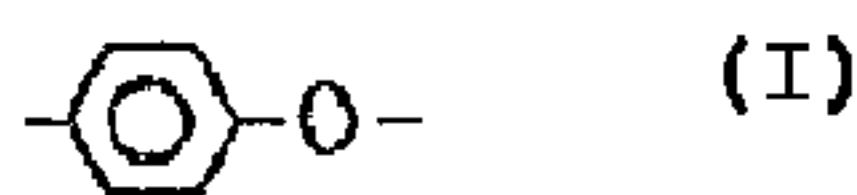
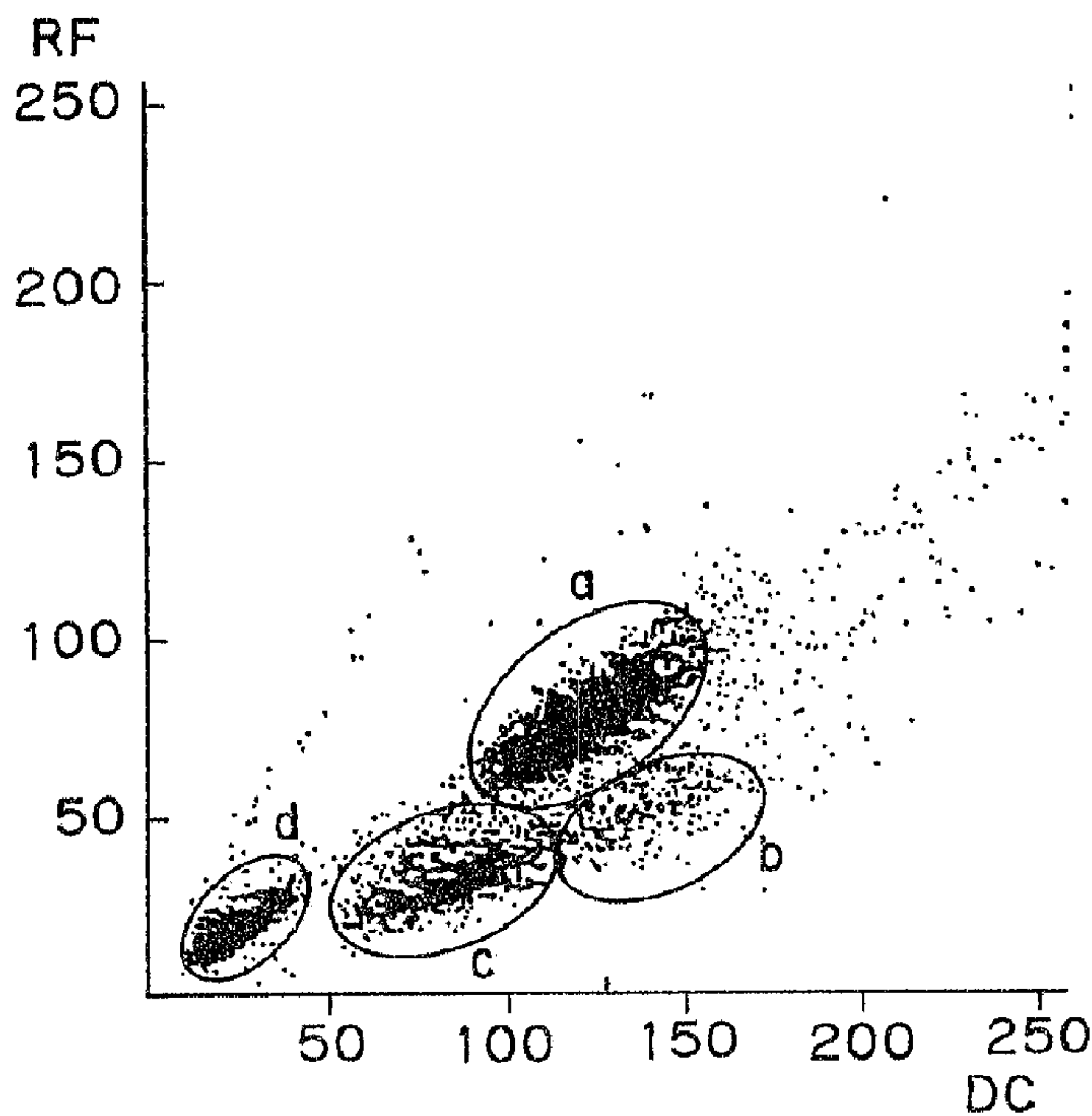




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(54) Titre : METHODE DE CLASSIFICATION DES LEUCOCYTES ET REACTIF UTILISE A CETTE FIN
 (54) Title: METHOD FOR CLASSIFYING LEUKOCYTES AND A REAGENT USED THEREFOR



(57) Abrégé/Abstract:

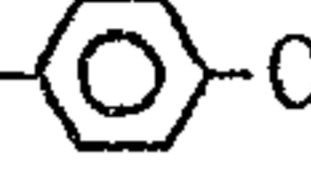
A reagent for enabling leukocytes to be classified and measured by lysing erythrocytes and acting on leukocytes, which has a pH of 1.5 - 5.0, and an osmolarity of 10 - 120 mOsm/kg and contains a polyoxyethylene based nonionic surfactant represented

(57) **Abrégé(suite)/Abstract(continued):**

by the general formula: $R_1-R_2-(CH_2CH_2O)_n-H$ (where R_1 is an alkyl, alkenyl or alkynyl group having 12 - 22 carbon atoms; R_2 is -O-, (see formula I) or -COO-; and n is an integer of 20 - 100) or a reagent for the same purpose, which comprises a first solution which is the reagent described above and a second solution to be added to the first solution, and is a pH of 5.0 - 12.0 and has an osmolarity of 150 - 2000 mOsm/kg after addition of the second solution to the first solution; and methods for classifying leukocytes into at least three types; that is, lymphocytes, monocytes and granulocytes and for detecting abnormal cells by using a reagent described and particulate analysis methods consisting of RF method and DC method are disclosed.

A METHOD FOR CLASSIFYING LEUKOCYTES AND
A REAGENT USED THEREFOR

ABSTRACT OF THE DISCLOSURE:

A reagent for enabling leukocytes to be classified
5 and measured by lysing erythrocytes and acting on leuko-
cytes, which has a pH of 1.5 - 5.0, and an osmolarity of
10 - 120 mOsm/kg and contains a polyoxyethylene based
nonionic surfactant represented by the general formula:
R₁-R₂-(CH₂CH₂O)_n-H (where R₁ is an alkyl, alkenyl or alkynyl
10 group having 12 - 22 carbon atoms; R₂ is -O-, -O- or
-COO-; and n is an integer of 20 - 100) or a reagent for the
same purpose, which comprises a first solution which is the
reagent described above and a second solution to be added
to the first solution, and is a pH of 5.0 - 12.0 and has
15 an osmolarity of 150 - 2000 mOsm/kg after addition of the
second solution to the first solution; and methods for
classifying leukocytes into at least three types; that is,
lymphocytes, monocytes and granulocytes and for detecting
abnormal cells by using a reagent described and particulate
20 analysis methods consisting of RF method and DC method are
disclosed.

A METHOD FOR CLASSIFYING LEUKOCYTES AND
A REAGENT USED THEREFOR

The present invention relates to methods for classifying blood cells in the practice of clinical testing and, more particularly, to methods for classifying, discriminating and counting leukocytes in a blood sample and reagents used in the methods.

Leukocytes in the peripheral blood of normal subjects can be classified as being of five types consisting of lymphocytes, monocytes, neutrophils, eosinophils, and basophils. Different leukocyte types have different functions and counting of leukocytes in the blood according to their type will provide valuable information for diagnostic purposes. For instance, an increase in the number of neutrophils is associated with such diseases as inflammations, myocardial infraction and leukemia, and a decrease in their number is associated with viral diseases, hypoplastic anemia, agranulocytosis, etc. On the other hand, an increase in the number of eosinophils is found in such diseases as parasitosis, Hodgkin's disease and allergosis. An increased number of monocyte occurs either during the convalescence period of patients suffering from infectious diseases or in such diseases as monocytic leukemia.

Lymphocytes, monocytes neutrophils, eosinophils and basophils are normal cells present in blood. In certain blood diseases, however, cells other than these cells, that is, abnormal cells appear in the peripheral blood. For example, blast cell appear in the peripheral blood of a patient with leukemia.

Since abnormal cells include various different kinds of cells, it is clinically important to classify them and to count cells belonging to each abnormal cell group.

Classification and counting of leukocytes have been made most commonly by the differential counting method which is also referred to as the visual counting method which is also referred to as the visual counting method or simply as the manual method. In this method, a blood sample is spread on a glass slide and the blood cells in the smear are fixed

and stained for examination by microscopy. The technician identifies the type of individual leukocytes according to their morphological features (e.g., their size, the morphology of their nucleus and cytoplasm, and the presence or absence of granules) or the degree of dye uptake and performs classification and counting of them. Under ordinary circumstances, 100 - 200 leukocytes are usually counted for each sample and the percentage of the total leukocyte count occupied by each type of cell is recorded as a measured value.

The differential counting method has several disadvantages. First, microscopic observation must be preceded by cumbersome procedures for preparing a specimen that involve such steps as smearing a blood sample on a glass slide, fixing the cells and staining them. Secondly, it is difficult for the technician to identify subtle differences between cells by microscopic classification and counting. Thirdly, it is difficult even for a skilled technician to yield consistent counts by the manual methods since aside from the small number of leukocytes computed, the smeared sample often has an uneven distribution of blood cells.

Various methods have been proposed for eliminating these disadvantages of the manual method of leukocyte classification by developing automated techniques and such automated techniques may be roughly divided into two types. The first method consists of recording the images of cells with a video camera or some other suitable imaging device and classifying the leukocytes by means of image processing on a computer. The operating principle of this method is similar to that of the conventional visual counting method but primarily due to the existence of many cells that defy classification by processing with a computer, this method has not yet become an ideal alternative to the manual method. Furthermore, this method is not economically feasible since it requires sophisticated equipment which is large and costly.

The other approach toward automatic classification and counting of leukocytes is based on a flow system. In

this method, a blood sample having cells suspended in a diluent is permitted to flow in such a way that the cells will individually (one by one) pass through a constricted detector and leukocyte classification is done by analyzing the signal generated by the detector. This second method of leukocyte counting which makes use of a flow system is further subdivided into two categories.

In a method of the first category, all red cells present in an electrolyte are destroyed with a lyzing agent so that only suspended leukocytes are permitted to flow through an orifice and the change in electrical impedance that occurs at the orifice when each cell passes through it is detected, with the magnitude of the detected signal being used as a basis for classification of leukocytes.

The first category includes a method comprising applying a high-frequency current to the orifice and detecting the change in permittivity brought about at the orifice when the blood cells pass through it. This method is referred to as the "RF method" and its basic principle is disclosed in Japanese Patent No. 508789 and U.S. Patent No. 3,390,326. According to the RF method, structures of blood cells and properties of substances constructing the cells in addition to sizes of blood cells can be determined.

Moreover, in the first category including a method comprising applying a direct current to the orifice and detecting the change in permittivity caused when the blood cells pass through it. This method is referred to as the "DC method" and is disclosed in Japanese Patent No. 217947 and U.S. Patent No. 2,656,508. In the DC method, magnitudes of detected signals are almost relative to volume of blood cells.

An apparatus is disclosed in Japanese Patent No. 785859 and U.S. Patent No. 3,502,974 for classifying populations of different kinds of particulates in a suspension by using the RF method in combination with the DC method. However, in these patent specifications, no suggestion is given for the possibility of classifying and counting populations of different kinds of leukocytes.

A method of the second category is characterized by the use of a flow cytometer that comprises a light source, a flow cell that permits the blood cells in a sample to flow one by one through a constricted channel, a photometric unit
 5 that detects light issuing from each blood cell, and an analyzer for analyzing the detected signals. In this method, the cells in the sample which are stained are illuminated under a light and the fluorescence emitted from the irradiated cells is detected, optionally together with scattered
 10 light with leukocyte classification being made in accordance with the intensity of the detected signals.

Methods belonging to the second category require complicated staining procedures and include many practical problems such as use of a complex and expensive apparatus
 15 including an optical system.

On the other hand, cytolytic agents used in a method of the first category include known saponins or quaternary ammonium salts. Since, however, saponins do not exhibit stable hemolytic activity and quaternary ammonium salts
 20 exhibit strong hemolytic activity which cause significant damage to leukocytes, there has been a problem that leukocytes could be classified into at most three types by using these hemolytic agents.

According to the applicant's invention of
 25 PCT/JP88/00514, neither saponin nor quaternary ammonium salts is used as a cytolytic agent, however, a method for classifying normal leukocytes into five types and classifying abnormal cells comprising using polyoxyethylene nonionic or anionic surfactant to hemolyze erythrocytes and to appropriately injure leukocytes in a short time and analyzing
 30 the results by combining the RF and DC methods of the first category is provided with.

On the other hand, Japanese Patent Public Disclosure No. 134957/88 (EP Patent Publication No. 259833) discloses
 35 a process for classifying leukocytes (a method of the second category) comprising hemolyzing erythrocytes in the first solution which is acidic and hyposmotic, neutralizing the acidity of the first solution and adjusting the osmolarity

5 by using the second solution, and measuring leukocytes by a flow cytometer to classify them. Moreover, WO 88/07187 (published on September 22, 1988) discloses a process (a method of the first category) for classifying leukocytes comprising lyzing erythrocytes in the first solution which is acidic and hyposmotic, neutralizing the acidity of the first solution and adjusting the osmolarity by using the second solution, and classifying and counting leukocytes by using the RF and DC methods in combination.

10 However, ghosts due to erythrocytes (hereinafter referred to as "erythrocyte ghosts") cannot be sufficiently reduced only by hemolyzing erythrocytes in an acidic and hyposmotic solution as conducted in the above two methods. Therefore, when measuring leukocytes by using RF and DC
15 methods, said ghosts and the intensity of the detected signs partially overlap and as a result, cannot be definitely distinguished. Since either of these two methods requires a reagent consisting of two solutions, an automatic blood analyzer wherein such methods are practised necessarily
20 becomes complicated.

Incidentally, although it is described in a reference [Clinical Analysis (Rinshō Kensa) Book, Additional Volume 1, "Pretreatment for Examination of Samples", page 3, published by Kanahara Shuppan] that smear must be prepared within 4
25 hours after blood gathering, smears are often prepared many hours after blood gathering in a big institution such as a clinical examination center. In such cases, injury of leukocytes increases at time passes and particularly neutrophils and monocytes are remarkably injured. Therefore,
30 results of the classification of leukocytes obtained from blood gathered 24 hours previously are largely different from those of leukocytes obtained immediately after blood gathering.



Therefore, there has been a need for a method for
35 accurately classifying leukocytes in blood gathered many hours ago.

The object of this invention is to provide a process wherein neither saponin nor quaternary ammonium is used as

a cytolytic agent but another cytolytic agent exhibiting stable hemolytic activities is used to hemolyze erythrocytes and to stabilize leukocytes in a short time, wherein erythrocyte ghosts can be distinguished with lymphocytes and normal leukocytes are classified into five types and abnormal cells are classified, and wherein accurate values of the classified leukocytes can be obtained for blood gather many hours ago; and a reagent used therein.

The problems mentioned above can be solved by using the reagent of this invention and the process for classifying leukocytes of this invention wherein said reagent is used.

The reagent and the process are as shown in the following:

- 15 (1) A reagent for enabling leukocytes to be classified and measured by lyzing erythrocytes and acting on leukocytes, which has a pH of 1.5 - 5.0, and an osmolarity of 10 - 120 mOsm/kg and contains a polyoxyethylene based nonionic surfactant represented by the general formula:
20 $R_1-R_2-(CH_2CH_2O)_n-H$ (where R_1 is an alkyl, alkenyl or alkynyl group having 12 - 22 carbon atoms; R_2 is -O-, -O- or -COO-; and n is an integer of 20 - 100).
- (2) A reagent for enabling leukocytes to be classified and measured by lyzing erythrocytes and acting on leukocytes, which comprises a first solution which is the reagent claimed in (1) and a second solution to be added to the first solution, and is a pH of 5.0 - 12.0 and has an osmolarity of 150 - 2000 mOsm/kg after addition of the second solution to the first solution.
- 30 (3) The reagent according to (2) wherein the second solution contains a polyoxyethylene based nonionic surfactant represented by the general formula: $R_1-R_2-(CH_2CH_2O)_n-H$ (where R_1 is an alkyl, alkenyl or alkynyl group having 12 - 22 carbon atoms; R_2 is -O-, -O- or -COO-; and n is an integer of 20 - 100).
- 35 (4) The reagent according to (2) wherein the second solution contains at least one solubilizing agent selected from the group consisting of the following five subgroups:

Subgroup 1 (ureas):

- urea
- thiourea
- 1,1-dimethylurea
- 5 ethyleneurea
- methylurethane
- 1,3-dimethylurea
- urethane ($\text{H}_2\text{NCOOC}_2\text{H}_5$)

Subgroup 2:

- 10 N-octyl β -D-glucoside
- CHAPS(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)
- CHAPS0(3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate)
- 15 MEGA8,9,10(octanoyl-, nonanoyl- or decanoyl-N-methylglucamide)
- sucrose monocaprato
- N-formylmethyllleucylalanine

Subgroup 3 (guanidines):

- 20 guanidine thiocyanate
- guanylguanidine
- guanidine hydrochloride
- guanidine rhodanate
- guanidine nitrate
- 25 1,1,3,3-tetraguanidine
- guanidine carbonate
- guanidine phosphate
- guanidine sulfate

Subgroup 4 (cholic acids):

- 30 sodium deoxycholate
- taurocholic acid
- cholic acid

Subgroup 5 (halogen substituted acetic acids):

- sodium trichloroacetate
- 35 sodium tribromoacetate
- sodium dichloroacetate
- sodium dibromoacetate
- sodium monochloroacetate

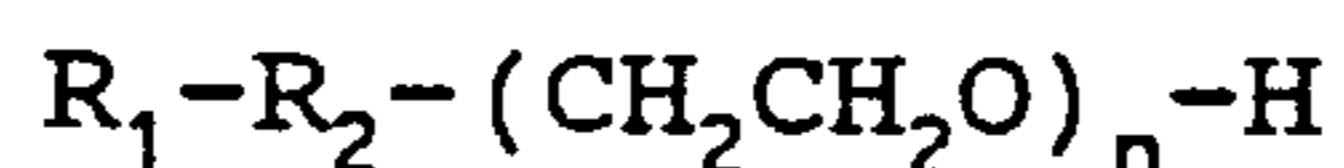
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sodium monobromoacetate.

- (5) A method for classifying leukocytes into at least three types; that is, lymphocytes, monocytes and granulocytes by using a reagent described in any one of (1) - (4) and particulate analysis methods consisting of RF method and DC method.
- (6) A method for detecting abnormal cells by using a reagent described in any one of (1) - (4) and particulate analysis methods which are consisting of RF method and DC method.

In another aspect, the present invention provides a kit for enabling leukocytes to be classified and counted by lysing erythrocytes and acting on leukocytes, which comprises:

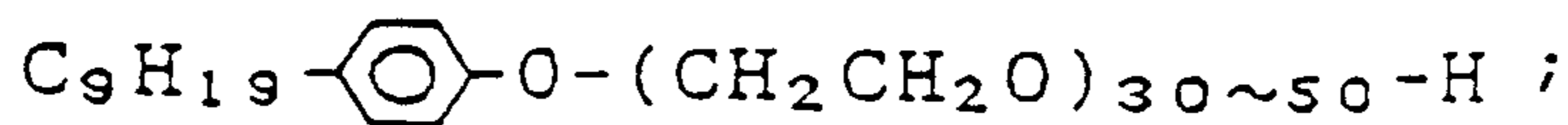
- (i) a first solution having a pH of 1.5-5.0, and an osmolarity of 10-120 mmol/kg (10-20 mOsm/kg) and containing a polyoxyethylene based nonionic surfactant represented by the general formula:



wherein R_1 is an alkyl, alkenyl or alkynyl group having 12-22 carbon atoms; R_2 is -O-,



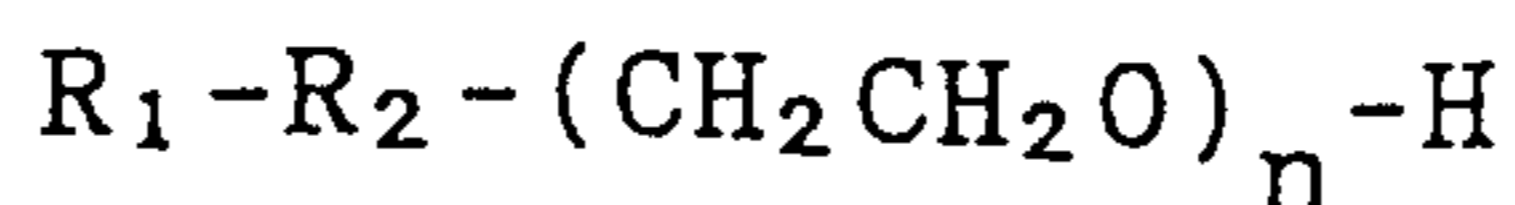
or -COO-; and n is an integer of 20-100; or by the formula

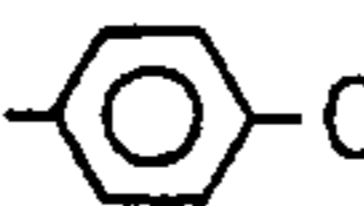


and

- (ii) a second solution containing a buffer and an osmolarity adjusting agent, which after addition to the first solution, results in a mixture having a pH of 5.0-12.0 and an osmolarity of 150-2000 mmol/kg (150-2000 mOsm/kg).

In yet another aspect, the present invention provides a reagent for enabling leukocytes to be classified into at least lymphocytes, monocytes and granulocytes and measured by lysing erythrocytes and acting on leukocytes, which has a pH of 1.5 - 5.0, and an osmolarity of 10 - 120 mOsm/kg and contains a polyoxyethylene based nonionic surfactant represented by the general formula:



(where R_1 is an alkyl, alkenyl or alkynyl group having 9 - 22 carbon atoms; R_2 is -O-, -O- or -COO-; and n is an integer of 20 - 100).

It is well known that erythrocytes are hemolyzed in a hyposmotic solution but the process of this invention is characterized in that erythrocytes can be hemolyzed under hyposmotic (10 - 120 mOsm/kg) and acidic conditions in a solution containing a nonionic surfactant.

If the solution is neutralized, damage to leukocytes becomes severer and erythrocyte ghosts do not sufficiently decrease. On the other hand, if the solution is made alkaline, damage to leukocytes becomes very severe. Therefore, it is essential to hemolyze erythrocytes under acidic condition. Erythrocyte ghosts cannot be decreased only when the acidic and hyposmotic conditions are combined.

Fig. 1 is a chart showing distribution of results obtained by measuring leukocytes immediately after blood gathering in the working examples concerning the reagent consisting of one solution.

Fig. 2 is a chart showing distribution of results obtained by measuring leukocytes 24 hours after blood gathering in the working examples concerning the reagent consisting of one solution.

Fig. 3 is a chart showing distribution of results obtained by measuring leukocytes immediately after blood gathering in the working examples concerning the reagent consisting of two solutions.

Fig. 4 is a chart showing distribution of results obtained by measuring leukocytes 24 hours after blood gathering in the working examples concerning the reagent consisting of two solutions.

Fig. 5 is a chart indicating distribution of results obtained by measuring blood of a subject suffering from acute myelocytic leukemia in the working examples wherein the reagent consisting of two solutions.

5 Figs. 6 and 7 are charts showing distributions of results obtained by measuring blood merely hemolyzed in an acidic and hyposmotic solution in the reference example. Fig. 6 is a chart relating to measurement immediately after blood gathering and Fig. 7 is one relating to measurement
10 24 hours after blood gathering.

Fig. 8 is a chart as a reference indicating distribution wherein the addition number (n) of polyethylene is less than that defined in this invention (n=10).

This invention is illustrated in the following
15 examples but it should not be intended to be limited to them.

Reference Example

Erythrocytes in a blood sample were hemolyzed and leukocytes in the sample were determined by using a reagent
20 solution wherein the pH was kept at 2.0 and the osmolarity was adjusted to 80 mOsm/kg. The results are indicated in Fig. 6. The transversal axis of the two dimensional distribution shown in Fig. 6 indicates relative signal strength determined by the DC method and the longitudinal axis of
25 said distribution indicates relative signal strength determined by the RF method. Each spot given in Fig. 6 corresponds to each cell which showed each DC signal strength and each RF signal strength.

The signs a - d in Fig. 6 have the following means:
30 a: granulocytes; b: monocytes; c: lymphocytes;
d: erythrocyte ghosts.

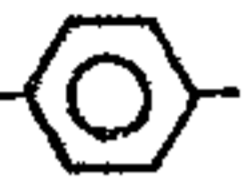
Incidentally, the longitudinal and transversal axes and the signs given in the other figures have the same meanings as in Fig. 6.

35 Fig. 6 shows the results obtained from blood immediately after gathering. In this figure, distribution of erythrocyte ghosts d and that of lymphocytes c are overlapped in part and distributions of granulocytes a,

monocytes b and lymphocytes c are not good in separation (are poorly separated).

Fig. 7 indicates the results obtained by determination of blood 24 hours after gathering but by using the same reagent as Fig. 6. Distributions of the three kinds of leukocytes are more poorly separated from each other.

Example 1

The results obtained when determining blood immediately after gathering by using the reagent claimed in Claim 1 are indicated in Fig. 1. The pH of the solution was 2.0 and the osmolarity thereof was 80 mOsm/kg. As a polyoxyethylene based nonionic surfactant 1.5 g/l of Emulsit 9 [C₉H₁₉--O-(CH₂CH₂O)_{30~50}-H; manufactured by Daiichi Kogyo Seiyaku Kabushiki Kaisha] was used. Distribution of erythrocyte ghosts d and that of lymphocytes c are clearly separated without overlapping and distributions of granulocytes a, monocytes c are well separated.

Example 2

Fig. 2 indicates the results obtained when determining blood 24 hours after gathering by using the same reagent as Fig. 1. As shown in Fig. 2, distributions of the three kinds of leukocytes and distribution of erythrocyte ghosts are well separated. Thus, use of the reagent claimed in Claim 1 can make erythrocyte ghosts well minimize and can accomplish a stable counting and classification of leukocytes in a blood sample gathered a long time ago.

Fig. 8 indicates the results obtained by determining the same blood as in Fig. 1 by using as a polyoxyethylene based nonionic surfactant Actinol L10 [C₁₂H₂₅-O-(CH₂CH₂O)₁₀-H; manufactured by Matsumoto Yushi Seiyaku Kabushiki Kaisha] in place of Emulsit 9. According to Fig. 8, classification and counting of leukocytes became impossible because not only erythrocytes but also leukocytes destroyed or remarkably contracted. As is already known, the smaller the addition mole number of polyoxyethylene is, the stronger the hemolytic activity thereof is. Actinol L10 wherein n is 10 destroyed or contracted not only erythrocytes but also leukocytes.

On the contrary, the larger the addition mole number (n) of polyoxyethylene of the surfactant is, the weaker or zero its hemolytic activity becomes. The surfactant wherein n is large has so weak hemolytic activity that damage to leukocytes is remarkably reduced. Therefore, use of such a surfactant makes possible classification and counting of leukocytes even in blood which was gathered a long time ago. On the other hand, if hemolytic activity of the surfactant is too weak, erythrocyte ghosts cannot be sufficiently reduced so that the ghosts cannot be well distinguished with lymphocytes.

However, surprisingly the inventors found that the erythrocyte ghosts can be sufficiently reduced without causing undesirable leukocytes damage by using the nonionic surfactant wherein the addition mole number of polyoxyethylene is 20 - 100.

Moreover, the inventors also found that the surfactant protects leukocyte membranes and can provide definite separation into three groups of leukocytes: granulocytes a; monocytes b; and lymphocytes c on a two dimensional distribution chart.

An example of said protecting effects is given below:

When hemolyzation was conducted in an acidic and hyposmotic solution, leukocytes are damaged and contracted in about 5 minutes. But when a nonionic surfactant wherein the addition mole number of polyoxyethylene is 20 - 100 is added to the solution, leukocytes do not contract even after 20 minutes has passed.

As indicated above, the invention of Claim 1 can be practised in the form of one solution. One-solution type reagent is better than the conventional two-solution type reagent in using in an automatic blood analyzer because the construction of the apparatus can be simplified and then cost thereof can be reduced.

This invention, however, is not limited only to a one-solution type reagent. Reagents of the two-solution type are claimed in Claims 2 - 4 and described as follows:

The first solution included in the two-solution type

reagent is identical to the reagent claimed in Claim 1 and is reacted with blood to make erythrocytes hemolyze under an acidic and hyposmotic condition with leukocytes being injured. The second solution is added to the reaction solution containing the first solution and the blood, whereby pH and osmolarity of the resulting mixture are adjusted to neutral (pH 5.0 - 12.0) and equivalent to high osmolarity (150 - 2000 mOsm/kg). Thus, the second solution has effects of adjustment of pH and osmolarity of the reaction solution. By adding the second solution, separation of groups of lymphocytes, monocytes and granulocytes is more improved, whereby analysis of a two-dimensional distribution by a computer to fractionate the distribution into each group is made easy.

Incidentally, separation of said groups can be still more improved by incorporating a polyoxyethylene based non-ionic surfactant claimed in Claim 3 and a solubilizing agent claimed in Claim 4.

Example 3

Reaction of the two-solution is illustratively described below.

Preparation of the first solution

To 1 ℓ of a solution kept at pH 2.0 and adjusted to 80 mOsm/kg with a potassium chloride, hydrochloric acid buffer 1.5 g of Emulsit 9 was added.

Preparation of the second solution

2 ℓ of a sodium primary phosphate/sodium secondary phosphate buffer was added to 100 g of formalin. After the resulting solution was adjusted to pH 7.5 and 1100 mOsm/kg, 2.5 g of β-phenetyl alcohol as a preservation, 10 g of triethanolamine as an antioxidant and 0.025 g of Emulgen 420 [CH₃(CH₂)₇CH=CH(CH₂)₈-O-(CH₂CH₂O)₁₃H; a polyoxyethylene based nonionic surfactant manufactured by Kao Kabushiki Kaisha] as an agent for constructing erythrocyte ghosts were added to said solution.

Reaction Condition

To 5 ml of the first solution 60 μℓ of blood was added and the mixture was reacted at 33°C for 30 seconds.

Thereafter, the second solution was added to the mixture and the resulting mixture was reacted at 33°C for 30 seconds.

The two dimensional distribution obtained by subjecting blood to the above two step reaction immediately after blood gathering are indicated in Fig. 3. As shown in Fig. 3, erythrocyte ghosts d were sufficiently contracted and granulocytes a, monocytes b and lymphocytes c were well separated. The sign g represents a platelet aggregation.

Fig. 4 indicates the results obtained by determining in the same manner as in Fig. 3 the same blood as in Fig. 3 except that 24 hours passed after blood gathering. As clearly shown in Fig. 4, even if determination was carried out 24 hours after blood gathering, substantially the same two dimensional distribution as in Fig. 3 was obtained. This proves that the reagent of this invention could realize a stable determination of blood a long time after gathering. This depends on stabilization of leukocytes with the reagent of this invention.

Leukocytes were classified into three fractions and, as described in the specification of PCT/JP88/00514, a sample for determination of eosinophils and a sample for determination of basophils were prepared to measure number of eosinophils and that of basophils. By the procedures, the leukocytes could be classified into five types, that is, lymphocytes, monocytes, neutrophils, eosinophils and basophils and cells of each type could be counted to obtain total number of cells of each type of leukocytes and ratios thereof. Coefficients of correlation for ratios of five types of leukocytes obtained by comparing the results from determination of 20 blood samples according to the above method with the obtained by measuring 200 cells per each of said samples by visual counting method are indicated in Table 1.

Table 1

lymphocytes	0.987
monocytes	0.900
neutrophils	0.985
eosinophils	0.979
basophils	0.525

In the same manner, blood of a patient with acute myelocytic leukemia (AML) was determined and the results are indicated as a two-dimensional distribution given in Fig. 5.

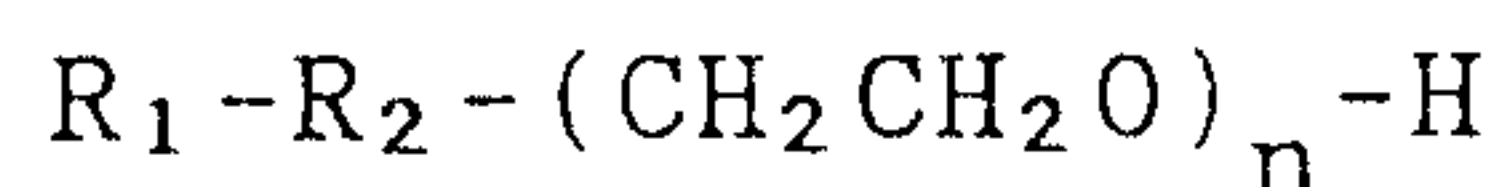
Signs e and f mean leukemia cells and nucleate erythrocytes respectively. In Fig. 5, abnormal cells such as leukemia cells appear at the locations at which no cell is present in Figs. 3 and 4. Thus, according to this invention, not only normal cells but also abnormal cells can be detected.


10 In accordance with the reagent and process of this invention, erythrocytes can be hemolyzed by using a cytolytic agent having a stable hemolytic activity; leukocytes can be stabilized in a short time; and erythrocyte ghosts and lymphocytes can be distinguished clearly; and combina-
 15 tion of the DC method and the RF method can realize classification of leukocytes blood into five types of normal leukocytes and abnormal cells; and accurate classification values can be obtained even for blood tested a long time after being gathered.

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The embodiment of the invention in which an exclusive property or privilege are claimed are defined as follows:

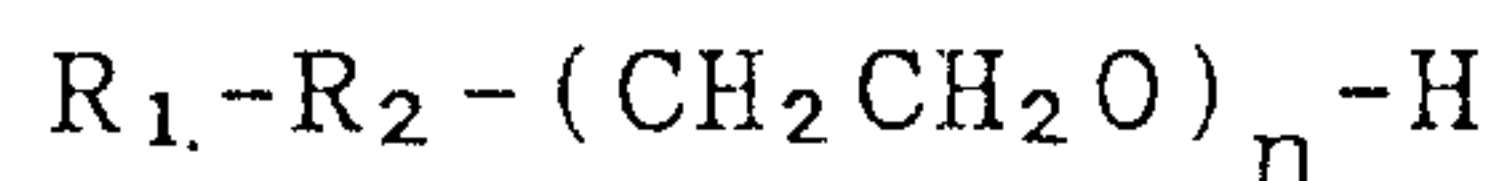
1. A reagent for enabling leukocytes to be classified into at least lymphocytes, monocytes and granulocytes and measured by lyzing erythrocytes and acting on leukocytes, which has a pH of 1.5 - 5.0, and an osmolarity of 10 - 120 mOsm/kg and contains a polyoxyethylene based nonionic surfactant represented by the general formula:




(where R_1 is an alkyl, alkenyl or alkynyl group having 9 - 22 carbon atoms; R_2 is -O-, -O- or -COO-; and n is an integer of 20 - 100).

2. A reagent for enabling leukocytes to be classified and measured by lyzing erythrocytes and acting on leukocytes, which comprises a first solution which is the reagent claimed in Claim 1 and a second solution to be added to the first solution, and has a pH of 5.0 - 12.0 and has an osmolarity of 150 - 2000 mOsm/kg after addition of the second solution to the first solution.

3. The reagent according to Claim 2 wherein the second solution contains a polyoxyethylene based nonionic surfactant represented by the general formula:



(where R_1 is an alkyl, alkenyl or alkynyl group having 12 - 22 carbon atoms; R_2 is -O-, -O- or -COO-; and n is an integer of 20 - 100).

4. The reagent according to Claim 2 wherein the second solution contains at least one solubilizing agent selected from the group consisting of the following five subgroups:

Subgroup 1 (ureas):

urea
thiourea
1,1-dimethylurea
ethyleneurea
methylurethane
1,3-dimethylurea
urethane ($H_2NCOOC_2H_5$)

Subgroup 2:

N-octyl β -D-glucoside

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CHAPS(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)

CHAPSO(3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate)

5 MEGA8,9,10(octanoyl-, nonanoyl- or decanoyl-N-methylglucamide)

sucrose monocaprinate

N-formylmethyllleucylalanine

Subgroup 3 (guanidines):

10 guanidine thiocyanate
 guanylguanidine
 guanidine hydrochloride
 guanidine rhodanate
 guanidine nitrate
 15 1,1,3,3-tetraguanidine
 guanidine carbonate
 guanidine phosphate
 guanidine sulfate

Subgroup 4 (cholic acids):

20 sodium deoxycholate
 taurocholic acid
 cholic acid

Subgroup 5 (halogen substituted acetic acids):

25 sodium trichloroacetate
 sodium tribromoacetate
 sodium dichloroacetate
 sodium dibromoacetate
 sodium monochloroacetate
 sodium monobromoacetate.

30 5. A method for classifying leukocytes into at least three types; that is, lymphocytes, monocytes and granulocytes by using a reagent described in any one of Claim 1 -4 and particulate analysis methods selected from the group consisting of Radio Frequency (RF) method and Direct Current (DC) method.

35 6. A method for detecting abnormal cells by using a reagent described in any one of Claims 1 - 4 and particulate analysis methods selected from the group consisting of Radio Frequency (RF) method and Direct Current (DC) method.

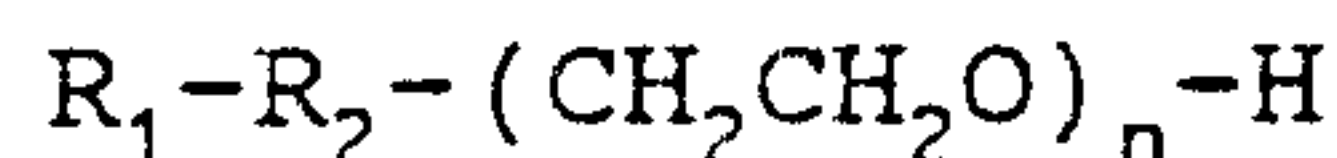
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7. The reagent according to any one of Claims 1 to 4, wherein said R_1 is an alkyl, alkenyl or alkynyl group having 12 - 22 carbon atoms.

5 8. The method according to Claim 5 or 6, wherein said R_1 is an alkyl, alkenyl or alkynyl group having 12 - 22 carbon atoms.

9. A kit for enabling leukocytes to be classified and
10 counted by lyzing erythrocytes and acting on leukocytes, which comprises:

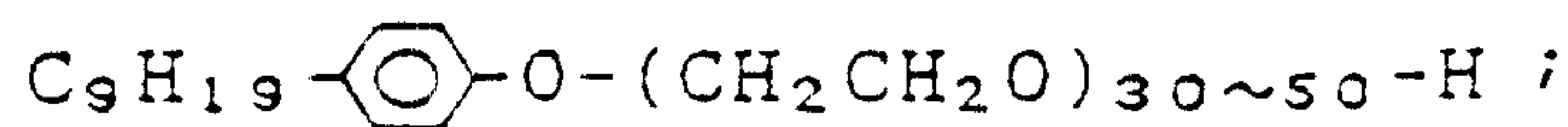
(i) a first solution having a pH of 1.5-5.0, and an osmolarity of 10-120 mmol/kg (10-20 mOsm/kg) and containing a polyoxyethylene based nonionic surfactant
15 represented by the general formula:



wherein R_1 is an alkyl, alkenyl or alkynyl group having
20 12-22 carbon atoms; R_2 is -O-,



or -COO-; and n is an integer of 20-100; or by the
25 formula



and

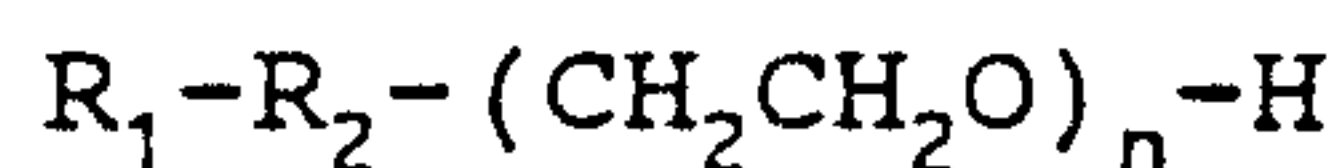
30 (ii) a second solution containing a buffer and an osmolarity adjusting agent, which after addition to the first solution, results in a mixture having a pH of 5.0-12.0 and an osmolarity of 150-2000 mmol/kg (150-2000 mOsm/kg).

35

10. A kit according to Claim 9, wherein the second

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solution additionally contains a polyoxyethylene based nonionic surfactant represented by the general formula:



5

wherein R_1 is an alkyl, alkenyl or alkynyl group having 12 - 22 carbon atoms; R_2 is -O-



10

or -COO-; and n is an integer of 20 - 100.

11. A kit according to Claim 9, wherein the second solution contains at least one solubilizing agent selected from the group consisting of the following five subgroups:

-Subgroup 1 (ureas):

20 urea
 thiourea
 1,1-dimethylurea
 ethyleneurea
 methylurethane
 25 1,3-dimethylurea
 urethane ($H_2NCOOC_2H_5$)

Subgroup 2:

30 N-octyl β -D-glucoside
 CHAPS(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)
 CHAPSO(3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate)
 35 MEGA8,9,10(octanoyl-, nonanoyl- or decanoyl-N-methylglucamide)

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sucrose monocaprato
 N-formylmethyllleucylalanine

Subgroup 3 (guanidines):

5

guanidine thiocyanate
 guanylguanidine
 guanidine hydrochloride
 guanidine rhodanate
 10 guanidine nitrate
 1,1,3,3-tetraguanidine
 guanidine carbonate
 guanidine phosphate
 guanidine sulfate

15

Subgroup 4 (cholic acids):

sodium deoxycholate
 taurocholic acid
 20 cholic acid

Subgroup 5 (halogen substituted acetic acids):

sodium trichloroacetate
 25 sodium tribromoacetate
 sodium dichloroacetate
 sodium dibromoacetate
 sodium monochloroacetate
 sodium monobromoacetate.

30

12. A method for classifying leukocytes into at least three
 types; that is, lymphocytes, monocytes and granulocytes by
 using a kit according to any of Claims 9 to 11 and
 particulate analysis methods selected from the group
 consisting of Radio Frequency (RF) method and Direct Current
 35 (DC) method.

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13. A method for detecting abnormal cells by using a kit according to any of Claims 9 to 11 and particulate analysis methods selected from the group consisting of Radio Frequency (RF) method and Direct Current (DC) method.

14. A method for classifying leukocytes into at least three types; that is, lymphocytes, monocytes and granulocytes, which comprises the steps of:

- (a) classifying leukocytes into the at least three types of leukocytes by using a reagent described in any one of Claims 1 - 4, and
- (b) counting each type of the leukocytes by particulate analysis methods selected from the group consisting of Radio Frequency (RF) method and Direct Current (DC) method.

15. A method for detecting abnormal cells, which comprises the steps of:

- (a) treating a blood sample with a reagent described in any one of Claims 1 - 4, and
- (b) detecting populations of abnormal cells by particulate analysis methods selected from the group consisting of Radio Frequency (RF) method and Direct Current (DC) method.

16. A method for classifying leukocytes into at least three types; that is, lymphocytes, monocytes and granulocytes, which comprises the steps of:

- (a) classifying leukocytes into the at least three types of leukocytes by using a kit described in any one of Claims 9 - 11, and
- (b) counting each type of the leukocytes by particulate analysis methods selected from the group consisting of Radio Frequency (RF) method and Direct Current (DC) method.

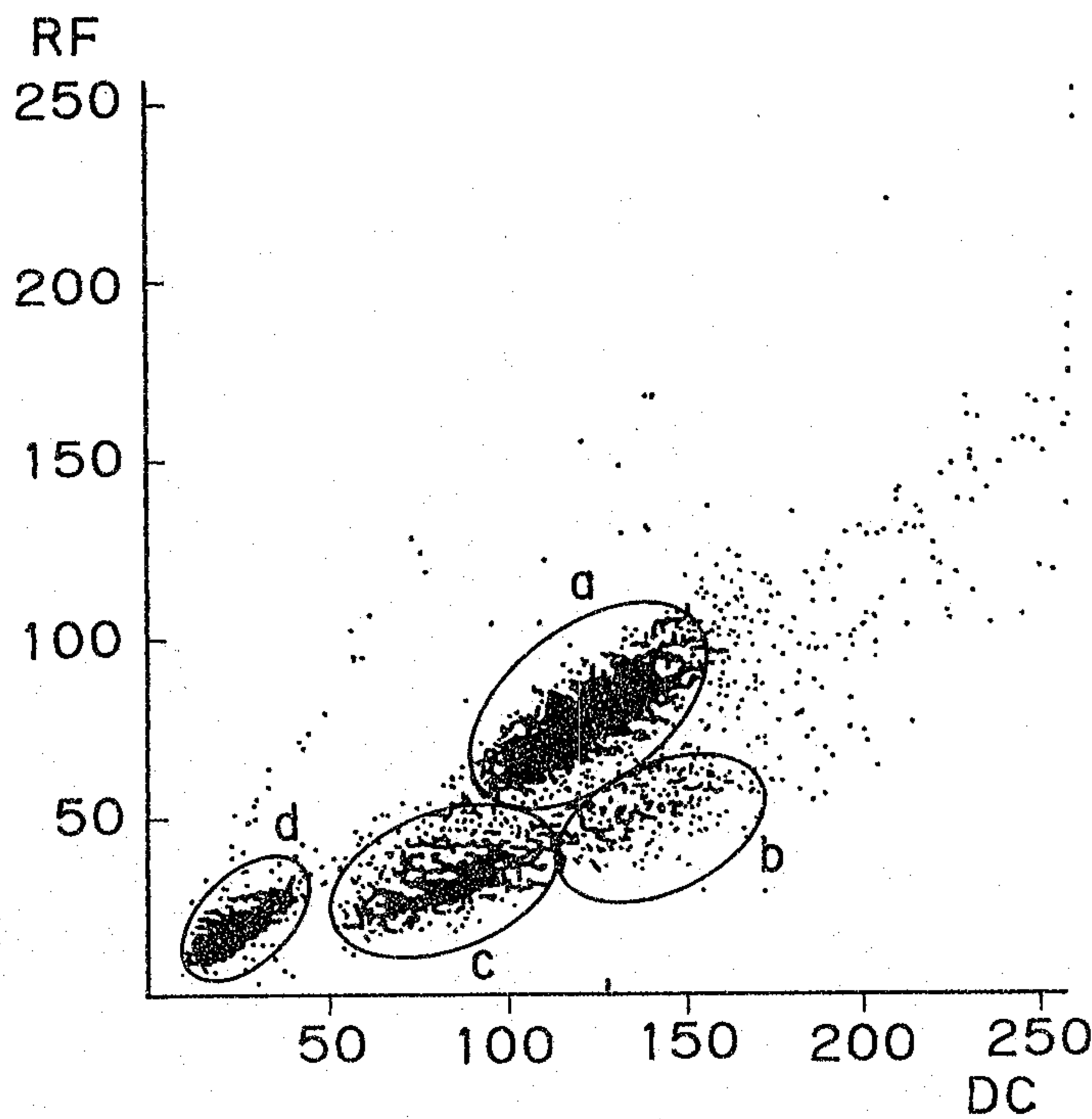
17. A method for detecting abnormal cells, which comprises the steps of:

- (a) treating a blood sample with a reagent included in a kit described in any one of Claims 9 - 11, and

-21-

(b) detected populations of abnormal cells by particulate analysis methods selected from the group consisting of Radio Frequency (RF) method and Direct Current (DC) method.

Fig. 1

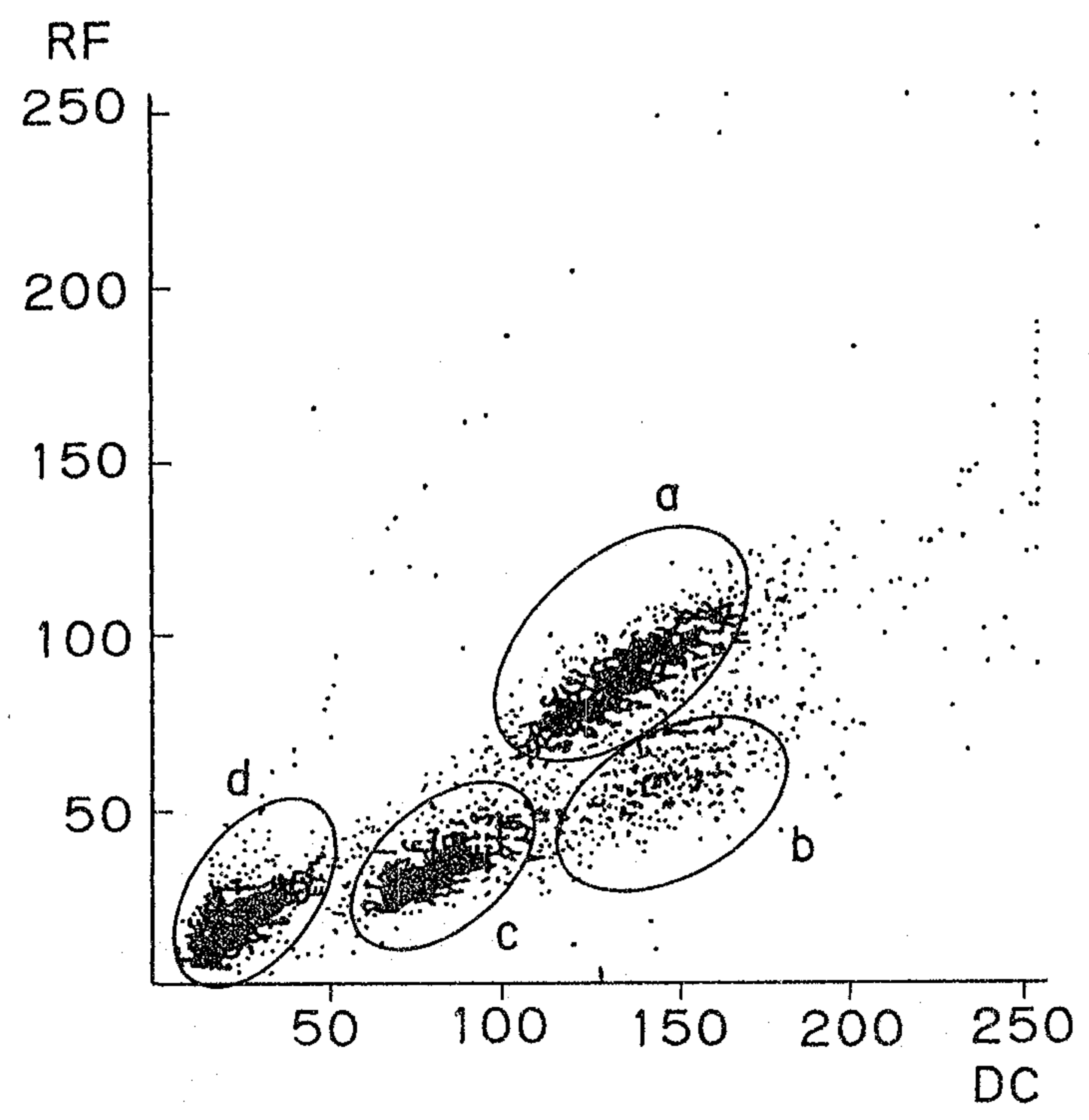


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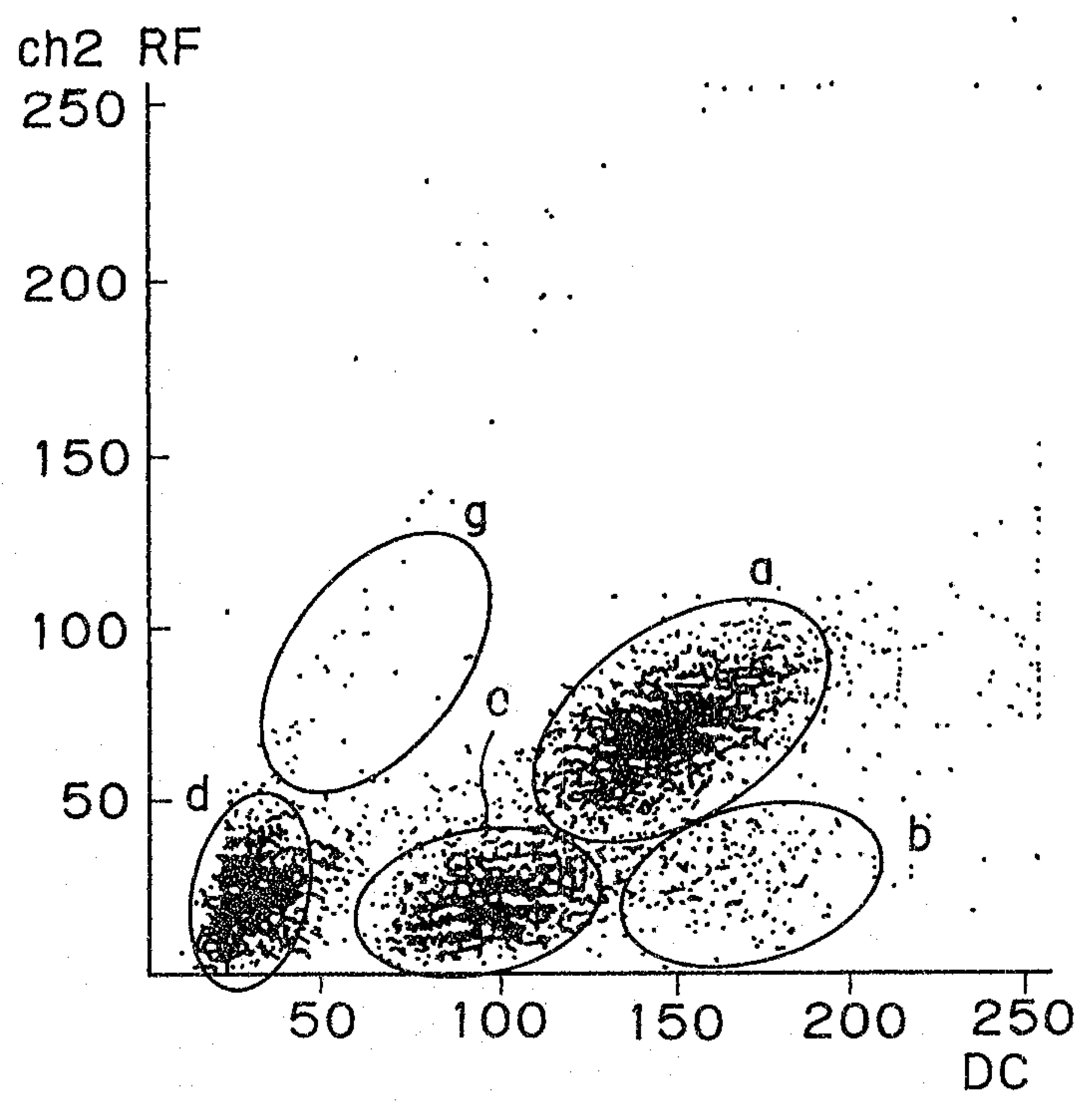
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Fig. 2



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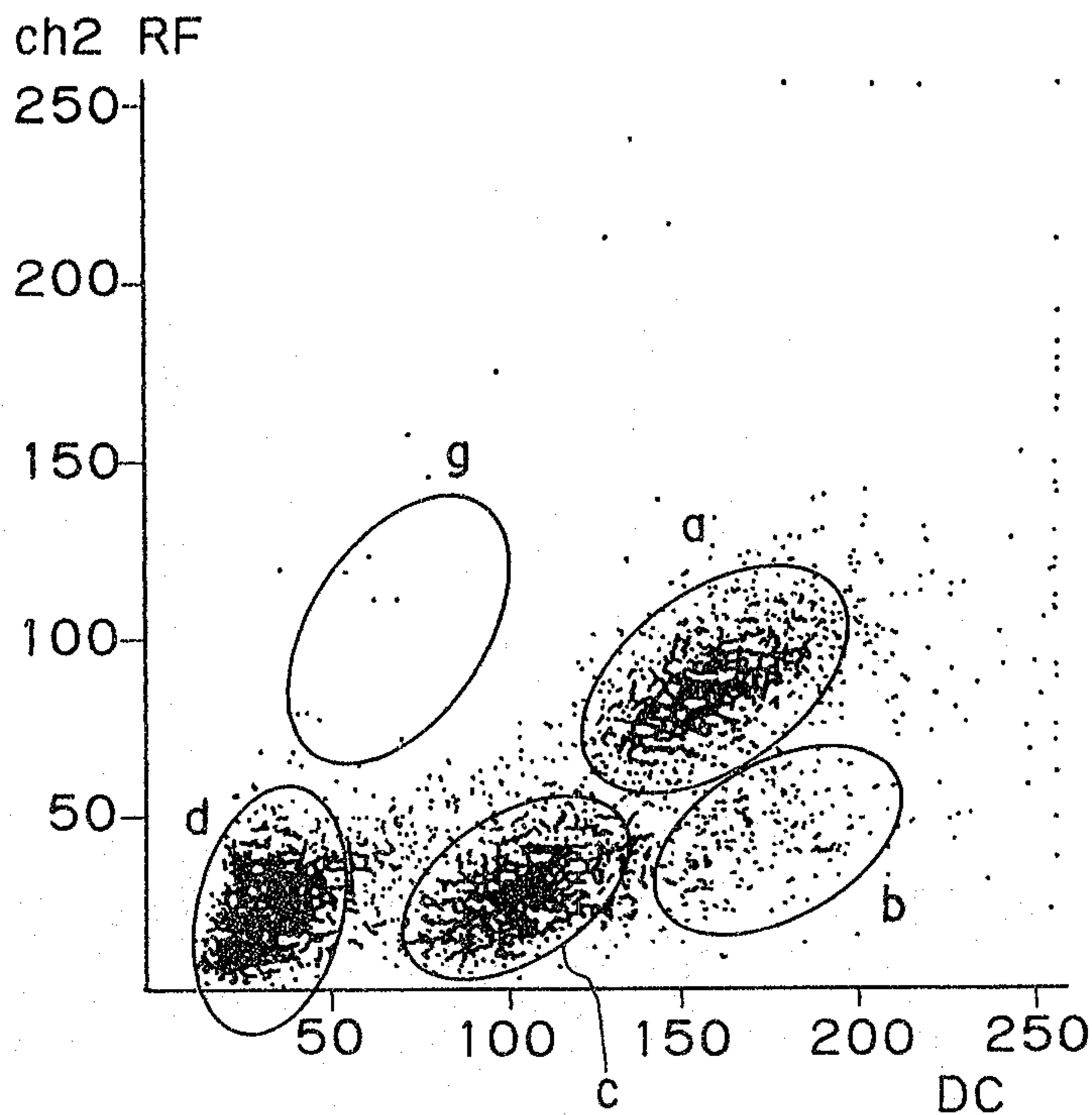
Fig. 3



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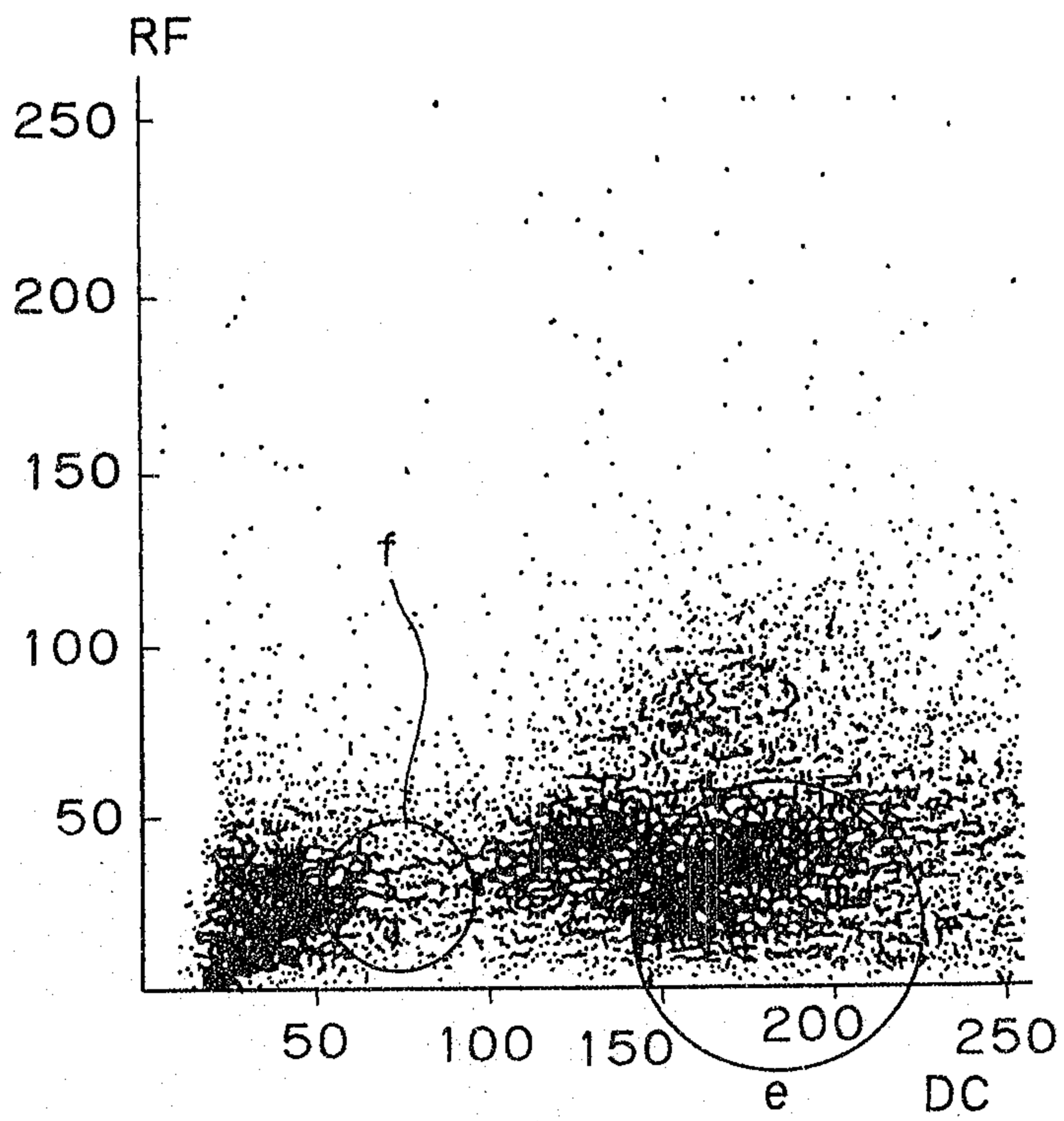
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Fig. 4



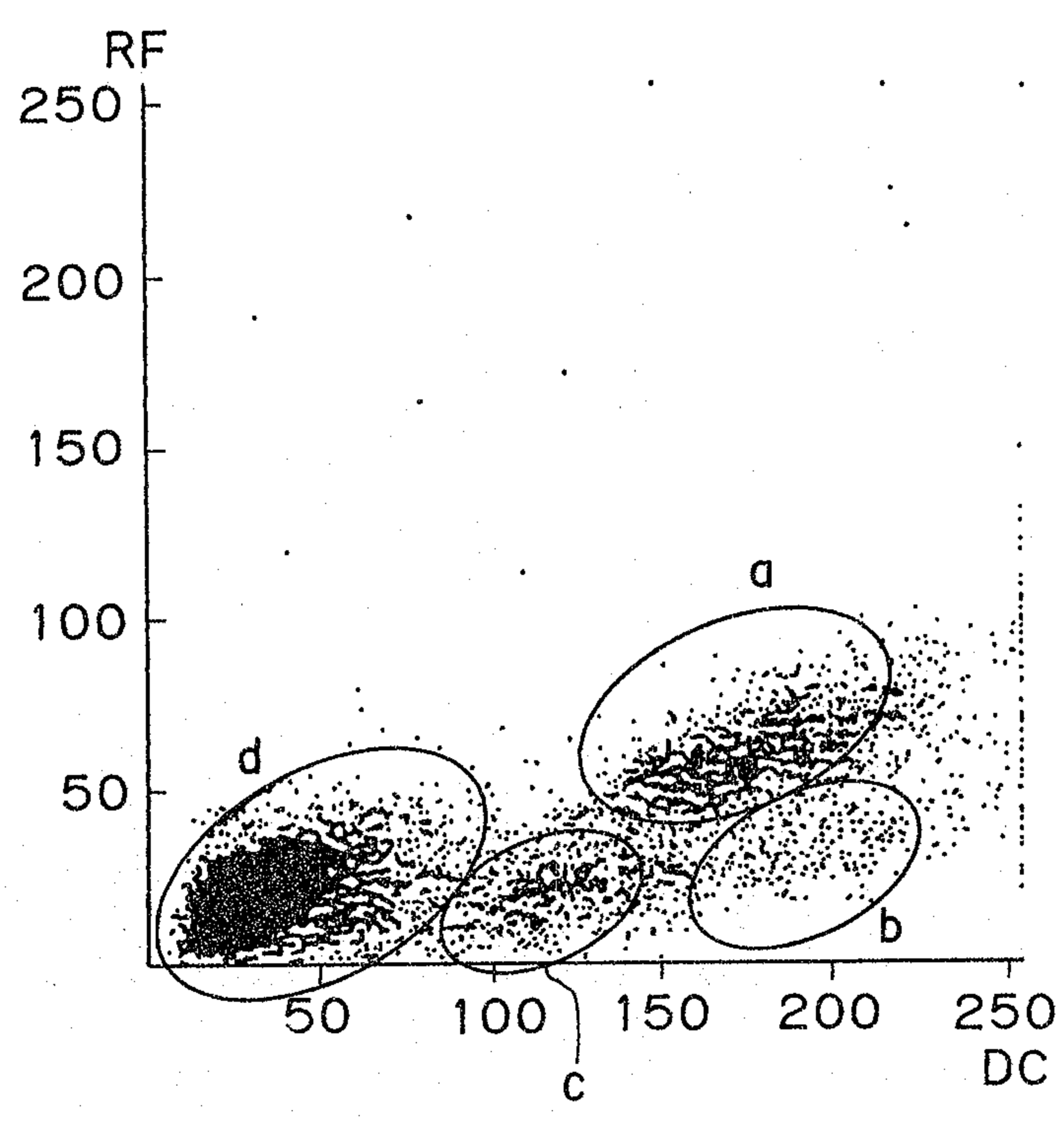
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Fig. 5



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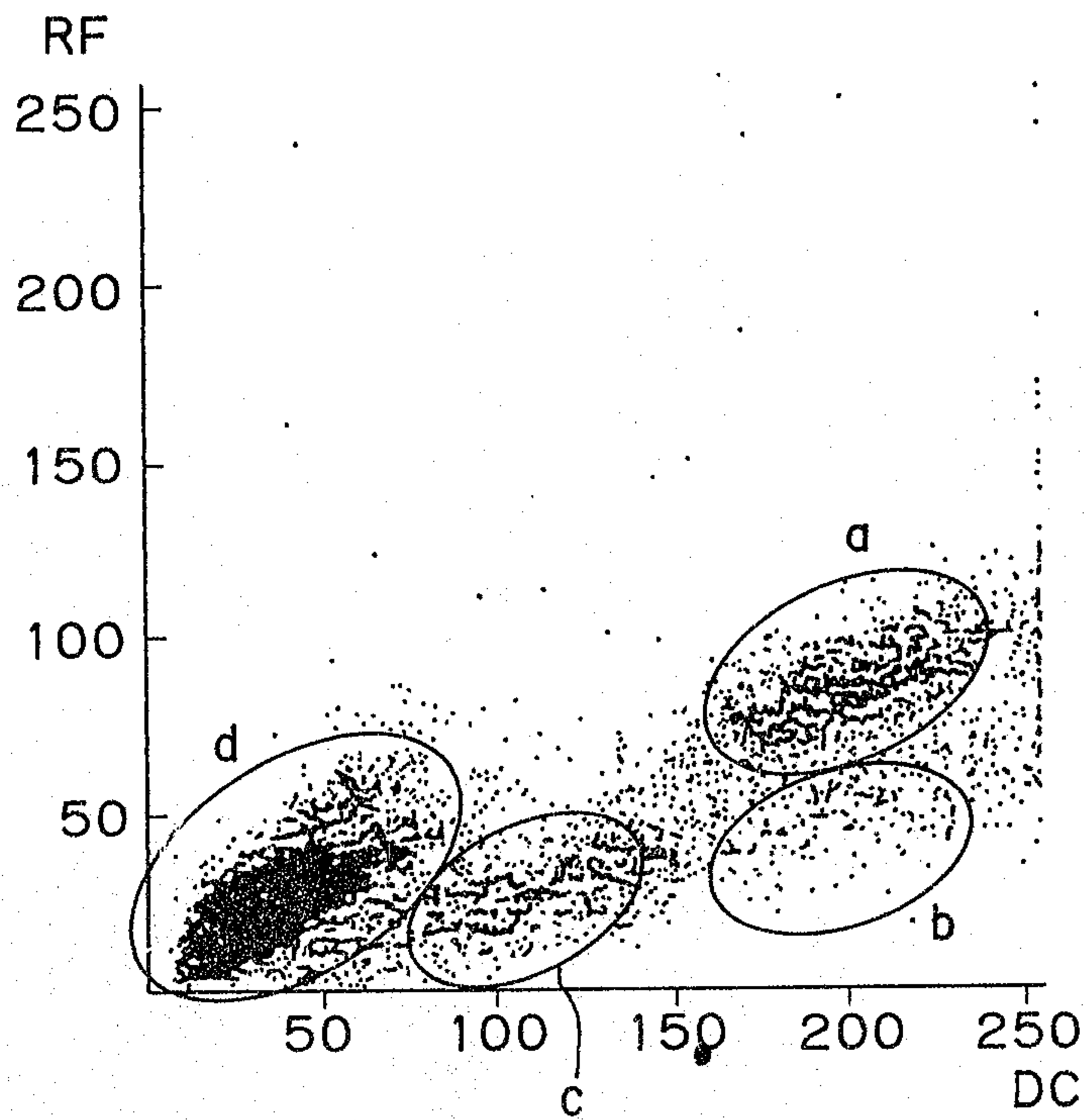
Fig. 6



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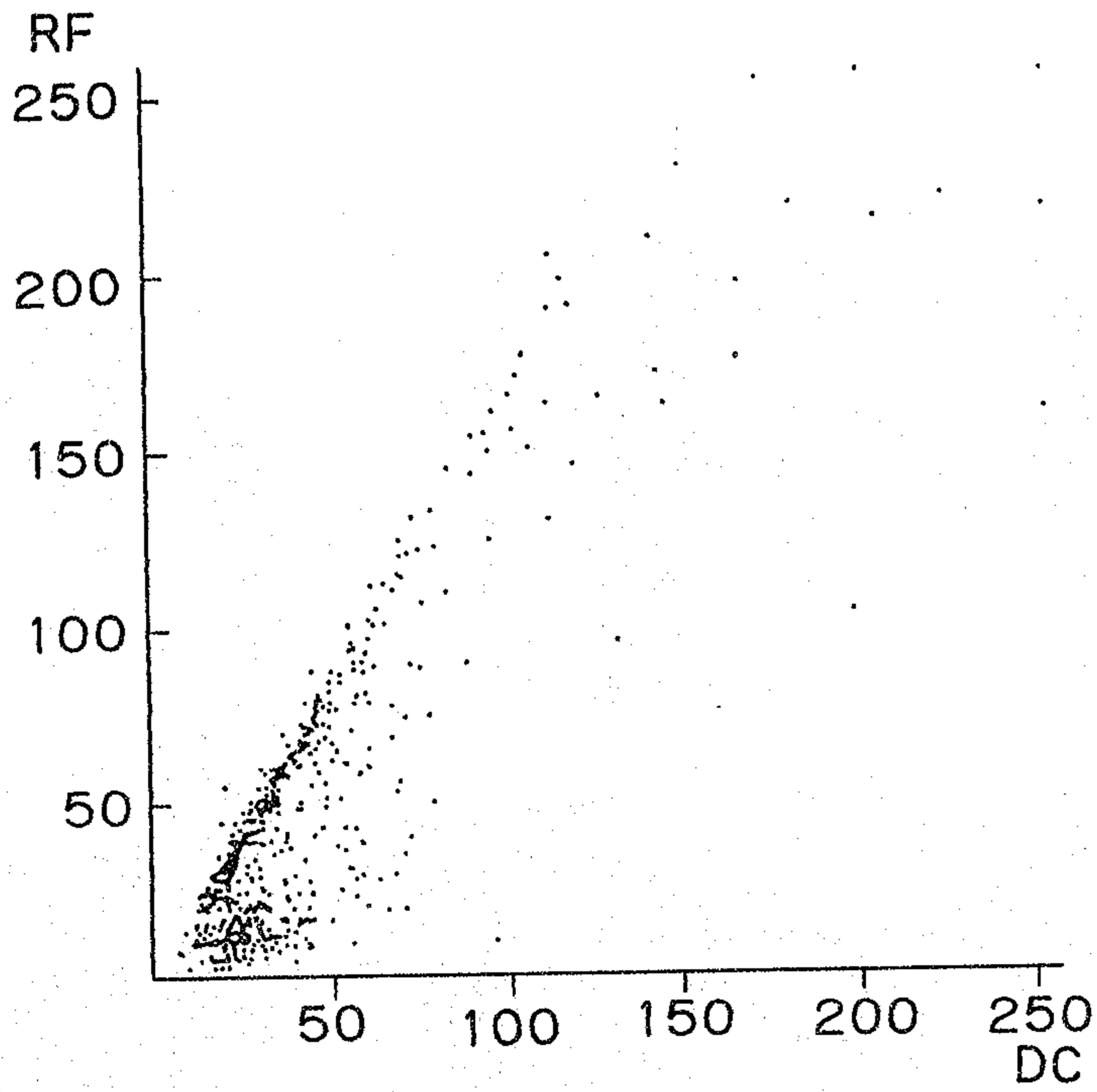
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Fig. 7

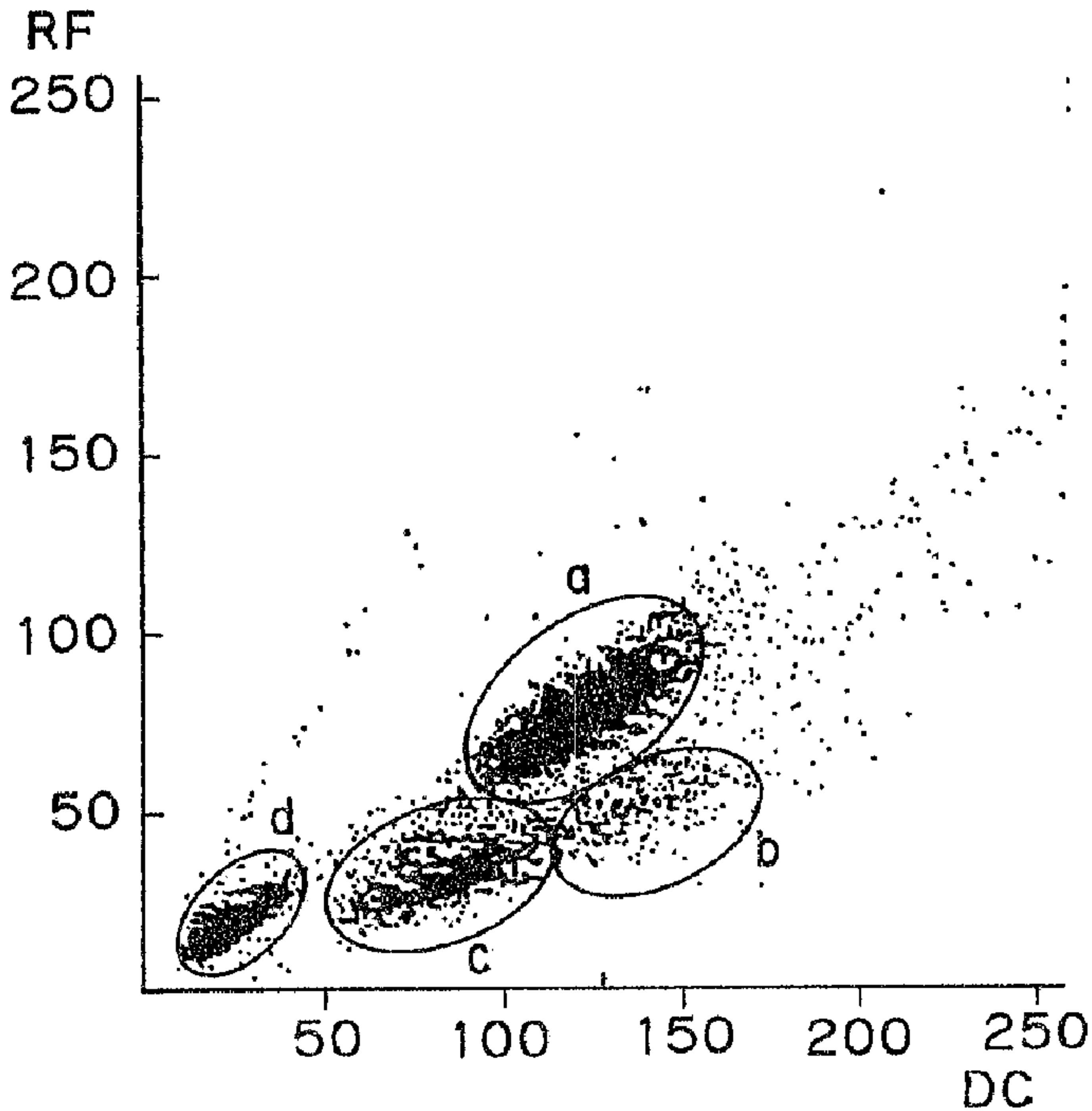


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Fig. 8



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