The determination of probiotics specific activity	GPhM (General Pharmacopoeial Monograph) 1.7.2.0009.15 First introduced

This General Pharmacopoeial Monograph covers the test methods of specific activity testing of probiotic master seed strains and probiotics on their basis determined by viable bacteria number and acid-production activity or their antagonistic activity against test strains.

Therefore, the microbiological methods described herein allow to determine the following parameters of test product or strain specific activity:

- Viable bacteria number in 1 dose[1] of immunobiological medicinal product (IMP) (section 1);
- Acid-production activity (section 2);
- Antagonistic activity (section 3).

THE GENERAL PART

The requirements for test product specific activity are related to the determination of viable bacteria number in 1 dose of a medicinal product, acid-production activity of strains-producers being the part of probiotics for medical use containing lactic acid bacteria and bifid bacteria, and to the determination of test product antagonistic activity level.

Test product control in terms of “Viable bacteria number in one dose of a medicinal product” in the development of new technologies for manufacturing a medicinal product, preliminary and certification control, quality evaluation, and stability testing of dosage forms in the process of shelf-life determination. The number identification of viable microorganisms in the test dose is carried out by serial dilution method, if necessary, with subsequent inoculation on the cultural media (Human dose is the dose of a test product specified on the label or in the instruction for medical use (package insert)).

It is necessary to take into account the number and the proportion of all types

of species or strains included in the product in the course of monitoring the multi-component or combined probiotics.

"Acid-production activity" is an obligatory parameter for controlling the strains-producers being the part of probiotics for medical use containing lactic acid bacteria and bifid bacteria, during preliminary and certification control, quality evaluation, and stability testing of the dosage forms in the process of shelf-life determination.

"Antagonistic activity" is an obligatory parameter for controlling the coli-containing and sporous probiotics for medical use and master seed strains. Antagonistic activity level of IMP related to the strains of pathogenic and opportunistic microorganisms is determined by deferred antagonism method on the solid medium according to the growth inhibition zones of test-strains.

Master seed strains are monitored at least once a year.

Cultural media used in the tests

For cultivating the microorganisms included in the probiotics for medical use, the cultural medium adequate for this type of bacteria and for this methodology should be used (unless otherwise specified in the normative documentation):

- For lactic acid bacteria– MPC-2, MPC-4, MPC-5, MPC-1; for acidophilus lactic acid bacteria – sterile nonfat milk;
- For bifid bacteria – Blaurock semi-liquid modified hepatic medium, Blaurock medium with sodium azide, MPC-5;
- For *Escherichia coli* – Endo medium, meat-and-peptone agar (MPA), Gauze medium No. 2 agarized;
- For *enterococci* – meat-and-peptone agar, medium No. 1;
- For bacteria of *Bacillus* – Gauze medium No. 2 agarized, meat-and-peptone agar, semi-synthetic medium with yeast dialysate.

Cultural media for testing are prepared according to the guidelines below. Equivalent commercially available media can also be used, provided that they pass the tests on the growth properties. The required pH value of cultural media are set

at the temperature of 25 ± 2°C.

MPC-1 medium

The following agents are dissolved subsequently in 200 ml of purified water:

- Manganese sulfate 0.05 g
- Cysteine hydrochloride or L-cystine - 0.10 g
- Magnesium sulfate 0.200 g
- Dibasic potassium hydrophosphate trihydrate 2.00 g
- Ammonium citrate 2.00 g
- Sodium acetate (sodium hydroxyacetate) 5.00 g
- Liver extract [a)] (1:1) 100.0 ml
- Yeast autolysate [b)] with content of amine nitrogen (0.15 ± 0.03)% 50.0 ml
- Peptone enzymatic dry 10.00 g
- Hydrolysate of nonfat milk[c)] 500.0 ml
- Polysorbate 80 1.00 ml
- Glucose 20.00 g

The medium is brought to volume of 1,000 ml with purified water; pH value of medium is 6.4 ± 0.2. The cultural medium is poured into the tubes of 10 ml, 25 ml or 30 ml and sterilized at the temperature of 120 ± 2°C for 15 ± 1 min. Shelf-life of ready sterile medium is 2 months at the temperature of 2 to 8°C or not more than 1 month at the temperature of 18 to 25°C.

Note

[a)] Preparation of liver extract containing amine nitrogen (0.050 ± 0.005) %.

- Beef liver 1.0 kg
- Purified water 1,000 ml

Sterilization in an autoclave at the temperature of 112 ± 5°C for 20 ± 1 min (sterilization process is validated).

Preparation: 1.0 kg of fresh beef liver is cleaned from fat, membranes, and ducts, cut into cubes with size of (3×3×3) cm, covered with 1 liter of purified water and boiled for 1.5 - 2 h. The cooled decoction is filtered through a gauze filter

("calico" fabric), the decoction amount is brought to 1 L with purified water, poured into the bottles and sterilized at 120 ± 2°C for 15 ± 1 min. Liver extract shelf-life is not more than 6 months at the temperature of 2 to 8°C or not more than 3 months at the room temperature.

[b]) Preparation of yeast autolysate with amine nitrogen content (0.15 ± 0.03) %.

- Yeast baking 1,000 g
- Drinking water 4,000 ml
- Chloroform 40 ml

Sterilization in an autoclave at the temperature of 112 ± 5°C for 20 ± 1 min (sterilization process is validated).

[c]) Preparation of nonfat milk hydrolysate under Bogdanov.

- Cow milk-pasteurized, low-fat (pH 6.7 ± 0.1) 1,000 ml
- Pancreatin 1.0 g
- Chloroform 5.0 ml

Sterilization in an autoclave at the temperature of 112 ± 5°C for 20 ± 1 min.

MPC-2 medium

- Manganese sulfate 0.05 g
- Cysteine hydrochloride or L-cystine 0.10 g
- Magnesium sulfate 0.20 g
- Dibasic potassium hydrophosphate trihydrate 2.00 g
- Ammonium citrate 2.00 g
- Sodium acetate 5.00 g
- Liver extract (1:1) 100.0 ml
- Yeast autolysate with content of amine nitrogen (0.15 ± 0.03) % 50.0 ml
- Peptone enzymatic dry 10.00 g
- Hydrolysate of nonfat milk 500.0 ml
- Polysorbate 80 1.00 ml
- Glucose 20.00 g

- Agar microbiologic 1.00 g
- Purified water Up to 1,000 ml

pH value of ready medium is 6.4 ± 0.2 The cultural medium is poured into the tubes of 10 ml and sterilized at the temperature of 120 ± 2°C for 15 ± 1 min. Cultural medium shelf-life is 2 months at the temperature of 2 to 8°C or not more than 1 month at the temperature of 18 to 25°C.

MPC-4 medium

The following agents are dissolved subsequently in 200 ml of purified water:

- Manganese sulfate 0.05 g
- Cysteine hydrochloride or L-cystine 0.10 g
- Magnesium sulfate 0.200 g
- Dibasic potassium hydrophosphate trihydrate 2.00 g
- Ammonium citrate 2.00 g
- Sodium acetate 5.00 g
- Liver extract (1:1) 100.0 ml
- Yeast autolysate with content of amine nitrogen (0.15 ± 0.03) % 50.0 ml
- Peptone enzymatic dry 10.00 g
- Nonfat milk hydrolysate 500.0 ml
- Polysorbate 80 1.00 ml
- Glucose 20.00 g
- Agar microbiologic 19.0 g

The medium is brought to volume 1,000 ml with purified water with pH 6.4 ± 0.2. The cultural medium is sterilized in an autoclave at the temperature of 120 ± 2°C for 15 ± 1 min. Cultural medium shelf-life is 6 months at the temperature of 2 to 8°C in sterile sealed vials or not more than 3 months at the temperature of 18 to 25°C.

MPC-5 medium

- Manganese sulfate 0.05 g
- Cysteine hydrochloride or L-cystine - 0.10 g
- Magnesium sulfate 0.200 g
- Dibasic potassium hydrophosphate trihydrate 2.00 g
- Liver extract (1:1) 100.0 ml
- Yeast autolysate with content of amine nitrogen (0.15 ± 0.03) % 50.0 ml
- Peptone enzymatic dry 10.00 g
- Nonfat milk hydrolysate 500.0 ml
- Polysorbate 80 1.00 ml
- Glucose 20.00 g
- Agar microbiologic 15.00 g
- Purified water Up to 1,000 ml

The cultural medium (pH 7.0) is sterilized in an autoclave at the temperature of 120 ± 2°C for 15 ± 1 min. Cultural medium shelf-life is 6 months at the temperature of 2 to 8°C in sterile sealed containers (vials) or not more than 3 months at the temperature of 18 to 25°C.

Note

Preparation of sterile nonfat milk (pH 6.7 ± 0.3); acidity value is not more than 18 °T:

- Dry nonfat milk 80 g
- Purified water Up to 1,000 ml

It is poured into the tubes of 10 ml, 25 ml or 30 ml and sterilized at the temperature of 120 ± 5°C for 15 ± 1 min (sterilization process is validated). Sterile nonfat milk shelf-life is 2 months at the temperature of 2 to 8°C or not more than 1 month at the temperature of 18 to 25°C.

Blaurock semi-liquid modified hepatic medium

- Liver water (1:2) containing amine nitrogen 100 ± 20 mg% 1,000 ml
- Dry peptone 10 g
- Sodium chloride 5 g
- Lactose 10 g
- Cysteine 0.1 g
- Agar microbiologic 0.75 g

The required pH value (7.2 ± 0.2) is set using 20% Sodium hydroxide solution. The cultural media are poured into the tubes of 10 ml, 25 ml or 30 ml and sterilized at the temperature of 112 ± 5°C for 60 ± 1 min (sterilization process is validated). Cultural medium shelf-life is 2 months at the temperature of 2 to 8°C or not more than 1 month at the temperature of 18 to 25°C. In 14 days of storage prior the use, and for minimizing dissolved oxygen, the medium should be once regenerated by heating the tubes in water bath at the temperature of 70-80°C for 10-15 min.

Note

Preparation of liver water containing amine nitrogen 100 ± 20 mg %.

- Beef liver 0.5 kg
- Purified water 1,000 ml

Sterilization in an autoclave at the temperature of 112 ± 5°C for 20 ± 1 min (sterilization process is validated).

0.5 kg of fresh beef liver is cleaned from fat, membranes and ducts, cut into cubes with the size of (3×3×3) cm, covered with 1 liter of purified water, and boiled for 1.5 - 2 h. The cooled decoction is filtered through a gauze filter (“calico” fabric), brought the decoction volume to 1 L with purified water, poured into the bottles and sterilized at 112 ± 5°C for 20 ± 1 min. Shelf-life is not more than 6 months at the temperature of 2 to 8°C or not more than 3 months at the temperature of 18 to 25.

Blaurock medium with amine nitrogen

- Blaurock medium 1,000 ml
- Amine nitrogen 0.1 g

The medium is mixed, poured into the tubes of 10 ml and sterilized at the temperature of 112 ± 5°C for 20 ± 1 min. Cultural medium shelf-life is 1 month at the temperature of 2 to 8°C.

Gauze medium No 2 agarized

- Hottinger broth[1)] containing amine nitrogen 700 mg% 30 ml
- Dry peptone 5 g
- Sodium chloride 5 g
- Glucose 10 g
- Agar microbiologic 30 g
- Purified water Up to 1,000 ml

Note
Preparation of Hottinger broth (containing 700 mg% of amine nitrogen)

- Hottinger hydrolizate 24.0 g
- Sodium chloride 5 g
- Purified water Up to 1,000 ml

Sterilization in an autoclave at the temperature of 112 ± 5°C for 20 ± 1 min. Cultural medium shelf-life is 6 months at the temperature of 2 to 8°C in sterile sealed containers (vials).

Agarized semi-synthetic medium with yeast dialysate

- Manganese chloride tetrahydrate or Manganese sulfate 0.01 g
- Ferrous (III) sulphate heptahydrate 0.01 g
- Magnesium sulphate heptahydrate or Magnesium chloride hexahydrate 0.1 g

- Calcium chloride 0.08 g
- Dry peptone 0.5 g
- Yeast dialysate containing amine nitrogen 150 mg% 5.0 ml
- Glucose 10.0 g
- Agar microbiologic 20-30 g
- Purified water Up to 1,000 ml

Sterilization in an autoclave at the temperature of 112 ± 5°C for 20 ± 1 min. Cultural medium shelf-life is 6 months at the temperature of 2 to 8°C in sterile sealed containers (vials).

Meat-and-peptone agar (MPA)

- Peptone 10.0 g
- Sodium chloride 5.0 g
- Meat water 300-500 ml
- Agar microbiologic 13-20 g

10 g of peptone and 5 g of Sodium chloride are added to meat water, boiled on slow fire with constant stirring until the complete dissolution of added ingredients. The prepared broth is filtered, pH values of 7.2-7.4 are set and the ground agar is added in the volume dependent on its quality and medium destination. After agar adding, the medium is boiled on slow fire with constant stirring until agar complete dissolution. It is poured into the tubes and bottles, sterilized at 120°C for 20 ± 1 min. After sterilization, while cooling down, the medium is consolidated.

Shelf-life of ready medium (pH 7.3 ± 0.2) is 6 months at the temperature of 2 to 8°C or not more than 1 month at the temperature of 18 to 25°C.

Meat-and-peptone broth (MPB)

- Peptone 10.0 g

- Sodium chloride 5.0 g
- Meat water 1,000 ml

10 g of peptone and 5 g of Sodium chloride are added to meat water, boiled on slow fire with constant stirring until the complete dissolution of added ingredients. The prepared broth is filtered, pH values of 7.2-7.4 are set and is poured out into the tubes and vials. It is sterilized at 120°C for 20 ± 1 min. Shelf-life of cultural medium is 6 months at the temperature of 2 to 8°C or not more than 1 month at the temperature of 18 to 25°C.

Note
Preparation of meat water (1:2).

- Meat-beaf (farce) 500 g
- Purified water 1,000 ml

Sterilization in an autoclave at the temperature of 121°C for 20 min. Shelf-life is 2 months at the temperature of 2 to 8°C and not more than 1 month at the temperature of 18 to 25°C.

Before testing, in case of solid cultural medium use, 10-15 or 20-25 ml of molten agarized cultural medium (at the temperature of about 45°C) were poured out into sterile Petri dishes with the diameter of 9 or 12 cm (respectively), left on a horizontal surface and allowed the medium to solidify. Before inoculation the surfaces of agarized media are dried to remove the water condensation and to check the sterility. Closed and turned upside down Petri dishes with cultural media are placed in an oven at 37 ± 1°C for 48 h. Endo agar is dried for 40 min in a laminar flow hood with open covers.

Section 1. Determining the viable bacteria number in one dose of IMP

This section contains General principles of determining viable bacteria number in probiotics for medical use by tenfold dilution method with the

inoculation in solid cultural media (Koch method) and by deep plate method or in liquid and semi-liquid media (method of limited dilutions). The results of quantitative determination of microorganisms are expressed in colony forming units (CFU).

The selection and preparation of samples for the analysis

Three tested samples are selected from each test batch of probiotic product by random sampling method.

The tests are carried out following aseptic regulations.

Each sample is separately diluted with sterile 0.9% Sodium chloride solution and stirred 8-10 times with a pipette.

The characteristics of preparing the test sample depending on the dosage form (unless otherwise specified in the normative documentation):

1. *Lyophilisates (vials)* – each sample is re-suspended separately with sterile 0.9% Sodium chloride solution (on the basis of 1 ml per dose) and mixed 8-10 times with a pipette, obtaining the background dilution.
2. *Powders* – the content of a packet (sachet) is aseptically poured into the sterile flask of 250 ml, added with 100 ml of 0.9% Sodium chloride solution and mixed by vigorous shaking for 10 min, obtaining the dilution of 1:100 (10^{-2}).
3. *Tablets* – each sample is separately pre-pulverized in a sterile mortar with sterile pestle until homogeneous state and is added fractionally with 0.9% Sodium chloride solution at the rate of 1 ml per dose and mixed 8-10 times with a pipette, obtaining the background dilution.
4. *Capsules* – each sample is separately placed into the sterile tubes, added with 10 ml of sterile 0.9% Sodium chloride solution. The tubes with capsules are placed in water bath at the temperature of 39 ± 1°C. In 10-30 min the tube contents are stirred until homogeneous state, obtaining the dilution of 1:10 (10^{-1}).
5. *Suppositories* - each sample is separately placed in the sterile test tubes and added with sterile 0.9% Sodium chloride solution preliminary heated to the

temperature of 37 ± 1°C to the total volume of 10 ml, depending on the value of suppository average of a test batch (for example, at the weight of 1.1 g, 8.9 ml of 0.9% solution of sodium chloride are added, and at the weight of 2 g, 8 ml of saline are added). The tubes with suppositories are placed in water bath at the temperature of 39 ± 1°C. In 10-30 min the content of vials is stirred until homogeneous state, obtaining the dilution of 1:10 (10^{-1}).

Testing method

The test is carried out by the method of serial ten-fold dilutions, following aseptic regulations. For this purpose, 1 ml of microbial suspension of test sample (initial dilution, dilutions of 10^{-1} or 10^{-2}) is introduced into the test tube containing 9 ml of 0.9% Sodium chloride solution with a pipette. Then, it is required to stir 10-15 times with a new pipette, obtaining the next dilution (10^{-2} or 10^{-3}), and then 1 ml of the suspension is transferred to the next dilution. The titration is carried out to the dilutions from which the inoculation on cultural media will be made. For each dilution, a separate pipette is used.

If the sample contains only one dose, 0.5 ml of microbial suspension of test sample (initial dilution) with a pipette is introduced into the tube containing 4.5 ml of 0.9% Sodium chloride solution. Then, it is required to stir 10-15 times with a new pipette, obtaining the next dilution (10^{-1}), and 1 ml of suspension is transferred into the tube with 9 ml 0.9% of Sodium chloride solution, obtaining the dilution of 10^{-2}. The titration is carried out to the dilutions from which the inoculation on cultural media will be made. For each dilution, a separate pipette is used.

Depending on bacterial biological properties contained in the test sample, the appropriate testing method is applied.

1. Inoculation on solid media

1.1 Koch method

In the series of consecutive tenfold dilutions of the test sample from the last 2 dilutions (dilution degree depends on CFU number in 1 dose of a test product), 0.1 ml of microbial suspension are inoculated on Petri dishes with cultural medium (2 dishes for each dilution). The suspension is spread evenly over the medium surface with Drygalski spatula or using glass beads until suspension full absorption (drying). The closed dishes are placed upside-down in an oven for incubating.

The inoculates are incubated at 37 ± 1°C for 24-96 h in the appropriate conditions, depending on microorganism type (aerobic, microaerophilic or anaerobic). After incubating under anaerobic or microaerophilic conditions the Petri dishes with inoculates are placed in an anaerobic jar and the required gas atmosphere is created.

This method can be used to determine the number of viable bacteria of each type in multi-component probiotics, provided the bacteria included in the composition of the product, form the colonies visually distinguished by shape and other colony characteristics.

1.2. Deep plate method

In a number of consecutive tenfold dilutions of the test sample from the last 2 dilutions (dilution degree depends on CFU number in the test product), 1.0 ml of the microbial suspension are introduced in Petri dishes with the diameter of 90 mm (2 dishes for each dilution) with a sterile pipette of 1.0 ml. 20-25 ml of molten agarized cultural medium cooled to temperature of 42.5 ± 2.5°C should be closed and quickly and gently mixed via rotational-translational motions, without taking the bottom off a dish from the table surface and avoiding medium contact of the with the cup of a dish. After agar solidification, the Petri dishes are turned upside down and incubated in appropriate conditions at 37 ± 1°C during the necessary time period for this microorganism.

Accountability of the results

At the end of incubation, the count of the grown colonies on Petri dishes is performed and viable bacteria content in one dose of the test sample is calculated. When calculating, the dishes with not less than 15 grown colonies are taken into

account.

Example of calculation of the content of viable microbial cells in 1 dose of the test sample:

- 42 and 45 colonies were grown from the dilution of 10^{-7}; the arithmetic mean is equal to (42+45) : 2 = 43.5;

- 410 and 450 colonies were grown from the dilution of 10^{-6}; the arithmetic mean is equal to (410+450) : 2 = 430;

- viable bacteria number per 1 dose is equal to:

$(43.5 \times 10^7 + 430 \times 10^6) : 2 \times 10 = 4{,}325{,}000{,}000 = 4.3 \times 10^9$.

The arithmetic mean of the number of colonies is multiplied by the dilution degree and if the inoculation on a Petri dish was made in the volume of 0.1 ml, it is multiplied by factor 10 for conversion to 1.0 ml.

2. The method of limited dilutions with subsequent inoculation on liquid and semi-liquid cultural media

2.1. Test-tube method of the most probable numbers (determining the number of viable acidophilus Lactic acid bacteria)

In the series of consecutive tenfold dilutions of test sample of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} (dilution degree depends on CFU number in a test sample), 1 ml of microbial suspension from each dilution is inoculated in two test tubes with 9 ml of sterile nonfat milk. The dilution used for inoculation should be marked. The dilution of 10^{-6} of the product in 0.9% Sodium chloride solution corresponds to the dilution of 10^{-6} in milk. For medium control, 4 non-inoculated test tubes with milk should be added. Inoculated and controlled tubes are incubated at the temperature of 38 ± 1°C for 3-4 days.

At the end of incubation, the number of tubes with clotted milk is determined. The smears are prepared from the clot with culture and stained by Gram. If specific bacteria in smears are visible and extraneous microflora is absent, this dilution is considered as one containing microorganisms of this type. In case of milk clotting in control tubes and the extraneous microflora detection in smears,

the control is performed on the new medium batch.

Accountability of the results

When counting the number of viable species, Mac-Credy table is used (Table 1). First, a numerical characteristic is made. It consists of three digits: the digit equal to the number of tubes with clotted milk taken in the last dilution where the milk is clotted in all tubes (e.g., 2 tubes of the dilution of 10^{-6}) are put on the first (left) place.

The next two digits indicate the number of the tubes with clotted milk in two subsequent dilutions (for example, in one tube of the dilution of 10^{-7} and in one tube of the dilution of 10^{-8}).

A numerical characteristic of the result will be 211.

According to Mak-Credy table, the probable number is found corresponding to the obtained numerical characteristics (it is 13 in our case), multiplied by the dilution corresponding to the first digit of a numerical characteristics (it is 10^{-6} in this example). Then the number of viable species of microorganisms in 1 dose is $13 \times 10^6 = 1.3 \times 10^7$.

Table 1. Mac-Credy table used for counting the number of viable Lactic acid acidophilus bacteria

A numerical characteristic	The most probable number of microorganisms while inoculating in 2 parallel tubes
200	2.5
201	5.0
202	-
210	6.0
211	13.0
212	20.0
220	25.0
221	70.0

2.2. Test-tube method of accounting the colonies in semi-liquid medium

From the product dilutions of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} (dilution degree depends on CFU number in a test sample), 1 ml of microbial suspension is

inoculated in the tubes with 9 ml of semi-liquid cultural medium. The dilution used for inoculation should be marked. The product dilution of 10^{-6} in 0.9% Sodium chloride solution corresponds to the dilution of 10^{-6} in semi-liquid cultural medium.

The inoculations are incubated at the temperature of 37 ± 1°C depending on microorganism type for 1-6 days. At the end of incubation, the dilutions with the colony growth typical for this microorganism type should be marked.

Accountability of the results

In the series of consecutive tenfold dilutions of the test sample in semi-liquid nutrient medium, 10-fold decrease in the number of microorganism colonies should be observed.

The number of viable bacteria in the dose of test product is calculated by multiplying the colony number in vitro by the value of dilution of microbial suspension in this tube (the introduced material volume in 1.0 ml gives the multiplication factor 1, which does not affect the final result in the calculation).

3. Determining the number of viable bacteria in polycomponent probiotics

3.1 Combined method (the determination of bifida bacteria and coli bacteria quantity)

To determine the number of viable bacteria in the composition of probiotic product from the appropriate subsequent tenfold dilutions of a test sample in 0.9% Sodium chloride solution (10^{-1} to 10^{-9}), the inoculations on Blaurock medium with sodium azide (accountability of bifida bacteria) and on Endo medium (accountability of coli bacteria) are carried out.

To determine the bifida bacteria number, 1 ml of microbial suspension from the dilutions of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} are inoculated in the tubes containing 9 ml of semi-liquid cultural Blaurock medium with sodium azide (marking the dilution used for inoculation). Thus, it should be taken into account that the product dilution of 10^{-6} in 0.9% Sodium chloride solution corresponds to the dilution of 10^{-}

^{6} in Blaurock medium with sodium azide. The inoculations in Blaurock medium with sodium azide are incubated at the temperature of 38 ± 1°C for 4-5 days.

To determine the number of E. coli, 0.1 ml of microbial suspension from test sample dilutions of 10^{-5}, 10^{-6} are inoculated on Petri dishes with Endo medium (2 dishes from each dilution). The suspension is spread evenly over the surface of Endo medium with Drygalski spatula until full absorption (drying) of the suspension. The dishes are closed and placed upside-down in a thermostat for the incubation. The inoculations on Endo medium are incubated at the temperature of 37 ± 1^{o}C for 18-24 h.

Accountability of the results

After incubation completion, on Blaurock medium with sodium azide there are the dilutions in which the colonies are growing in the form of "studs". The viable bacteria number in the dose is calculated by multiplying the number of colonies in vitro by the dilution value of the microbial suspension in this tube.

After incubation completion, on Endo medium, the grown colonies on Petri dishes are counted and viable bacteria number is calculated in one dose of the product. When calculating, the dishes with not less than 15 grown colonies are taken into account. The calculation example is similar to that described in subsection 1.

Section 2. Acid-production activity

Acid-production activity of the strains-producers being the part of probiotics for medical use containing lactic acid bacteria and bifida bacteria is determined by titratable acidity while culturing the bacteria in an appropriate cultural medium. Testing the quantitative determination of acid-production activity is carried out by acid-base titration.

For bifida bacteria cultivation the modified semi-liquid hepatic Blaurock medium for lactic acid bacteria – MPC-1 or the sterile nonfat milk is used, unless otherwise specified in the normative documentation.

The reagents used in the test:

- titrated 0.1 M Sodium hydroxide solution;
- indicator, 1% phenolphthalein.

The characteristics of selecting and preparing the samples for analysis

Two samples are selected from each test batch of the medicinal product independently on its volume by random sampling method. If the test sample (i.e. tablet/capsule/suppository) contains 1 dose, 1 sample should be comprised from 3 tablets/capsules/suppositories.

The tests are carried out following aseptic regulations.

The characteristics of test sample preparation depending on the dosage form (unless otherwise specified in the normative documentation):

1. *Lyophilisates* – each test sample (each sample separately) is diluted with sterile 0.9% Sodium chloride solution on the basis of 1 ml per dose and mixed 8-10 times with a pipette.

   2.5 ml of obtained suspension of each sample are introduced into 50 ml tubes, containing 25 ml of the cultural medium. The vials with inoculations of test samples are placed in a thermostat at the temperature of 38 ± 1°C and incubated for 48-72 h (incubation time depends on microorganism species).
2. *Tablets* – each sample is separately pre-pulverized (aseptically) in a sterile mortar until homogeneous state and is added fractionally with 0.9% Sodium chloride solution at the rate of 1 ml per dose and mixed 8-10 times with a pipette.

   2.5 ml of obtained suspension of each sample are introduced into 50 ml tubes, containing 25 ml of the cultural medium. The vials with inoculations of test samples are placed in a thermostat at the temperature of 38 ± 1°C and incubated for 48-72 h (incubation time depends on microorganism species).
3. *Powders* – the content of a packet (sachet) is aseptically poured out into 50 ml tube, containing 25 ml of cultural medium and mixed. The tubes with inoculations of test samples are placed in a thermostat at the temperature of 38 ± 1oC and incubated for 48-72 h (incubation time depends on

microorganism species).

4. *Capsules* – each sample is separately introduced into 50 ml sterile tubes, containing 30 ml of cultural medium, appropriate for microorganism type included in product composition. The tubes are placed in water bath at the temperature of 39 ± 1°C. In 20-30 minutes the obtained suspension is stirred until homogeneous state. The tubes with test sample inoculations are placed in a thermostat at the temperature of 38 ± 1°C and incubated for 48-72 h (incubation time depends on microorganism species).
5. *Suppositories* - each sample is separately placed in 50 ml tubes containing 30 ml of the cultural medium, appropriate for microorganism type included in product composition. The tubes are placed in water bath at the temperature of 39 ± 1°C. In 20-30 min the obtained suspension is stirred until homogeneous state. The tubes with test sample inoculations are placed in a thermostat at the temperature of 38 ± 1°C and incubated for 48-72 h (incubation time depends on microorganism species).

Note

After incubation completion, the suspension in tubes is liberated from lipophilic suppository basis in one of the following ways:

- immediately after incubation completion (when inoculation temperature is 38 ± 1^{o}C) the melted top layer of fat is removed with a sterile pipette;
- the tubes are cooled at the temperature of 2 to 8 ^{o}C for 20 ± 5 min, and then the frozen suppository basis is removed aseptically with a pasteur pipet with the curved end or with a bacteriological loop.

Testing method

Acid-production level is determined in every 2 samples.

After incubation completion, the tube content is mixed 8-10 times with a separate pipette. Each sample in the volume of 10 ml, is introduced in a separate conical flask or a beaker of 100 ml.

Strains-producers grown in sterile nonfat milk form the clot. The formed clot is broken by thorough mixing (10-12 times) with 10 ml pipette. Then 10 ml of microbial suspension are transferred from the tube with culture medium to 100 ml conical flask containing 20 ml of purified water. The flask is intensively shaken for uniform mixing of contents, and then 2-3 drops of 1% phenolphthalein solution are added.

The resulting suspension is titrated with 0.1 M Sodium hydroxide solution until persistent weak-pink color appearance and achievement of pH 8.5±0.1 (controlled by potentiometer), while taking into account the number of milliliters of 0.1 M Sodium hydroxide solution used for titration.

Accountability of the results

Acidity is expressed in Turner degrees (°T) and calculated according to the formula:

$$T = A \times K \times 10,$$

where: A – the amount of milliliters of 0.1 M Sodium hydroxide solution used for the titration of 10 ml of test microbial suspension;

K – amendment to the titer of 0.1 M Sodium hydroxide solution;

10 – the volume of microbial suspension, ml

Example of calculation: 10.6 ml of 0.1 M Sodium hydroxide solution was used for the titration of 10 ml of microbial suspension, where K = of 1.03, then:

$T = 10.6 \times 1.03 \times 10 = 109.18\ ^{o}T$

The average value of 2 parallel samples from each sample (tube) and the average value of 2 samples (test tubes) are calculated, provided that the indicator of acid-production activity of each of them was not less than specified one.

Section 3. Antagonistic activity

Antagonistic activity against pathogenic and opportunistic microorganisms is estimated in both strains-producers and the products of probiotics obtained on their basis. The determination of antagonistic activity of strains-producers included in probiotics for medical use is carried out using deferred antagonism method.

When testing, the cultural medium, appropriate for this type of bacteria (if there are no other indications in normative documents) is used:

- for lactic acid bacteria and bifida bacteria – MPC-5 medium;
- for *Escherichia coli* and sporous probiotics – Gauze medium No. 2.

Test strains of microorganisms

Test strains of microorganisms used in the testing are presented in Table 2. They are obtained in lyophilized form (in ampoules) from the State collection of pathogenic microorganisms of Federal State Budget Institution Scientific Center of Assessment of Medical Products under Roszdravnadzor (unless otherwise specified in the normative documentation) with Certificate of compliance (passport on strain).

Table 2. Microorganism test-strains, used in the studies on delayed antagonism

Microorganism test-strain	Strain number
Staphylococcus aureus	ATCC 6538 (FDA 209P)
Escherichia coli	O157
Shigella flexneri	170, 337
Shigella sonnei	5063
Proteus mirabilis	H-237 or 56/10
Proteus vulgaris	177 or 401
Candida albicans	ATCC10231

Recovering the lyophilized test strains of microorganisms.

The ampoules with test cultures of microorganisms are opened under aseptic conditions in accordance with the manufacturer's instructions.

To restore the culture viability, it is required to conduct at least two re-inoculations in the appropriate cultural medium (appropriate to the needs of used microorganism) with the incubation under standard conditions. For obtaining isolated colonies, the second re-inoculation of test strain culture is carried out on the appropriate solid cultural medium while the incubation is under standard

conditions.

Incubation temperature of bacteria cultures is 32.5 ± 2.5°C, fungi – 22.5 ± 2.5°C unless otherwise required in the normative documents.

After incubation completion, the morphology of grown colonies is investigated, the Gram stained smears are examined microscopically, the biochemical properties are studied using the test systems approved for the use. Microorganism of a test-strain should have typical morphological, tinctorial, culture, biochemical properties, in accordance with the presented collection certificate.

Test strains of microorganisms in lyophilized form are stored at the temperature 6 ± 2°C for 24 months.

It is admissible to make not more than five passages form original culture.

The preparation of test strains of microorganisms for testing. For testing the cultures grown for 22 ± 2 h, of second or third passages are used, and they are incubated on appropriate solid cultural medium under adequate conditions. The grown pure culture is washed with 0.9% Sodium chloride solution from the surface of solid cultural medium. The resulting suspension is collected in a sterile container (vial, tube etc.). The concentration of microbial cells in the resulting suspension is adjusted in accordance with the standard sample of turbidity 5 AU (Absorbance Unit) (according to General Pharmacopoeial Monograph "Determining the concentration of microbial cells").

Selecting and preparing the samples for analysis

The samples are selected from each test batch of the product by random sampling method.

Probiotic master seed strains-producers (lyophilisates) are recovered to the original dried volume (but not less than 10^7 microbial cells/ml) by adding 0.9% Sodium chloride solution to the sterile lyophilisate.

The characteristics of preparing the probiotic product test sample

depending on the dosage form (unless otherwise required in the normative documentation):

1. *Lyophilisates* are diluted with sterile 0.9% Sodium chloride solution on the basis of 1 ml per dose and mixed 8-10 times with a pipette.
2. *Tablets* are pre-pulverized in a mortar (aseptically) until homogeneous condition and is added fractionally with 0.9% Sodium chloride solution at the rate of 1 ml per dose and mixed 8-10 times with a pipette.
3. *Powders* – the content of a packet (sachet) is poured out into the tube containing 25 ml of sterile 0.9% Sodium chloride solution and mixed by vigorous shaking for 10 min.
4. *Capsules* – the capsule content is introduced in a tube, added with 1 ml of sterile 0.9% Sodium chloride solution and stirred until homogeneous state.
5. *Suppositories* are placed in a tube and added with sterile 0.9% sodium chloride solution pre-heated to temperature of 37 ± 1°C to the total volume of 10 ml. The tubes with suppositories are placed in water bath at the temperature of 39 ± 1°C. In 10-30 min the tube contents are stirred until homogeneous state and liberated from fat basis (as above mentioned in section 2).

Testing method

The resulting suspension of test sample is mixed and inoculated with a bacteriological loop with the diameter of 3.5 ± 0.5 mm on 6 Petri dishes with the cultural medium, making two parallel streaks with the length equal to the diameter of a dish.

After incubating for 48-96 h at the temperature of 37 ± 1°C under appropriate conditions (aerobic, microaerophilic or anaerobic), depending on the type of microorganism, the test sample grown culture is overseeded with the culture of test strains (in accordance with the requirements of normative documents). The overseeding of test strains is carried out with a loop of 1.75+0.25

mm in diameter at the direction perpendicular to the growth zone of the studied microorganism, not touching it. Petri dishes turned upside down are incubated at the temperature of 37 ± 1°C for 18-20 h (unless otherwise specified in the normative documentation).

The parallel inoculation on the dishes with the same cultural medium without test culture is served as the growth control of test strains.

Accountability of the results

The size of zones with no growth of test strain, which is expressed in mm should be taken into account. The greater the growth inhibition of test cultures, the higher the test strain antagonistic activity.

Unless otherwise required in the normative documentation, the antagonistic activity of strains-producers included in the probiotics for medical use, is considered as high under following conditions:

- the zones of growth inhibition of test strains are not less than 20 mm – for the strains-producers included in the lactocontaining probiotics;
- the zones of growth inhibition of test strains are not less than 15 mm – for the strains-producers included in probiotics containing coli bacteria, bifida bacteria;
- the zones of growth inhibition of test strains are not less than 10 mm – for the strains-producers included in sporous probiotics