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METHOD FOR RECOGNIZING PERIPHERAL BLOOD CIRCULATING TUMOR CELL OF TUMOR PATIENT BASED ON ISOLATION BY SIZE OF EPITHELIAL TUMOR CELLS

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Abstract

The invention provides a method for recognizing a peripheral blood circulating tumor cell (CTC) of a tumor patient based on isolation by size of epithelial tumor cells (ISET), wherein a cell immunochemical method is used to detect whether CD45 and CD31 on the suspected CTC are expressed or not so as to exclude a leukocyte and an endothelial cell.

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DRAWINGS

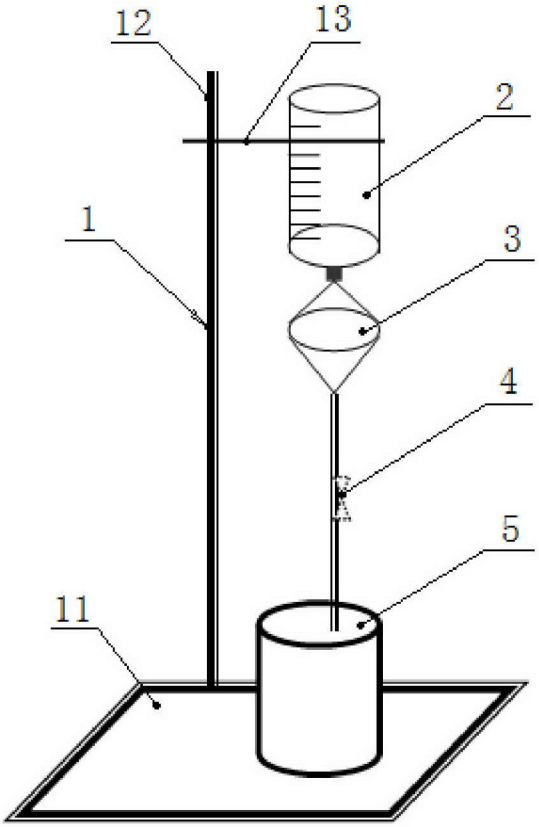

FIG. 1

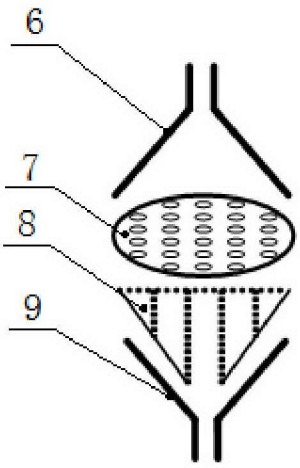

FIG. 2

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METHOD FOR RECOGNIZING PERIPHERAL BLOOD CIRCULATING TUMOR CELL OF TUMOR PATIENT BASED ON ISOLATION BY SIZE OF EPITHELIAL TUMOR CELLS

TECHNICAL FIELD

[01] The present disclosure relates to a method for recognizing a peripheral blood circulating tumor cell (CTC) of a tumor patient based on isolation by size of epithelial tumor cells (ISET), in particular for recognizing the CTC which is quite difficult to be captured by a physical method and recognized by cell morphology. The present disclosure uses a CD45 and CD31 cell immunochemical method for detection, and belongs to the technical field of molecular biology.

BACKGROUND ART

[02] A CTCBIOPSY® detection system separates circulating tumor cells (CTCs) based on a cell size and uses a Wright-Giemsa staining method to stain cells on a filter membrane. The cells detected by the method in strict accordance with SOP operation requirements can be divided into the following categories: CTCs, blood cells, heteromorphic cells and naked nuclear cells. According to cell morphology standards, typical CTCs, blood cells, endothelial cells and naked nuclear cells can be distinguished. However, the heteromorphic cells and suspected CTCs found during a detection process are difficult to be recognized by using a CTC morphology criteria (Hoffman criteria). It is extremely difficult to tell whether such cells are CTCs or other abnormal cells. A method for recognizing the cells is urgently needed.

[03] A CD45 molecule is expressed on all leukocytes and called leukocyte common antigen (LCA). The CD45 molecule is highly expressed in lymphocytes and all other hematopoietic cells except red blood cells and platelets ($>10^6$ molecules/cell).

[04] CD31 is also known as a platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and has a molecular weight of 130 kDa. The CD31 structurally belongs to a member of the immunoglobulin superfamily. The CD31 is important for eliminating aged neutrophils in the body. The CD31 is found on surfaces of platelets, neutrophils, monocytes and certain T cells, and tight junctions between endothelial cells. In immunohistochemistry, the CD31 is mainly used to prove an existence of endothelial cell tissues and to assess tumor angiogenesis, which may mean a rapid growing extent of tumors. Malignant vascular endothelial cells usually also retain antigens, so that the CD31 immunohistochemical method can also be used to prove hemangioma and angiosarcoma. It is proved that the CD31 also exists in small lymphocytes and lymphocytic lymphomas, but is not their specific marker molecule. In the prior art, there is no related reports on identifying circulating tumor cells in peripheral blood of tumor patients based on isolation by size of epithelial tumor cells (ISET).

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SUMMARY

[05] A circulating tumor cell (CTC) falls off from a solid tumor and enters a peripheral blood circulation. Since it was found in 1989, there have been a variety of methods for detecting the CTC in peripheral blood. Recent studies have shown that the detection of the CTC is important for evaluating prognosis of tumor patients, especially patients with advanced cancers, and selecting appropriate personalized treatment. Since the CTC detection is minimally invasive and real-time, it is called a "liquid biopsy" of tumors.

[06] In order to relatively accurately recognize the CTC detected by a CTCBIOPSY® detection system based on isolation by size of epithelial tumor cells (ISET), the present disclosure provides a recognizing method by using a cell immunochemical technology to detect whether CD45 and CD31 on the suspected CTC are expressed or not so as to exclude a leukocyte and an endothelial cell.

[07] To achieve the above objective, the technical solution of the present disclosure is as follows:

[08] The present disclosure provides a method for recognizing a peripheral blood circulating tumor cell (CTC) of a tumor patient based on isolation by size of epithelial tumor cells (ISET), where a cell immunochemical method is used to detect whether CD45 and CD31 on the suspected CTC are expressed or not so as to exclude a leukocyte and an endothelial cell.

[09] Further, the method may specifically include the following steps:

[10] (1) collecting peripheral blood of patients with middle and advanced cancers: collecting 5 ml of peripheral blood from a median cubital vein;

[11] (2) pretreating a peripheral blood sample: adding the collected peripheral blood sample to a diluent for a 10-fold dilution and adding paraformaldehyde to fix the peripheral blood sample for 10 min after the dilution with a final fixing concentration of 0.25%;

[12] (3) filtering the peripheral blood sample by using a device for separating a tumor cell via a membrane filtration to separate and obtain the peripheral blood CTC: adding the pretreated peripheral blood sample to a blood sample container of the device for separating a tumor cell via a membrane filtration and conducting natural filtering by gravity;

[13] (4) after the filtration, taking out a filter from the device for separating a tumor cell by a membrane filtration, adding 0.5 ml of a staining solution A for a CTC to the filter, and conducting staining for 3 min and rinsing with a PBS buffer; after a filtrate is filtered completely, adding 1 ml of a solution B, conducting staining for 2 min and rinsing with 1 ml of pure water twice, taking out a filter membrane and placing on a glass slide, and conducting observation under a microscope after drying to determine whether the CTC exists; and

[14] (5) detecting expressions of the CD45 and CD31 on the CTC by using the cell immunochemical technology.

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[15] Further, the diluent may be composed of 1 mmol/L of EDTA+0.1% of BSA+0.1% of trehalose+0.2% of a polyoxyethylene-polyoxypropylene ether block copolymer and a base solution may be a Tris-HCl buffer.

[16] Further, the staining solution A may be a diaminobenzidine (DAB) staining solution; and the staining solution B may be a hematoxylin staining solution.

[17] Specific processes of the cell immunochemical technology for detecting the expressions of the CD45 and CD31 on the CTC are as follows:

[18] (1) decolorization: taking out a filter membrane with the CTC from the glass slide, soaking the CTC in a decolorization solution for 4-6 h, and removing the CTC staining solution;

[19] (2) adding 100 μl of 0.1% of Triton X-100 dropwise and conducting incubation at room temperature for 15 min and washing with deionized (DI) water for 3 times with 2 min for each time;

[20] (3) adding 100 μl of 0.3% of H2O2 dropwise and conducting incubation at room temperature for 10 min and washing with PBS for 3 times with 2 min for each time;

[21] (4) adding 100 μl of CD45 (human) and CD31 (human) primary antibodies dropwise and conducting incubation at room temperature for 2 h (or overnight at 4°C) and washing with PBS for 3 times with 2 min for each time;

[22] (5) adding 100 μl of goat anti-human IgG/HRP dropwise and conducting incubation at room temperature for 20 min and washing with PBS for 3 times with 2 min for each time;

[23] (6) adding 100 μl of a diaminobenzidine (DAB) color developing solution, conducting incubation at room temperature and observing color development under a microscope at any time;

[24] (7) after the color development is completed, discarding the DAB color development solution and conducting rinsing with running water for 5 min and staining with hematoxylin for 5 min;

[25] (8) conducting differentiation with hydrochloric acid and alcohol for 8 s and blue turning with tap water for 5 min;

[26] (9) conducting a gradient ethanol dehydration with 75% ethanol (1 min), 95% ethanol (1 min) and 100% ethanol (1 min), air-drying, and sealing with neutral resin; and

[27] (10) conducting a microscopic examination under an optical microscope.

[28] Further, the decolorization solution may be prepared by evenly mixing 95% alcohol and 100% xylene in a volume ratio of 1:1.

[29] A device for separating a tumor cell by a membrane filtration used in the present disclosure includes a filter, a blood sample container, a waste liquid tank, and an iron stand. The iron stand is provided with a base, a vertical stand and a bracket. The blood sample container is arranged on an upper part of the iron stand through the bracket. The filter is arranged below the blood sample container and connected to the waste liquid tank through an infusion apparatus. The waste liquid tank is arranged on the base.

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[30] The filter includes a filter upper mouth, a filter membrane, a filter membrane carrier platform and a filter lower mouth. The filter membrane is placed on the filter membrane carrier platform. The filter upper mouth is connected to the blood sample container and the filter lower mouth is connected to the waste liquid tank through the infusion apparatus.

[31] The filter membrane is made of a hydrophobic material and filter holes with a diameter of 8 mm are evenly distributed in the filter membrane.

[32] The filter membrane obtained by a CTCBIOPSY® detection system is stained, CTC and suspected CTC and heteromorphic cells identified by cell morphology are observed and imaged, immunocytochemical staining of common antigens CD45 and CD31 on surfaces of leukocytes is used to distinguish the CTC and hematogenous and endothelial cells so as to reduce a false positive rate of the CTCBIOPSY® in detecting the CTC. The method improves specificity, more accurately detects the CTC and has important clinical guidance significance in evaluating an illness state and a curative effect by clinically applying CTC detection.

[33] The present disclosure has the following beneficial effects:

[34] (1) The detection method can accurately recognize the CTC in peripheral blood of tumor patients, belongs to the field of minimally invasive technologies and can be used for real-time dynamic detection.

[35] (2) The method provided by the present disclosure can more accurately detect the CTC, reduces a false positive rate of CTC detection, and improves specificity. The method has important clinical guidance significance in evaluating an illness state and a curative effect by clinically applying CTC detection.

BRIEF DESCRIPTION OF DRAWINGS

[36] FIG. 1 is a structural schematic diagram of a membrane filtration device of the present disclosure;

[37] FIG. 2 is a structural schematic section diagram of a filter of a membrane filtration device of the present disclosure;

[38] FIG. 3 is a structural schematic diagram of a filter membrane of a filter of a membrane filtration device of the present disclosure;

[39] FIG. 4 is an image of a circulating tumor cell separated and obtained from peripheral blood of a patient with a liver cancer (A) and a staining image of CD45 and CD31 in a circulating tumor cell in peripheral blood (B);

[40] FIG. 5 is an image of a circulating tumor cell separated and obtained from peripheral blood of a patient with a kidney cancer (A) and a staining image of CD45 and CD31 in a circulating tumor cell in peripheral blood (B);

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[41] FIG. 6 is an image of a circulating tumor cell separated and obtained from peripheral blood of a patient with a lung cancer (A) and a staining image of CD45 and CD31 in a circulating tumor cell in peripheral blood (B); and

[42] FIG. 7 shows criteria on combination of an image of a circulating tumor cell and immunohistochemistry.

[43] Reference numerals: 1 iron stand, 2 blood sample container, 3 filter, 4 infusion apparatus, 5 waste liquid tank, 6 filter upper mouth, 7 filter membrane, 8 filter membrane carrier platform, 9 filter lower mouth, 10 filter hole, 11 base, 12 vertical stand and 13 bracket.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[44] The present disclosure will be described in combination with the drawings and embodiments.

[45] Examples by using the method to separate, obtain and identify a circulating tumor cell in peripheral blood of 8 cases of tumor patients (8 cases of normal human samples are detected at the same time as a negative control) are as follows.

[46] **1. Separation and obtaining of CTC in peripheral blood of patients with middle and advanced tumors by using membrane filtration device**

[47] Five milliliter of fasting blood was collected from a median cubital vein after subjects fasted for 8-12 h, the peripheral blood was diluted by using 45 ml of a diluent (components are 1 mmol/L of EDTA+0.1% of BSA+0.1% of trehalose+0.2% of a polyoxyethylene-polyoxypropylene ether block copolymer and a base solution is a Tris-HCl buffer), and 3 ml of 4% of paraformaldehyde was added to fix the diluted blood sample for 10 min;

[48] in a fixing period, a membrane filtration device is assembled: as shown in FIGs. 1, 2, and 3, the filtration device is composed of a filter 3, a filter membrane 7, a blood sample container 2, a waste liquid tank 5, and an iron stand 1;

[49] 10 ml of PBS was used to wet the filter 3, the fixed peripheral blood sample was added to the blood sample container 2 of the membrane filtration device to be filtered naturally by gravity, and a circulating tumor cell (CTC) was trapped on the filter membrane 7;

[50] where, a diameter of a tumor cell is generally greater than 15 microns, while a diameter of a blood cell (including a red blood cell and a leukocyte) is generally less than 8 microns, so that after the peripheral blood containing the CTC was filtered, the blood cell can be filtered out since the diameter of the blood cell is smaller than that of filter holes 10, while the CTC was trapped on the filter membrane 7 since the diameter of the CTC is larger than that of the filter holes 10;

[51] after the filtration, the filter 3 was taken from the filtration device and opened, a filter upper mouth 6 was removed, 0.5 ml of a staining solution A for the CTC was added to the filter, staining was conducted for 3 min, and the filter 3 was rinsed clean with PBS buffer; after a filtrate was

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filtered completely, 1 ml of a solution B was added, staining was conducted for 2 min, the filter 3 was rinsed clean with 1 ml of pure water and PBS buffer, and the filter membrane 7 was taken out with ophthalmic tweezers and placed on a glass slide with a cell face facing upward; and

[52] observation was conducted under a microscope after the filter membrane was dried to determine whether the CTC existed.

[53] Through observation, no CTC was found in 8 healthy volunteers. Except 1 case of a tumor patient, the CTC was detected in the other 7 cases of tumor patients. Expressions of CD45 and CD31 were negative in all cases (Table 1). A positive rate of the detection was 87.5%.

[54] Table 1 Detection result of CTC in an example

Sample NO.	Sample Source	Detection Result (number of CTC)
1	Healthy volunteer	0
2	Healthy volunteer	0
3	Healthy volunteer	0
4	Healthy volunteer	0
5	Healthy volunteer	0
6	Healthy volunteer	0
7	Healthy volunteer	0
8	Healthy volunteer	0
9	Patient with advanced liver cancer	2
10	Patient with advanced gastric cancer	1
11	Patient with advanced colon cancer	3
12	Patient with middle kidney cancer	1
13	Patient with middle lung cancer	0
14	Patient with advanced esophagus cancer	5
15	Patient with middle gastric cancer	1
16	Patient with advanced liver cancer	2

[55] 2. Detection of expressions of CD45 and CD31 on CTC by using cell immunochemical technology

[56] A filter membrane 7 with CTC was taken from a glass slide, the CTC was soaked in a decolorization solution prepared by evenly mixing 95% of ethanol and 100% of xylene at a volume ratio of 1:1 for 4-6 h, and the CTC staining solution was removed; 100 μl of 0.1% of Triton X-100 was added dropwise, incubation was conducted at room temperature for 15 min, and washing was conducted with deionized (DI) water for 3 times with 2 min for each time; 100 μl of 0.3% of H_2O_2 was added dropwise, incubation was conducted at room temperature for 10 min and washing was

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conducted with PBS for 3 times with 2 min for each time; 100 μl of CD45 (human) and CD31 (human) primary antibodies were added dropwise, incubation was conducted at room temperature for 2 h (or overnight at 4°C) and washing was conducted with PBS for 3 times with 2 min for each time; 100 μl of goat anti-human IgG/HRP was added dropwise, incubation was conducted at room temperature (18-26°C) for 20 min and a developing condition was observed under a microscope at any time (generally 3-10 min and no more than 10 min); after the color development, a diaminobenzidine (DAB) color development solution was discarded, rinsing was conducted with running water for 5 min and staining was conducted with hematoxylin for 5 min; differentiation was conducted with hydrochloric acid and alcohol for 8 s and blue turning was conducted with tap water for 5 min; a gradient ethanol dehydration with 75% ethanol (1 min), 95% ethanol (1 min) and 100% ethanol (1 min), air-drying, and sealing with neutral resin were conducted; and a microscopic examination was conducted under an optical microscope, and films were read by cytopathologists and expressions of CD45 and CD31 were determined according to a degree of cell membrane staining. Immunocytochemical staining of common antigens CD45 and CD31 on surfaces of leukocytes was used to distinguish the CTC and hematogenous and endothelial cells so as to reduce a false positive rate of a CTCBIOPSY® in detecting the CTC. The method improves specificity, more accurately detects the CTC and has important clinical guidance significance in evaluating an illness state and a curative effect by clinically applying CTC detection.

[57] In FIGs. 4-6, A represented an image of a circulating tumor cell separated and obtained from peripheral blood of a tumor patient, a cell nucleus was atypia, a nucleo-cytoplasmic ratio was greater than 0.8, a diameter of the cell is larger than 15 μm, the nucleus was deeply and unevenly stained, or the cell and chromatin shifted, or abnormal mitosis existed, and positive CTC was determined; B represented an immunocytochemical staining of CD45 and CD31, where, if a brown-yellow appeared around a cell membrane, it was determined to be positive and CTC was excluded according to criteria of immunohistochemistry; and the specific criteria were shown in FIG. 7.

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WHAT IS CLAIMED IS:

1. A method for recognizing a peripheral blood circulating tumor cell (CTC) of a tumor patient based on isolation by size of epithelial tumor cells (ISET), wherein a cell immunochemical method is used to detect whether CD45 and CD31 on the suspected CTC are expressed or not so as to exclude a leukocyte and an endothelial cell.

2. The preparation method according to claim 1, specifically comprising the following steps:

(1) collecting peripheral blood of patients with middle and advanced cancers: collecting 5 ml of peripheral blood from a median cubital vein;

(2) pretreating a peripheral blood sample: adding the collected peripheral blood sample to a diluent for a 10-fold dilution and adding paraformaldehyde to fix the peripheral blood sample for 10 min after the dilution with a final fixing concentration of 0.25%;

(3) filtering the peripheral blood sample by using a device for separating a tumor cell via a membrane filtration to separate and obtain the peripheral blood CTC: adding the pretreated peripheral blood sample to a blood sample container of the device for separating a tumor cell via a membrane filtration and conducting natural filtering by gravity;

(4) after the filtration, taking out a filter from the device for separating a tumor cell by a membrane filtration, adding 0.5 ml of a staining solution A for a CTC to the filter, and conducting staining for 3 min and rinsing with a PBS buffer; after a filtrate is filtered completely, adding 1 ml of a solution B, conducting staining for 2 min and rinsing with 1 ml of pure water twice, taking out a filter membrane and placing on a glass slide, and conducting observation under a microscope after drying to determine whether the CTC exists; and

(5) detecting expressions of the CD45 and CD31 on the CTC by using the cell immunochemical technology.

3. The method according to claim 2, wherein the diluent is composed of 1 mmol/L of EDTA+0.1% of BSA+0.1% of trehalose+0.2% of a polyoxyethylene-polyoxypropylene ether block copolymer and a base solution is a Tris-HCl buffer.

4. The method according to claim 2, wherein the staining solution A is a diaminobenzidine (DAB) staining solution; and the staining solution B is a hematoxylin staining solution.

5. The method according to any one of claims 2-4, wherein specific processes of the cell immunochemical technology for detecting the expressions of the CD45 and CD31 on the CTC are as follows:

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(1) decolorization: taking out a filter membrane with the CTC from the glass slide, soaking the CTC in a decolorization solution for 4-6 h, and removing the CTC staining solution;

(2) adding 100 μl of 0.1% of Triton X-100 dropwise and conducting incubation at room temperature for 15 min and washing with deionized (DI) water for 3 times with 2 min for each time;

(3) adding 100 μl of 0.3% of H_2O_2 dropwise and conducting incubation at room temperature for 10 min and washing with PBS for 3 times with 2 min for each time;

(4) adding 100 μl of CD45 (human) and CD31 (human) primary antibodies dropwise and conducting incubation at room temperature for 2 h (or overnight at 4°C) and washing with PBS for 3 times with 2 min for each time;

(5) adding 100 μl of goat anti-human IgG/HRP dropwise and conducting incubation at room temperature for 20 min and washing with PBS for 3 times with 2 min for each time;

(6) adding 100 μl of a diaminobenzidine (DAB) color developing solution, conducting incubation at room temperature and observing color development under a microscope at any time;

(7) after the color development is completed, discarding the DAB color development solution and conducting rinsing with running water for 5 min and staining with hematoxylin for 5 min;

(8) conducting differentiation with hydrochloric acid and alcohol for 8 s and blue turning with tap water for 5 min;

(9) conducting a gradient ethanol dehydration with 75% ethanol (1 min), 95% ethanol (1 min) and 100% ethanol (1 min), air-drying, and sealing with neutral resin; and

(10) conducting a microscopic examination under an optical microscope;

wherein the decolorization solution is prepared by evenly mixing 95% alcohol and 100% xylene in a volume ratio of 1:1.

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DRAWINGS

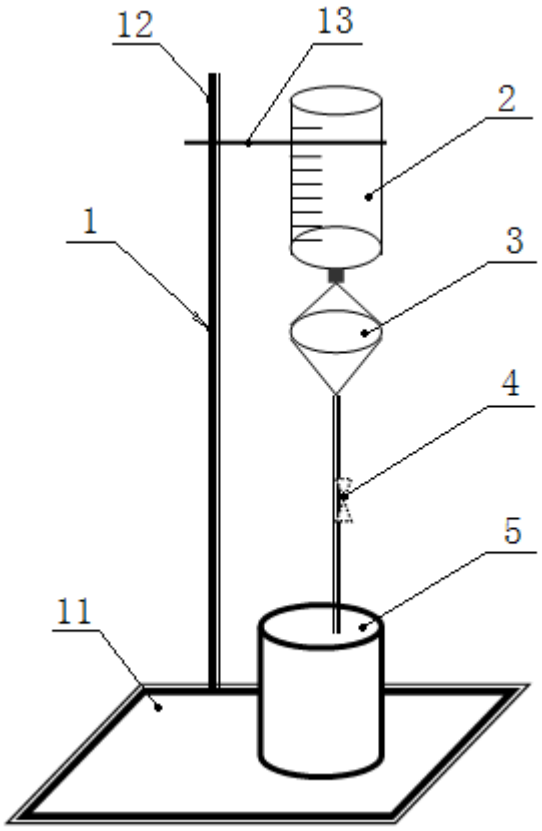

FIG. 1

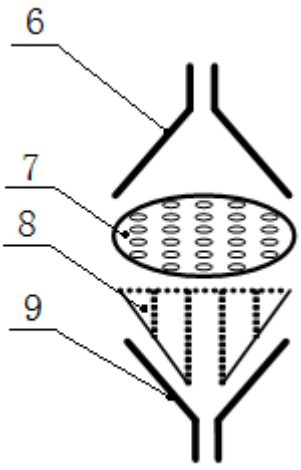

FIG. 2

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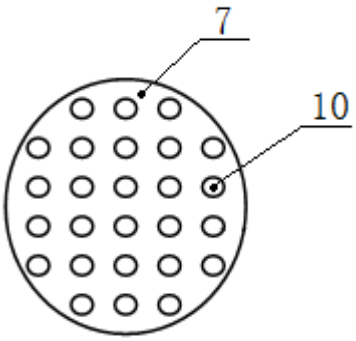

FIG. 3

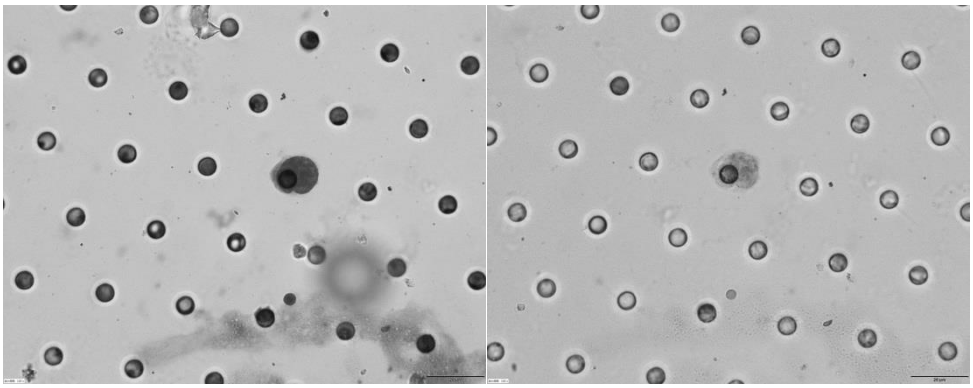

FIG. 4

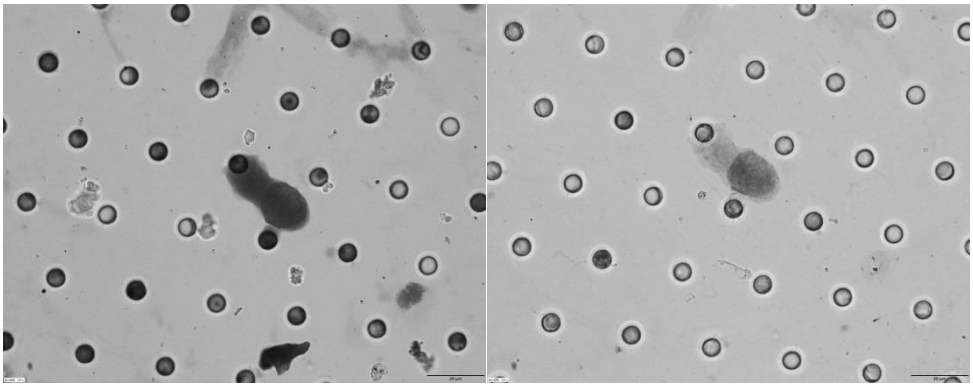

FIG. 5

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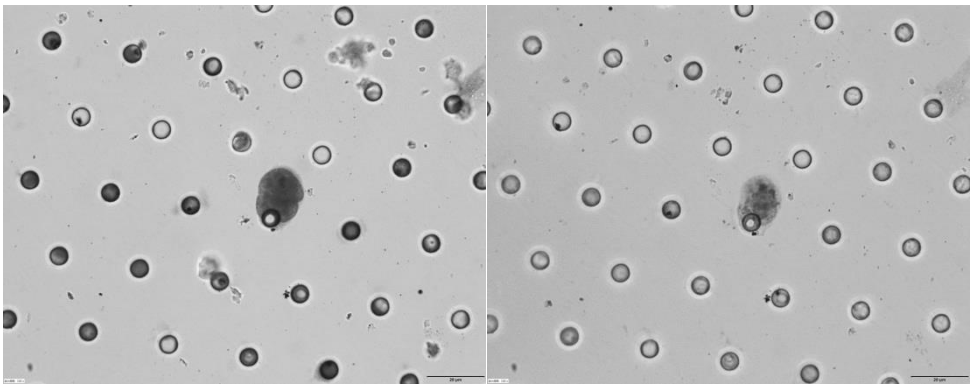

FIG. 6

Conclusion: final determination is primary based on an optical result and an immunohistochemical result is considered.

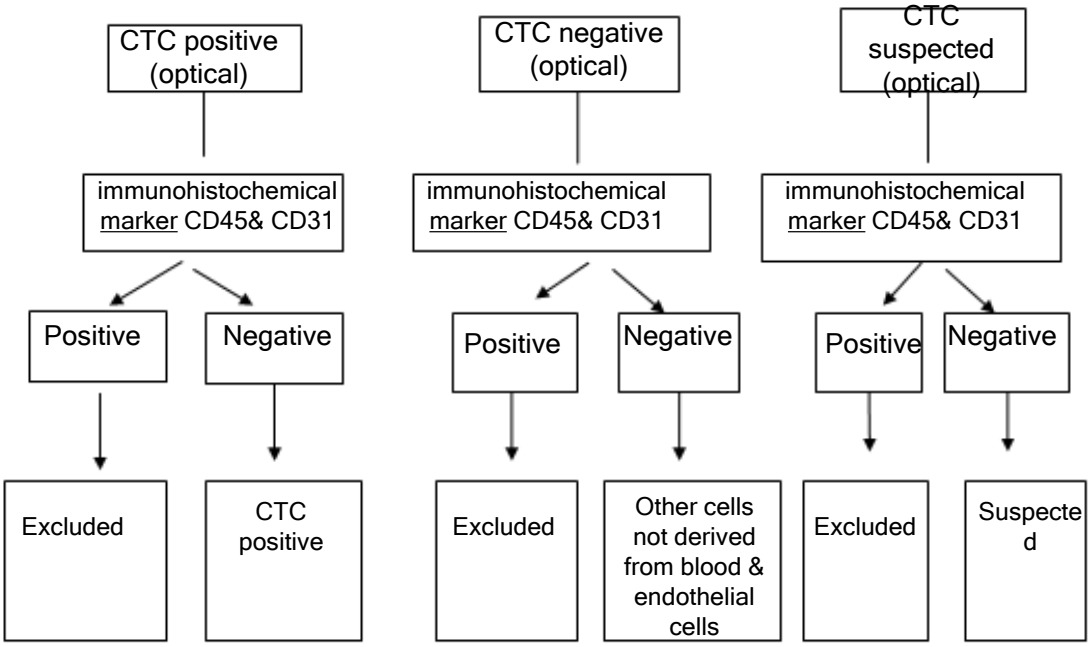

FIG. 7