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(54) **DEVICE AND METHOD FOR FLUID SEPARATION BY DENSITY GRADIENT CENTRIFUGATION**

VORRICHTUNG UND METHODE ZUR TRENNUNG VON FLÜSSIGKEITEN IN ABHÄNGIGKEIT IHRER DICHTEN MITTELS ZENTRIFUGATION

DISPOSITIF ET PROCÉDE DE SEPARATION DE LIQUIDES PAR CENTRIFUGATION EN DEPENDANCE DE LEUR DENSITÉ

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Description

[0001] The invention relates to a centrifugation container with an insert, and a method of fluid separation using a density gradient centrifugation. In particular, the invention is used in the separation of body fluids, e.g. blood of animals, including humans, for diagnostic purposes. The solution which is the subject of the invention belongs to the field of containers for laboratory purposes, and especially to tubes specifically adapted for centrifugation purposes. Another aspect of the invention relates to the field of testing or analyzing materials by determining their chemical, physical or biological properties, and particularly includes analyzing liquid biological material, e.g., blood.

State of the art

[0002] Collection, purification, fractionation and/or fixation of body fluid samples, including blood, play an important role, e.g. in medical diagnostics and clinical trials. For conventional systems and methods for collecting blood samples on a large scale, blood samples collected from the patient can be separated into different fractions by centrifugation, filtration or elutriation, and then stored for later use or further testing. Separated blood components usually contain fractions of red blood cells, white blood cells, platelets and plasma. Separation of blood into its fractions can be carried out continuously, during blood collection or in the stages following its collection. Separating blood into different components under highly sterile conditions is critical for many therapeutic applications and for clinical research purposes.

[0003] There are many methods for separating blood into its fractions. The methods known in the state of the art require using state-of-the-art specialized medical/research devices and highly qualified personnel for their proper operation.

[0004] From International Patent Application No. WO8805331, there is a technique known for separating white blood cells (leukocytes) from red blood cells (erythrocytes), which consists of mixing a blood sample with a working solution that aggregates red blood cells and thus increases their sedimentation rate. The density of the working separation fluids is selected so that the sedimentation of white blood cells is minimally changed and that the white blood cells do not sediment to the bottom, and as a result could be taken from the upper part of the separated liquid after the red blood cells have sediment to the bottom.

[0005] In another technique, in which the working solution that aggregates red blood cells is not mixed with blood, the blood is layered precisely on the surfaces of separation fluids, after which the red blood cells agglutinate or aggregate under the effect of surface contact with the working separation fluids, as a result of which they sediment to the bottom of that tube. There are several well-known multi-polymer compounds that agglutinate

red blood cells, e.g. FICOLL 400 (Pharmacia Fine Chemicals, Sweden). Blood separation can occur under the influence of gravity or under the influence of centrifugation. Most white blood cells remain at the phase boundary, but these previously developed systems are not effective in separating white blood cells into subpopulations, i.e., into peripheral blood mononuclear cell (PBMC) and polymorphonuclear leukocytes (PMN) populations. In particular, there is an ongoing search for a single-stage method that uses a density separation medium that would enable separation of whole blood cells into subpopulations.

[0006] To perform the aforementioned separation of white blood cells into subpopulations, one known method is to isolate peripheral blood mononuclear cell (PBMC) based on a centrifugation process, wherein the first stage uses the Isopaque-Ficoll mixture (Nyegaard & Co., Norway) having the sodium metrizoate component, in the next stage, polymorphonuclear neutrophils are isolated using dextran or gelatin, which cause sedimentation of red blood cells. Another method uses discontinuous density gradients where two or more working separation fluids are poured in layers on top of each other. The densities are selected so that the (discontinuous) gradient is in the appropriate/required range - adjusted to the density of the separated substances.

[0007] On the other hand, U.S. Patent Application No. US4824560 A discloses methods and means of centrifuging in a tubular container having at least two adjacent chambers that are connected to each other by a narrow, essentially capillary opening. For operation, the working fluid is placed in the bottom chamber, while the fluid to be separated into fractions is placed in the upper chamber, and there is no need for special precautions to avoid liquid mixing before starting centrifugation. This method has several advantages over the manual methods described above, it also has the disadvantage due to the narrow connection of both chambers which constitutes a partial barrier, even during centrifugation, This barrier prevents effective passage of blood cells between the chambers and precludes separation of the blood into fractions.

[0008] Patent application US2014087360 A1 discloses an insert for a centrifuge tube suitable for use in density gradient separation. The insert includes a member sized to fit within the tube for dividing the tube into a top portion and a bottom portion. Optionally the insert has a support extending or depending from the member for positioning the member within the tube. At least two openings are located on the member so that a first opening is closer to a bottom end of the tube relative to a second opening when the insert is positioned in the centrifuge tube.

[0009] Patent US5314074 A discloses layering insert within the vessel that prevents back mixing after density gradient centrifugation. The insert is capable of supporting the body of liquid dispersion to be centrifuged under regular gravity condition and enables bi-directional crossflow during centrifugation.

[0010] Patent US5132232 A discloses apparatus for collecting liquid analytical samples for examination, and reproducibly separating them into two or more fractions. An apparatus is utilizing a container and a petter, said petter provided with an upper portion which enables attachment to the container, a lower retainer portion including a peripheral aperture, the non-apertured portion of which enables wedge-fit thereof into the container, and a body portion connecting the upper petter portion and lower retainer portion.

[0011] Patent US5648223 A discloses a cell-trap centrifugation tube containing a specific density gradient solution adjusted to a specific density to enrich for breast tumor cells from a cell mixture. The tube allows the desired cell population to be collected by decantation after centrifugation to minimize cell loss and maximize efficiency.

[0012] The problem arising in the manual separation methods described above is the preparation of the sample for this process, and in particular in layering of poured liquids used for different density separation and the material being tested, e.g. blood. It is important that fluids do not mix with each other and that interface is established between fluids of different densities. To achieve this state, various techniques and skills have been developed that allow for the proper layering of separation fluids and blood, which are most often carefully pipetted into a container for further separation into fractions using density gradient centrifugation. Unfortunately, these procedures are cumbersome, difficult to perform, introduce the possibility of uncontrolled human errors, and additionally require highly qualified personnel, which is associated with high operating costs, reduces the repeatability of the procedure and precludes large-scale separation/division.

[0013] The aim of the solution according to the invention was to obtain a tool for fast and partially automated separation of fluids into fractions of different density, e.g. biological fluids, including blood, which additionally enable the purification, isolation and fixation of biological samples.

Definitions

[0014] Within the description of the invention and the claims, the following terms shall be understood in accordance with the following definitions:

the "container" is any liquid storage container that is adapted to centrifugation, e.g. centrifugation tubes,

the "guide" is an element of the insert that directs liquid flow from the upper chamber to the bottom chamber through the opening in the partition, the guide should be of such a size that enables the test liquid to layer on the separation medium at the bottom of the container. The guide, in accordance with foregoing definition, may be the container wall or a

different structure inside the container e.g. spiral, elongated sleeve, etc.

Detailed description of the invention

[0015] The essence of the invention is a centrifugation container comprising an insert, especially a test tube, for separation of liquids into fractions of the desired density range by density gradient centrifugation, especially liquids constituting suspensions and/or biological fluids, the insert is equipped with a partition suitable for dividing the container's interior into at least two chambers in a vertical arrangement - the upper chamber and the bottom chamber, characterized in that the partition having an opening into which a guide adjoins, on which liquids, especially biological fluid, flow into the bottom chamber of the centrifugation container, and said partition is made of two adjacent surfaces with openings, especially in the shape of flattened discs fitted to the cross-section of the container with a cross-section similar to the circle, the surfaces being movably connected with each other, they can be freely positioned with each other enabling closing of the opening lumen.

[0016] Preferably, the guide is spiral, funnel or vertical elements in the shape of an elongated cylinder.

[0017] Preferably, the upper chamber of centrifugation container has additionally a vertical partition or partitions separating it into sub-chambers, each of the sub-chambers having an opening.

[0018] The invention also includes a method of separating a fraction with a desired density range from a sample containing fractions of different density, especially from a biological sample, comprising:

a) providing a centrifugation container with an insert for a centrifugation container, especially a test tube, for the separation of liquids into fractions of the desired density range by density gradient centrifugation, especially liquids constituting suspensions or biological fluids,

b) filling the bottom chamber of the container with a medium for separation on a density gradient or filling the top chamber of the container with this medium, which then flows through the opening in the partition along the guide to the bottom chamber;

c) pouring liquid for separation containing fractions of different density to the bottom chamber by filling the upper chamber or at least one sub-chamber or attaching the upper chamber to the partition, so that the liquid can flow through the opening in the partition on the guide and layer on the surface of the separation media in bottom chamber;

d) centrifugation of the container until the test sample is separated into fractions of different density.

[0019] Preferably, the step (b) is followed by an additional step or steps b) consisting of adding an additional density gradient separation medium, wherein the addi-

tion of subsequent media is from the highest to the lowest density.

[0020] It is equally advantageous if, after step (d), selected fractions of different density from the liquid being separated are subjected to tests and analysis, including the possibility of being fixed, especially by the freezing method.

[0021] Preferably, when the fraction is separated from the blood liquid, the individual fractions of the desired density range contain various blood components, including: leukocytes (lymphocytes and granulocytes), platelets, erythrocytes, bone marrow cells (megakaryocytes, erythroblasts), cells suspended in homogenate including endothelial cells, neurons, fungi, viruses, microparticles including exosomes, cell fragments, cell organelles including nuclei, mitochondria, chloroplasts.

[0022] The invention also relates to a kit comprising:

a) insert (6) for the container (1) for centrifugation, in particular the tube, for the separation of liquids into fractions of the desired density range by density gradient centrifugation, especially liquids constituting suspensions or biological fluids, this device is equipped with a partition (7) dividing the inside of the container (1) into the upper chamber (2) and the bottom chamber (3), the partition (7) having an opening (4), and at the opening there is a guide (12) on which fluids flow, especially separated fluid, into the bottom chamber (3) of the container (1) for centrifugation,

b) at least one density gradient separation medium.

[0023] For a better understanding, the invention is illustrated in the embodiment illustrated in the drawing which is not a limitation of the protection applied for, in which:

Fig. 1 illustrates a container in the shape of a centrifugation tube with an insert, intended for collecting liquids, in particular biological material, and then for its separation, which - according to the invention - allows layered arrangement of liquids placed in the container before centrifugation;

Fig. 2 and 3 illustrate a longitudinal section and a side view of a centrifugation tube container, respectively, in which, for a better understanding of the essence of the invention, the disc and the partition of the insert are spaced apart;

Fig. 4 and 5 illustrate, the side view and longitudinal section of the tube-shaped container, respectively, with a visible narrowing tube lumen as the wall thickness increases,

Fig. 6 illustrates a cross section through a tube-shaped container in an example without a vertical partition, but with an air channel visible,

Fig. 7a and 7b illustrate a side view and cross-section of the upper part of the insert in the form of a disc with an incomplete partition, respectively.

Fig. 8a and 8b illustrate a side view and cross-section of the upper part of the insert in the form of a disc with a rectangular partition, respectively.

Fig. 9a and 9b illustrate, a side view and cross-section of the upper part of the insert in the form of a disc with a partition in the shape of three rectangles, respectively.

Fig. 10a and 10b illustrate, a side view and cross-section of the upper part of the insert in the form of a disc with a partition in the shape of two cross intersecting rectangles, respectively.

Fig. 11a and 11b illustrate, a cross section and a side view of a disc-shaped partition with a notch, respectively.

Fig. 12 illustrates one embodiment of the solution of the invention in which the insert is placed on a centrifugation container,

Fig. 13 and 13a illustrates the insert, a side and top cross-section adapted for connecting the upper chamber equipped with a guide in the shape of an elongated cylinder, respectively.

Fig. 14 and 14 a illustrates the insert, a cross-section from the side and top, equipped with a guide in the form of eight elongated cylinders, respectively.

Fig. 15 and 15a illustrate the insert, a side and top cross section equipped with a spiral-shaped guide, respectively.

Fig. 16, 16a and 16b illustrate the insert cross-section from the side and top view, and enlarged guide, equipped with a funnel-shaped guide, respectively.

Example 1

[0024] As shown in Fig. 1, in the first embodiment the tube insert 6 for the centrifugation container consists of a partition 7 in the form of a flat disc tightly adhering to the inner walls of the container 1, and a disc 8 equipped with a full vertical partition 11. The insert in this example is placed inside a container 1 constituting a 0.23" diameter centrifugation tube. Insert 6 in this example is made of plastic, but could also be made of other materials. As shown in Fig. 12, the insert 6 can also be placed in an additional container that can be attached to the centrifugation container 1, then the insert 6 is outside the container 1.

[0025] The wall of the tube-shaped container 1 forms

a guide 12 and widens gradually into the container 1 (Fig. 4 and 5), at the same time the lumen of the tube gradually decreases towards its bottom. In this embodiment, the inner wall of the container 1 is a guide 12 that allows liquids to flow from the upper chamber 2 to the bottom chamber 3 through the opening 4. Liquids - especially liquids that are biological fluids for separation - flow down to the bottom of the container 1 along the guide 12, constituted by container wall, and become arranged in layers on the bottom of the container 1. The liquid flow down along the guide 12 prevents agitation of the separation liquids, which could cause errors in the separation of the tested liquids.

[0026] In this example, the partition 7 has the shape of a flat disc with a circular cross-section (Fig. 11a, Fig. 11b) and is closely fitted in shape to the cross-sectional shape of the container 1, so the diameter on its top side is greater than on the bottom side, and the longitudinal section of the partition 7 is close to a flattened inverted trapezoid. The partition 7 divides the container 1 into an upper chamber 2 and a bottom chamber 3. The partition in the example has an opening 4 constituting an indentation in the shape similar to a semi-circle.

[0027] As shown in Fig. 8a and 8b, the vertical partition 11 may be in the shape of a rectangle that closely adheres to the inner wall of the container 1, then the vertical partition 11 located on the disc 8 divides the upper chamber 2 of the container 1 in the shape of a tube into two sub-chambers 10a, 10b. In each of the halves of the disc 8 formed by the partition 11, there is one opening 5 in the shape of a notch that can be closed with the disc 8. In the embodiment, the indentation-shaped openings 5 in the disc 8 are semicircular. In other implementations of the solution according to the invention, it is also possible to use discs 8 with other shapes of openings 5. The liquid flow rate from the upper chamber 2 to the bottom chamber 3 depends on the shape of the openings 4, 5 and their mutual arrangement with respect to each other.

[0028] In this example, the indentation-shaped openings 4, 5 with an indentation radius of 0.115" are of the same shape. In other versions of the embodiment, the openings 4, 5 may have different shapes and may differ in shape, but their diameter should be greater than 0.1". With the partition 7 and disc 8 arranged in such a way that the openings 4, 5 do not overlap, the flow of fluids between the upper chamber 2 and the bottom chamber 3 is blocked and proper fluid down flow cannot take place.

[0029] In this embodiment, the container 1 is provided with a lid 9. In one version of the example, the lid 9 has a gap in which the upper part of the vertical partition 11 of the insert 6 fits and passes through. Such a position of the vertical partition 11 allows changing the position of the disc 8 relative to the partition 7 by turning the protruding part of the partition, and thus the movable part of the lid 9. The container 1 and the lid 9 have a thread and form a screw cap. Alternatively, the use of the lid without a gap 91, where the vertical partition 11 of the insert is adapted to the length of the container 1 so that, after

screwing the lid 9, the vertical partition 11 would tightly adhere to the inner surface of the lid 9. The lid 9 may be made of plastic and may have a calibrated turning/twisting scale. Markings on the centrifugation container 1 and lid 9 may be provided to facilitate correct alignment/arrangement of the openings 4, 5 in relation to each other.

[0030] Alternatively, different shapes and arrangements of the vertical partition 8 are possible in other versions of the embodiment. As shown in Fig. 7a and 7b, the vertical partition 11 may not adhere to the walls of the container 1, then the vertical partition 11 located on the disc 8 divides the tube into two chambers only - the upper chamber 2 and the bottom chamber 3, and the upper chamber 2 is not divided into additional sub-chambers. In this version, disc 8 is equipped with one indentation-shaped opening 4, wherein the shape of the disc 8 in another version of the example could be limited to closing the openings 4 in the partition 7.

[0031] As shown in Fig. 9a and 9b, the vertical partition 11 may consist of three rectangular elements connected together by longer sides, the sides of which are closely adjacent to the inner wall of the container 1, then the vertical partition 11 located on the disc 8 divides the upper chamber 2 of the container 1 in the shape of a tube into three sub-chambers. In this version, disc 8 is equipped with three indentations 5, one in each of the sub-chambers.

[0032] As shown in Fig. 10a and 10b, the vertical partition 11 may consist of four rectangles connected together, the sides of which are closely adjacent to the inner wall of the container 1, then the vertical partition 11 located on the disc 8 divides the upper chamber 2 of the container 1 in the shape of a tube into four sub-chambers. In this version, disc 8 is equipped with four indentations 5, one in each of the sub-chambers.

[0033] Insert 6 can also be used in containers 1 with shapes other than the centrifugation tube shown in the embodiment, but these containers must be suitable for centrifugation.

Example 2

[0034] Fig. 13 and 13a shows another embodiment of the solution according to the invention, in which the insert 6 has a partition 7, which does not have an upper chamber, but allows connection, through a tube 16, to the upper chamber partition in the form of a container (e.g. test tube, pouch) with a medium for separation or with liquid for separation. Next, the partition is equipped with a guide 12 in the shape of an elongated cylinder, which is attached to the partition 7 and is located at such a distance from the opening 4 that allows liquid to flow from the upper chamber through the tube 16, then through the opening in the partition and along the guide to the bottom chamber 3. In this embodiment, the elongated cylinder constituting the guide 12 is of such a length that the test material layers onto the surface of the gradient medium without causing significant disturbances in the separation

medium.

Example 3

[0035] Fig. 14 and 14a shows another embodiment of the solution according to the invention, in which the insert **6** has a partition **7**, equipped with a guide **12** in the shape of 8 elongated cylinders, which are attached to the partition **7** and are at a distance from the opening **4** allowing liquid to flow from the upper chamber through the opening in the partition following the guide to the bottom chamber **3**. In this embodiment, the elongated cylinders constituting the guide **12** are of such a length that the test material layers onto the surface of the gradient centrifuging medium without causing significant disturbances of the separation medium.

Example 4

[0036] On the other hand, Fig. 15 and 15a show yet another example of the solution according to the invention, in which the insert **6** has a partition **7** equipped with a spiral-shaped guide **12**. Similarly, to example 2, the spiral length should be such that the test material layers onto the surface of the gradient centrifuging medium without causing significant disturbances in the separation medium.

Example 5

[0037] Fig. 16, 16a and 16b show yet another example of the solution according to the invention, in which the insert **6** has a partition **7** equipped with a funnel-shaped guide **12**. At the same time, openings **4** in the partition **7** direct liquids from the upper chamber so that they flow down along the outer surface of the funnel to the bottom of the chamber **3**. Similarly, to example 2, the spiral length should be such that the test material layers onto the surface of the gradient centrifuging medium without causing significant disturbances in the separation medium.

Example 6

[0038] The method of separating the fraction with the desired density range from a sample containing fractions of different density according to the invention can be carried out in such a way that in different sub-chambers **10a**, **10b** of upper chamber **2**, there are two different separation media placed in a density gradient, the first medium has a density of 1.119 g/mL, the second medium has a density of 1.077 g/mL (Histopaque 1.119 and Histopaque 1.077 from Sigma Aldrich, respectively), with the openings **4,5** constituting indentations - of the partition **7** and disc **8**, respectively - not overlapping when remaining in the closed position. Next, by changing the position of the disc **8** by turning it, the indentations **4, 5** overlap enough to allow the medium to flow from the upper chamber **2** to the bottom chamber **3**. The liquids flow along the inner

wall of the container **1** what constitutes the guide **12**. The position of the openings **4,5** constituting an indentation in the partition **7** and the disc **8** from the example is adjacent to the the wall of the container **1** what constitutes the guide **12**. The media are added in order from highest to lowest density, and a interphase is formed between the media of different density. Next, a liquid or mixture intended for separation into fractions of different density under the influence of centrifugation, e.g. native or diluted blood, is added to one of the empty sub-chambers **10** with a closed flow between the upper chamber **2** and the bottom chamber **3**.

[0039] The size of the clearance formed by the openings **4, 5** being cutouts of the partition **7** and the disc **8**, respectively, can be controlled by adjusting the mutual position of the partition **7** and the disc **8**. Slowly twisting the upper part of the insert **11**, and thus the disc **8**, gradually increases the fluid flow rate until the assumed liquid flow velocity between the chambers **2, 3** is achieved. By adjusting the amount of mutual overlap between the opening **4** and the opening **5**, the liquid flow can be controlled to create a continuous laminar flow along the wall **12** of the centrifugation container **1**. The construction of the partition **7** and disc **8** according to the invention ensures very smooth down flow of fluid from the upper chamber **2** into the bottom chamber **3** of the centrifugation container **1** in such a way that the interface between liquids is intact and the subsequent liquid poured from the upper chamber **2** does not mix with the liquid present in bottom chamber **3**.

[0040] After pouring two working density gradient separation media, these fluids are arranged in layers one on top of the other due to different density, then the test sample in the form of blood is added, it is also possible to use different types of fluids for separation, including native or diluted biological samples. The blood was first placed in the chamber **10a**, and then after turning the disc **8** of the insert **6** in such a way that the opening **4** of the partition **7** matches at least in part with the corresponding opening **5** of the disc **8** of the insert **6**, and allows blood to flow along the wall **12** of the container **1** from the sub-chamber **10a** to the bottom chamber **3** and to layer on the surface of separation media placed there previously. Due to the structure of the insert **6**, it is not necessary for the biological specimen to be placed in container **1** with extreme precision and care.

[0041] The blood in the bottom chamber **3** of the container **1** is then centrifuged according to methods generally known in the art. During centrifugation, two-way fluid flow occurs within different compartments formed by separation fluids of different density in the bottom chamber **3**, and a continuous density gradient is formed at the end of centrifugation, with red blood cells settling on the bottom forming the lowest-located layer, the higher layer is a liquid with a density of 1.119 g/mL, the next layer located above is white blood cells with segmented nuclei, above is a layer formed by liquid with a density of 1.077 g/mL, and a layer of white mononuclear cells above that, finally

the plasma is located above them as the highest layer. After removing the insert, each of the cell layers/or liquids can be removed by aspiration using a pipette or decantation.

Example 7

[0042] The insert and method of the invention is used, for example, to separate a desired subset of blood cells. In an embodiment, ten blood samples were collected from healthy volunteers (20 mL of venous blood) into commercially available ethylenediaminetetraacetic acid (EDTA) tubes (EDTA tube, Becton Dickinson). In this experiment, the volume of the centrifugation tube 1 constituting the invention was 50 mL, two separation media of different density (Histopaque 1.119 and Histopaque 1.077 Sigma Aldrich) were also used. The separation media used had a neutral pH, were isotonic to body fluids, the first separation medium had a density of 1.119 g/mL, while the second had a density of 1.077 g/mL.

[0043] Next, 10 mL of separation medium with a density of 1.119 g/mL was placed in the sub-chamber **10a** of the upper chamber **2** of the centrifugation container **1** equipped with the insert **6** according to the invention. A second medium with a density of 1.077 g/mL with a volume of 10 mL was placed in the sub-chamber **10b** of the upper chamber **2**, and then layered on the first medium using the insert **6** according to the invention described above. In the experiment, the partition wall was 0.08" thick and the indentations **4,5** of partition **7** and disc **8** had a radius of 0.115". Next, the collected blood with ethylenediaminetetraacetic acid (EDTA) was placed in sub-chamber **10a** of upper chamber **2**. Each blood sample was layered on the surfaces of the separation media with the insert **6** of the invention described above.

[0044] In the next step, all tubes were centrifuged at 700g (with minimal acceleration and no active braking) for 30 minutes at room temperature. As an effect of density gradient centrifugation, blood was separated into four fractions: plasma, peripheral blood mononuclear cells (PBMC), polymorphonuclear leukocytes (PMN), and red blood cells. The purity of the PBMC and PMN fractions was confirmed by flow cytometry. The purity of PBMC and PMN in their fractions was 95% and 92%, respectively. PBMC and PMN cells were not detectable in the plasma fraction. Isolated plasma, PBMC and PMN were suitable for further analysis, including but not limited to: RNA, micro-RNA, mitochondrial DNA, nuclear DNA, protein and cell phenotyping.

Claims

1. A centrifugation container (1) comprising an insert (6), especially a test tube, for separation of liquids into fractions of the desired density range by density gradient centrifugation, especially liquids constituting suspensions and/or biological fluids, the insert is

equipped with a partition (7) suitable for dividing the container's (1) interior into at least two chambers in a vertical arrangement - the upper chamber (2) and the bottom chamber (3), **characterized in that** the partition (7) has an opening (4) into which a guide (12) adjoins, on which liquids, especially biological fluid, flow into the bottom chamber (3) of the centrifugation container (1), and said partition (7) is made of two adjacent surfaces with openings, especially in the shape of flattened discs fitted to the cross-section of the container (1) with a cross-section similar to the circle, the surfaces being movably connected with each other, they can be freely positioned with each other enabling closing of the opening (4) lumen.

2. The centrifugation container (1) comprising the insert (6) according to claim 1 **characterized in that** the guide (12) is a spiral, a funnel or vertical elements in the shape of an elongated cylinder.

3. The centrifugation container (1) comprising the insert (6) according to claim 1 **characterized in that** the upper chamber (2) has additionally a vertical partition (11) or partitions separating it into sub-chambers (10), each of the sub-chambers (10) having an opening (4, 5).

4. A method of separating a fraction with a desired density range from a sample containing fractions of different density, especially from a biological sample, comprising:

- a) providing a centrifugation container (1) of any claim from 1 to 3 for the separation of liquids into fractions of the desired density range by density gradient centrifugation, especially liquids constituting suspensions or biological fluids,
- b) filling the bottom chamber (3) of the container (1) with a medium for separation in a density gradient or filling the top chamber (2) of the container (1) with this medium, which then flows through the opening in the partition along the guide (12) to the bottom chamber;
- c) pouring liquid for separation containing fractions of different density to the bottom chamber (3) by filling the upper chamber (2) or at least one sub-chamber (10a, 10b) or attaching the upper chamber to the partition, so that the liquid can flow through the opening (4) in the partition on the guide (12) and spill on the surface of the bottom chamber (3) separation media;
- d) centrifuging the container (1) until the test sample is separated into fractions of different density.

5. The method according to claim 4, **characterized in that** after the step (b) is followed by an additional

step or steps b) consisting of adding an additional density gradient separation medium, wherein the addition of subsequent media is from the highest to the lowest density.

6. The method according to claim 4, **characterized in that** after step (d), selected fractions of different density from the liquid being separated are subjected to tests and analysis, including the possibility of being fixed, especially by the freezing method.
7. The method according to claim 6, **characterized in that** when the fraction is separated from the blood liquid, the individual fractions of the desired density range contain various blood components, including: leukocytes (lymphocytes and granulocytes), platelets, erythrocytes, bone marrow cells (megakaryocytes, erythroblasts) suspended in cell homogenate including endothelial cells, neurons, fungi, viruses, microparticles including exosomes, cell fragments, cell organelles including nuclei, mitochondria, chloroplasts.

Patentansprüche

1. Zentrifugationsbehälter (1) mit einem Einsatz (6), insbesondere einem Reagenzglas, zur Trennung von Flüssigkeiten in Fraktionen des gewünschten Dichtebereiches durch Dichtegradientenzentrifugation, insbesondere Flüssigkeiten, die Suspensionen und/oder biologische Flüssigkeiten darstellen, der Einsatz ist ausgestattet mit einer Trennwand (7), die geeignet ist, das Innere des Behälters (1) in mindestens zwei Kammern in vertikaler Anordnung zu unterteilen - die obere Kammer (2) und die untere Kammer (3), **dadurch gekennzeichnet, dass** die Trennwand (7) eine Öffnung aufweist (4) an die sich eine Führung (12) anschließt, an der Flüssigkeiten, insbesondere biologische Flüssigkeit, in die Bodenkammer (3) des Zentrifugierbehälters (1) einströmen, und die Trennwand (7) aus zwei benachbarten Flächen mit Öffnungen besteht, insbesondere in Form von an den Querschnitt des Behälters (1) angepassten, abgeflachten Scheiben mit kreisähnlichem Querschnitt, deren Flächen beweglich miteinander verbunden sind, frei zueinander positionierbar sind und ein Verschließen ermöglichen NS e Öffnung (4) Lumen.
2. Zentrifugationsbehälter (1) mit Einsatz (6) nach Anspruch 1, **dadurch gekennzeichnet, dass** die Führung (12) eine Spirale, ein Trichter oder vertikale Elemente in Form eines länglichen Zylinders ist.
3. Zentrifugationsbehälter (1) mit Einsatz (6) nach Anspruch 1, **dadurch gekennzeichnet, dass** die obere Kammer (2) zusätzlich eine vertikale Trennwand

(11) oder diese in Teilkammern (10) unterteilende Trennwände aufweist, die jeweils Unterkammern (10) mit einer Öffnung (4, 5).

4. Verfahren zum Trennen einer Fraktion mit einem gewünschten Dichtebereich aus einer Probe enthaltend Fraktionen unterschiedlicher Dichte, insbesondere aus einer biologischen Probe, umfassend:
 - a) Bereitstellen eines Zentrifugationsbehälters (1) nach einem der Ansprüche 1 bis 3 zur Trennung von Flüssigkeiten in Fraktionen des gewünschten Dichtebereiches durch Dichtegradientenzentrifugation, insbesondere Flüssigkeiten, die Suspensionen oder biologische Flüssigkeiten darstellen,
 - b) Füllen der unteren Kammer (3) des Behälters (1) mit einem Medium zur Trennung in einem Dichtegradienten oder Füllen der oberen Kammer (2) des Behälters (1) mit diesem Medium, das dann durch die Öffnung in Trennwand entlang der Führung (12) zur unteren Kammer;
 - c) Eingießen von Trennflüssigkeit mit Fraktionen unterschiedlicher Dichte in die untere Kammer (3) durch Befüllen der oberen Kammer (2) oder mindestens einer Unterkammer (10a, 10b) oder Anbringen der oberen Kammer an der Trennwand, so dass die Flüssigkeit kann durch die Öffnung (4) in der Trennwand an der Führung (12) fließen und an der Oberfläche der Bodenkammer (3) Trennmedium verschütten;
 - d) Zentrifugieren des Behälters (1), bis die Testprobe in Fraktionen unterschiedlicher Dichte getrennt ist.
5. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** nach dem Schritt (b) ein weiterer Schritt oder die Schritte b) folgen, die aus der Zugabe eines zusätzlichen Dichtegradienten-Trennmediums bestehen, wobei die Zugabe der nachfolgenden Medien vom höchsten bis zum geringste Dichte.
6. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** nach Schritt (d) ausgewählte Fraktionen unterschiedlicher Dichte aus der abzutrennenden Flüssigkeit einer Prüfung und Analyse unterzogen werden, einschließlich der Möglichkeit der Fixierung, insbesondere durch das Gefrierverfahren.
7. Verfahren nach Anspruch 6, **dadurch gekennzeichnet, dass** beim Trennen der Fraktion von der Blutflüssigkeit die einzelnen Fraktionen des gewünschten Dichtebereiches verschiedene Blutbestandteile enthalten, darunter: Leukozyten (Lymphozyten und Granulozyten), Thrombozyten, Erythrozyten, Knochenmark Zellen (Megakaryozyten, Erythroblasten), die in Zellhomogenat suspendiert

sind, einschließlich Endothelzellen, Neuronen, Pilze, Viren, Mikropartikel einschließlich Exosomen, Zellfragmente, Zellorganellen einschließlich Kerne, Mitochondrien, Chloroplasten.

5

Revendications

1. Récipient de centrifugation (1) comprenant un insert (6), notamment un tube à essai, pour la séparation de liquides en fractions de la gamme de densité souhaitée par centrifugation à gradient de densité, notamment de liquides constituant des suspensions et/ou des fluides biologiques, l'insert est équipé avec une cloison (7) adaptée pour diviser l'intérieur du conteneur (1) en au moins deux chambres disposées verticalement - la chambre supérieure (2) et la chambre inférieure (3), **caractérisée en ce que** la cloison (7) présente une ouverture (4) auquel se raccorde un guide (12) sur lequel des liquides, notamment du fluide biologique, s'écoulent dans la chambre inférieure (3) du récipient de centrifugation (1), et ladite cloison (7) est constituée de deux surfaces adjacentes percées d'ouvertures, notamment en forme de disques aplatis adaptés à la section transversale du conteneur (1) de section similaire au cercle, les surfaces étant reliées mobiles entre elles, elles peuvent être librement positionnées l'une avec l'autre permettant la fermeture de l'ouverture (4) lumière. 10 15 20 25 30
2. Récipient de centrifugation (1) comportant l'insert (6) selon la revendication 1 **caractérisé en ce que** le guide (12) est une spirale, un entonnoir ou des éléments verticaux en forme de cylindre allongé. 35
3. Récipient de centrifugation (1) comportant l'insert (6) selon la revendication 1 **caractérisé en ce que** la chambre supérieure (2) comporte en outre une cloison verticale (11) ou des cloisons la séparant en sous-chambres (10), chacune des sous-chambres (10) ayant une ouverture (4, 5). 40
4. Procédé de séparation d'une fraction avec une gamme de densité souhaitée à partir d'un échantillon contenant des fractions de densité différente, notamment à partir d'un échantillon biologique, comprenant : 45
 - a) fournir un récipient de centrifugation (1) selon l'une quelconque des revendications 1 à 3 pour la séparation de liquides en fractions de la plage de densité souhaitée par centrifugation à gradient de densité, en particulier de liquides constituant des suspensions ou des fluides biologiques, 50
 - b) remplir la chambre inférieure (3) du conteneur (1) avec un milieu de séparation dans un gradient de densité ou remplir la chambre supérieure (2) du conteneur (1) avec ce milieu, qui s'écoule ensuite par l'ouverture dans le cloison le long du guide (12) jusqu'à la chambre inférieure ; 55
 - c) verser du liquide de séparation contenant des fractions de densité différente dans la chambre inférieure (3) en remplissant la chambre supérieure (2) ou au moins une sous-chambre (10a, 10b) ou en attachant la chambre supérieure à la cloison, de sorte que le liquide peut s'écouler à travers l'ouverture (4) de la cloison sur le guide (12) et se répandre à la surface de la chambre inférieure (3) du média de séparation ;
 - d) centrifugation du récipient (1) jusqu'à ce que l'échantillon d'essai soit séparé en fractions de densité différente.
5. Procédé selon la revendication 4, **caractérisé en ce qu'**après l'étape (b) est suivie d'une ou des étapes supplémentaires b) consistant à ajouter un milieu supplémentaire de séparation à gradient de densité, dans lequel l'ajout de milieux ultérieurs se fait du plus haut au plus densité la plus faible.
6. Procédé selon la revendication 4, **caractérisé en ce qu'**après l'étape (d), des fractions sélectionnées de densité différente du liquide à séparer sont soumises à des tests et analyses, incluant la possibilité d'être fixées, notamment par la méthode de congélation.
7. Procédé selon la revendication 6, **caractérisé en ce que** lorsque la fraction est séparée du liquide sanguin, les fractions individuelles de la gamme de densité souhaitée contiennent divers composants sanguins, notamment : leucocytes (lymphocytes et granulocytes), plaquettes, érythrocytes, moelle osseuse cellules (mégacaryocytes, érythroblastes) en suspension dans un homogénat cellulaire comprenant des cellules endothéliales, des neurones, des champignons, des virus, des microparticules comprenant des exosomes, des fragments cellulaires, des organites cellulaires comprenant des noyaux, des mitochondries, des chloroplastes.

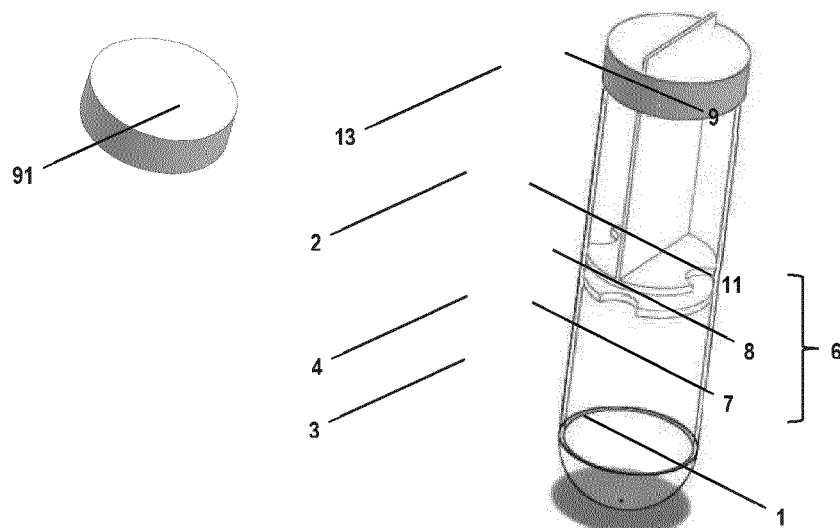


Fig. 1

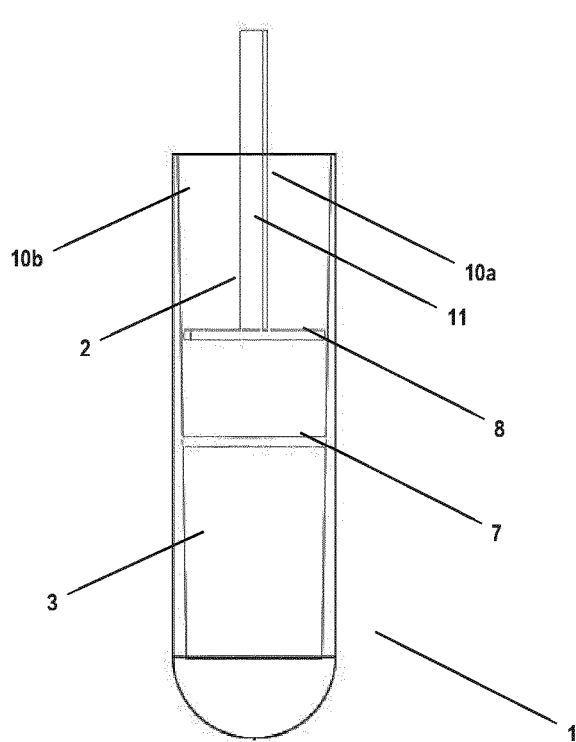


Fig. 2

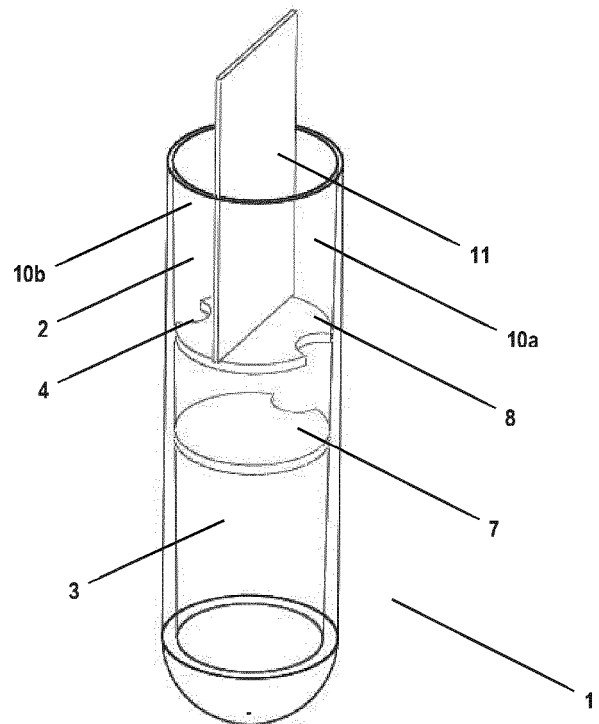


Fig. 3

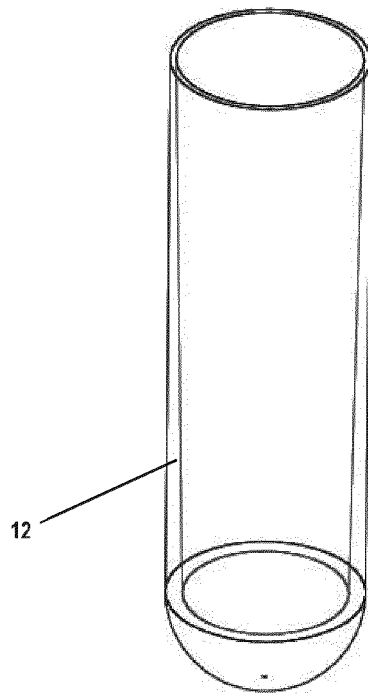


Fig. 4

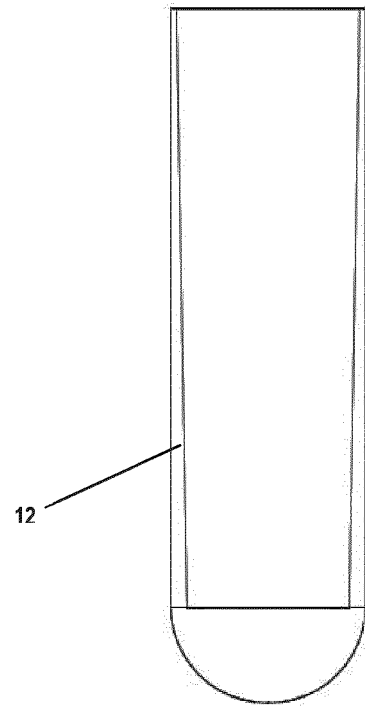


Fig. 5

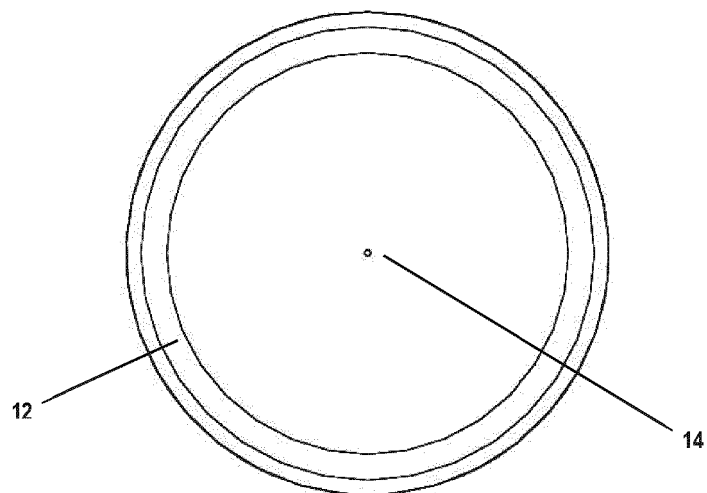


Fig. 6

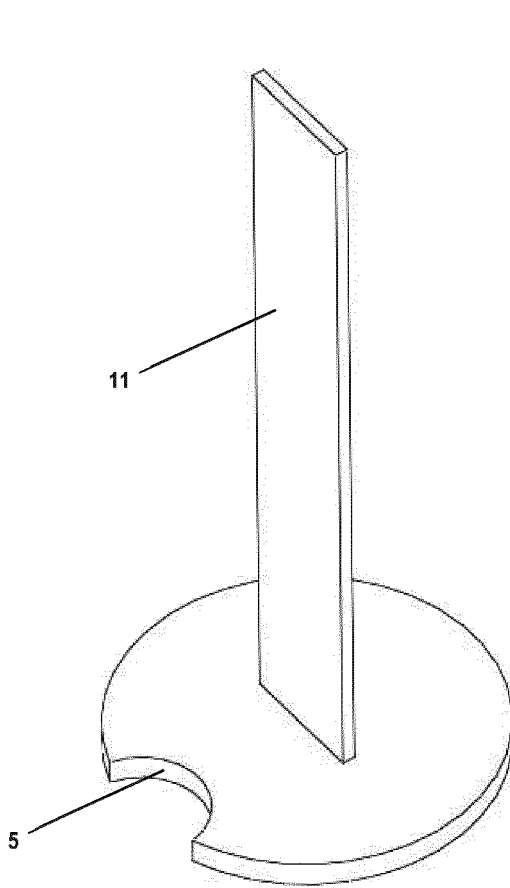


Fig. 7a

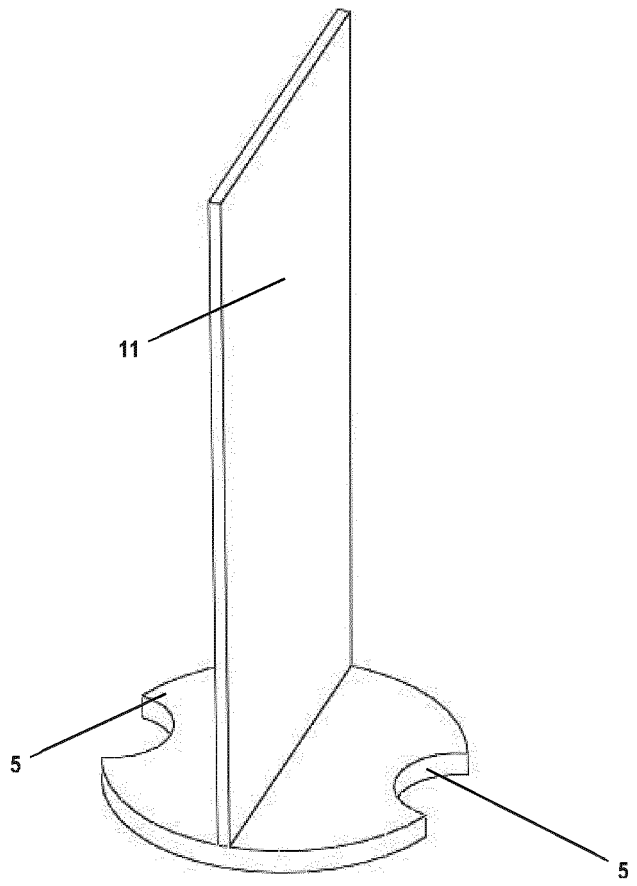


Fig. 8a

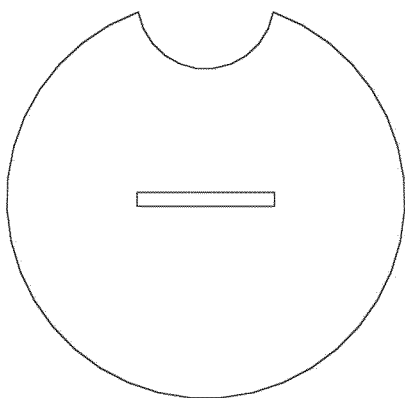


Fig. 7b

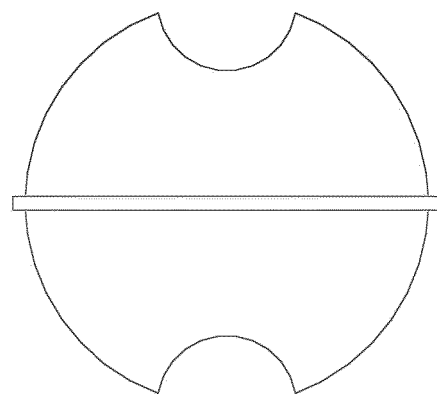


Fig. 8b

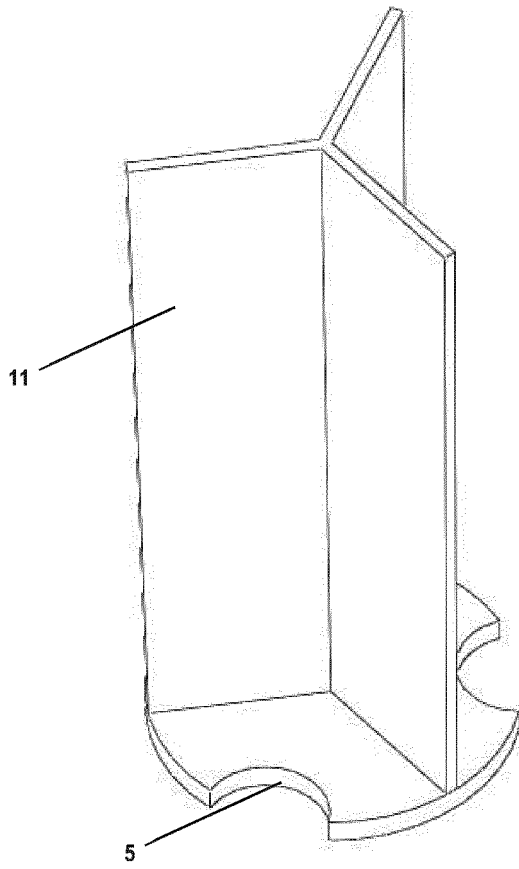


Fig. 9a

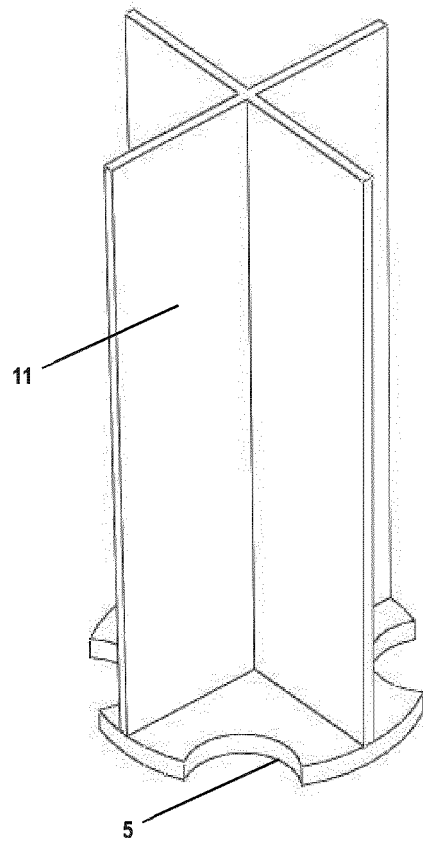


Fig. 10a

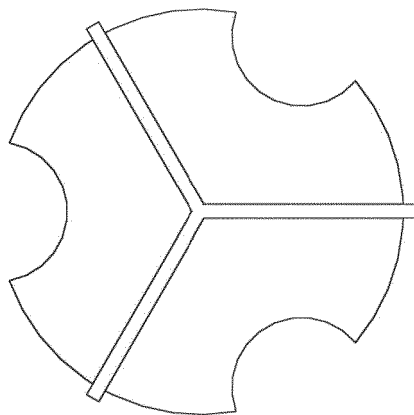


Fig. 9b

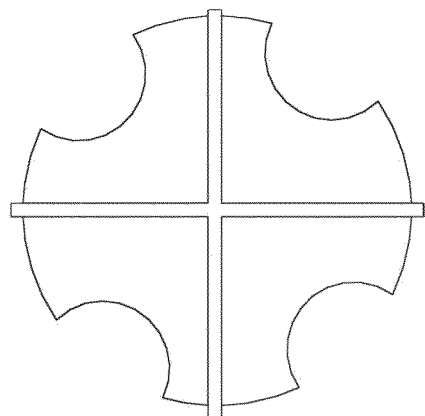


Fig. 10b

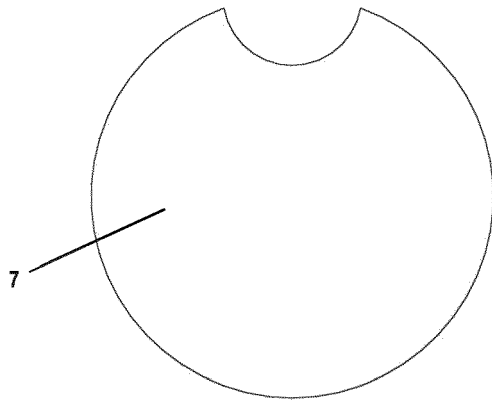


Fig. 11a

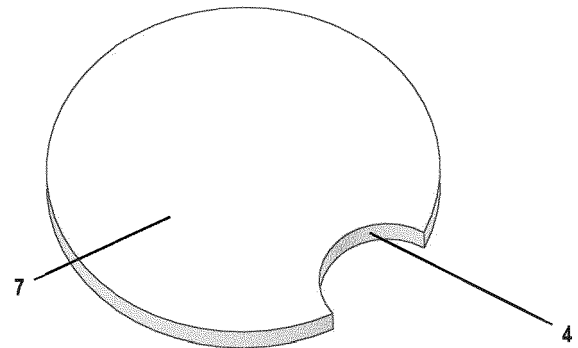


Fig. 11b

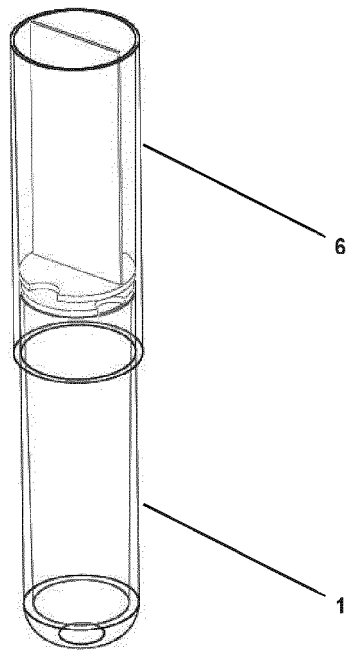


Fig. 12

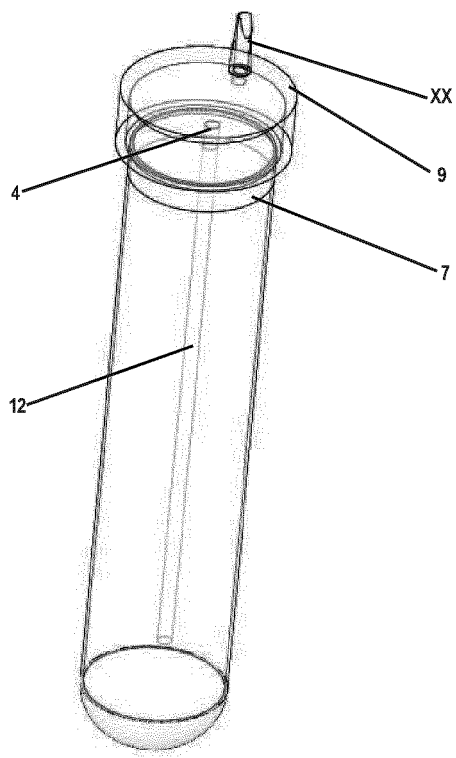


Fig. 13

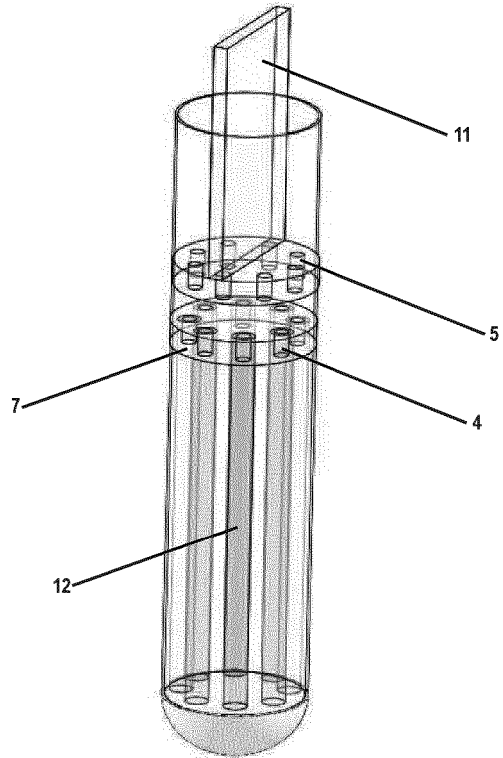


Fig. 14

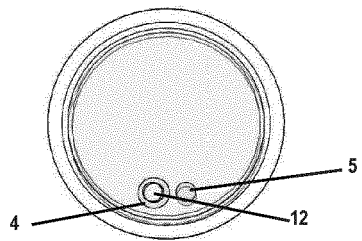


Fig. 13a

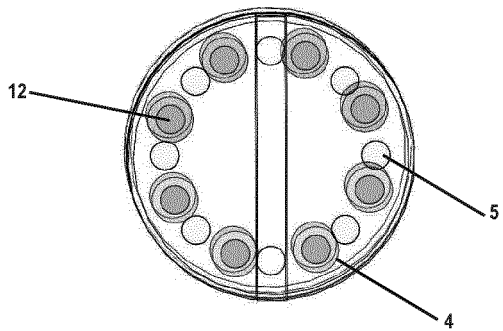


Fig. 14a

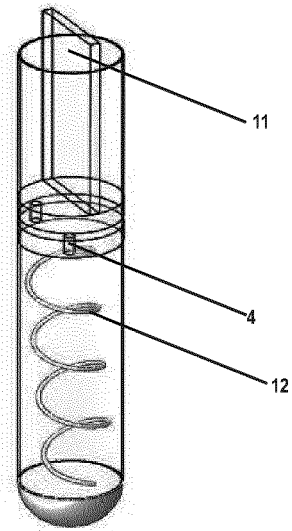


Fig. 15

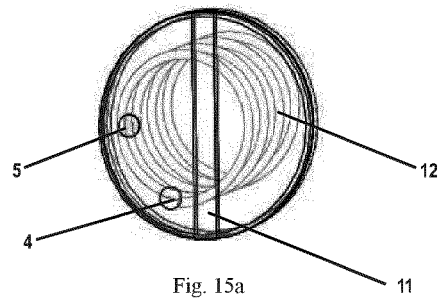


Fig. 15a

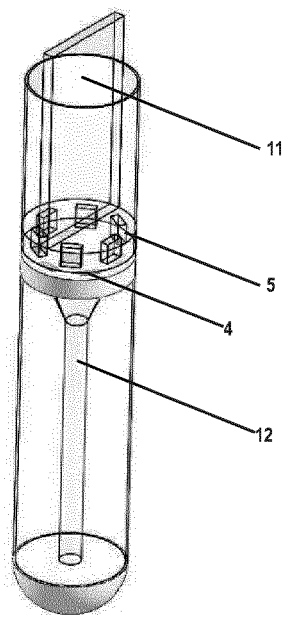


Fig. 16

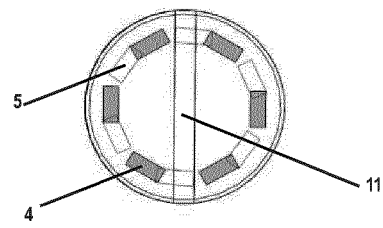


Fig. 16a

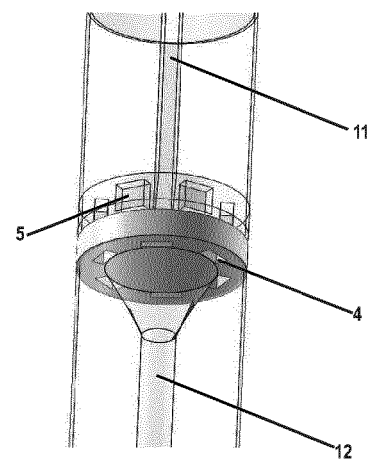


Fig. 16b

REFERENCES CITED IN THE DESCRIPTION

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