(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 763 201 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
28.08.2002 Bulletin 2002/35

(21) Application number: **95920628.5**

(22) Date of filing: **23.05.1995**

(51) Int Cl.[7]: **G01N 33/50**, G01N 15/14

(86) International application number:
PCT/US95/06537

(87) International publication number:
WO 95/032424 (30.11.1995 Gazette 1995/51)

(54) **Detection de réticulocytes avec la Coriphosphine O**

Nachweis von Retikulozyten mit Coriphosphin O

Detection of reticulocytes with coriphosphine O

(84) Designated Contracting States:
AT BE CH DE DK FR GB IE IT LI LU MC NL PT SE

(30) Priority: **23.05.1994 US 247379**

(43) Date of publication of application:
19.03.1997 Bulletin 1997/12

(73) Proprietor: **Coulter International Corporation**
Miami, Florida 33196 (US)

(72) Inventor: **SHENKIN, Mark, Lee**
Pembroke Pines, FL 33029 (US)

(74) Representative: **Perry, Robert Edward et al**
GILL JENNINGS & EVERY
Broadgate House
7 Eldon Street
London EC2M 7LH (GB)

(56) References cited:
EP-A- 0 226 272
US-A- 3 887 812
US-A- 3 899 297
US-A- 4 571 388

- **Bullough et al., Inhibition of the bovine heart mitochondrial F1-ATPase by cationic dyes and amphiphatic peptides. Biochim. Biophys. Acta, 1989,; 975(3):377-383.**

EP 0 763 201 B1

Description

Technical Field

[0001] The present invention relates to the detection and enumeration of reticulocytes in a blood sample. More particularly, the present invention relates to a dye which is suitable for staining ribonucleic acid (RNA) and ribonucleic acid polymers and is particularly suitable for detecting reticulocytes by fluorescence flow cytometry techniques.

Background Art

[0002] Reticulocytes are immature red blood cells (RBC) from which the nucleus has been lost. Reticulocytes are known to contain RNA, and detection and enumeration of reticulocytes in a blood sample is of value to clinicians. The reticulocyte count of a blood sample has been used as an indicator of erythropoietic activity, diagnostic and prognostic value in acute hemorrhage, hemolytic anemia, and bone marrow transplantation, and as a measure of response to iron, vitamin B_{12} and folic acid therapy. As known in the art, reticulocytes are precursors to mature red blood cells, and hence the term reticulocyte embraces the evolution and development of the cell whereby a mature red blood cell is generated.

[0003] In the past, reticulocytes in a blood sample have been determined by both manual and automated methods by using appropriate stains such as new methylene blue (NMB), brilliant cresyl blue (BCB), acridine orange, and pyronin Y, and thiazole orange.

[0004] Vital staining with the dye new methylene blue is considered to be the reference method for reticulocyte determinations, and in use this dye precipitates RNA. The method is manual, requires counting large numbers (for example, 500 to 1,000) of cells with a microscope, is slow, tedious, and subject to statistical errors. New methylene blue is nonfluorescent and true precipitated RNA is often difficult to differentiate from precipitated stain.

[0005] Acridine orange has had some use in staining reticulocytes by both manual and automated procedures. Acridine orange precipitates RNA; this prevents quantitative estimates of RNA content because of potential quenching. Moreover, acridine orange does not lead to a diffuse fluorescent distribution of stained cells. Age profiles of the cells (based on RNA content being proportional to fluorescence) are not reliable. Acridine orange has a great affinity for the plastic tubing in flow cytometers, which leads to increased background and lengthy procedures for removing the dye from the flow cytometer tubing. In addition, acridine orange stained cells are difficult to separate from the autofluorescent red cell peak, and the reticulocyte count is usually lower than that obtained with new methylene blue.

[0006] The use of pyronin Y requires prior fixation of the erythrocytes with formalin; this is cumbersome, time consuming, and generally yields poor results. Moreover, pyronin Y has very low quantum efficiency, leading to very low fluorescent signals.

[0007] An example of using thiazole orange for detecting reticulocytes may be found in United States Patent No. 4,883,867, issued November 28, 1989, which is a Continuation-In-Part of Application Serial No. 793,813, filed November 1, 1985, now abandoned.

[0008] An example of using thioflavin T for detecting reticulocytes may be found in United States Patent No. 4,571,388, issued February 18, 1986.

[0009] Shapiro, Howard M., Practical Flow Cytometry, p.144, Alan R. Liss, Inc. 1985, at Table 7-3 lists tricyclic heteroaromatic compounds used for staining DNA and/or RNA. While Table 7-3 lists coriphosphine O (CPO), it does not, however, include CPO as a reticulocyte or RNA stain.

[0010] Bullough *et al*, Biochim. Biophys. Acta 975 (1983) 377-383, discloses aqueous compositions of coriphosphine O, at concentrations of 480 and 16 μM, pH 8.0.

Summary of the Invention

[0011] According to a first aspect of the present invention, a process for detecting reticulocytes in a sample, comprises staining the sample with coriphosphine O; exciting said sample with light of excitation wavelength; and measuring fluorescence emitted from said sample.

[0012] According to a second aspect of the present invention, a process of generating a stable RNA-dye complex in a sample containing reticulocytes, comprises mixing the sample with a reagent comprising an aqueous solution of coriphosphine O, and allowing the sample and the reagent to react for sufficient time so that the reagent is effectively taken up by the reticulocytes, wherein the RNA-dye complex is stable for 8 to 24 hours.

[0013] According to a third aspect of the present invention, a method for quantitating reticulocytes in a whole blood sample by flow cytometry, comprises the steps of:

(a) mixing a sample of whole blood to be tested with a reagent comprising an aqueous solution of coriphosphine O;
(b) allowing the sample and the reagent to react for sufficient time so that the reagent is effectively taken up by the reticulocytes;
(c) passing the mixture through a flow cytometer;
(d) measuring the intensity of red fluorescence against green fluorescence of the red blood cell population gated on light scatter; and
(e) determining the amount or percentage of reticulocytes in the sample from said measurement.

[0014] The present invention provides a method of differentially staining cells causing fewer interference by

platelets, nucleated red blood cells, and Howell-Jolly Bodies. Further, the present invention provides a method of staining cells, to produce an RNA-dye complex which is more stable than complexes produced by other known methods, and which results in an increase in the time during which one can look at the total color generated by the RNA-dye complex.

[0015] A novel reagent, for staining reticulocytes in a whole blood sample for quantitation, includes an aqueous solution of coriphosphine O at a concentration of 5-10 mg/L, and has a pH of 6.0 to 8.0. This optionally includes a buffer system.

Brief Description of Drawings

[0016]

Figure 1 shows a schematic diagram of the optics of a flow cytometer that may be employed in implementing the method of the present invention;
Figure 2 shows an example of a flow cytometric histogram from a normal donor;
Figure 3 shows an example of a flow cytometric histogram from an abnormal (high) patient;
Figure 4 shows a dose/response curve of RNA and coriphosphine O reagent;
Figure 5 shows the correlation of a CPO method of the present invention against a thiazole orange method;
Figure 6 shows the stability of CPO;
Figure 7 gives an analysis of red blood cells and platelets; and
Figure 8 gives an analysis of red blood cells and white blood cells.

Modes for Carrying out the Invention

[0017] For convenience, the dye of the invention for staining reticulocytes is referred to as coriphosphine O (CPO), also known as basic yellow seven. Coriphosphine O is available from Pfaltz & Bauer, Inc. Division of Aceto Corporation, Waterbury, Connecticut.

[0018] Applicant has found that coriphosphine O is an effective dye for staining reticulocytes. The function of the reticulocyte stain is to further delineate the reticulocyte for light scatter enumeration in a flow cytometer. Thus, by using coriphosphine O as the stain, it is possible to detect and enumerate reticulocytes in a whole blood sample. Coriphosphine O is a fluorochrome dye that does not precipitate intracellular ribonucleic acid of the reticulocyte.

[0019] The use of coriphosphine O offers the advantage of differentially staining cells causing less interference by platelets, nucleated red blood cells, and Howell-Jolly Bodies. Coriphosphine O offers the further advantage of increasing stability of RNA-dye complex, thereby increasing the time during which one can look at the total color generated by the RNA-dye complex. The color generated is thus stable for a much longer period of time than dyes used in the art, for example the color generated by the use of thiazole orange is stable for about 2 hours, whereas the color generated by the use of coriphosphine O is stable for about 8 to 24 hours.

[0020] In accordance with the present invention, when staining reticulocytes in a blood sample, coriphosphine O is preferably employed as an aqueous solution, preferably in an isotonic saline solution, and most preferably in ISOTON® II, U.S. Patent No. 3,962,125, Coulter Corporation, Miami, Florida, at CPO concentration of 5-10 mg/L., which solution may contain a minor amount of methanol. The CPO reagent is buffered at a pH of 6 to 8. The blood sample, which may be whole blood or a blood fraction, is stained with the coriphosphine O solution by mixing the blood sample with the solution of coriphosphine O. The volumes of blood sample and solution used are such that the concentrations of RBC are sufficient to run through the instrument. Thus, the concentration is in the range of 1:50 - 1:5000, preferably 1:100 - 1:1000, most preferably 1:200 - 1:800. The sample is then incubated for a minimum of about 60 seconds to about 8 hours, preferably 30 minutes, and then run through a flow cytometer. Applicant has found that CPO is a vital stain, and, accordingly, fixation is not required.

[0021] Coriphosphine O when unbound from ribonucleic acid (RNA) provides little or no red fluorescence, and exhibits a strong absorption peak at about 491.5 nm. When coriphosphine O is bound to RNA in the raticulocytes, the optical properties thereof change dramatically. In particular, coriphosphine O when bound to RNA in the reticulocytes exhibits a strong red fluorescence. The excitation maximum is at about 491.5 nm and the emission maximum is at about 630-700 nm, giving a Stokes shift of about 160 nm. As a result of the excitation peak of the bound coriphosphine O being in the order of about 490 nm, in using the automatic flow cytometer the light source may be a mercury lamp which has an energy line at about 485 nm or an argon ion laser which has strong emission at about 488 nm. Although excitation may be effected at other wavelengths, reticulocytes stained with coriphosphine O are preferably excited at a wavelength of from about 450 nm to about 500 nm.

[0022] Coriphosphine O when unbound to deoxyribonucleic acid (DNA) in the white blood cells provides little or no green fluorescence, whereas coriphosphine O when bound to DNA in the white blood cells exhibits a strong green fluorescence. The lack of fluorescence of the coriphosphine O dye when not bound to nucleic acid provides low background and allows an operator to select a fluorescent threshold (or "gates") for an automatic flow cytometer.

[0023] Because CPO when bound to RNA emits red fluorescence and when bound to DNA emits green fluorescence, the use of CPO otters the advantage of differentially staining cells causing fewer interference by

platelets, nucleated red blood cells, white blood cells, and Howell-Jolly Bodies. This enables one to gate-in the red blood cells and thus obtain a more accurate count.

[0024] Further, when CPO is bound, the amount or intensity of green fluorescence is proportional to the amount of background or nonspecific staining due to the binding of CPO to "other" structures. These cellular structures or elements include DNA and subcellular vesicle such as lysosomes, endosomes, and granules. Each of these elements binds the CPO differently, resulting in different amounts of fluorescence. However, only single-stranded RNA will bind CPO and fluoresce only red. We determine the maximum amount of green fluorescence of mature red blood cell population as the "threshold" for both red blood cells and reticulocytes. All other cells will fluoresce green above this threshold. The difference in intensity of green fluorescence offers the advantage of differentially staining cells enabling one to gate-out non-specific cells, such as platelets and white blood cells.

[0025] Coriphosphine O dye does not precipitate RNA and, as a result, reticulocytes stained with coriphosphine O maintain a relatively homogeneous distribution of intracellular RNA, whereby there is a nearly linear relationship between the fluorescent signal measured for an individual reticulocyte and its RNA content. Clinically, this provides the physician with additional information beyond the reticulocyte count in that RNA content is a function of reticulocyte age. Accordingly, by using coriphosphine O, a clinician has the ability to obtain reticulocyte age profiles as well as simple reticulocyte counts.

[0026] In the use of coriphosphine O for staining reticulocytes in a blood sample the fluorescent signals from the stained reticulocytes are well separated from those of the mature erythrocytes, whereby results can be directly read in an automatic flow cytometer without extensive data manipulation.

[0027] Reticulocytes, RNA or DNA stained with CPO, although preferably enumerated in an automatic flow cytometer, can also be counted by a manual procedure or automated microscopy.

[0028] The fundamental concept of flow cytometry is essentially the passing of cells, one at a time, through a specific sensing region. By means of hydrodynamic focusing, single cells are passed through the sensing zone, which consists of a focused laser light source and a detection system for the measurement of scattered and fluorescent light.

[0029] Automatic flow cytometers are well known in the art, and the present invention is not limited to the use of any particular flow cytometer.

[0030] A specific example of the optics of a flow cytometer employed in the present invention is hereunder described with reference to Figure 1. The optics shown in Figure 1 are used in a flow cytometer designed for measuring right-angle scattered light, red fluorescence and green fluorescence. The optic generally indicated by **10** uses an argon ion laser **12** as a light source and it operates at a wavelength of 488 nm, producing an output of 15 mW. Light emitted from the laser **12** is converged by a cylindrical lens **16** and illuminates a blood sample flowing through a flow cell **14** in a conventional means.

[0031] When the stained red blood cells in the sample are irradiated by the laser light, they produce scattered light and fluorescence. The right-angle scattered light and the fluorescence are converged with a condenser lens **18** and pass through an aperture **20** to fall upon a dichroic mirror **22**. The dichroic mirror **22** reflects the right-angle scattered light **24** and transmits the fluorescence **26.** The right-angle scattered light **24** reflected from the dichroic mirror **22** is detected in a photomultiplier tube or photodiode **28.** Of the fluorescence **26** that passes through the dichroic mirror **22**, green fluorescence **32** is reflected by a dichroic mirror **30** and red fluorescence **38** is transmitted through that mirror. The reflected green fluorescence **32** passes through a color filter **34** and is detected in a photomultiplier tube **36**. The transmitted red fluorescence **38** passes through a color filter **40** and is detected in a photomultiplier tube **42**.

[0032] Thus, for example, reticulocytes stained with coriphosphine O may be detected and enumerated in the COULTER® XL flow cytometer sold by Coulter Corporation, Miami, Florida. In using such automatic flow cytometers, fluorescent gates are set by use of the position of the mature red cells in the sample, and the fluorescent gates are then set to enumerate reticulocytes.

[0033] The use of an automatic flow cytometer for detection and enumeration of reticulocytes stained with coriphosphine O provides results which closely correlate with results obtained by a known standard method for enumerating reticulocytes which uses methylene blue or acridine orange, or thiazole orange.

[0034] The use of reticulocytes stained with coriphosphine O in an automatic flow cytometer is particularly advantageous in that there is low fluorescence background and fluorescent gates may be easily selected. Moreover, there is no precipitation of intracellular reticulocyte RNA, whereby the cells need not be fixed. In addition, there is a linear relationship between the fluorescent signal for an individual reticulocyte, which provides information as to reticulocyte age.

[0035] Reticulocytes stained with coriphosphine 0, although preferably enumerated in an automatic flow cytometer, can also be counted by a manual procedure or automated microscopy.

[0036] Accordingly, the subject method includes the steps of:

(a) mixing a sample of blood to be tested with the subject reagent composition including the subject derivative dye composition to form a suspension of cells;

(b) incubating said suspension of cells for a time period of not less than 1 minute and not more than 24 hours at a temperature of not less than 2°C and not

more than 25°C;
(c) measuring the derived fluorescence of the cells on a flow cytometer;
(d) generating the correlated data histograms of red fluorescence vs green fluorescence gated and light scatter (LFS vs SS);
(e) selecting the fluorescence threshold of the reticulocyte population; and
(f) calculating the total reticulocyte as percentage reticulocyte x total RBC (total RBC in billions/mL from a hematology analyzer such as the COULTER STKS (Coulter Corporation, Miami, Florida).

[0037] The following non-limiting example illustrates various features of the present invention. The following example of the staining is utilized in obtaining the results illustrated in Figures 4-8.

EXAMPLE 1

[0038] Specimen was collected into triphosphate EDTA (K3EDTA). 0.002 mL of patient whole blood specimen was added to 1.0 mL of reagent. The sample was mixed and allowed to incubate at room temperature a minimum of 15 minutes but no more than 8 hours. The specimen was then mixed again just prior to analysis on a calibrated XL flow cytometer.

[0039] Figures 2-4 show data for reticulocyte analysis of normal and abnormal blood using CPO. In particular, Figure 2 shows a fluorescence histogram of a normal person's blood demonstrating the distribution of erythrocyte events detected by the 525 nm photomultiplier tube and by the 630 nm photomultiplier tube. As shown in Figure 2, region E delineates the reticulocyte separate from white blood cells and platelets.

[0040] Figure 3 shows a fluorescence histogram of an abnormal blood demonstrating the distribution of erythrocyte events detected by the 525 nm photomultiplier tube and by the 630 nm photomultiplier tube. As can be seen in Figure 3, there is an increased number of events in the reticulocyte area (region E). As shown in Figure 3, CPO reacts specifically with RNA, and an increase in the amount of RNA in the sample results in an increase in fluorescence.

[0041] Figure 4 shows a graph of the dose response for the reagent when it is mixed with increasing amounts of ribonucleic acid (RNA). The more RNA that is added, the more the fluorescence intensity increases.

[0042] Figure 5 thus shows the correlation of a CPO method of the present invention against a thiazole orange method (reference method). As shown in Figure 5, the results are the same for CPO as with the reference method. Thus, CPO is a measure of reticulocytes.

[0043] Figure 6 demonstrates the stability of CPO, as a function of time versus percent reticulocytes. Figure 6 shows that after mixing blood with reagent, it takes about 15 minutes to establish equilibrium. Once equilibrium is established, the CPO-RNA complex remains stable for at least 8 hours. Thiazole orange, acridine orange and thioflavin T, on the other hand, have a stability of less than 2 hours.

[0044] Figure 7 gives an analysis of red blood cells and platelets. Samples with an increased amount of platelets were used. Analysis of red blood cells and platelets shows that platelets distribution is different from reticulocytes distribution.

[0045] Figure 8 gives an analysis of red blood cells and white blood cells. Samples with an increased amount of white blood cells were used. Analysis of red blood cells and white blood cells shows that white blood cells distribution is different from red blood cells and reticulocytes distribution.

Claims

1. A process for detecting reticulocytes in a sample, which comprises staining the sample with coriphosphine O; exciting said sample with light of excitation wavelength; and measuring fluorescence emitted from said sample.

2. A process according to claim 1, wherein the sample is excited and fluorescence is measured by means of a flow cytometer.

3. A process according to claim 1, wherein the sample is excited and fluorescence is measured by means of fluorescence microscopy.

4. A process according to claim 2, wherein the sample is excited in the flow cytometer with light from a mercury arc lamp.

5. A process according to claim 2, wherein the sample is excited in the flow cytometer with light from an argon laser.

6. A process according to any preceding claim, wherein the sample comprises whole blood.

7. A process according to any preceding claim, wherein the reticulocytes are detected without fixation thereof.

8. A process according to any preceding claim, wherein the reticulocytes are differentially stained.

9. A reagent for staining reticulocytes in a whole blood sample for quantitation, which reagent includes an aqueous solution of coriphosphine O at a concentration of 5-10 mg/L, and which has a pH of 6.0 to 8.0.

10. A process of generating a stable RNA-dye complex in a sample containing reticulocytes, which com-

prises mixing the sample with a reagent comprising an aqueous solution of coriphosphine 0, and allowing the sample and the reagent to react for sufficient time so that the reagent is effectively taken up by the reticulocytes, wherein the RNA-dye complex is stable for 8 to 24 hours.

11. A method for quantitating reticulocytes in a whole blood sample by flow cytometry, which comprises the steps of:

(a) mixing a sample of whole blood to be tested with a reagent comprising an aqueous solution of coriphosphine O;
(b) allowing the sample and the reagent to react for sufficient time so that the reagent is effectively taken up by the reticulocytes;
(c) passing the mixture through a flow cytometer;
(d) measuring the intensity of red fluorescence against green fluorescence of the red blood cell population gated on light scatter; and
(e) determining the amount of percentage of reticulocytes in the sample from said measurement.

Patentansprüche

1. Verfahren zum Nachweis von Retikulozyten in einer Probe, das das Anfärben der Probe mit Coriphosphin O; die Anregung der Probe mit Licht der Anregungswellenlänge; und die Messung der Fluoreszenz, die von der Probe emittiert wird, umfasst.

2. Verfahren nach Anspruch 1, wobei die Probe angeregt wird und die Fluoreszenz gemessen wird, und zwar mittels eines Durchflusszytometers.

3. Verfahren nach Anspruch 1, wobei die Probe angeregt wird und die Fluoreszenz gemessen wird, und zwar mittels eines Fluoreszenzmikroskops.

4. Verfahren nach Anspruch 2, wobei die Probe in dem Durchflusszytometer mit Licht aus einer Quecksilberbogenlampe angeregt wird.

5. Verfahren nach Anspruch 2, wobei die Probe in dem Durchflusszytometer mit Licht aus einem Argonlaser angeregt wird.

6. Verfahren nach einem der vorstehenden Ansprüche, wobei die Probe Vollblut umfasst.

7. Verfahren nach einem der vorstehenden Ansprüche, wobei die Retikulozyten ohne Fixierung nachgewiesen werden.

8. Verfahren nach einem der vorstehenden Ansprüche, wobei die Retikulozyten differenziell angefärbt werden.

9. Reagenz zur Anfärbung von Retikulozyten in einer Vollblutprobe zur quantitativen Bestimmung, wobei das Reagenz eine wässrige Lösung von Coriphosphin O in einer Konzentration von 5 bis 10 mg/l enthält und einen pH-Wert von 6,0 bis 8,0 aufweist.

10. Verfahren zur Erzeugung eines stabilen RNA-Farbstoff-Komplexes in einer Retikulozyten enthaltenen Probe, das es umfasst, die Probe mit einem Reagenz, das eine wässrige Lösung von Coriphosphin O umfasst, zu mischen und die Probe und das Reagenz für eine ausreichende Zeit reagieren zu lassen, so dass das Reagenz in wirksamer Weise von den Retikulozyten aufgenommen wird, wobei der RNA-Farbstoff-Komplex für 8 bis 24 Stunden stabil ist.

11. Verfahren zur quantitativen Bestimmung von Retikulozyten in einer Vollblutprobe mittels Durchflusszytometrie, das folgende Stufen umfasst:

(a) eine Probe von Vollblut, die getestet werden soll, wird mit einem Reagenz, das eine wässrige Lösung von Coriphosphin O umfasst, gemischt;

(b) man lässt die Probe und das Reagenz für eine ausreichende Zeit reagieren, so dass das Reagenz in wirksamer Weise von den Retikulozyten aufgenommen wird;

(c) das Gemisch wird durch ein Durchflusszytometer geleitet;

(d) die Intensität der roten Fluoreszenz wird gegen die grüne Fluoreszenz der roten Blutzellpopulation, mit einem Gate auf Lichtstreuung, gemessen; und

(e) die Menge oder der Prozentsatz an Retikulozyten in der Probe wird aus der Messung bestimmt.

Revendications

1. Procédé pour détecter des réticulocytes dans un échantillon, qui comprend les étapes consistant à colorer l'échantillon avec de la coriphosphine O; à exciter le dit échantillon avec une lumière d'une longueur d'onde d'excitation; et à mesurer la fluorescence émise par le dit échantillon.

2. Procédé selon la revendication 1, dans lequel

l'échantillon est excité, et la fluorescence est mesurée au moyen d'un cytomètre en flux.

3. Procédé selon la revendication 1, dans lequel l'échantillon est excité, et la fluorescence est mesurée par microscopie en fluorescence.

4. Procédé selon la revendication 2, dans lequel l'échantillon est excité dans le cytomètre en flux avec la lumière d'un lampe à arc au mercure.

5. Procédé selon la revendication 2, dans lequel l'échantillon est excité dans le cytomètre en flux avec la lumière d'un laser à argon.

6. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'échantillon comprend du sang complet.

7. Procédé selon l'une quelconque des revendications précédentes, dans lequel les réticulocytes sont détectés sans qu'ils soient fixés.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel les réticulocytes sont colorés de manière différenciée.

9. Réactif pour colorer des réticulocytes dans un échantillon de sang complet pour quantification, lequel réactif comprend une solution aqueuse de coriphosphine O à une concentration de 5-10 mg/L, et qui a un pH de 6,0 à 8,0.

10. Procédé de génération d'un complexe stable d'ARN-colorant dans un échantillon contenant des réticulocytes, qui comprend les étapes consistant à mélanger l'échantillon à un réactif comprenant une solution aqueuse de coriphosphine O, et à laisser l'échantillon et le réactif réagir pendant un laps de temps suffisant pour que le réactif soit efficacement absorbé par les réticulocytes, dans lequel le complexe d'ARN-colorant est stable pendant 8 à 24 heures.

11. Méthode pour quantifier des réticulocytes dans un échantillon de sang complet par cytométrie en flux, qui comprend les étapes consistant à:

(a) mélanger un échantillon de sang complet à tester à un réactif comprenant une solution aqueuse de coriphosphine O;
(b) laisser l'échantillon et le réactif de réagir pendant un laps de temps suffisant pour que le réactif soit efficacement absorbé par les réticulocytes;
(c) faire passer le mélange à travers un cytomètre en flux;
(d) mesurer l'intensité de la fluorescence dans le rouge par rapport à la fluorescence dans le vert de la population des globules rouges du sang consignées dans la diffusion de la lumière; et
(e) déterminer à partir de la dite mesure le pourcentage de réticulocytes dans l'échantillon.

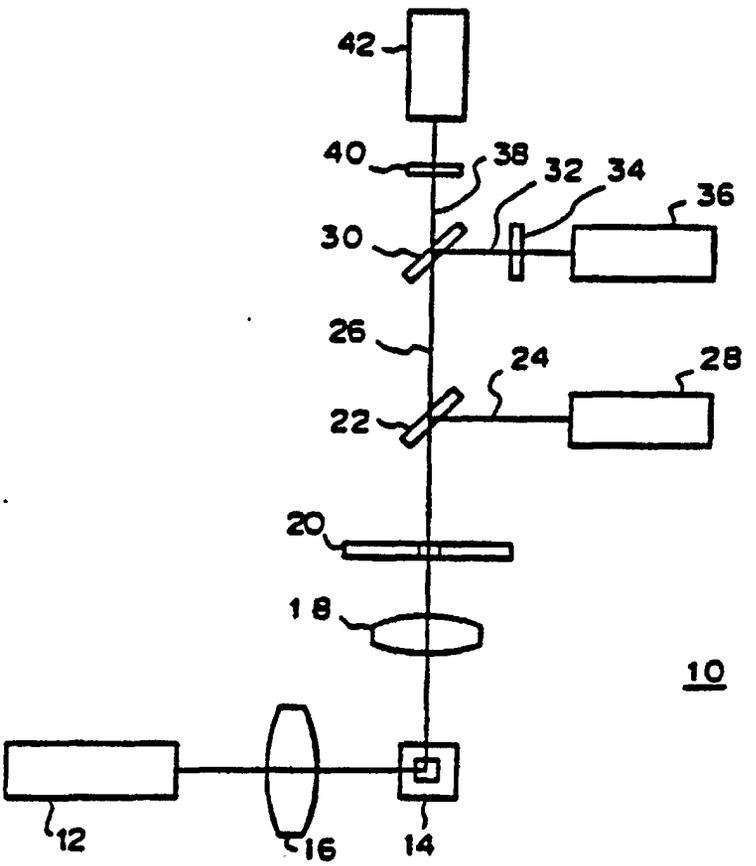

Fig. 1

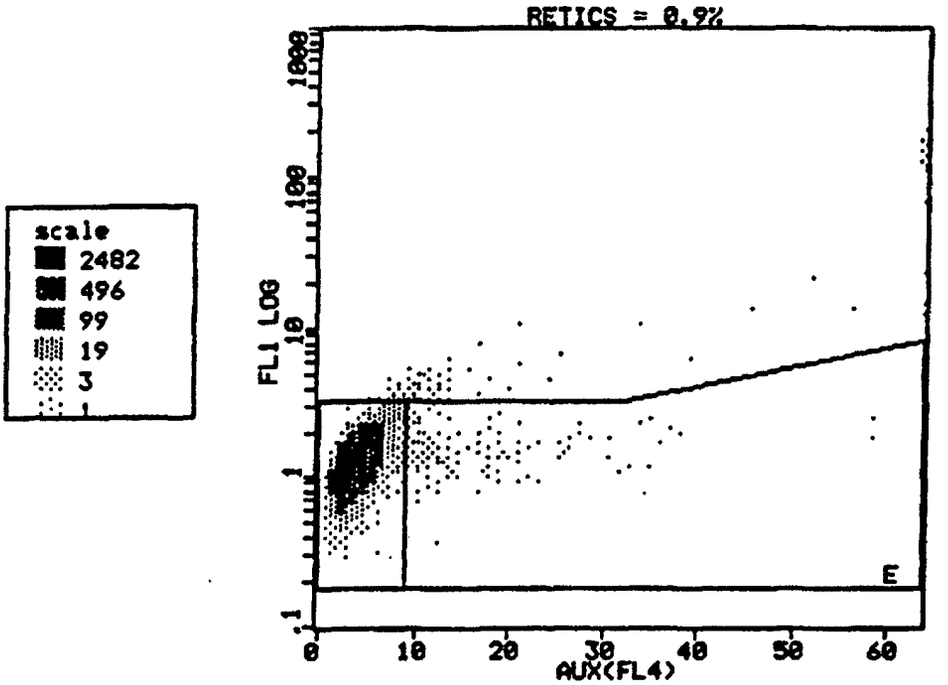
RETICS = 0.9%
scale
2482
496
99
19
3
1
FL1 LOG
1000
100
10
1
-1
E
0
10
20
30
40
50
60
AUX(FL4)

Fig. 2

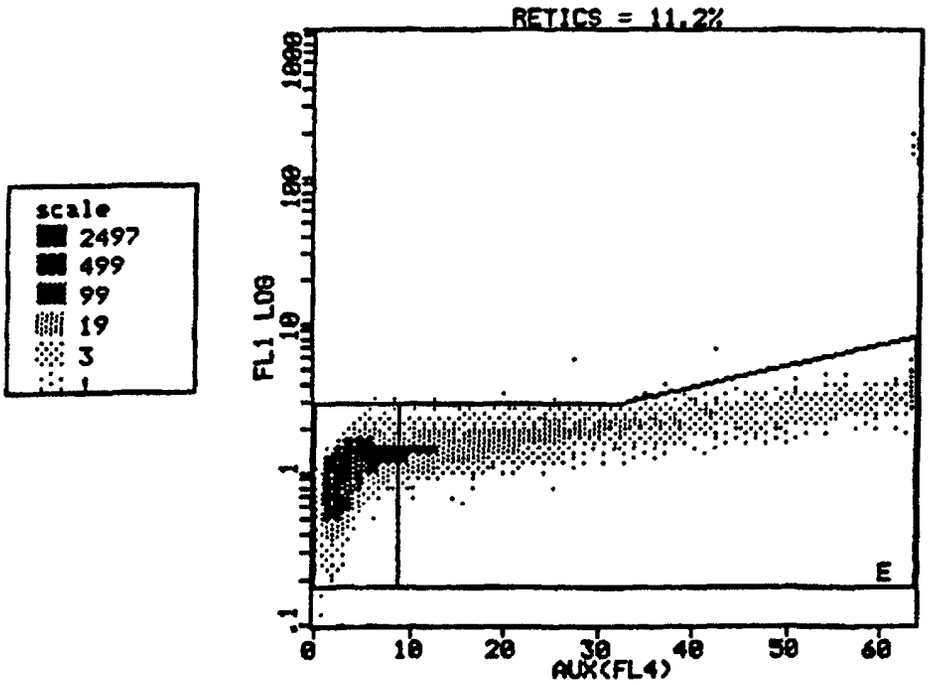
RETICS = 11.2%
scale
2497
499
99
19
3
1
FL1 LOG
1000
100
10
1
-1
E
0
10
20
30
40
50
60
AUX(FL4)

Fig. 3

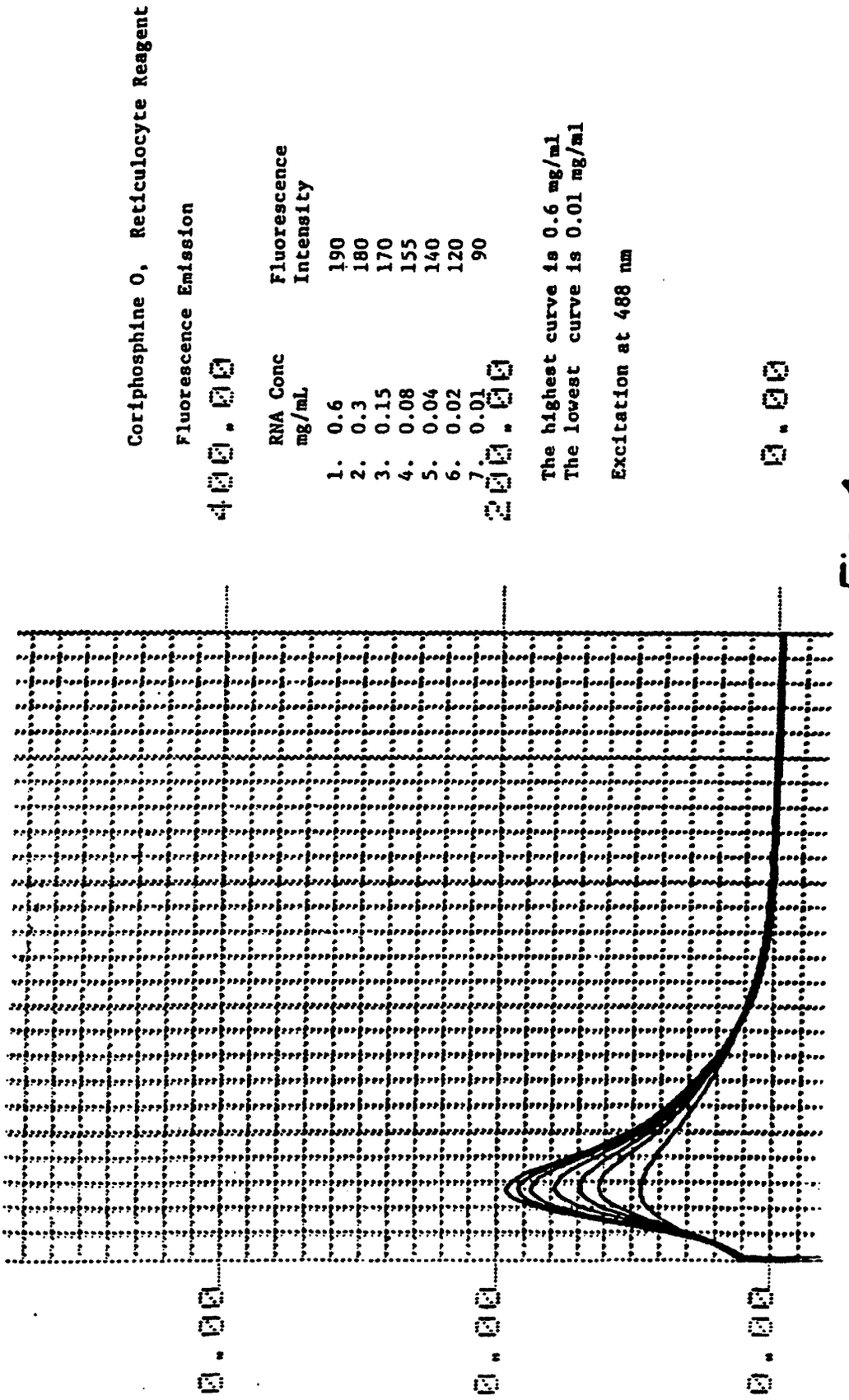
Coriphosphine O, Reticulocyte Reagent
Fluorescence Emission
400.00
200.00
0.00
RNA Conc mg/mL
Fluorescence Intensity
1. 0.6 190
2. 0.3 180
3. 0.15 170
4. 0.08 155
5. 0.04 140
6. 0.02 120
7. 0.01 90
The highest curve is 0.6 mg/ml
The lowest curve is 0.01 mg/ml
Excitation at 488 nm

Fig. 4

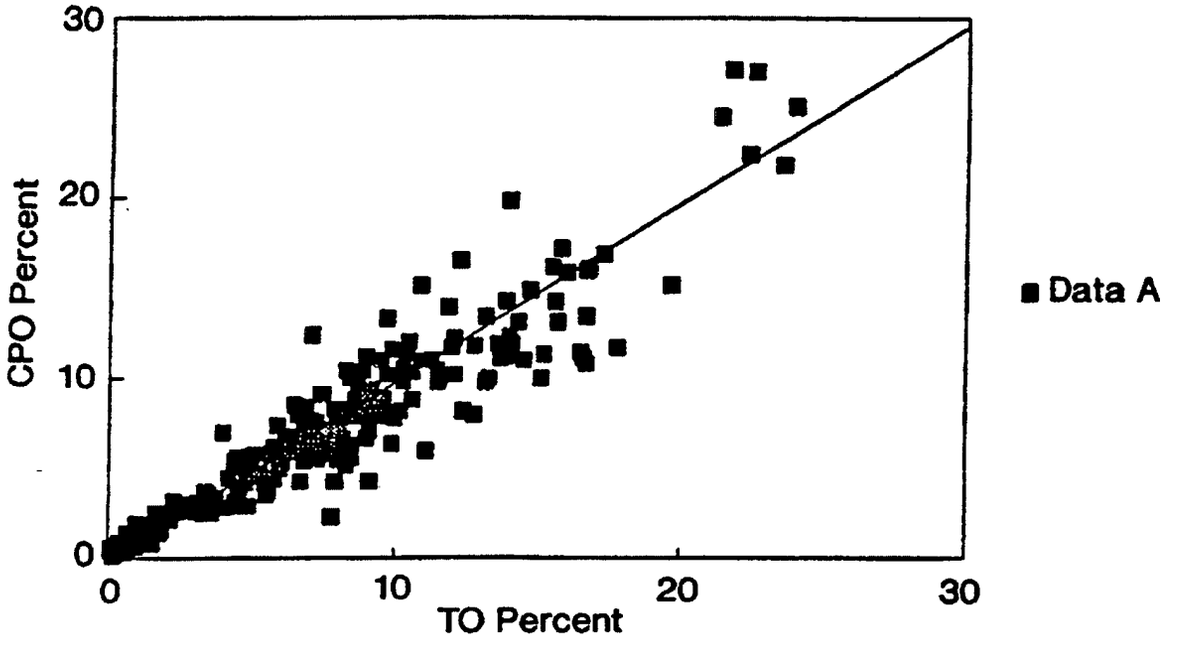
30
20
10
0
CPO Percent
0
10
20
30
TO Percent
Data A

Fig. 5

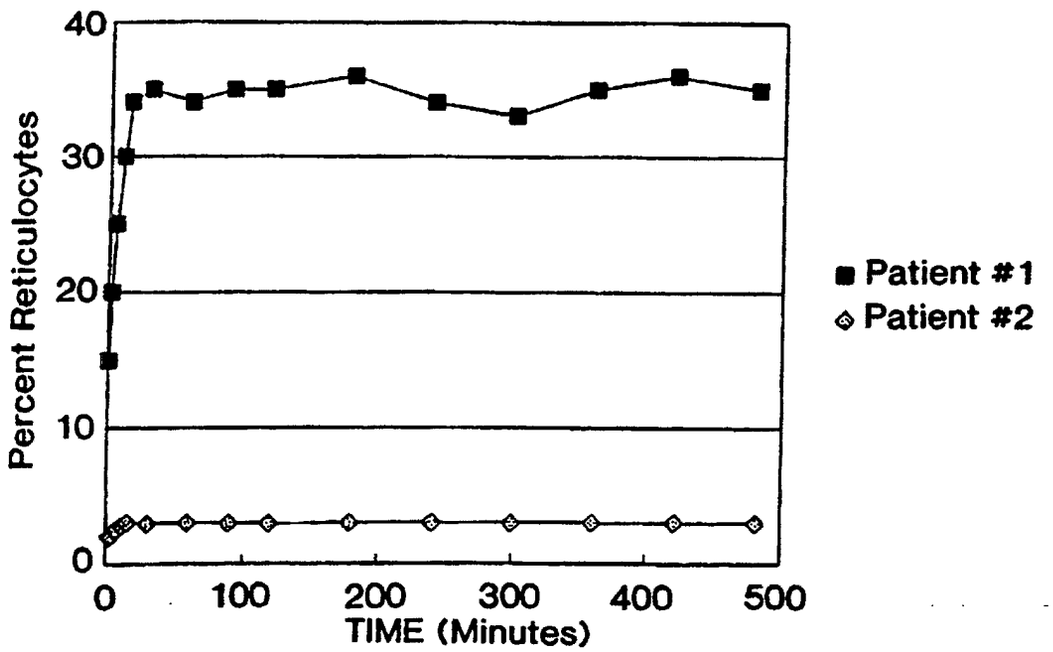
40
30
20
10
0
Percent Reticulocytes
0
100
200
300
400
500
TIME (Minutes)
Patient #1
Patient #2

Fig. 6

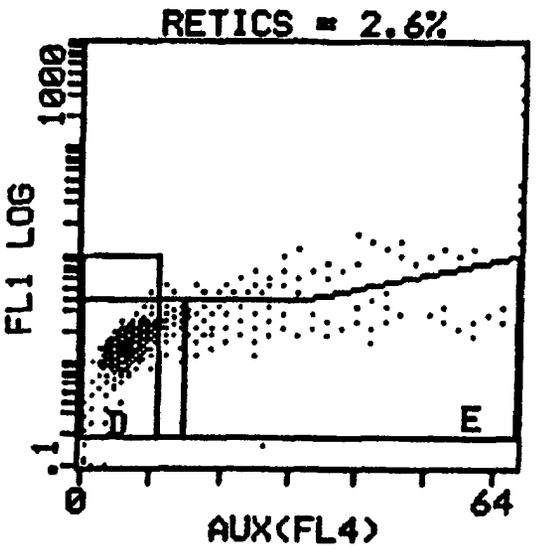
RETICS = 2.6%
1000
FL1 LOG
1
0
D
E
64
AUX(FL4)

Fig. 7

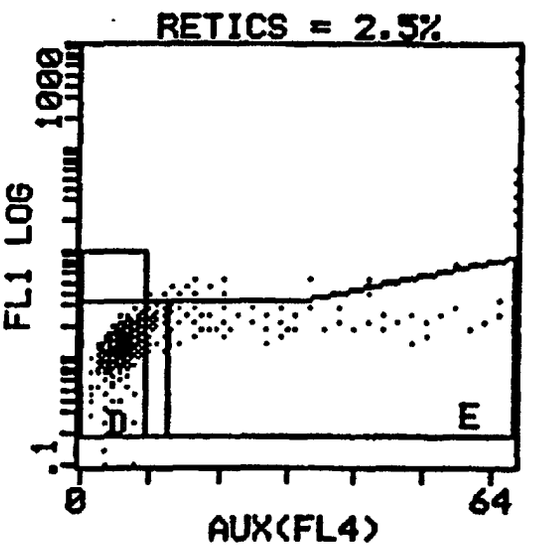
RETICS = 2.5%
1000
FL1 LOG
1
0
D
E
64
AUX(FL4)

Fig. 8