Part 2. Biotechnology

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STUDY OF MORPHOLOGICAL, KARYOLOGICAL, BIOLOGICAL CHARACTERISTICS OF VIRUS-PRODUCING CELL LINES FLK-BLV (FLK-POL, FLK-71, FLK 50/100, FLK-SBBL) DURING LONG-TERM STORAGE IN THE CRYOBANK OF THE NATIONAL COLLECTION OF THE NSC 'IECVM'

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Summary. Studies have shown that, morphologically, the virus-producing cell line (FLK 50/100) consists of epithelial-like cells with a perinuclear space around the nucleus, while the FLK-71 line is represented by fibroblast-like cells with large nuclei. The karyology of FLK-71 cell lines after long-term storage was characterized by an increase in the range of chromosome number fluctuations from 40 to 64 from the data sheet of these cells before freezing (from 42 to 56). The limits of chromosome number fluctuation in FLK 50/100 decreased and ranged from 40 to 68. The antigen-producing biological activity of the subline of the reinoculated FLK-BLV culture (FLK-Pol) was preserved from passage 5 and further up to 22 passages (passage period) during the studied storage periods, namely from 55.5 months (FLK-Pol) to 201 months of storage (FLK-SBBL) and 206.5 months (FLK 50/100) in the conditions of the NSC 'IECVM' cryobank. However, for the strain FLK 50/100, up to 10 passages after thawing are required to fully recover the titers of its biological activity

Keywords: virus antigens, mitotic activity, immunodiffusion test

Introduction. Increasing the yield of bovine leukemia virus and its antigens produced in FLK-BLV cell culture as a result of leukemia virus replication in cell culture remains an urgent task for the timely diagnosis of this disease (Stegniy M., 2018; Dyakonov, 2009; Stegniy B. et al., 2019). For the diagnosis of bovine leukemia, an immunodiffusion (ID) test is used with the use of leukemia antigen, the producer of which is continuous cell culture FLK-BLV. Bovine leukemia is a chronic malignant infectious disease in which the integration of the pathogen into the body of susceptible animals causes its lifelong persistence (Van Regenmortel et al., 2000; Mamoun et al., 1983) and causes huge economic losses. The International Office for Epizootics (OIE) considers the ID test to be the main method in bovine leukemia (BLV) prevention and eradication programs today, also because the level of precipitating antibodies to the gp51 glucoprotein in cows reaches ID-positive levels 1–3 weeks after infection. The FLK-BLV cell culture was obtained in 1976 by Van der Maaten and Miller in the USA from softened fetal sheep kidney (fetal lamb kidney) cultured in monolayer (Van Der Maaten and Miller, 1976). Then, leukocytes from animals with viral lymphosarcoma were inoculated into the FLK cell culture.

Since continuous cell lines are composed of genetically heterogeneous cells, their long-term continuous passaging is often accompanied by undirected selective processes that lead to a decrease and sometimes complete loss of the necessary biological properties of the cell population (Stegniy B. et al., 2000). In order to increase the stability of the continuous lines and select the most efficient cell subpopulations, cloning is performed (Altaner et al., 1985), but the yield of fertile clones is very low. Therefore, cryopreservation with subsequent storage in the cryobank of the Pathogen Collection of the NSC 'IECVM' is very important for preserving the original properties of FLK-BLV cell lines (Stegniy M., 2009; Stegniy B., Stegniy M. and Stetsenko, 2011).

The aim of the research was to study the morphological, karyological, biological properties and antigen-producing activity of FLK-BLV virus-producing cell culture during long-term storage in the cryobank 'Collection of Pathogens of Animal Infectious Diseases', which was classified as a national heritage object by the Cabinet of Ministers of Ukaraine.

Materials and methods. The cell culture strains FLK-BLV (FLK-Pol; FLK-71; FLK 50/100; FLK-SBBL) were the study targets. To study the morphological characteristics of the continuous cell culture lines (FLK-71) and (FLK 50/100) after long-term storage in a cryobank at minus 196 °C for 17 years 9 months (FLK-71) and 19 years 8 months (FLK 50/100), they were thawed and then grown in culture mattresses or on coverslips in penicillin vials at 37 °C for one to four days, respectively, then fixed in 70% alcohol and stained with

Karachi stain, followed by visualization with an immersion light microscope.

Strains of the continuous culture FLK-BLV (FLK-Pol; FLK 50/100; FLK-71 FLK-SBBL) after long-term storage in the cryobank of the National Collection of Pathogens of the NSC 'IECVM' were thawed using a water bath at a temperature of 35–37 °C. Then, the total concentration of cells in all quadrants of the Goryaev chamber at low magnification and the concentration of preserved cells, excluding those stained with 0.2% trypan blue, were calculated. For this purpose, up to 1 cm^3 of cell suspension was added to an equal volume of 0.2% trypan blue solution, mixed thoroughly, and the Goryaev chamber was filled. The number of cells in 1 cm^3 of the suspension was determined by the formula (1):

$$X = \frac{A \times B}{0.9} \times 1{,}000 \quad (1)$$

where X — is the number of cells in 1 cm^3 of the suspension to be examined;

A — the number of counted living cells in the Goryaev chamber;

B — the dilution factor (for example, 20);

1,000 — the number of cubic millimeters in 1 cm^3;

0.9 — the volume of the Goryaev counting chamber in cubic millimeters.

Next, the cells were resuspended in a growth medium at a sowing concentration of 1.8×10^5 cells/cm^3 and seeded into culture dishes. The growth medium consisted of equal volumes of Eagle's and 199 media with 10% bovine serum.

The morphological properties of continuous cells and the dynamics of their monolayer formation after long-term storage in liquid nitrogen were studied both by daily microscopy under an inversion microscope and by making cytological preparations on coverslips. For this purpose, after defrosting, the cells were grown in penicillin vials on coverslips at 37 °C for four days. After 24, 48, 72, 96 hours of cultivation, the grown monolayer of cells was removed, rinsed in warm saline, dried with filter paper, and fixed in an ethanol-acetic solution. Cells fixed on coverslips were stored in 70% alcohol before staining with Karachi stain. Then they were rinsed with distilled water and stained with Karachi stain for 10 min. After staining, the slides were rinsed with ammonia water, dehydrated with high-concentration ethanol, finally with high-concentration ethanol-xylene, and then with pure xylene for 3 min. After air drying, the cell preparations were covered with Canadian balsam or polystyrene, followed by examination with an immersion light microscope. Mitotic activity was determined by the number of dividing cells per 1,000 cells counted and expressed in (‰) ppm (Stegniy B. et al., 2008).

In the process of cell growth, changes in the mitotic regime (mitotic activity, percentage of pathological mitoses, etc.) (Mamaeva, 1984), cytogenetic parameters and morphological state of cells were determined. The mitotic activity was determined by the number of dividing cells in relation to the total number of cells per 1,000 counted and expressed in ppm (‰).

Pathological mitoses were taken into account simultaneously with the analysis of mitotic activity (Mamaeva, 1988). The percentage number of pathological mitoses was determined by the ratio of the detected number of mitoses, and certain forms of mitotic pathology — by the ratio of the total number of pathological mitoses, which was taken as 100%.

The contamination of continuous cell cultures with fungal and bacterial microflora was checked in accordance with DSTU 4483:2005 'Veterinary Immunobiological Preparations. Methods for Determination of Bacterial and Fungous Contamination' (DSSU, 2005). For this purpose, samples of the cell suspension were taken and 0.2 cm^3 of the test sample was added to tubes with thioglycol medium and Sabouraud. Cultures on these media were incubated in a thermostat at 37 °C, and on Sabouraud medium — at room temperature (21–22 °C) for 7 and 14 days, respectively. The cultures were visually monitored daily for the growth of bacterial or fungal microflora. The absence of growth on bacterial media indicates the possibility of further use of these cell cultures for the development of biomass necessary for conservation (Stegniy B., Belokon' and Lavrik, 2005).

The study of the preservation of FLK-BLV continuous culture strains was carried out using trypan blue after thawing the cell culture; the morphology, mitotic cycle, and dynamics of the formation of a monolayer of continuous cell culture were investigated (Stegniy M. and Stegniy B., 2019; Stegniy M., Magats and Borodai, 2019).

The absence of growth on bacterial media indicates the possibility of further use of these cell cultures to develop the biomass necessary for subsequent cryopreservation to replenish the Collection and determine the antigen-producing biological activity of FLK-BLV.

After excluding contamination, it was expected that the biomass of cells would accumulate and a part of their number would be required for plating for further storage in liquid nitrogen (Belokon' et al., 1990). Virological control for the presence of diarrhea virus was carried out with specific antibodies of immune serum in a neutralization reaction according to the generally accepted method (Dyakonov, 2009).

Next, the biomass of the FLK-BLV cells was developed by repeated successive passages in the amount from 0 to 20. The leukemia virus was purified and concentrated from the culture fluid sample on ultrafiltration modules with hollow fibers. The evaluation of bovine leukemia virus antigen was carried out by activity in the immunodiffusion (ID) test and quantitative dose yield (doses of AG from 1 dm^3 of virus-

culture fluid). For this purpose, the concentrated antigen was resuspended in a phosphate-saline physiological solution with a pH of 7.0–7.2.

The reaction was performed and the results were recorded according to the 'Leaflet-inlay to the kit of components for the serological diagnosis of bovine leukemia in the immunodiffusion reaction' (No. 3272-14-0525-04/08-1/0 of 18.03.08) in 2 replicates on Petri dishes using a rectangular stamp. To determine the final titer of the antigen, serial dilutions of the experimental series were prepared from the native titer to 1:6. Phosphate-saline physiological solution with a pH of 7.0–7.2 was used as an antigen solvent. The reaction components were added to the agar wells in a volume of 40 μL.

A positive antigen titer was considered a dilution at which a clear line of precipitation with a positive control serum of two ++ was observed in the ID test. In order to compare the activity of the obtained antigens, the final antigen yield per 1 dm^3 of culture fluid was calculated. For this purpose, the term 'working titer of antigen' was used, which was considered to be the dilution of the antigen at which a positive reaction with positive serum was at ++++.

The formula (2) was used to calculate the final antigen yield per 1 dm^3 of culture fluid (X):

$$X = \frac{V_1 \times [A] \times T}{V_2} \times 100 \qquad (2)$$

where X — the amount of antigen obtained from 1 dm^3 of culture fluid, thousand doses;

V_1 — the volume of antigen actually obtained, cm^3;

[A] — the degree of concentration of the obtained antigen;

T — working titer of the obtained antigen;

V_2 — the volume of culture fluid used for antigen production

100 — coefficient for conversion to thousands of doses.

Statistical processing of the research results, mean values (M), standard deviation of the mean (m) and degree of reliability (p) was performed using the Student-Fisher method.

Results and discussions. The study of the morphological characteristics of the continuous cell culture line (FLK-71) on the third and fifth passages after thawingshowed that on the first day of cultivation, 30 to 35% of the monolayer was formed by fibroblast-like cells with large nuclei. In addition, several morphological types of cells were observed: spindle-shaped and polygonal. The morphology of the line (FLK 50/100) was represented by epithelial-like cells with a perinuclear space around the nucleus. On the first day of growth (FLK 50/100), 60% of the monolayer was formed (Fig. 1).

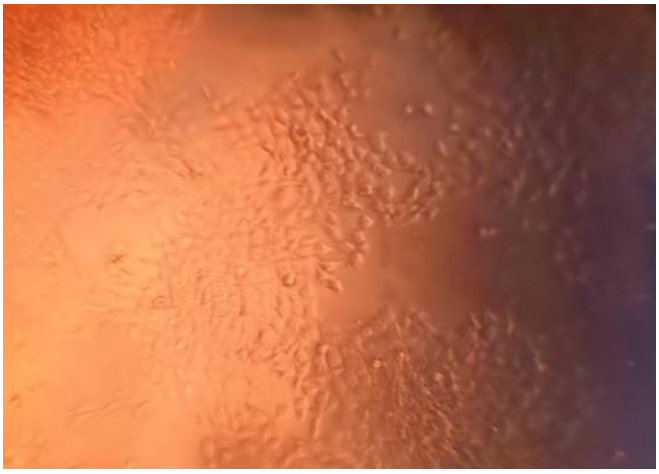

Figure 1. FLK 50/100 cell culture morphology at the third passage after thawing (magnification × 60).

In addition, 20% of the cells were at different stages of cell division, for example, metaphase plates, anaphase and telophase were observed.

After 48 hours of growth, the cells (FLK-71) formed 49% of the monolayer, and the cell cytoplasm was finely meshed. Cells in anaphase and telophase of division, as well as single multinucleated cells were observed.

The line (FLK 50/100) after 48 hours of growth formed 98% of the monolayer, which consisted of rounded cells with fine-grained cytoplasm, which occupied 35% of the cell volume, 65% — the nucleus. After 72 hours of growth, (FLK-71) cells formed 90% of the monolayer, and (FLK 50/100) cells formed 100%. The analysis of the total mitotic activity of the FLK 50/100 cell culture on the 3rd and 5th passages of cultivation showed that it was higher in the first two days of cultivation than in FLK-71 with a gradual decrease on the 3rd–4th day (Tabs 1, 2).

Table 1 — Total mitotic activity of FLK 50/100 cell culture on the 3rd passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
30.00 ± 0.82	4.95 ± 0.87	41.50 ± 0.65	6.58 ± 1.08	31.25 ± 0.75	3.96 ± 0.70	19.00 ± 0.71	3.97 ± 2.63

Table 2 — Total mitotic activity of FLK-71 cell culture on the 3rd passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
23.75 ± 1.03	2.23 ± 1.29	37.75 ± 0.48	5.28 ± 1.04	43.50 ± 1.44	4.56 ± 0.84	30.75 ± 0.95	4.96 ± 1.07

The total mitotic activity of the FLK-71 cell culture on the 3rd and 5th passages after thawing was lower than that of FLK 50/100 in the first two days of cultivation with a maximum on the third day (Tabs 3, 4).

The beginning of the monolayer destruction 30–35% was observed after 96 hours of cell growth (FLK-71), while the monolayer destruction was not observed in the (FLK 50/100) line, only an increase in the granularity of the cell cytoplasm was observed.

Table 3 — Total mitotic activity of FLK-71 cell culture on the 5th passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
26.25 ± 0.63	2.86 ± 0.96	39.25 ± 0.48	3.82 ± 0.72	44.75 ± 0.85	4.45 ± 0.85	24.75 ± 1.25	3.18 ± 1.07

Table 4 — Total mitotic activity of FLK 50/100 cell culture on the 5th passage after thawing (M ± m, n = 4)

Cell growth period, hours							
24		48		72		96	
Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses	Mitotic activity,‰	% pathological mitoses
31.00 ± 0.82	4.01 ± 0.87	43.50 ± 1.08	8.58 ± 0.88	30.25 ± 0.75	5.96 ± 0.90	22.00 ± 0.71	5.97 ± 2.33

In addition, it should be noted that the level of mitotic activity (FLK-71) on the fifth passage after thawing was reliably 2‰ higher in the first two days of cultivation than its level on the third passage. Whereas the level of mitotic activity (FLK 50/100) on the third passage of cultivation did not differ reliably from the level of the fifth passage of this cell culture in the first three days of cultivation.

The karyologic characteristics of FLK-71 cell lines after long-term storage were characterized by an increase in the range of chromosome number fluctuations from 40 to 64 on the third passage of cultivation with a modal class of 56, and according to the passport of these cells before freezing (from 42 to 56) and 54, respectively. In the line (FLK 50/100), on the third passage of cultivation after thawing, the range of chromosome number fluctuations decreased and ranged from 40 to 68 with a modal class of 56, indicating the stabilization of culture cells (Fig. 2).

The karyological analysis of the virus-producing cell culture (FLK-Pol) on the first, second and fifth passages after thawing (from 7 months to 6 years and 7 years 2 months) of storage in the cryobank of the National Collection of Cell Cultures showed the stability of the modal class of chromosomes, which was equal to 54 from the first to the fifth passage of cultivation.

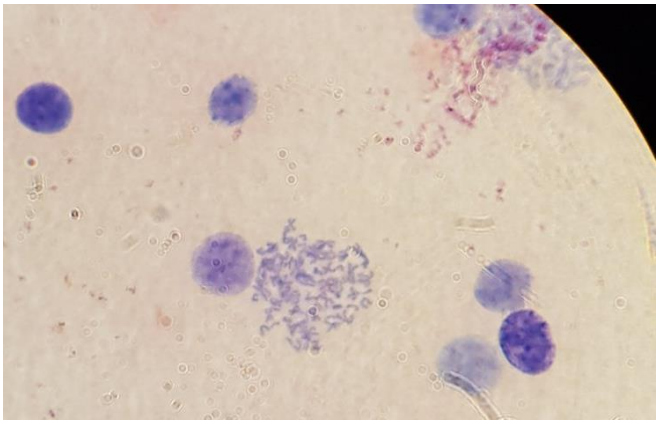

Figure 2. FLK 50/100 chromosomes on the third passage of cultivation after thawing.

The range of fluctuations in the number of chromosomes of the continuous virus-producing cell culture (FLK-Pol) on the first passage of cultivation after thawing and long-term storage in a cryobank at a temperature minus 196 °C was from 38 to 60; on the second passage — from 40 to 62; on the fifth passage — from 40 to 62.

It was proved that the addition of dimethyl sulfoxide to the culture medium accelerated the monolayer formation: on the first day of cultivation, the monolayer

was 95–100% complete, unlike the control variant, where the formation of a complete monolayer was observed only on the second day. It should be noted that in the control, monolayer destruction occurred 2–3 days earlier than in the dimethyl sulfoxide variants. All experimental groups (except for the variant with the addition of 3.0% dimethyl sulfoxide) maintained the integrity of the monolayer on the 7th day of cultivation and had a high potential for further antigen accumulation.

The titers of antigen-producing biological activity of the FLK 50/100 subline during long-term storage for 206.5 months of storage in cryobank conditions ranged from 1:1.5 to 1:4. Working titers (++++) of strain FLK 50/100 were at the same level (1:1.5 to 1:4) from 11 to 20 passages of cultivation after thawing (Table 5).

Studies have shown that the duration of storage under liquid nitrogen conditions (minus 196 °C) of a chronically infected strain (FLK-SBBL) from 4 months to 10 years did not affect the production of bovine leukemia virus after thawing and cultivation, leukemia antigen titers, its yield and volume from the first to the fifteenth passage of cultivation.

Table 5 — Activity of BLV antigens (ID) during the cultivation of FLK-BLV strains after thawing at long-term storage

Sublines FLK-BLV	Antigen titer in the immunodiffusion (ID) test	Working titer	The volume of antigen actually obtained, cm^3	Antigen yield, thousand doses per 1 dm^3
FLK 50/100 11 to 20 pas.	1:1.5 ++++ 1:2 ++++ 1:4 ++++	1:4	400	4.0
FLK 50/100 1 to 10 pas.	1:1.5 ++++ 1:2 +++ 1:3 +++	1:1.5	300	1.50
FLK-SBBL 1to10 pas.	1:2 ++++ 1:2.5 +++ 1:3 ++	1:2	420	2.00
FLK-SBBL 1 to 20 pas.	1:1.5 ++++ 1:2 +++	1:1.5	530	1.50
FLK-Pol 1 to 10 pas.	1:1.5 ++++ 1:2 ++++ 1:3 +++	1:2	385	2.00
FLK-Pol 11 to 22 pas.	1:1.5 ++++ 1:2 +++ 1:3 +++	1:1.5	440	1.50
FLK-Pol 5 to 17 pas.	1:2 ++++ 1:3 ++++ 1:4 +++ 1:6 ++	1:3	500	3.00
FLK-71 29.03.2002 11.01.2020 213	1–9	1:1.5	850	1.50
FLK-71 29.03.2002 28.01.2021	1–22	1:2	450	2.00

The antigen-producing biological activity of virus-producing strains of the FLK-SBBL cell culture after long-term storage for 201 months in the cryobank of the National Collection of Pathogens of the NSC 'IECVM' was 1:3 (++), and the working antigen titer was 1:1.5 (++++) (Table 5).

The titer of biological activity of leukemia antigen in the immunodiffusion (ID) test of FLK 50/100 strain from the 1st to the 10th passages of cultivation after thawing was 1:3 (+++), while the working titer was 1:1.5 (++++). This fact indicates that for the FLK 50/100 strain, up to 10 passages after thawing are required to fully restore the titers of biological activity.

The study of the antigen-producing biological activity of FLK-BLV strains during long-term storage in the cryobank of the National Collection of Pathogens of the NSC 'IECVM' showed that the working titers of FLK-Pol strain after 55.5 months of storage in liquid nitrogen from the 5th to the 17th passage after thawing ranged from 1:1.5 to 1:3 (++++).

The studies revealed that the preservation of biological activity titers in ID test FLK-Pol after 55.5 months of storage in liquid nitrogen ranged from 1:1.5 to 1:6 (Figs 3–5), and FLK-SBBL from the 1st to the 10th passages after 81.5 months of storage was 1:2, after 129.5 months of storage in liquid nitrogen — 1:1.5.

Figure 3. Lines of precipitation in ID test of the strain FLK-Pol after 55.5 months of storage in cryobank conditions from passage 5 to 17 after thawing. Dilutions from 1:1.5 to 1:3 (++++).

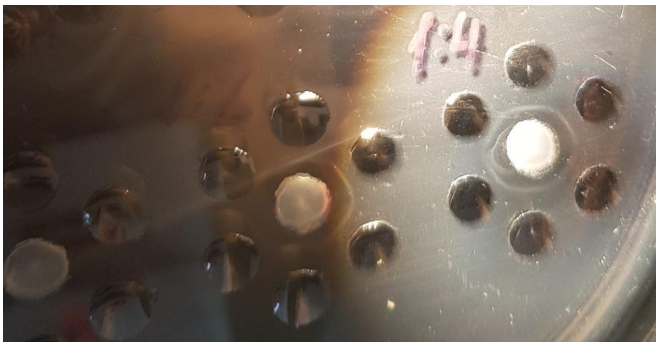

Figure 4. Lines of precipitation in ID test of the strain FLK-Pol after 55.5 months of storage in cryobank conditions from 5 to 17 passages after thawing. Dilution 1:4 (+++).

Figure 5. Lines of precipitation in ID test strain FLK-Pol after 55.5 months of storage in cryobank conditions from passage 5 to 17 after thawing. Dilution 1:6 (++).

Thus, the antigen-producing biological activity of the FLK-BLV strain (FLK-Pol) was preserved from the 5th passage and further up to 22 passages (passage period) during the studied storage periods, namely from 55.5 months (FLK-Pol) to 201 months of storage (FLK-SBBL) and 206.5 months (FLK 50/100) in the conditions of the NSC 'IECVM' cryobank. However, for the FLK 50/100 strain, up to 10 passages after thawing are required to fully restore the titers of its biological activity.

Conclusions. 1. It has been demonstrated that the virus-producing cell line (FLK 50/100) morphologically consists of epithelial-like cells with a perinuclear space around the nucleus, whereas the FLK-71 line is represented by fibroblast-like cells with large nuclei.

2. The karyology of FLK-71 cell lines after long-term storage was characterized by an increase in the range of chromosome number fluctuations from 40 to 64 compared to the passport data of these cells before freezing (from 42 to 56). The range of chromosome number fluctuations in FLK 50/100 decreased and ranged from 40 to 68.

3. The karyological analysis of the virus-producing cell culture (FLK-Pol) on the first, second and fifth passages after thawing showed a stable fluctuation in the number and modal class of chromosomes, which was 54 from the first to the fifth passage of the cultivation.

4. Studies have shown that the duration of storage under liquid nitrogen conditions (minus 196 °C) of a chronically infected strain (FLK-SBBL) from 4 months to 201 months of storage did not affect the production of bovine leukosis virus after thawing and cultivation, leukemia antigen titers, its yield and volume from the first to the fifteenth passage of cultivation.

5. The antigen-producing biological activity of the continuous culture of FLK-BLV (FLK-Pol) was preserved from the 5th passage and further up to 22 passages (passage period) during the studied storage periods, namely from 55.5 months (FLK-Pol) in the conditions of the NSC 'IECVM' cryobank. The antigen-producing biological activity of the strain (FLK 50/100) was fully restored only after 10 passages of its cultivation after thawing.

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