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PATENTS ACT, 1978

CERTIFICATE

In accordance with section 44 (1) of the Patents Act, No. 57 of 1978, it is hereby certified that:

OBSCHESTVO S OGRANICHENNOI OTVETSTVENNOSTYU "NEW MEDICAL TECHNOLOGIES"

Has been granted a patent in respect of an invention described and claimed in complete specification deposited at the Patent Office under the number

2014/05954

A copy of the complete specification is annexed, together with the relevant Form P2.

In testimony thereof, the seal of the Patent Office has been affixed at Pretoria with effect from the **25** day of **November 2015**

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FORM P2

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METHOD FOR ASSESSING THE VIABILITY OF VIRUSES WITH LYMPHOTROPISM

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(54) **Title**: METHOD FOR ASSESSING THE VIABILITY OF VIRUSES WITH LYMPHOTROPISM

(54) **Название изобретения** : СПОСОБ ОЦЕНКИ ЖИЗНЕСПОСОБНОСТИ ВИРУСОВ, ОБЛАДАЮЩИХ ЛИМФОТРОПИЗМОМ

(57) **Abstract**: Use: medicine and biotechnology. Aim: to increase the reliability of determining infection by viruses with lymphotropism, to eliminate false negative reactions in testing blood for the presence of lymphotropic viruses during EIA and PCR testing, and to detect viruses with lymphotropism in biological material having a concentration of virus particles lower than the sensitivity threshold of EIA or PCR methods. Essence of the invention: the method for assessing the viability of viruses with lymphotropism comprises collecting biological material and determining whether said material contains virus RNA or DNA by means of conducting a polymerase chain reaction (PCR reaction). In addition, a lymphocyte suspension is taken from the blood of healthy people, to which lymphocytes an equal volume of biological material is added. This combination is then mixed, incubated at a temperature of 37°C for a period of 6-8 hours, and the lymphocytes are washed of plasma and broken down. The lymphocyte cytoplasm is then subjected to PCR testing. The detection of virus RNA or DNA in the lymphocyte cytoplasm indicates that the viruses have retained their viability. The absence of virus RNA or DNA in the lymphocyte cytoplasm indicates the inactivation of the viruses.

(57) **Реферат**: Использование: медицина, биотехнология. Задача: повышение достоверности определения инфицированности вирусами, обладающих лимфотропизмом, исключение ложноотрицательных реакций при тестировании крови на наличие лимфотропных вирусов при ИФА и ПЦР-исследовании, обнаружение вирусов, обладающих лимфотропизмом, в биологическом материале с концентрацией вирусных частиц ниже порога чувствительности методов ИФА или ПЦР. Сущность изобретения: способ оценки жизнеспособности вирусов, обладающих лимфотропизмом, включает забор биологического материала, Определение наличие в нем РНК или ДНК вирусов путем проведения полимеразной цепной реакции (ПЦР-реакция). При этом, дополнительно из крови здоровых людей получают взвесь лимфоцитов, к которым добавляется равный объем биологического материала. После чего смесь перемешивают, инкубируют при температуре 37°C в течение 6-8 часов, производят отмывание лимфоцитов от плазмы и разрушение лимфоцитов. Цитоплазму лимфоцитов подвергают ПЦР-исследованию. Обнаружение в цитоплазме лимфоцитов РНК или ДНК вирусов свидетельствует о сохранении жизнеспособности вирусов. Отсутствие в цитоплазме лимфоцитов РНК или ДНК вирусов свидетельствует об инактивации вирусов.

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METHOD FOR EVALUATION OF VIABILITY OF VIRUSES WITH LYMPHOTROPISM PROPERTIES

The invention relates to the methods of detection of viruses with lymphotropism properties in biological substrates with low concentration of viral particles, evaluation of their viability and elimination of false-negative results of EIA and PCR test and may be used in the medical industry and biotechnology.

The detection of virus in biological substrates by its isolation in cell culture is a well-known method. Viruses isolated by cell culture method are identified by haemadsorption, hemagglutination or indirect immunofluorescence methods. Proper sampling and short-time transportation to the laboratory venue on appropriate media are essential for effective isolation of virus isolation in culture. It preserves virus viability and restricts bacteria and fungi reproduction (Laboratory diagnostic of virus infections, Online: http://www.center-hc.ru/). Many viruses, in particular hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV), are anthroponotic viruses affecting human cells only thus causing disease of human solely. There are no experimental models of these infections. Also, there are no cultivated cell cultures, particularly in the republic of Uzbekistan, on which one may adequately study cytopathogenic properties and viability of these viruses *in vitro*. Moreover, because of its complexity the method of isolation of viruses on cell cultures is not used for diagnostic purposes.

The immunological method for detection of viruses in biological material is known as enzyme-linked immunosorbent assay (ELISA) is based on using of specific viral proteins extracted from infected cells or produced by genetic engineering by the detection of antibodies to the number of virus antigens (Ilyina, E.N. *et. al.* Chronic virus liver diseases "Khronicheskiye virusnye zabolevaniya pecheni" Methodological manual for physicians "Metodicheskoye posobiye dlya vrachej" Moscow, 2001, p. 7-11).

In some virus infections, e.g. HCV, EIA detects the antibodies only, thus substantially restricting the evaluation capability of the progress and activity of infection. Moreover, EIA has sensitivity threshold, below which the detection of virus is impossible.

Most close to declared methods of detection of viruses with lymphotropism properties in the biological materials, virus viability assessment and the exclusion of false-negative results of EIA and PCR is the detection of viral RNA or DNA by sampling of biological material and detection of presence of viral RNA or DNA by polymerase chain reaction (PCR) (Ilyina, E.N. *et. al.* Chronic virus liver diseases "Khronicheskiye virusnye zabolevaniya pecheni" Methodological manual for physicians "Metodicheskoye posobiye dlya vrachej" Moscow, 2001, p. 7-11).

The method relates to direct methods of the detection of the pathogen in the biological material thus permitting to evaluate the activity of viral process. Positive PCR-reaction confirms the presence of the virus in the liver and in the blood with high probability. PCR of the biological samples (plasma or blood proteins, tissue or organs' biopsy materials) not always allow detecting infection caused by viruses with lymphotropism properties, though such viruses may persist in substantially high concentrations in the lymphoid tissue (false-negative results of PCR) and *vice versa* positive PCR may be obtained without persistence of viruses. Furthermore, PCR has the sensitivity threshold, below which virus presence is not detectable by this method.

The goal of presented invention is increasing of reliability of infection detection caused by viruses with lymphotropism properties, elimination of false-negative results testing the blood for the presence of lymphotropic viruses by EIA and PCR, detection of viruses with lymphotropism properties in the biological material with virus concentration below the threshold of IFA or PCR methods sensitivity.

Assigned goal is being resolved by the evaluation of viability of the viruses with lymphotropism properties by sampling of the biological material, detection of presence of viral RNA or DNA by the polymerase chain reaction (PCR); additionally lymphocytes suspension is obtained from the healthy human blood and the equal amount of biological material is added; the mixture is stirred, and incubated at 37°C for 6-8 hours; washing-out of lymphocytes from the plasma and the lymphocytes is being destructed; lymphocytes' cytoplasm is subjected for PCR test. The detection of viral RNA or DNA in the cytoplasm of lymphocytes indicates preserved viability of viruses; the absence of viral RNA or DNA viruses in the cytoplasm of lymphocytes indicated inactivation of viruses. Plasma or blood serum, biopsy samples of tissue or organs, the washouts from the medical instruments may be used as the biological samples. This method allows assessment of the viability of HBV, HCV or HIV viruses.

Assigned objective is being resolved by elimination of EIA and PCR false-negative results by obtaining blood from patients suspected being infected by lymphotropic viruses, and the 6-8 ml of the blood is being sampled into test tubes, containing 2,0 ml normal saline and 2-3 heparin drops; lymphocytes are separated from the blood and incubated at 37°C for 6-8 hours; lymphocytes are washing-out from the plasma and destruction, and the cytoplasm of lymphocytes is subjected to PCR; the detection of viral RNA or DNA in lymphocytes' cytoplasm indicates the presence of viruses; the absence of viral RNA or DNA in lymphocytes' cytoplasm indicates the absence of viruses in the blood. The content of patient's lymphocyte is subjected to PCR-testing.

It is known that many viruses particularly those of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) can replicate in mononuclear blood cells, particularly, in the lymphocytes and the macrophages. It is known, that under HBV- and HCV-infections simultaneously cause inflammatory processes in the liver with all subsequent hepatitis, as well as secondary immunodeficiency with various degrees of T- lymphopenia and B-lymphopenia, imbalance of regulatory subpopulations of T-lymphocytes (T-helpers and T-suppressors), reduction of immune regulatory index (IRI) and dysgammaglobulinemia. The degree and grade of immunodeficiency has no relation with the degree of the pathologic process in the liver. Patients with chronic HBV and HCV infections have different intensity of pathological process in the liver tissue after some time, from weak to expressed, but regardless of this, stable and steady aggravation of secondary immunodeficiency.

The dissociation of degree of liver tissue injury and degree of secondary immunodeficiency in various nosological forms of chronic virus hepatitis supported the idea that the hepatitis and secondary immunodeficiency in HBV and HCV infections are associated, mutually aggravating, but not mutually conditional: that is, HBV and HCV along with hepatotropic property possess expressed lymphotropic property – direct property to cause secondary immunodeficiency. Thr differences in clinical appearances of the liver tissue injuries and degree of immunodeficiency in HBV and HCV infections are due to differences in degree of hepatotropic and lymphotropic properties of these viruses. Namely the differences in degrees of hepatotropic and lymphotropic properties of viruses determine the differences of the pathogenesis, clinical appearances and the pattern of antiviral therapy effect in chronic HBV and HCV infections in various stages of these diseases.

The identity of the lymphotropic properties of HBV and HCV, and HIV besides secondary immunodeficiency forming is confirmed also by commonality of their epidemiological features, mechanism of transfer, the progress of associated opportunistic infection (frequent respiratory diseases, intestinal infections), and particularly the development of the lymphogranulomatosis in different tissues of the organism. The development of lymphoid follicles' clusters, which is the lymphogranulomatosis, in various organs and tissues of the organism is supposed to be intrinsic for viral infections of the lymphoid cell system.

Considering lymphotropic properties of HBV, that regardless of serum titer, HBV can permanently persist in essentially high concentrations in the cytoplasm of lymphoid elements. This phenomenon is used in this method for reliable increasing and elimination of false-negative results by EIA and PCR, and detection of lymphotropic viruses in the biological material with concentration of viral particles below the threshold of test-sensitivity of EIA or PCR.

We used the lymphotropic properties of HBV, HCV and HIV in this evaluation method of virus viability – the ability of these viruses to penetrate and persist intracellularly in the healthy human lymphocytes during their *in vitro* incubation.

The evaluation of viability of viruses with lymphotropism properties, in particular HBV, HCV and HIV, required after long term storage of viruses, for the control of the antiviral efficiency of various disinfecting chemical and physical factors against these viruses, as well as for the control of antiviral therapy.

Below is the description of method for evaluation of viability of viruses with lymphotropism properties is performed as described.

I. Producing of suspension of viruses with lymphotropism properties

For producing of suspension of viruses, the biological material (plasma or blood serum, biopsy samples of tissue or organs, the wash-outs from medical instruments) is obtained. Then the biological material is subjected to quantitative PCR for the verification for the presence of viruses with lymphotropism properties and quantification of titer of viruses. Virus comprising biological material is kept in the frozen state in the refrigerator at below – 25°C temperature.

II. Producing of lymphocytes suspension from healthy human subject:

a) healthy volunteers are tested for infection with lymphotropic viruses by ElA. Lymphocytes from healthy people with a negative result for study viruses are used in investigations;

b) to receive a sufficient amount of lymphocyte the blood is taken from ulnar vein in an amount of 20-30 ml in the morning from fasting healthy human subject. Then the blood per 7-8 ml is transferred to the centrifuge tubes containing 2 ml normal saline and 3 heparin drops ("Heparin" concentration of 5000 ME/ml; 3 drops contain 750 ME/ml of heparin). The resulting solution is stirred thoroughly;

c) the lymphocytes are separated from the whole heparin containing blood in ficoll-verografin gradient with density d=1.077 g/ml according to previously described method (Garib, F.Yu., Gurary, N.I., Garib, V.F. "Sposob opredeleniya subpopulyatsij limfotsitov (Determination method of lymphocytes' subpopulations)" // Rasmij Akhborotnoma. Tashkent, 1995, #1, p. 90 – UZ DP 2426). 2 ml of ficoll-verografin gradient is poured into the clean centrifuge tube, then the heparinized blood lays on its surface and the tube is centrifuged at 1500 RPM for 20 minutes. During centrifugation all blood cells, excluding lymphocytes, penetrate through ficoll-verografin gradient. Blood plasma remains above the gradient. In the border of ficoll-verografin gradient and plasma peculiar turbid ring consisting from pure lymphocytes is formed. The ring with lymphocytes is carefully pumped with a pipette and transferred to the clean centrifugal tube;

d) the lymphocytes are washed-out with 10 ml of normal saline 2-3 times with further centrifugation at 1500 RPM for 20 minutes.

e) after last centrifugation the supernatant is removed. The sediment containing lymphocytes is diluted and re-suspended in 600 µl of normal saline. Lymphocytes suspension may be stored no more than 1 day at temperature + 4°C.

III. Evaluation of viruses with lymphotropism properties to penetrate and persist intracellularly in the human lymphocytes *in vitro*.

1) Biological material containing viruses with lymphotropism properties is taken from refrigerator and thawed at room temperature.

2) equal amount (300 µl) of virus containing biological material and the suspension of healthy human lymphocytes is transferred with pipette to the clean centrifugal tube; the contents mixed and placed for the incubation (incubation of viruses with lymphocytes *in vitro*) into the

thermostat at +37°C for 6-8 hours. The testing tube is mixed with shacking every 1.5-2 hours.

3) The washing-out of lymphocytes. Testing tube is removed from the thermostat. 6-8 ml of normal saline is added, mixed and centrifuged at 1500 RPM for 20 minutes. The lymphocytes are sediment on the bottom of the tube. The supernatant (mixture of plasma with saline) is entirely removed. The lymphocytes are washed out in normal saline and sediment 2-3 times. After last centrifugation, the supernatant is removed the suspension of the lymphocytes (sediment) is diluted with 500 µl of normal saline and transferred to plastic 1.5 lock tube (Eppendorf tube).

4) Thereafter the tube is placed into the freezer of house grade refrigerator overnight. The lymphocytes are destructed under slow freezing condition.

5) The removal of the membrane of destroyed lymphocytes. Next day the tubes from the freezer thawed at room temperature. Then the membranes of destroyed lymphocytes are removed by centrifugation at 3000 RPM for 30 minutes. Membranes are precipitated on the bottom of the tube and lymphocytes' cytoplasm content remains in the supernatant.

6) The supernatant from the tube is transferred and subjected to quantitative PCR of viral RNA or DNA in the cytoplasm of lymphocytes that previously was in infected patient's plasma.

IV. Assessment of results.

1. Positive PCR for the presence of viral RNA or DNA in the cytoplasm of lymphocytes indicates the remaining virus viability, i.e. ability to penetrate and persist in human lymphocytes *in vitro*.

2. Negative PCR for the presence of viral RNA or DNA in the cytoplasm of lymphocytes indicates the loss (inactivation) of virus viability, i.e. loss of their ability to penetrate and persist inhuman lymphocytes *in vitro*.

Clamming methods are certified by the following examples:

Example #1.

The assessment of viability of viruses with lymphotropism properties

The blood is obtained from ulnar vein from the patient after receiving antiviral therapy for hepatitis C. The plasma separated from whole blood and subjected to the quantitative PCR for the verification of HCV presence and quantification virus titer. The PCR is negative. Tested plasma is kept in the freezer at below -25°C temperature.

Simultaneously the 20-30 ml of blood from healthy human volunteers is obtained in the morning from ulnar vein. The part of blood plasma is subjected to PCR for viruses with lymphotropism properties infection. The lymphocytes from the healthy humans with negative results of testing for infection are used for further investigation. Then the 7-8 ml blood aliquots transferred to the centrifuge tubes containing 2 ml of normal saline and 3 heparin drops ("Heparin" concentration is 5000 ME/ml, 3 drops contain 750 ME/ml of heparin). The solution in the tube is mixed thoroughly. The lymphocytes separated from the whole heparinized blood in ficoll-verografin gradient with d=1.077 g/ml density according to Garib, F.Yu. *et al* method. 2 ml the ficoll-verografin gradient poured into clean centrifuge tube, heparinized blood lays on the surface of gradient and centrifuged at 1500 RPM for 20 minutes. All blood cells excluding lymphocytes penetrate the ficoll-verografin gradient and sediment under it. The blood plasma located above the gradient. On the border between ficoll-verografin gradient and plasma, the peculiar turbid ring with pure lymphocytes suspension is formed. The ring with lymphocytes carefully sucked with a pipette and transferred to the clean centrifuge tube. The lymphocytes are washed out in normal saline and sediment 2-3 times. After last centrifugation, the supernatant is removed. The sediment containing lymphocytes is diluted with 600 µl saline and re-suspended. The lymphocytes suspension may be stored no more than 1 day at + 4°C temperature.

The testing plasma from the freezer is thawed at room temperature. Equal volumes (300 µl) of plasma and the suspension of lymphocytes are transferred to clean centrifuge tube with pipette, mixed and placed for incubation in thermostat at +37°C temperature for 6-8 hours. The tube is mixed by shaking every 1.5-2 hours.

After incubation the tube is removed from the thermostat. 6-8 ml saline is added, mixed and centrifuged at 1500 RPM for 20 minutes. The lymphocytes sediment on the bottom of the tube. The supernatant (mixture of plasma and normal saline) is removed. Then 2-3 times wash-out in normal saline and the lymphocytes sedimentation is performed *ditto*. After last centrifugation, the supernatant is removed, and suspension of lymphocytes (sediment) is diluted by adding 300 µl of normal saline, and transferred to the 1.5 ml lock tube (Eppendorf tube).

Thereafter lymphocyte membranes are destructed by overnight placing into house grade freezer. Next day the tubes are thawed at the room temperature. Then the membranes of destroyed lymphocytes are removed from suspension by centrifugation of tube at 3000 RPM for 30 minutes. The membranes are precipitated on the bottom of the tubes and lymphocyte cytoplasm contents remain in the supernatant. The supernatant is transferred from the tube and subjected to quantitative PCR for the presence of HCV virus in the cytoplasm of lymphocytes. Positive PCR test for HCV indicates the preservation of the HCM viability and the requirement of further antiviral therapy.

Example #2.

The liver tissue sampled by liver puncture from a patient, who was given antiviral therapy for hepatitis B. Liver biopsy sample is homogenized in 1.5 ml normal saline; transferred to the centrifuge tube, centrifuged at 1500 RPM for 20 minutes; and the supernatant transferred to the tube. One part of supernatant is subjected to quantitative PCR testing for the presence of HCV virus and quantification of virus titer. PCR test for HCV is positive. The biopsy sample is kept in the freezer in the refrigerator at below -25°C temperature.

The lymphocyte suspension from healthy human is made as described in example #1. The supernatant from liver biopsy sample homogenate is thawed at room temperature. The equal volumes (300 µl) of the supernatant and lymphocyte suspension is added to the tube by automatic pipette; the resulting solution is mixed and placed for incubation into thermostat at +37°C temperature for 6-8 hours. Testing tube is mixed by shaking every 1.5-2 hours.

The tube is removed from thermostat. 6-8 ml of normal saline is added, mixed and centrifuged at 1500 RPM for 20 minutes. The lymphocytes sediment on the bottom of the tube. The supernatant (mixture of plasma with normal saline) is removed entirely. The supernatant (mixture of plasma and normal saline) is removed. Then 2-3 times wash-out in normal saline and the lymphocytes sedimentation is performed *ditto*. After last centrifugation, the supernatant is removed and suspension of lymphocytes (sediment) is diluted by adding 300 µl of normal saline. Thereafter the destruction of lymphocyte membranes is performed by putting testing tube into house grade freezer overnight.

Thereafter lymphocyte membranes are destructed by overnight placing into house grade freezer. Next day the tubes are thawed at the room temperature. Then the membranes of destroyed

lymphocytes are removed from suspension by centrifugation of tube at 3000 RPM for 30 minutes. The membranes are precipitated on the bottom of the tubes and lymphocyte cytoplasm contents remain in the supernatant. The supernatant is transferred from the tube and subjected to quantitative PCR for the presence of HBV virus in the cytoplasm of lymphocytes. Negative PCR for HBV indicates the lost virus viability (inactivation).

Example #3.

The detection of viruses with lymphotropism properties in biological material with the concentration of virus below EIA and PCR sensitivity threshold.

In blood center, the blood plasma from 6-8 ml of blood is being tested for viruses with lymphotropism properties. One part of plasma is subjected to quantitative PCR for testing the presence of HBV, HCV or HIV viruses and the quantification of virus titer. PCR for the presence of viruses is negative. Tested plasma is stored in the freezer at below -25°C.

The lymphocyte suspension from healthy human subject is performed as described in example #1.

The testing plasma from the freezer is thawed at room temperature. Equal volumes (300 µl) of plasma and the suspension of lymphocytes is transferred to clean centrifuge tube with automatic pipette, mixed and placed for incubation in thermostat at +37°C for 6-8 hours. The tube is mixed by shaking every 1.5-2 hours.

The tube is removed from thermostat. 6-8 ml of normal saline is added, mixed and centrifuged at 1500 RPM for 20 minutes. The lymphocytes sediment on the bottom of the tube. The supernatant (mixture of plasma with normal saline) is removed entirely. The supernatant (mixture of plasma and normal saline) is removed. Then 2-3 times wash-out in normal saline and the lymphocytes sedimentation is performed *ditto*. After last centrifugation, the supernatant is removed and suspension of lymphocytes (sediment) is diluted by adding 300 µl of normal saline.

Thereafter lymphocyte membranes are destructed by overnight placing into house grade freezer. Next day the tubes are thawed at the room temperature. Then the membranes of destroyed lymphocytes are removed from suspension by centrifugation of tube at 3000 RPM for 30 minutes. The membranes are precipitated on the bottom of the tubes and lymphocyte cytoplasm contents remain in the supernatant. The supernatant is transferred from the tube and subjected to

quantitative PCR for detection of HBV, HCV and HIV viruses in the content of lymphocytes' cytoplasm. Positive PCR for HCV indicates the presence of HCV virus in the donor plasma its ineligibility for the transfusion.

Example #4.

The elimination of EIA and PCR false-negative results.

The 6-8 ml of blood obtained in the morning from fasting donor from the ulnar vein. Whole blood transferred to the tube, subjected to the sedimentation, and the serum is obtained; one part of serum is subjected to PCR for the presence of HBV, HCV or HIV viruses and quantification of virus titer. The PCR tests are negative. The rest of the blood serum is stored in the tube. The lymphocyte suspension from healthy human subject is performed as described in example #1.

The lymphocytes separated and destructed by overnight freezing in house grade refrigerator. Next day the tube is thawed at room temperature. Then the membranes of destroyed lymphocytes are removed from suspension by centrifugation at 3000 RPM for 30 minutes. The membranes precipitate on the bottom of the tube, and lymphocyte cytoplasm contents remain in the supernatant. The supernatant is transferred from the tube and subjected to quantitative PCR for detection of HBV, HCV and HIV viruses in the cytoplasm of lymphocytes. Positive PCR for HBV indicated the presence of HBV in the donor blood.

In standard PCR, the detection rate of HBV and HCV in the Republic is 2.7%. According to the epidemiological data new cases of hepatitis B (HBV) and hepatitis C (HCV) transfer occur due to the transfusion of infected blood or its components in 2.2%-5.6%. To reveal the reasons behind and elimination of HBV infection in recipients the lymphotropic properties of the virus were used.

The serums from 309 donor blood samples were tested by PCR for HBV markers detection rate.

PCR revealed HBV in 6 out of 209 serum samples that estimated 1.94% of all number of donors' sample. The same PCR study (study of lymphocytes content from the same donors) revealed HBV in 17 out of 309 samples, estimated 7.44% of all donors' samples. Thus, the standard PCR testing of blood serum was false-negative in 5.50% of samples, thus being the reason of HBV infection in recipients by transfusion of infected blood or it components.

The implementation of PCR testing of lymphocytes for detection of viruses in blood centers will allow significantly reduction of frequency or eliminate the cases of HBV, HCV and HIV after transfusion of blood or it components.

Claims:

1. A method for evaluation of viability of viruses with lymphotropism properties, comprising sampling of the biological material detection of presence of viral RNA or DNA by polymerase chain reaction (PCR), ***characterized in that*** additional acquisition of lymphocyte suspension from healthy human subjects mixed with an equal amount of biological material, mixed and incubated at 37°C for 6-8 hours, lymphocytes washed-out from plasma and destructed, with subjecting of lymphocytes' cytoplasm for PCR, detection of viral RNA or DNA in the cytoplasm of lymphocytes that indicate the viability of viruses while the absence of viral RNA or DNA indicates the inactivation of viruses.

2. The method of claim 1, ***characterized in that*** plasma or blood proteins, biopsy tissues or organs, and medical instrument wash-outs serving as the biological materials.

3. The method of claim 1, ***characterized in that*** assessment of HBV, HCV or HIV viability.

4. A method of detection of viruses with lymphotropism properties in the biological material with virus concentration below the EIA and PCR sensitivity thresholds consists of sampling of biological material, ***characterized in that*** acquisition of the lymphocyte suspension from the healthy human blood, adding an equal amount of the biological material, mixing and incubation at 37°C for 6-8 hours, washing-out the lymphocytes from the plasma, destruction of lymphocytes, and subjecting the cytoplasm of lymphocyte to the PCR, with detection of viral RNA or DNA in cytoplasm indicating the presence of viruses, while the absence of viral RNA or DNA indicates the absence of viruses in tested biological sample.

5. The method of claim 4, ***characterized in that*** plasma or blood proteins serve as biological materials.

6. The method of claim 4, ***characterized in that*** presence of HBV, HCV or HIV is being detected.

7. A method of elimination of false-negative EIA and PCR results includes sampling of the blood from patients suspected for infection by lymphotropic viruses, ***characterized in that*** 2.0 ml samples of blood taken into a test tube containing 2.0 ml of normal saline and 2-3 drops of heparin, isolation of lymphocytes from 6-8 ml of blood, incubation at 37°C for 6-8 hours, washing of lymphocytes from the plasma, destruction of lymphocyte, and subjecting the cytoplasm of lymphocytes to PCR, detection of viral RNA or DNA that indicates the presence of viruses in the patient's blood while the absence of viral RNA or DNA indicates the absence of viruses in patient's blood.

8. The method of claim 7, ***characterized in that*** content of patient's lymphocyte tested by PCR.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/UZ 2013/000001

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/04 *(2006.01)* ***G01N 33/483*** *(2006.01)*
C12Q 1/68 *(2006.01)* *G01N 33/569 (2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/04, 1/68, G01N 33/483, 33/569, 33/49

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Google, PabMED, PatSearch

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KALLE SAKSELA et al. Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the numbers of CD4+ lymphocytes. Proc. Natl. Acad. Sci. USA, 1994, Vol. 91, pp. 1104-1108	1-8
Y	F. REGGETI et al. CD134 and CXCR4 expression corresponds to feline immunodeficiency virus infection of lymphocytes, macrophages and dendritic cells, Journal of General Virology, 2008, Vol. 89, pp. 277-287	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2013 (20.09.2013)	24 October 2013 (24.10.2013)
Name and mailing address of the ISA/ RU Facsimile No.	Authorized officer Telephone No.

TRANSLATION

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Form PCT/IPEA/416
PAT/191-13		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/UZ2013/000001	21.05.2013	18.06.2012

International Patent Classification (IPC) or national classification and IPC

C12Q1/04(2006.01), C12Q1/68(2006.01), G01N33/483(2006.01), G01N33/569(2006.01)

Applicant

OBSCHESTVO S OGRANICHENNOI OTVETSTVENNOSTYU "NEW MEDICAL TECHNOLOGIES"

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

3. This report is also accompanied by ANNEXES, comprising:

   a. ☐ *(sent to the applicant and to the International Bureau)* a total of _____ sheets, as follows:

   ☐ sheets of the description, claims and/or drawings which have been amended and/or sheets containing rectifications authorized by this Authority, unless those sheets were superseded or cancelled, and any accompanying letters (see Rules 46.5, 66.8, 70.16, 91.2, and Section 607 of the Administrative Instructions).

   ☐ sheets containing rectifications, where the decision was made by this Authority not to take them into account because they were not authorized by or notified to this Authority at the time when this Authority began to draw up this report, and any accompanying letters (Rules 66.4*bis*, 70.2(e), 70.16 and 91.2).

   ☐ superseded sheets and any accompanying letters, where this Authority either considers that the superseding sheets contain an amendment that goes beyond the disclosure in the international application as filed, or the superseding sheets were not accompanied by a letter indicating the basis for the amendments in the application as filed, as indicated in item 4 of Box No. I and the Supplemental Box (see Rule 70.16(b))

   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s))

   _____, containing a sequence listing, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see paragraph 3*bis* of Annex C of the Administrative Instructions).

4. This report contains indications relating to the following items:

- ☒ Box No. I — Basis of the report
- ☐ Box No. II — Priority
- ☐ Box No. III — Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV — Lack of unity of invention
- ☒ Box No. V — Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI — Certain documents cited
- ☐ Box No. VII — Certain defects in the international application
- ☐ Box No. VIII — Certain observations on the international application

Date of submission of the demand	Date of completion of this report
Name and mailing address of the IPEA/RU Facsimile No.	Authorized officer Telephone No.

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/UZ2013/000001

Box No. I Basis of the report

1. With regard to the **language**, this report is based on:

☒ the international application in the language in which it was filed

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of:

☐ international search (Rules 12.3(a) and 23.1(b))

☐ publication of the international application (Rule 12.4(a))

☐ international preliminary examination (Rule 55.2(a) and/or 55.3(a))

2. With regard to the **elements** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

☒ the international application as originally filed/furnished

☐ the description:

pages _____ as originally filed/furnished.

pages* _____ received by this Authority on _____

pages* _____ received by this Authority on _____

☐ the claims:

nos. _____ as originally filed/furnished.

nos.* _____ as amended (together with any statement) under Article 19

nos.* _____ received by this Authority on _____

nos.* _____ received by this Authority on _____

☐ the drawings:

pages _____ as originally filed/furnished.

pages* _____ received by this Authority on _____

pages* _____ received by this Authority on _____

☐ a sequence listing – see Supplemental Box Relating to Sequence Listing.

3. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheets/figs _____

☐ the sequence listing *(specify)*: _____

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since either they are considered to go beyond the disclosure as filed, or they were not accompanied by a letter indicating the basis for the amendments in the application as filed, as indicated in the Supplemental Box (Rule 70.2(c) and (c-*bis*)):

☐ the description, pages _____

☐ the claims, Nos. _____

☐ the drawings, sheets/figs _____

☐ the sequence listing *(specify)*: _____

5. ☐ This report has been established:

☐ taking into account **the rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rules 66.1(d-*bis*) and 70.2(e)).

☐ without taking into account **the rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rules 66.1*bis* and 70.2(e)).

6. ☐ Supplementary international search report(s) from Authority(ies) _____ has/have been received and taken into account in establishing this report (Rule 45*bis*.8(b) and (c)).

* *If item 4 applies, some or all of those sheets may be marked "superseded."*

Box No. V **Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-8	YES
	Claims		NO
Inventive step (IS)	Claims		YES
	Claims	1-8	NO
Industrial applicability (IA)	Claims	1-8	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

D1: KALLE SAKSELA et al. Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the numbers of CD4+ lymphocytes. Proc. Natl. Acad. Sci. USA, February 1994, Vol. 91, pp. 1104-1108

D2: F. REGGETI et al. CD134 and CXCR4 expression corresponds to feline immunodeficiency virus infection of lymphocytes, macrophages and dendritic cells, Journal of General Virology, 2008, Vol. 89, pp. 277-287

D1 is the prior art closest to the claimed inventions.

D1 (pages 1104-1108) describes a method for detecting human immunodeficiency virus in samples of peripheral blood mononuclear cells, commonly known to include lymphocytes, by performing real-time PCR analysis.

The methods according to claims 1 and 4 differ from the method known from D1 in that healthy human blood is used

in the production of a suspension of lymphocytes, to which an equal volume of biological material is added, the mixture is stirred and then incubated at a temperature of 37°C for 6-8 hours, the lymphocytes are washed and broken down, and the lymphocyte cytoplasm is subjected to PCR analysis.

The method according to claim 7 differs from the method known from D1 in that 6-8 ml of blood is collected in test tubes containing 2.0 ml of a physiological solution and 2-3 drops of heparin, lymphocytes are separated from the blood and incubated at a temperature of 37°C for 6-8 hours, the lymphocytes are washed and broken down, and the lymphocyte cytoplasm is subjected to PCR analysis.

Thus, the inventions according to claims 1-8 satisfy the criterion of novelty.

However, collecting blood, obtaining lymphocytes therefrom and subsequently incubating and breaking down said lymphocytes is a method conventionally known in the art for obtaining lymphocyte cytoplasm.

Furthermore, the possibility of infecting lymphocytes with a lymphotropic virus and subsequently incubating said lymphocytes in order to confirm the presence of the virus in a sample is known from D2 (pages 277-287).

In the light of the above, the possibility of detecting lymphotropic viruses by studying infected lymphocytes using PCR methods is obvious to a person skilled in the art.

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Furthermore, the application does not describe an unexpected effect, nor does it contain reliable data confirming the reliable detection of infection by viruses that exhibit lymphotropism in a biological material, the prevention of false negative reactions during blood testing, and the detection of viruses that exhibit lymphotropism when the concentration of viral particles is below the threshold of sensitivity of IFA and PCR methods.

On the basis of the above, the methods according to claims 1, 4 and 7 do not satisfy the criterion of inventive step.

The features of dependent claims 2, 3, 5, 6 and 8 are disclosed in D1.

Consequently, the inventions according to the aforesaid claims also do not satisfy the criterion of inventive step.

The inventions satisfy the criterion of industrial applicability.

FORM P16

AGENT CODE – DGI001
AMOUNT: R
QUOTE REF: 267026

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION TO RECORD A TRANSACTION AFFECTING THE RIGHTS IN A PATENT APPLICATION OR A PATENT

[Sections 53, 55, 56 & 60 – Regulations 58-60, 62 and 64(1)]

21	01	**Patent application No:** 2014/05954	**DrG Ref.:** 267026
71		**Full name(s) of applicant(s):** OBSCHESTVO S OGRANICHENNOI OTVETSTVENNOSTYU "NEW MEDICAL TECHNOLOGIES"	
54		**Title of Invention:** METHOD FOR ASSESSING THE VIABILITY OF VIRUSES WITH LYMPHOTROPISM	

Section of Act under which recording is required:

60

Nature of transaction:

Assignment to Ovik Leonardovich MKRTCHYAN

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Mirzo-Ulugbek district
Tashkent
100187
Republic of Uzbekistan

GERNTHOLTZ
2016 -09- 02
Received / Empfangen

Documents lodged in support of this application:

1. General Power of Attorney
2. Assignment dated: May 26, 2016

Date:

DR GERNTHOLTZ INC
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267026 P16.doc

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The above transaction has been recorded in the patents register.

Official date stamp

2016 -07- 06