sodium chloride, glucose, water soluble yeast extract, and pancreatic digest of casein and heats until complete dissolution. One then adds sodium thioglycollate or thioglycol acid, and if necessary, the medium pH is adjusted with sodium hydroxide solution to the desired value. The Resazurin sodium solution is added, stirred, poured into test tubes of appropriate volume and sterilized.

Liquid casein soya bean medium

- Pancreatic digest of casein - 17.0 g
- Papaic digest of soya bean - 3.0 g
- Sodium chloride - 5.0 g
- Potassium phosphate dibasic - 2.5 g
- Glucose - 2.5 g
- Purified water - 1,000.0 mL

pH after sterilization 7.3±0.2

The components are dissolved in water (if necessary with heating), cooled at a room temperature. If required, 1M sodium hydroxide solution is added, to make the medium pH 7.3±0.2 after sterilization, then is filtered to obtain transparent medium, poured into test tubes and sterilized.

Sabouraud liquid medium

- Fermentation peptone - 10.0 g
- Glucose monohydrate - 40.0 g
- Purified water - 1,000.0 mL

pH after sterilization 5.6±0.2

Peptone and glucose are added to purified water and completely dissolved with gentle heating, cooled to a room temperature and the pH is adjusted to the desired value, if necessary, filtered, poured into test tubes and sterilized.

It is assumed the composition of dry and ready for use media of industrial production varies, provided that they meet the requirements for the growth properties.

3.2. Sterility of culture media

After sterilization, at least 5% containers from each batch of the culture medium are placed in an oven and incubated for at least 14 days for the sterility control together with the inoculation of a test sample for sterility. There should be no growth of microorganisms.

3.3. Determination of growth properties of culture media

Growth properties of the medium are determined for each series of the culture medium delivered by the industry and having a number, and for each batch of the medium made in the laboratory.

Each kind of microorganism in the amount of 10-100 CFU/mL is placed in a separate portion of the test medium (in 2 test tubes), is then incubated in accordance with the conditions specified in Table 3. If during the required incubation time in inoculated media one visually observes the growth of microorganisms, the medium is considered suitable for use.

3.3.1. Preparation of test-strains of microorganisms

Use the test-strains of bacteria and fungi from specialized collections that should be typical by cultural, morphological and biochemical properties.

The quantity of reference culture passages should not exceed five.

Prior to testing, aerobic bacteria cultures are plated in sloped casein soya bean agar, medium No. 1 or other appropriate solid medium; *C. albicans* and *A. brasiliensis* fungi cultures are plated on a slope Sabouraud medium (or Medium No. 2); *Clostridium novyi* and *C. sporogenes** anaerobes are cultured in media for anaerobic microorganisms (e.g., liquid thioglycolic medium) and are incubated at an appropriate temperature.

* The culture inoculation in media for aerobic microorganisms is possible under incubation in an anaerobic jar.

3.3.2. Preparation of inoculum

Grown cultures of test-strains of bacteria (including *C. sporogenes* grown under anaerobic conditions) and *C. albicans* are washed from the surface of a slope agar with sterile 0.9% sodium chloride solution. One then prepares suspension of each test-strain corresponding to 10 Units on the optical turbidity standard sample.

Table 3: Test-strains of microorganisms used to determine the growth properties of media and the check of antimicrobial effect of a drug*

Culture media	Test-strains of microorganisms	Incubation conditions	
		Temperature	Time
Liquid thioglycolic medium	**Aerobic bacteria:**	32.5±2.5 °C	3 days
	Bacillus subtilis GKPM 010011, ATCC 6633 or *Bacillus cereus* GKPM 010014, ATCC 10702		
	Staphylococcus aureus GKPM 201108, ATCC 6538		
	Pseudomonas aeruginosa GKPM 190155, ATCC 9027		
	Alcaligenes faecalis 415** GKPM 300205		2 days
	Anaerobic bacteria:		3 days
	Clostridium sporogenes 272 GKPM 300524, ATCC 19404		
	Clostridium novyi 198** GKPM 242484		2 days
	Fungi:**	22.5±2.5 °C	5 days
	Candida albicans NCTC885-653, ATCC 10231		
Liquid casein soya beam medium	**Aerobic bacteria:**	32.5±2.5 °C	3 days
	Bacillus subtilis GKPM 010011, ATCC 6633 or *Bacillus cereus* GKPM 010014, ATCC 10702		
	Fungi:	22.5±2.5 °C	5 days
	Candida albicans NCTC 885-653, ATCC 10231		
	Aspergillus brasiliensis ATCC 9642, ATCC 16404		
Liquid Sabouraud medium	**Fungi:**	22.5±2.5 °C	5 days
	Candida albicans NCTC 885-653, ATCC 10231		
	Aspergillus brasiliensis ATCC 9642, ATCC 16404		

*****Other test-strains from different collections can be used that are typical by cultural, morphological, tinctorial, and biochemical properties. The set of test-strains may be modified depending on the method of application or the composition of the test drug.

****** The test-strains are marked if the thioglycolic medium is used as a universal one when testing an IBD. The cultivation is carried out at two temperature modes: 32.5±2.5 ºC and 22.5±2.5 ºC.

The *B. subtilis, C. albicans, A. brasiliensis* cells concentration are brought to $1 \cdot 10^7$ CFU/mL; *S. aureus, P. aeruginosa, C. sporogenes, A. faecalis* – to $1 \cdot 10^9$ CFU/mL. After centrifugation 3000 rpm/min for 20 minutes, the *C.novyi* culture grown in liquid medium of cultivation for anaerobic microorganisms (2 reinoculations) is diluted with sterile liquid of the composition as follows:

- Sodium chloride - 8.5 g,
- Thioglycolic acid - 0.3 mL,
- Purified water - 1,000 mL,

pH 7.2±0.2 after sterilization

To wash *A. Brasiliensis* conidia, one uses sterile 0.9 % sodium chloride solution that contains 0.05 % Tween-80. The quantity of conidia in 1 mL of swab is determined using the Goryaev's chamber or by inoculation of appropriate dilution in Sabouraud agar or medium No. 2.

The standardized suspensions of bacteria and fungi are brought by sterile 0.9% solution of sodium chloride by successive decimal dilutions to a concentration of 10-100 CFU/mL for plating in liquid and semi-liquid culture media for the determination of their growth properties.

To confirm the concentrations obtained, the bacteria inocula, including *C. sporogenes* (provided that the incubation of the latter in an anaerobic jar) are plated in casein soya bean agar (Medium No. 1 or specialized medium for Clostridia respectively) by 0.1 mL of suspension with a concentration of 103 CFU/mL, *C. novyi* are plated in special medium for Clostridia. Fungal inocula are plated in Sabouraud agar (or Medium No 2).

3.4. Determination of neutralizing properties of thioglycolic medium

When testing IBDs containing thiomersal, to determine thioglycolic medium neutralizing properties, one uses the test-strain *Alcaligenes faecalis* 415 (preparation of the inoculums, see para. 3.3.2.) Prior to culture inoculation, in each test tube in a column center with thioglycolic medium, one introduces by 0.5mL of fresh 0.01% thiomersal solution diluted with sterile 0.9% sodium chloride solution. Thioglycolic medium is recognized suitable by neutralizing properties, if within 5 days of inoculation incubation at a 32.5±2.5 °C temperature, one visually observes the growth of the test-strain *A. faecalis* 415.

3.5. Storage of culture media

The media prepared in the laboratory are kept at a temperature from 2 to 25 ºC in the dark place for 1 month max, or for another period of time, confirmed during the validation tests.

If when keeping thioglycolic medium that contains Resazurin, the top layer of the medium (more than 1/3 volume) turns pink, the medium may be regenerated by heating in boiling water for 10-15 minutes until the pink color disappears. If the stain does not disappear after heating, the medium is considered unsuitable for use. The medium can be regenerated only once.

Culture media of industrial production, ready for use, are kept in tightly closed containers provided that their sterility and growth properties are kept for the shelf life.

Dry culture media of industrial production are kept in accordance with the instruction for use and destroyed by the manufacturer after the expiry date specified.