

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

DECLARATION IN SUPPORT OF CONVENTION OR NON-CONVENTION APPLICATION FOR A PATENT

Insert title of invention.

In support of the Application made for a patent for an invention
entitled: "CONSERVATIVE WHOLE BLOOD SAMPLE PREPARATION
TECHNIQUE"

Insert full name(s) and address(es)
of declarant(s) being the appli-
cant(s) or person(s) authorized to
sign on behalf of an applicant
company.

I ~~We~~ Wayne A Barlin
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Cross out whichever of paragraphs
1(a) or 1(b) does not apply

1(a) relates to application made
by individual(s)
1(b) relates to application made
by company; Insert name of
applicant company.

do solemnly and sincerely declare as follows :-

1. (a) ~~the applicant~~
or (b) I am authorized by

Coulter Corporation

Cross out whichever of paragraphs
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2(a) relates to application made
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name(s) and address(es) of inven-
tors.

the applicant..... for the patent to make this declaration on ^{its} ~~their~~ behalf.

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^{is} ~~are~~ the actual inventor(s)..... of the invention and the facts upon which the applicant.....
^{is} ~~are~~ entitled to make the application are as follows :-

State manner in which applicant(s)
derive title from inventor(s)

The actual inventors assigned the invention
to the said applicant

Cross out paragraphs 3 and 4
for non-convention applications.
For convention applications,
insert basic country(s) followed
by date(s) and basic applicant(s).

3. The basic application..... as defined by Section 141 of the Act ^{was} ~~were~~ made
in United States of America on the 4 December 1987
by Jorge Angel Quintana, Thomas R. Russell, Ronald D. Paul
ix and Timothy J. Fischer ~~xxxx~~
by.....
in..... on the.....
by.....

4. The basic application..... referred to in paragraph 3 of this Declaration ^{was} ~~were~~
the first application..... made in a Convention country in respect of the invention the subject
of the application.

Insert place and date of signature.

Hialeah,
Declared at Florida this 5th day of July, 1989.
Coulter Corporation

Signature of declarant(s) (no
attestation required)

Note Initial all alterations.

by *Wayne A. Barlin*
Wayne A. Barlin, Secretary

DAVIES & COLLISON, MELBOURNE and CANBERRA.

(12) PATENT ABRIDGMENT (11) Document No. AU-B-28204/89
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 606493

(54) Title
CONSERVATIVE WHOLE BLOOD SAMPLE PREPARATION TECHNIQUE

International Patent Classification(s)
(51)⁴ A01N 001/02 A01N 001/00 G01N 001/00 G01N 001/28
G01N 001/34 G01N 033/49 G01N 033/569
(51)⁵ G01N 033/531

(21) Application No. : 28204/89 (22) Application Date : 01.12.88

(87) WIPO Number : WO89/05092

(30) Priority Data

(31) Number (32) Date (33) Country
128668 04.12.87 US UNITED STATES OF AMERICA

(43) Publication Date : 05.07.89

(44) Publication Date of Accepted Application : 07.02.91

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(56) Prior Art Documents
US 4637986
US 4521518

(57) Claim

1. A method for preparation of a whole blood sample for analysis by photooptical measurement techniques, which method includes enrichment of the non-erythrocyte cellular fraction of said sample and the labelling of one or more sub-populations of said fraction by immunochemical interaction of the cells of said fraction with an indicator labelled binding material which is specific for a characteristic cellular component of the sub-population of interest, the method comprising the steps of:

(a) providing a common reaction vessel of the type which is typically utilized as a sample container for photooptical instrumentation designed for quantitation and/or differentiation of particulate analytes from other particulates which are endogenous to the sample; said

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(10) 606493

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method characterized by:

(b) preparing the sample for analysis in said reaction vessel by subjecting the sample to selective stromatolysis conditions by a lytic reagent, so as to enable effective differentiation of the non-erythrocyte fraction of said sample from the erythrocyte fraction;

(c) labelling at least one of said sub-populations with a binding material prior to, concurrent with, or after said step of selective stromatolysis; and

(d) transferring an aliquot of said sample, from the ^{reaction vessel}~~incubation chamber~~, to the photooptical analyzer without prior separation of the labelled sub-population from unreacted and/or unconsumed reagents and/or binding material.

PCT

WORLD

OPI DATE 05/07/89

APPLN. ID

28204 / 89

AOJP DATE 27/07/89

PCT NUMBER PCT/US88/04330

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

600493

(51) International Patent Classification 4:

A01N 1/02, G01N 1/00

A1

(11) International Publication Number:

WO 89/ 05092

(43) International Publication Date:

15 June 1989 (15.06.89)

(21) International Application Number: PCT/US88/04330

(22) International Filing Date: 1 December 1988 (01.12.88)

(31) Priority Application Number: 128,668

(32) Priority Date: 4 December 1987 (04.12.87)

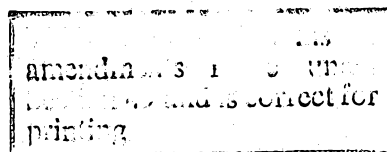
(33) Priority Country: US

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(81) Designated States: AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent).

Published*With international search report.**Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: CONSERVATIVE WHOLE BLOOD SAMPLE PREPARATION TECHNIQUE

(57) Abstract

A method for the rapid preparation of a whole blood sample for photooptical analysis. A whole blood sample, lytic reagent system and stain are contacted in a reaction vessel with gentle, asymmetric vortex mixing. This method conserves the endogenous constituents of the sample, since the preparation of the sample occurs within a common vessel and any excess, unconsumed or unreacted, reagents and stain are not separated from the sample during preparation; thus avoiding the multiple wash steps traditionally employed. The lytic agent preferably is acidic and stromatolyzes the erythrocyte fraction of the whole blood, leaving the leukocyte sub-populations in an unlysed, near native state. The lysing can occur, before, simultaneous with, or after the staining in an elapsed time of 90 seconds or less.

CONSERVATIVE WHOLE BLOOD SAMPLE PREPARATION TECHNIQUE

This invention is directed to a method. More specifically, this invention concerns a unique process for the rapid preparation of particulate analytes, notably cells, cell organelles and the like, for analysis by photooptical means. This process is particularly well suited for analysis of cellular analytes which are present in small concentrations and/or where a change in their relative proportion to another cell population is diagnostically significant. This process is particularly well suited for rapid preparation of whole blood samples for analysis by flow cytometry.

The traditional techniques for the isolation and staining of the leukocyte fraction of whole blood has typically involved numerous physical manipulations of the sample and an inordinate number of wash steps. As has been appreciated and reported in the technical literature, these traditional techniques inherently results in some finite depletion of the leukocyte population from the sample; at least some alteration in the gross morphology of the leukocyte population which remain in the sample; at least some alteration in the markers on the surface of the leukocyte population; and at least some displacement of the stain on the surface of the cell population of interest.

The techniques which have been used up to now in the preparation of leukocytes from whole blood samples for subsequent analysis, have traditionally involved: (1) the preparation of a buffy coat fraction by controlled centrifugation of the whole blood sample, followed by staining with a fluorochrome labelled conjugate; (2) concurrent treatment of a whole blood sample with a lytic reagent and a fluorochrome labelled conjugate; or (3) the preparation of a buffy coat fraction by controlled centrifugation of the whole blood sample and thereafter

subjecting the buffy coat sample to further enrichment by Ficoll-Paque density gradient centrifugation. Such enrichment permits the recovery of an interfacial layer containing enriched mononuclear cells, which are
5 thereafter stained with a fluorochrome labelled conjugate.

Each of the above preparative procedures is labor intensive, requires repeated physical manipulation of the fraction containing the leukocytes of interest and is time
10 consuming. Where conventional centrifugation techniques are used to obtain an enriched leukocyte sample, cell loss will invariably take place and, thus, accurate determination of the relative concentration of the cellular population of interest is virtually impossible.
15 The shortcomings in the above procedures have been recognized for some time and at least one alternative has been proposed in the technical literature: articles by Caldwell, appearing in American Journal of Clinical Pathology, Vol. 88:4 (1987) pp 447-456; and Vol. 86:5
20 (1986), pp 600-607. The Caldwell articles, however, focus only upon one facet of this problem, which he believes to be the principle detractor from prior procedures. More specifically, Caldwell emphasizes that excessive washing of the leukocytes samples subsequent to staining and prior
25 to analysis can introduce analytical error into the analysis, especially where leukocytes are observed in certain classes of diseased patients. Caldwell suggests adoption of a "no wash" technique to reduce processing time and analytical error which is inherent in the
30 extensive manipulation treatment of the leukocyte sample prior to its ultimate analysis. The "no wash" technique proposed by Caldwell involves retention of the traditional techniques for preparation of an enriched lymphocyte fraction by Ficoll-Paque density gradient centrifugation,
35 which itself employs multiple wash steps for removal of the Ficoll-Paque reagents. The enriched sample of lymphocytes is, thereafter, stained with a fluorochrome dye conjugated to a monoclonal antibody. Caldwell's

improvement resides in the observation of enhanced fluorescence intensity in both background and positive peaks of a histogram, when the unbound conjugate is allowed to remain in the sample. Caldwell attributes this observation of enhanced fluorescence intensity to his elimination of the wash steps after staining. He hypothesizes that the elimination of such wash step is less disruptive upon the stained lymphocyte fraction; thus enabling preservation of the immunochemical bond between the conjugate and the surface markers on the cell population of interest. As is clearly evident from review of Caldwell's "no wash" procedure, the repeated trauma of mixing, vortexing and centrifugation is only somewhat reduced.

The "enrichment" of the leukocyte sample through the use of lytic reagents has not, up to now, proven to be a viable alternative to the more traditional centrifugation/density gradient techniques for partitioning the whole blood sample into its various fractions. The reason for this limitation is the inability of prior lytic reagents to effect hemolysis of the erythrocyte fraction of whole blood without also traumatizing the leukocyte fraction of the sample. These lytic reagents typically cause both gross and subtle morphological changes in the leukocyte fraction and alteration in the surface markers on such cells. In the limited number of instances where lytic reagents have been used with some measure of success, additional reagents were required to be added to the sample to protect the leukocytes from lysis by the lytic reagent; see for example U.S. Patent 4,637,986 to Brown, et al. As is evident from the review of the Brown patent, the physiological environment of the sample is modified dramatically by the addition of both lytic reagent and the solutes which are used to protect the leukocytes from lysis by the lytic reagent. The samples prepared in accordance with Brown's reagents and technique can be

subjected to analysis by flow cytometry, where differentiation of the three principle sub-populations is accomplished by conventional light scattering measurements. Because of the alteration in the physiological environment of the sample, the integrity and immunochemical response of the surface markers on the leukocytes of the sample is altered as well. Thus, refinement in the analysis of the leukocyte fraction by immunochemical techniques is precluded. Moreover, where the leukocyte fraction is from a sample which is "aged" (not fresh) or a diseased patient sample, the sensitivity of the leukocytes to such harsh treatment is increased dramatically; thus further limiting the usefulness of the Brown reagents and technique.

As is evident from the above discussion, both the physical and chemical trauma which can be effected upon the leukocyte fraction by the above procedures, is likely to damage a number of the cells within this fraction. Even more unfortunate, these procedures are more destructive of the leukocytes, such as the lymphocyte sub-population of the disease state samples. Accordingly, the ability to analyze and monitor such disease state samples by traditional enrichment and staining techniques is severely limited. Where such analysis are to be performed, the more traditional centrifugation and density gradient enrichment protocols are preferred, since they are somewhat less disruptive of the lymphocytes than the effects of the lytic reagents.

The enhancement in accuracy and sensitivity observed by Caldwell in his "no-wash" procedure represents a step in the appropriate direction, however, further improvement is obviously necessary, particularly where relative cell population determinations are critical to an accurate analysis of the patients conditions. As is evident from the above discussion, none of the prior art techniques, even that described by Caldwell, provide a complete

solution to this problem, since virtually every procedure, even those utilizing lytic reagents, cause a statistically significant loss or destruction in one or more of the sub-populations of leukocytes, and at least 1 hour total
5 preparation time. This loss is statistically more significant in dealing with an aged sample, a sample essentially totally deficient in endogenous nutrient, and disease state samples, because of the relatively higher sensitivity of the nutritionally deprived cells and cells
10 indicative of the disease state to lysis by physical and chemical trauma.

The object of this invention is to provide a method and system for the conservation of the cellular analytes of
15 content of a sample to insure that the analysis thereof accurately reflects both the number of the cellular analytes of interest in the sample and, where appropriate, the relative concentration of such analytes to other cells of the sample. Thus, this invention is concerned with
20 preparation of the sample so as to insure that virtually all diagnostically relevant components of the sample are conserved.

The ability of this process to afford such advantage over
25 the more traditional techniques is based upon the conservative nature of the preparation process. The term "conservative" as used herein is intended as descriptive of the ability of this process to preserve virtually all of the blood components, with the exception of
30 erythrocytes, for later analysis. The integrity of the sample, with respect to the conserved components, also provides the first truly reliable means for the establishment of standards against which other samples can be measured. This process is particularly well adapted to
35 analysis of aged samples of whole blood, and to blood samples from disease state patients, wherein cell integrity is difficult to maintain and thus an accurate analysis heretofore impossible.

In the analysis of whole blood, a diagnostically relevant component of particular interest is the non-erythrocyte fraction. This invention provides the ability to effectively isolate and yet effectively conserve the non-erythrocyte fraction, without the washing and multiple transfer steps traditionally associated with that process. In the preferred embodiments of this invention, a whole blood sample is placed in a vessel or chamber. The chamber of choice is the same type of vessel which is used in the instrument selected for analysis of the sample. This process of preparation of the sample departs from the norm in that virtually all contact of the sample with reagents is performed in the chamber and the intimate interaction of the sample and such reagents is assured by asymmetric vortexing of the content of the chamber at appropriate intervals, frequency and for appropriate duration. By precisely controlling the reagent additions, reaction environment and the physical forces acting upon the sample, it is now possible to prepare a whole blood sample, even aged or disease state samples, without washing or statistically significant losses in any of the diagnostically significant non-erythrocyte blood components.

More specifically, this method and system provides a conservative method and system in which the mixing dynamics and reagents effect rapid and essentially complete hemolysis of the erythrocyte fraction of a whole blood sample while maintaining the non-erythrocyte fraction of the sample in its essentially native physiological state. This method and system are, thus, compatible with the selective staining of one or more subpopulations of the leukocyte fraction, either prior to, or concurrent with, hemolysis of the erythrocytes.

Subsequent to such staining, the sample can be subjected to analysis by semi-automated or automated techniques, for example by flow cytometry, for identification and quantification of the cell populations of interest. This

process of enrichment and staining of the leukocyte fraction of the sample can be performed within about sixty (60) seconds or less, provides improved clustering of the various sub-populations of the leukocytes within the sample, and is conservative of the integrity and of the total number of leukocytes present in the sample.

According to the invention there is provided a method for preparation of a whole blood sample for analysis by photooptical measurement techniques, which method includes enrichment of the non-erythrocyte cellular fraction of said sample and the labelling of one or more sub-populations of said fraction by immunochemical interaction of the cells of said fraction with an indicator labelled binding material which is specific for a characteristic cellular component of the sub-population of interest, the method comprising the steps of providing a common reaction vessel of the type which is typically utilized as a sample container for photooptical instrumentation designed for quantitation and/or differentiation of particulate analytes from other particulates which are endogenous to the sample; said method characterized by: preparing the sample for analysis in said reaction vessel by subjecting the sample to selective stromatolysis conditions by a lytic reagent, so as to enable effective differentiation of the non-erythrocyte fraction of said sample from the erythrocyte fraction; labelling at least one of said sub-populations with a binding material prior to, concurrent with, or after said step of selective stromatolysis; and transferring an aliquot of said sample, from the incubation chamber, to the photooptical analyzer without prior separation of the labelled sub-population from unreacted and/or unconsumed reagents and/or binding material.

The invention also provides a method for preparation of a whole blood sample for analysis by photooptical measurement techniques, which method includes enrichment

of the non-erythrocyte cellular fraction of said sample and the labelling of one or more sub-populations of said fraction by immunochemical interaction of the cells of said fraction with an indicator labelled binding material
5 which is specific for a characteristic cellular component of the sub-population of interest, the method comprising the steps of providing a common reaction vessel of the type which is typically utilized as a sample container for photooptical instrumentation designed for quantitation
10 and/or differentiation of particulate analytes from other particulates which are endogenous to the sample; said method characterized by: preparing the sample for analysis in said reaction vessel by subjecting the sample to selective stromatolysis conditions by an acidic lytic
15 reagent, so as to enable effective differentiation of the non-erythrocyte fraction of said sample from the erythrocyte fraction; then labelling at least one of said sub-populations with a binding material; and transferring an aliquot of said sample from said reaction vessel to the
20 photooptical analyzer.

By way of example, illustrated embodiments of the invention now will be described with reference to the accompanying drawings in which:

25 FIGURES 1-6 are: scattergrams 1A-6A and histograms 1B and 1C-6B and 6C, respectively, illustrating the Examples I-VI.

In one of the preferred embodiments of this invention, the
30 whole blood sample and stain are initially combined in a mixing chamber. A 12 x 75 mm test tube, of the type commonly used with an EPICS® brand flow cytometer, available from Coulter Corporation, is generally suitable for this purpose. The test tube and its contents can then
35 be agitated on a suitable mixing device, for example a Q-PREP™ mixer, also available from Coulter. This mixer has the capability for controlled mixing of the fluid contents

of the test tube and the automated introduction of additional reagents at appropriate pre-programmed intervals. Initially, an immunological stain can be contacted with the whole blood sample in the chamber, accompanied by gentle, asymmetric vortex mixing. After a suitable incubation period, generally less than sixty (60) seconds, a differentiation effective amount of a novel lytic reagent is contacted with the sample in the chamber and, thereafter, its lytic activity substantially retarded by the introduction of a suitable quench. This entire process of staining, lysing of the erythrocytes and quenching of the activity of the lytic reagent is performed without physical isolation of the cell types from one another, without transfer of the cells of the sample from one vessel to another and without the need for removal of unreacted stain and/or unconsumed reagents from the chamber prior to analysis or measurement of the sample. The stained leukocyte fraction now can be subjected to further analysis on instrumentation designed for that purpose, for example a COULTER® EPICS Model C or PROFILE flow cytometer. The analysis of the sample on such instrumentation enables the generation of a histogram with well-defined clustering of the stained leukocyte fraction of interest.

The method and system of this invention are unique in a number of significant respects: (a) the ability to, sequentially or concurrently, rapidly stain and enrich the non-erythrocyte fraction of a whole blood sample; (b) the conservation of the morphology and total number of leukocytes originally present in the sample; (c) the preservation of the immunochemical interaction between the stain and the characteristic cellular components of the cell population of interest; (d) the preservation of the stained, enriched sample for up to twenty-four (24) hours prior to analysis without material alteration of the characteristic size and/or shape of the sub-population of interest; and (e) the compatibility of the reagents

utilized in the staining sequence with the reagents used in both lytic sequence and quenching sequence so as to eliminate the necessity of washing of the sample between any or each such processing step.

- 5 Preliminary to entering into a detailed discussion of the method and system of this invention, it will be helpful to initially define a number of terms used herein.

10 The terms "stain" and "staining" are descriptive of a process for identification of a specific cell population of interest, so as to permit its differentiation from other cells which are also generally present in a sample undergoing analysis.

- 15 The term "conservative" is intended as descriptive of both the conditions and manipulative steps of the method of this invention which preserve not only the total number of leukocytes originally present in the sample, but also their gross morphology and their immunochemical
20 reactivity. Such conservation is achieved without prior fixation of the leukocyte population, but can include fixation subsequent to such conservation preparation.

25 The terms "nutative" and "nutative motion" are descriptive of an asymmetric or eccentric mixing action of a reaction vessel, wherein the sample and various reagents (stain, lytic reagent, quench and fixative-if any) are maintained in a mild state of agitation. This type of movement of the reaction vessel causes the fluid contents of the
30 vessel to asymmetrically distribute itself within the vessel, rising up to a greater height on one side of the vessel than the other. Because of this asymmetry, the vortex which forms in the vessel does not behave in the traditional manner.

- 35 The term "enrichment" is descriptive, in the context of the prior art, of the physical separation of the leukocyte

population, the buffy coat, from the other constituents of the whole blood sample. However, in the context of this invention, "enrichment" is the relative increase in ease of identification of the leukocyte population upon lysis
5 of the erythrocyte fraction of the sample.

The complement of chemicals, which are employed to effect both hemolysis of the erythrocyte fraction of the sample and subtle modification of the leukocyte fraction, and
10 thereby facilitate their subsequent isolation, identification and/or analysis, are collectively referred to as the "lytic reagent system". In the context of this invention, such system includes a lytic reagent and a companion quench, which is formulated for substantially
15 retarding the lytic activity of the lytic reagent and restoring the ionic balance of the sample.

The phrase "differentiation effective amount" is used through this disclosure as indicative of a concentration
20 of lytic reagent which is not only effective for hemolysis of the erythrocyte fraction of the sample, but also effects subtle changes in the leukocyte fraction to facilitate their subsequent isolation, identification and/or analysis.

25 As noted previously in the discussion of the relevant prior art, the preferred technique for staining of the leukocyte fraction involves an immunochemical interaction of an indicator, antibody conjugate with a surface marker, which is characteristic of the cell fraction of interest.
30 The relative avidity of the conjugate for a specific surface marker of interest can vary depending upon the precise location and conformation of the epitopic site on the cell surface. Where the cell population of interest, or its cell count, is indicative of a disease state, these
35 cells tend to be more fragile and, thus, their interaction with the stain more sensitive to physical trauma and to chemical imbalance in the sample environment. Thus, the

abbreviated contact with the lytic reagent, generally less than ten (10) seconds, and the subsequent restoration of the ionic balance of the leukocyte fraction, by the addition of the quench, is conservative of both the total
5 numbers and of the qualities of the cells of interest and, thus, facilitate their subsequent identification by immunochemical techniques. Since there is typically a hiatus between the time at which the sample is enriched and stained and its subsequent analysis, the sample is
10 preferably contacted with a fixative to preserve both the physical size and shape of the leukocyte fraction of interest. Such fixation must be compatible with the prior enrichment and staining procedures and be further compatible with the analytical means selected for the
15 determination, measurement of the relative concentration of the cell population of interest. Where the analytical method of choice is flow cytometry, the fixative selected must not exhibit any native or autofluoresce at the wavelength of excitation energy of the fluorochrome label
20 of the conjugate stain or at the emission wavelength of fluorochrome stain. The preferred fixative used in this invention is paraformaldehyde.

In the practice of the method of this invention,
25 peripheral blood is aseptically collected by standard venipuncture techniques in tubes containing an anticoagulant, EDTA or heparin. The specimen is transferred to a mixing chamber of the type typically used in conjunction with the analytical measuring device of
30 choice. In the semi-automated equipment contemplated for practice of this method of invention, the sample then is contacted with appropriate quantities of the stain, lytic reagent and fixative. During this period of contact with these various reagent materials, the sample is maintained
35 in mild agitation. In one of the preferred embodiments of this invention the stain is combined with a whole blood sample prior to addition of the lytic reagent system and fixative. The activity of the lytic reagent is

substantially retarded by the addition of a quench about ten (10) seconds after initial contact of the sample with the lytic reagent. The staining is allowed to continue, with the entire process being completed within about
5 ninety (90) seconds. An optional fixative is preferably added at the conclusion of the staining cycle and subsequent to the addition of the quench. In an alternative embodiment of this invention, the enrichment of the leukocyte fraction of interest can precede staining
10 of the sample. In this embodiment of the invention, the stain is simply added to the whole blood sample and allowed to interact with the cellular fraction of interest. Such interaction occurs, under mild agitation, for approximately 15 to 60 seconds, after addition of the
15 lytic agent, quench and optional fixative.

The preferred lytic reagent which is suitable for use in the method and system of this invention comprises an aqueous solution containing a differentiation effective
20 amount of a lytic reagent comprising a water soluble, organic carboxylic acid having a pK value >3.0 , a pH in the range of from about 2.6 to 4.0 and a counterion which does materially alter the ionic strength of the treated sample. The most preferred lytic reagents are selected
25 from the group consisting of formic acid, acetic acid and their respective mixtures. In the preferred embodiments of this invention, these lytic reagent mixtures will comprise formic acid, as the major functional component, with the acetic acid being present in only minor amounts,
30 if at all.

The lytic reagent employed in the method and system of this invention comprises an aqueous solution containing surprisingly low concentrations of the lytic reagent,
35 preferably less than 1.0% by volume. This aqueous solution is prepared by simple addition of the lytic reagent to deionized water. The amount of lytic reagent added to this diluent is sufficient to prepare a solution

preferably
containing from about 0.05 to about 0.5% (v/v) solution.
In the preferred embodiments of this invention, the concentration of lytic reagent will range from about 0.1 to about 0.25% (v/v).

- 5 When the lytic reagent comprises a mixture containing both formic and acetic acid, the acetic acid is preferably only present as a partial replacement for a definitive amount of formic acid and then only at a concentration in the range of from about 0.05 to 0.10% (v/v).
- 10 The lytic reagent system also can contain other traditional additives, to the extent their presence is not otherwise incompatible with the primary functional components of the system, for example, an anti-microbial preservative, such as sodium omadine.
- 15 This lytic reagent can be combined with a whole blood sample by simple manual or automated addition, allowed to briefly interact in a chamber, and the action of the lytic reagent substantially then retarded by addition of a suitable quench. The quench, to be effective in this
- 20 environment, must be capable of retarding the lytic activity of the lytic reagent immediately upon its addition to the aqueous mixture containing the blood sample and the lytic reagent. The precise formulation of the quench can vary, depending upon the composition of the
- 25 lytic reagent and other fluids which may be dictated by the requirements of the analytical measurement instrument. The quench is typically an aqueous solution containing soluble salts which are both effective to substantially retard and/or substantially neutralize the lytic activity
- 30 of the lytic reagent and restore the ionic balance to the sample. This restoration of the ionic balance will extend the longevity of the surviving cells and permit subsequent analysis on equipment which requires that the sample be electrically conductive, contain electrolytes, as for
- 35 example with COULTER VCS flow cytometer.



A quench which is suitable for use in conjunction with the lytic reagent composition can, and usually will, contain any combination of at least two of the following four ingredients; sodium chloride, sodium sulfate, sodium bicarbonate, and sodium carbonate; and, in addition, sodium azide as a preservative. The effectiveness of the quench upon the lytic reagent in the context of the method and system invention is determined by its ability to rapidly reduce the lytic activity of the acidic lytic reagent.

The method and equipment utilized in the differentiation of the leukocyte sub-population also can place certain requirements, conductivity, pH, etc., upon the precise formulation of the quench. More specifically, when such differentiation is performed in a focused flow aperture analysis system, such as the COULTER VCS, the composition and volume of quench can be important to optimal separation (differentiation) of the five (5) leukocyte subclasses from one another. In this type of analysis system, the ionic balance of the quench must also be adjusted to obtain a satisfactory conductivity match of the lysed blood sample to the sheath fluid. In the preferred quench formulation, the major ionic species and their relative ratio in the lysed-quenched blood sample should be essentially the same as the major ionic species and their relative ratio in the sheath fluid. Components of the sample, for example fibrin and platelets, are pH sensitive and can form aggregates under acidic conditions which can potentially interfere with differential analysis or physically obstruct the aperture of a focused flow aperture system. These considerations with respect to pH adjustment may also prove important in other analytical measurement environments.

The relative concentration of the functional components of the quench, which is to be used in conjunction with the lytic reagent system of this invention, will range from

about 1 to about 3% (w/v) sodium chloride, about 0.25 to about 0.8% (w/v) sodium carbonate or bicarbonate, and about 2 to about 4% (w/v) sodium sulfate. The relative quantities of ingredients of the optimum quench are generally determined empirically. The objective of such adjustment is to attain the pH of the lysed blood sample within the range of from about 6.0 to about 7.5 and a final osmolality of the stabilized lysed blood sample in the range of from about 300 to about 330 mOs. In a focused flow aperture system, optimal clustering of the leukocyte subclasses is achieved by adjustment in the osmolality of the final blood sample to about 310 mOs.

In the preferred embodiments of this invention, the duration of effective contact of the lytic reagent and the blood sample, from the time the two are combined, to the time when the quench is added, must be less than ten (10) seconds and most preferably six seconds or less. The interval of reactive contact of the lytic reagent and blood sample as specified above, presumes such reactive contact occurs at room temperature, 18-28°C.

The stain which is used in the method and system of this invention comprises an indicator which has been conjugated to a protein which is specific for binding to an epitope on the cell surface of a cellular analyte of interest, for example B-lymphocytes and T-lymphocytes. Antibodies specific for leukocytes surface markers are available from a number of commercial sources. In the preferred embodiments of this invention a monoclonal antibody is the reagent of choice. Monoclonals which are specific for surface markers on T-cell and B-cells can be readily obtained commercially and also have been conjugated to an appropriate fluorochrome.

A series of analysis, Examples I-V, were made in the evaluation of the method and reagent system of this invention by comparison to prior art techniques used in

enrichment and staining of a specific lymphocyte population of interest (B- and T-cells). In each instance, the whole blood sample was common to each, as was the method and equipment for measurement of the specific cell sub-populations of interest. The technique for separation, enrichment of the leukocyte fraction followed the protocol established for each of the reagent systems designed for that purpose. The method and reagent system of this invention is illustrated in Example VI. In each of these Examples, the analysis of the enriched sample was conducted on a COULTER EPICS Model C flow cytometer, following the standard operating protocol for this instrument. The results of each analysis is depicted in a series of scattergrams, FIGURES 1A-6A, and histograms, FIGURES 1B,C-6B,C, with FIGURES 1-5 representing the prior art and FIGURE 6 the method and reagent system of this invention.

Example I

The separation media and method used in the preparation of the sample for this Example involved separation, enrichment of the leukocyte fraction by traditional lysis techniques, including sample washing, preceeded by staining with fluorochrome labeled antibodies which are specific for B and T-cells, respectively; (Ortho Diagnostic System, procedure for preparation and staining of mononuclear cells for analysis on Ortho SPECTRUM III Laser Flow Cytometry System. CYTOFLUOROGRAFTM). Following preparation of the sample, the relative concentration of B and T-cells was determined in an EPICS Model C flow cytometer. FIGURE 1A represents a scattergram of the leukocyte sub-populations of whole blood sample from a healthy donor. FIGURE 1B is a histogram which represents the relative B-cell concentration of (12.20%) between the B-cells and the non B-cells. FIGURE 1C represents the relative T-cell concentration of (83.30%) between the T-cells and the non T-cells.

Example II

Sample preparation involved lysis of erythrocytes with a detergent, in accordance with the procedures described for the reagent system marketed under the Immunolyse label by Coulter Corporation Immunology Division. Sample analysis is performed in the same manner as for the sample of FIGURE 1. FIGURE 2A represents a scattergram of the leukocyte sub-population of a whole blood sample from the same healthy donor as depicted in the scattergram of FIGURE 1A. FIGURE 2B represents the relative concentration (15.40%) of B-cells in the sample. FIGURE 2C represents the relative concentration (79.59%) of T-cells in the sample. Note the variations in results in comparison of reported relative concentration of B cells and T cells with the values reported for the analysis conducted in accordance with FIGURE 1.

Example III

Sample preparation involved lysis of erythrocytes with diethylene glycol in accordance with the procedures described for the reagent system marketed under the FACS label by Becton-Dickinson. FIGURE 3A represents a scattergram of this leukocyte sub-population of a whole blood sample from the same healthy donor as in FIGURES 1A & 2A. FIGURE 3B is an enhanced histogram representing the relative concentration of the B-cells in sample (19.04%). FIGURE 3C is an enhanced histogram representing the relative concentration of the T-cells in the sample (69.17%). Note the lack of correlation of the results with those of FIGURES 1 and 2. Moreover, the relative concentration B to T-cells has also shifted dramatically, indicating that the sample preparation, lysis of RBC's with diethylene glycol, is unduly harsh upon T-cells.

Example IV

Sample preparation involved density gradient separation of primarily lymphocyte and monocyte sub-populations. This procedure is acknowledged to be highly technician dependant and reportedly prone to substantial cell losses. FIGURE 4A represents a scattergram of the leukocyte sub-populations of a whole blood sample from the same healthy donor as in FIGURES 1A, 2A and 3A. FIGURE 4B is an enhanced histogram representing the relative concentration of the B-cells in such sample (4.64%). FIGURE 4C is an enhanced histogram representing the relative concentration of the T-cells in such sample (88.55%). Note the lack of correlation of the results with those of FIGURES 1, 2 and 3. Moreover, the relative concentration of B-cells to T-cells has dramatically shifted, indicating that the repeated washing and physical manipulation of this procedure is more traumatic to B-cells than T-cells.

20

Example V

The procedures of Example IV were repeated, except that the unreacted stain was allowed to remain in the chamber and the analysis conducted as specified per Caldwell et al "no wash". The scattergram of FIGURE 5A represents the leukocyte sub-populations which were capable of differentiation. FIGURE 5B is an enhanced histogram representing the relative concentration of the B-cells (8.14%). FIGURE 5C is an enhanced histogram representing the relative concentration of the T-cells (87.75%). Note the increased relative concentration of the B-cell population, thus confirming the Caldwell et al observation regarding the advantages of elimination of the wash step following staining of the sample.

Example VI

Sample preparation involved rapid (less than 90 seconds total elapsed time) staining, lysis, quench and fixation of the sample. Sample preparation involved the automated dispensing and mixing of the following reagents, in prescribed sequence, with the a whole blood sample from the same donor as in Examples I-V.

10 I. Materials

1. Lyse: 1.2 mL formic acid
0.2 mL sodium omadine (40%)
998.6 mL deionized water

15 2. Quench: 31.30 grams sodium sulfate
14.50 grams sodium chloride
6.00 grams sodium carbonate
1 liter deionized water

20 3. Fixative: 6.05 grams TRIS (HCL) buffer
10.00 grams paraformaldehyde
00.05 mL sodium hydroxide (50%)
1 liter deionized water

25 4. Stain: Becton-Dickinson Immunocytometry
Systems
G1- FITC
HLE-FITC

30 Coulter Immunology Division
MIG-FITC TY-RD1
MIG-RD1 T1-FITC
T8-FITC B1-FITC

35 The COULTER Q-PREP device was used in the programmed addition of the stain, lytic reagent system and fixative, and delivered a pre-set amount of each of these reagents

at the appropriate times, with mild agitation of a 25x75 mm test tube, operating on the following cycle:

- (a) addition of fluorochrome labeled antibody to sample
- 5 (b) mild agitation of sample and stain for 60 seconds
- (c) addition of lytic reagent to stain and sample
- (d) agitate for 6 seconds
- (e) addition of quench
- (f) agitate for 10 seconds
- 10 (g) addition of fixative
- (h) place test tube in flow cytometer and analyze for relative population of B-cells and T-cells.

FIGURE 6A is a scattergram of the leukocyte sub-population
15 of a whole blood sample from the same healthy donor as in FIGURES 1A, 2A, 3A and 5A. FIGURE 6B is an enhanced histogram representing the relative concentration of the B-cells in such sample (19.79%). FIGURE 6C is an enhanced
20 histogram representing the relative concentration of T-cells in such sample (76.97%). Note the relative high concentration of B-cells, indicating that the method and reagent system of this invention are effective in the identification of the relevant sub-population of interest
25 and that this technique is efficacious with minimal technician involvement, without any washing steps.

CLAIMS:

22

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

1. A method for preparation of a whole blood sample for analysis by photooptical measurement techniques, which method includes enrichment of the non-erythrocyte cellular fraction of said sample and the labelling of one or more
5 sub-populations of said fraction by immunochemical interaction of the cells of said fraction with an indicator labelled binding material which is specific for a characteristic cellular component of the sub-population of interest, the method comprising the steps of:
 - 10 (a) providing a common reaction vessel of the type which is typically utilized as a sample container for photooptical instrumentation designed for quantitation and/or differentiation of particulate analytes from other particulates which are endogenous to the sample; said
15 method characterized by:
 - (b) preparing the sample for analysis in said reaction vessel by subjecting the sample to selective stromatolysis conditions by a lytic reagent, so as to enable effective
20 differentiation of the non-erythrocyte fraction of said sample from the erythrocyte fraction;
 - (c) labelling at least one of said sub-populations with a binding material prior to, concurrent with, or after said
25 step of selective stromatolysis; and
 - (d) transferring an aliquot of said sample, from the ^{reaction vessel} ~~incubation chamber~~, to the photooptical analyzer without prior separation of the labelled sub-population from
30 unreacted and/or unconsumed reagents and/or binding material.



2. A method for preparation of a whole blood sample for analysis by photooptical measurement techniques, which method includes enrichment of the non-erythrocyte cellular fraction of said sample and the labelling of one or more sub-populations of said fraction by immunochemical interaction of the cells of said fraction with an indicator labelled binding material which is specific for a characteristic cellular component of the sub-population of interest, the method comprising the steps of:
- 10 (a) providing a common reaction vessel of the type which is typically utilized as a sample container for photooptical instrumentation designed for quantitation and/or differentiation of particulate analytes from other particulates which are endogenous to the sample; said
- 15 method characterized by:
- (b) preparing the sample for analysis in said reaction vessel by subjecting the sample to selective stromatolysis conditions by an acidic lytic reagent, so as to enable
- 20 effective differentiation of the non-erythrocyte fraction of said sample from the erythrocyte fraction; then
- (c) labelling at least one of said sub-populations with a binding material; and
- 25 (d) transferring an aliquot of said sample from said reaction vessel to the photooptical analyzer.

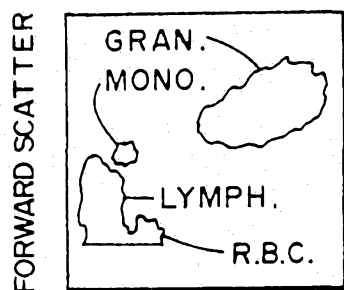
3. The method of claim 2, characterized wherein said step of transferring is accomplished without prior separation of the labelled sub-population from unreacted and/or unconsumed reagents and/or binding material.

4. The method of claim 2, characterized wherein said step of transferring is accomplished such that the relative concentration of lytic reagent and indicator labelled binding material, relative to each other and to the whole blood sample, enables effective differentiation of the labelled sub-population of interest from the other sample constituents, without prior separation of the labelled sub-population of interest from the unreacted and/or unconsumed lytic reagent system and/or indicator labelled binding materials, which remain free within the sample and other constituents of the sample.
5. The method of any one of claims 1, 2 or 3, characterized wherein said lytic reagent has a pH in the range of from about 2.6 to about 4.0.
6. The method of any one of claims 1, 2 or 3, characterized wherein the lytic reagent comprises a stromatolysis effective amount of an organic carboxylic acid having a pH in the range of from about 2.6 to about 4.0.
7. The method of any one of claims 1, 2 or 4, characterized wherein the lytic reagent is an organic acid selected from the group consisting of formic acid, acetic acid and their respective mixtures.
8. The method of any one of claims 1, 2 or 4, characterized by the further step of quenching the activity of said lytic reagent with a companion reagent which is effective in arresting the lytic activity of the lytic reagent and the restoration of the ionic balance of the sample.
9. The method of any one of claims 1, 2 or 3, characterized wherein the sample is contacted with a fixative subsequent to labelling with the binding material.

10. The method of any one of claims 1, 2 or 3,
characterized by contacting the whole blood sample with
the lytic reagent and with the indicator labelled binding
material in said reaction vessel by means of gentle vortex
5 mixing, to maintain the sub-populations of said fraction
of the sample at an essentially homogeneous concentration
throughout the sample.

11. The method of any one of claims 1, 2 or 4,
characterized wherein said step of preparing involves
contacting the sample with the lytic reagent and the
binding material, and the asymmetric movement of said
5 reaction vessel to cause gentle mixing, said preparing
requiring less than 90 seconds.

FIG. 1



90° SCATTER

FIG. 1A

FIG. 2

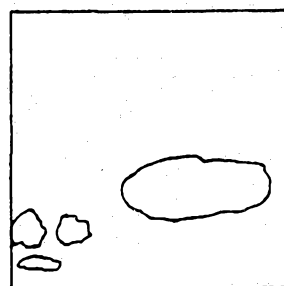
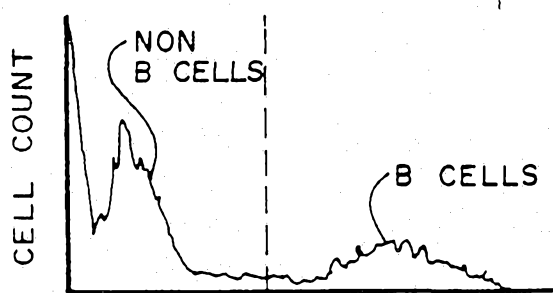


FIG. 2A



SIGNAL INTENSITY

FIG. 1B

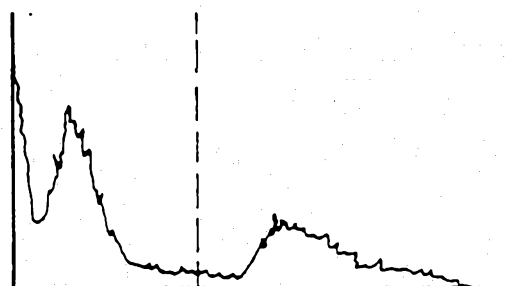
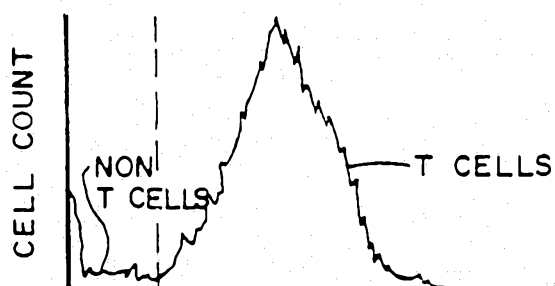


FIG. 2B



SIGNAL INTENSITY

FIG. 1C

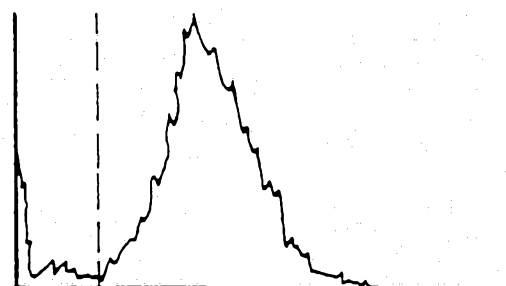


FIG. 2C

.2/3

FIG. 3

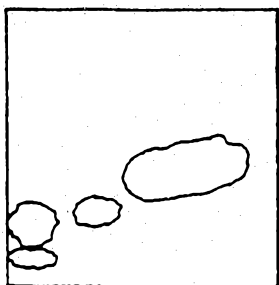


FIG. 4

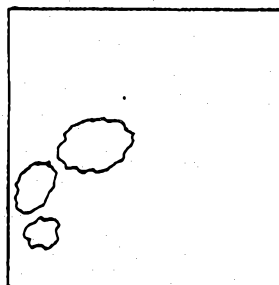


FIG. 3A

FIG. 4A

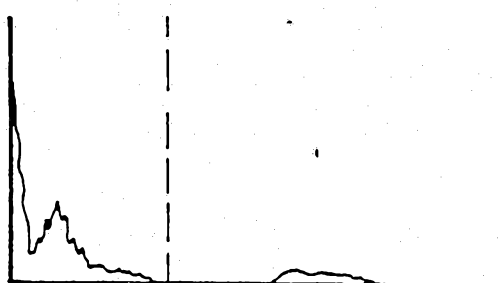
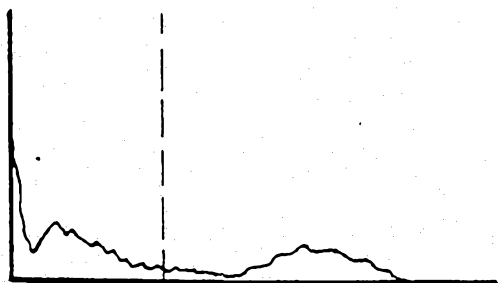


FIG. 3B

FIG. 4B

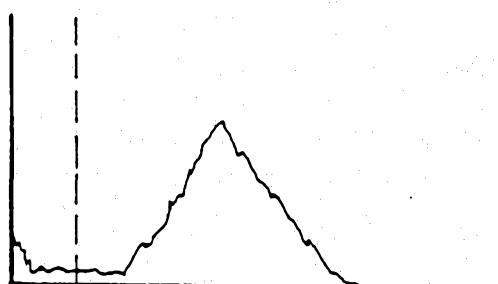
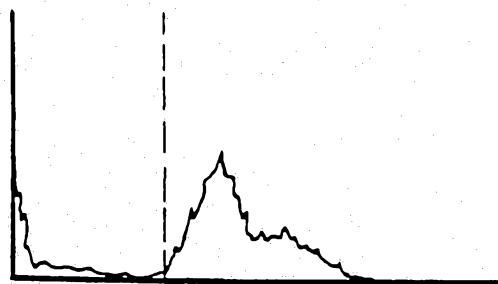


FIG. 3C

FIG. 4C

FIG. 5

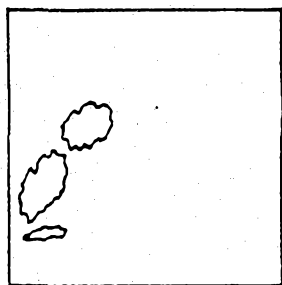


FIG. 5A

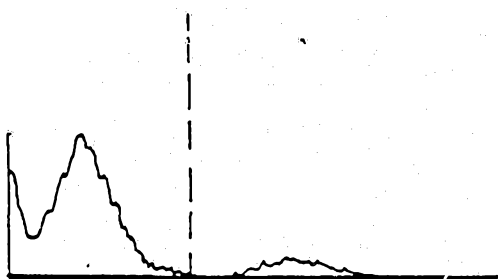


FIG. 5B

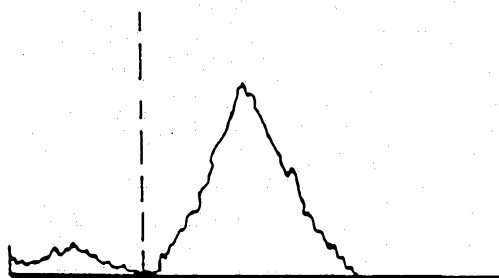


FIG. 5C

FIG. 6

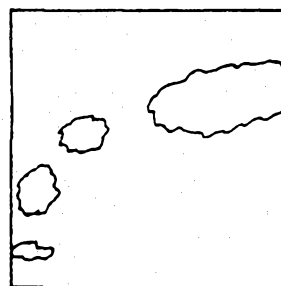


FIG. 6A

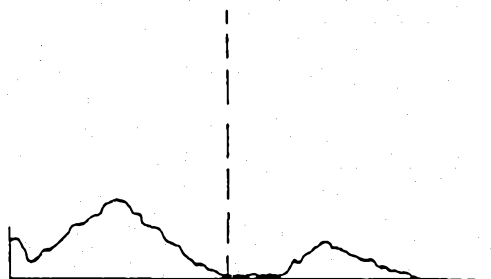


FIG. 6B

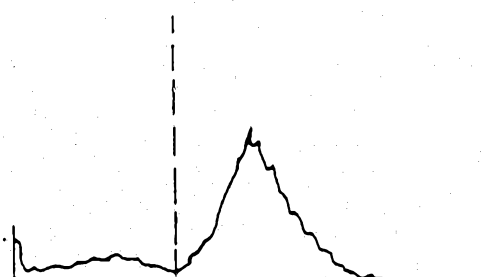


FIG. 6C

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US88/04330**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): A01N 1/02; G01N 1/00 U.S. CL.: 435/2; 436/63														
II. FIELDS SEARCHED Minimum Documentation Searched ⁷ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black;">Classification System</th> <th style="border: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">U.S.</td> <td style="border: 1px solid black;">435/2; 436/17, 63, 166, 172, 174, 175, 522</td> </tr> </table> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ Automated Patent System (File USPAT, 1975-1988).			Classification System	Classification Symbols	U.S.	435/2; 436/17, 63, 166, 172, 174, 175, 522								
Classification System	Classification Symbols													
U.S.	435/2; 436/17, 63, 166, 172, 174, 175, 522													
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; border: 1px solid black;">Category [*]</th> <th style="border: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 10%; border: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4,521,518 (CARTER) 04 June 1985, See column 1, lines 11-23, column 2, lines 16-48, column 3, lines 11-14, and column 7, lines 19-35.</td> <td style="text-align: center; vertical-align: top;">1-11</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4,637,986 (BROWN) 20 January 1987, See the Abstract and Column 2, lines 10-68.</td> <td style="text-align: center; vertical-align: top;">1-11</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Am. J. Clin. Pathol., Volume 86, No. 5, pp. 600-607, published 1986, (Philadelphia, Pennsylvania, USA) C.W. Caldwell, "A Rapid, No-Wash Technic for Immunophenotypic Analysis by Flow Cytometry" See page 600, column 2, first complete paragraph and page 601, column 1, final paragraph.</td> <td style="text-align: center; vertical-align: top;">1-11</td> </tr> </tbody> </table>			Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	US, A, 4,521,518 (CARTER) 04 June 1985, See column 1, lines 11-23, column 2, lines 16-48, column 3, lines 11-14, and column 7, lines 19-35.	1-11	Y	US, A, 4,637,986 (BROWN) 20 January 1987, See the Abstract and Column 2, lines 10-68.	1-11	Y	Am. J. Clin. Pathol., Volume 86, No. 5, pp. 600-607, published 1986, (Philadelphia, Pennsylvania, USA) C.W. Caldwell, "A Rapid, No-Wash Technic for Immunophenotypic Analysis by Flow Cytometry" See page 600, column 2, first complete paragraph and page 601, column 1, final paragraph.	1-11
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[*] Special categories of cited documents: ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the International filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the International filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family														
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 02 February 1989 </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report 18 APR 1989 </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority ISA/US </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer TONI R. SCHEINER </td> </tr> </table>			Date of the Actual Completion of the International Search 02 February 1989	Date of Mailing of this International Search Report 18 APR 1989	International Searching Authority ISA/US	Signature of Authorized Officer TONI R. SCHEINER								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Am. J. Clin. Pathol., Volume 88, No. 4, pp 447-456, published 1986, (Philadelphia, Pennsylvania, USA) C.W. Caldwell, "Fluorescence Intensity as a Quality Control Parameter in Clinical Flow Cytometry", See the paragraph bridging pages 448 and 449.	1-11
Y	Henry, J.B., "Clinical Diagnosis and Management by Laboratory Methods", 17th edition, published 1984, by W.B. Saunders Company (Philadelphia, Pennsylvania, USA), See page 590, Column 1, final paragraph.	1-11