PREPARATION AND STABILISATION OF CELL SUSPENSIONS

BACKGROUND OF THE INVENTION

This invention relates to the preparation and stabilisation of cell suspensions, and more particularly to a novel method for preparing and stabilising cell suspensions, and to the use of novel stabilised cell suspension preparations in the quality control of analytical techniques such as UV microscopy and flow cytometric leucocyte immunophenotyping techniques, immobilised antigen/antibody detection systems and haematology analysers, and blood monitoring techniques such as zinc protoporphyrin (ZPP), red cell folate and blood glucose measurements.

UV microscopy and flow cytometry are techniques used in the diagnosis of haematological malignancies. They are also used to monitor the progress of patients infected with the Human Immunodeficiency Virus (HIV), whether asymptomatic or suffering from ARC or full-blown AIDS. Quality control (QC) of these two techniques is extremely important to arrive at the correct diagnosis and to monitor effective therapeutic regimes. The current QC methods use freshly drawn blood or microspheres coated with a fluorochrome.

The use of fresh blood on a daily basis fails to provide the information on day to day variation of the technique or equipment. Furthermore, fluorochrome coated

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microspheres, though providing a day to day monitor of the flow cytometer's performance cannot be used for UV microscopy work. In addition, they cannot be used to provide quality control for the labelling techniques of leucocytes.

Fixation of normal leucocytes, utilising compounds such as paraformaldehyde, although giving stability for 5-7 days increases cellular autofluorescence. This makes the preparation unsuitable for use as a long term quality control material. Furthermore, the lysis of red cells by the whole blood lysing technique requires a preparation that will quality control this procedure. The current methods of fixing the leucocytes inhibit this lysing procedure resulting in a significant increase in debris that interferes with the tests.

Other QC equipment requiring the use of whole blood samples or blood products for calibration include haematology analysers where currently fixed blood from donkeys and turkeys is used because a suitable source of stabilised human blood is not available. Zinc protoporphyrin (ZPP) and red cell folate monitoring techniques also require a fresh suspension of red blood cells for calibration, again because a suitable stabilised source is not available. Finally the lack of a stabilised source of whole human blood for calibration purposes limits the possibility for diabetics to carry out blood sugar monitoring at home.

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In International Application No. PCT/US91/03236, the entire disclosure of which is incorporated herein by reference, there is described a blood diluent and lysing agent for differential determination of white blood cells (leucocytes) in which the stabilising agent is diazolidinyl urea. Such leucocyte preparations have not been suggested as flow cytometric preparations possibly because they have insufficient stability and lack certain specific antigenic activity for those routine quality control procedures which need to assess results from a large number of laboratories.

International Application No. PCT/US92/03758 discloses the use of diazolidinyl urea, imidazolidinyl urea, dimethylol-5,5-dimethylhydantoin, dimethylol urea, 2-bromo-2-nitropropane-1,3-diol and quaternary adamantane as tissue fixatives which are free of aldehydes. The formulations may contain inter alia mordants such as zinc, calcium, barium and chromium salts. It is not suggested, however, that any of these salts have stabilising properties.

GB1563839 provides a process for preparing a stabilised preparation of erythrocytes, wherein a suspension of erythrocytes in an aqueous isotonic solution is reacted with, simultaneously or successively in any sequence, an aliphatic aldehyde and a water-soluble salt of 2- or 3- valent chromium. In the examples, the erythrocytes are treated with formaldehyde

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and glutaraldehyde, followed by an aqueous solution of chromium III chloride.

It has been found that cell suspensions containing erythrocytes treated in this way are no longer suitable for use in a whole blood lysing technique, and thus the process of GB1563839 cannot be used to prepare stabilised whole blood samples, for example, for flow cytometry quality control.

GB2001757 describes a method of preparing a stable suspension of blood platelets which comprises adding chromium ions to a platelet-rich plasma and adding at least one organic aldehyde to the chromium-platelet-rich plasma mixture. In the examples, the platelet-rich plasma is treated with solutions of potassium dichromate and glutaraldehyde. This process, if used on leucocyte cells, substantially increases autofluorescence and renders the suspension unsuitable for use in flow cytometry.

The entire disclosures of the above-mentioned patent and applications are incorporated herein by reference.

It will be appreciated from the above, that there is a need for an improved method for stabilising cell suspensions, particularly of whole blood and blood products, giving a cell preparation that is still capable of lysis, for a variety of quality control, monitoring and calibration applications.

In UK Patent Application Nos. 9313962.4 and 9315871.5 it is disclosed that certain heavy metal

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compounds can stabilise leucocyte preparations formed from lysed whole blood, and that such stabilised preparations can be added to leucocyte-depleted blood to provide a stabilised whole blood preparation with high antigenic activity. The entire disclosures of these applications are incorporated herein by reference.

OBJECTS OF THE INVENTION

It is an object of the invention to provide an improved stabilised cell suspension preparation which is still capable of lysis and an improved method for producing such stabilised cell suspensions.

It is also an object of the invention to provide a stabilised whole blood preparation which is still capable of lysis and a method of manufacturing such a preparation.

It is a still further object of the invention to provide stable quality control materials which can be used in a wide spectrum of quality control, analysis and monitoring techniques.

SUMMARY OF THE INVENTION

It has now been discovered that a wide range of cell suspensions comprising cells capable of lysis can be stabilised by the sequential addition of a heavy metal compound and paraformaldehyde to the suspension in

effective amounts, and that such stabilised cell suspensions will remain active for much longer periods than those known hitherto.

In one aspect the invention provides a method of treating a suspension of cells capable of lysis, to produced a stabilised cell suspension still capable of lysis, which comprises:

treating the cell suspension, in an aqueous medium maintained at a pH of from 6.5 to 7.5, sequentially with,

1) an aged aqueous solution of a heavy metal compound, the pH of the solution being from 6.5 to 7.5, and the solution having been maintained at a pH of from 6.5 to 7.5 for a period of time sufficient to allow any precipitate to form, and

2) an aqueous solution of paraformaldehyde, the pH of the solution being from 6.5 to 7.5.

The invention is particularly applicable to the stabilisation of whole blood and of blood products and will be henceforth more particularly described with reference thereto. It is to be understood, however that the invention is not limited to the stabilisation of such materials, and is broadly applicable to a wide range of cell suspensions capable of lysis.

In a further aspect, therefore, the invention provides an unseparated stabilised whole blood preparation capable of lysis in which the stabilising agent comprises effective amounts of a heavy metal

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compound, particularly a heavy metal salt, and paraformaldehyde, and a method of stabilising a whole blood preparation capable of lysis by adding effective amounts of the compound and paraformaldehyde thereto.

DETAILED DESCRIPTION OF THE INVENTION

In UK Patent Applications Nos. 9313962.4 and 9315871.5, leucocyte preparations separated from lysed whole blood are stabilised by the addition of a stabilising agent, and then added back to leucocyte depleted whole blood (red blood cells) to from a stabilised whole blood preparation. The present invention provides an alternative method for the preparation of stabilised whole blood in which the lysing and separating steps can be omitted.

The invention will now be particularly described with reference to the manufacture of stabilised whole blood preparations for use in laboratory quality control procedures, although it will be appreciated that the invention is not limited thereto and that, for example, the stabilised whole blood preparations may find other uses, and that the stabilisation procedures described may find application in the manufacture of stabilised cell suspensions from sources other than blood, for example microbial cells, plant cells and similar materials derived from living tissue.

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In this specification, a suspension of cells capable of lysis is defined as: a suspension of cells, such as, for example, whole human blood, in which specific cell populations can be selectively lysed using erythrocyte lysing reagents. Lysis comprises rupture of the cell membrane and leakage of the cell contents. Lysing reagents and conditions suitable for the selective lysis of the erythrocyte component of whole human blood include, for example, treatment in aqueous solution with an ammonium salt, for example, ammonium chloride, or a quaternary ammonium salt and urea, or a substituted urea, or urea derivative, as described in UK Patent Applications Nos. 9313962.4 and 9315871.5.

Whole blood preparations in this specification include preparations containing substantially all the components of fresh blood, and preparations containing substantially all the components of fresh blood other than plasma.

The process by which the stabilisation of the whole blood is achieved uses a series of stages incorporating the addition of both organic and inorganic compounds. The initial process of drawing a unit of blood is well documented and can be any of those used by those employed in the art of venesection. Anticoagulated blood is preferably used, and several suitable anticoagulants are commercially available. However, the use of a potassium EDTA salt is preferred.

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The process of stabilising the whole blood sample is usually performed within 24 hours of venesection but is preferably performed within 2 hours.

The fresh whole blood, containing an anticoagulant, is first centrifuged, and the plasma removed and retained.

The remaining cells are washed and then treated with an aged aqueous solution of a heavy metal compound, and particularly a heavy metal salt. Suitable heavy metals are those with complexing properties and having an atomic weight greater than 20, for example, aluminium, and transition metals such as chromium and manganese. Suitable metal compounds include inorganic acid salts, and particularly chlorides.

Particularly good results have been obtained using chromium compounds, such as, for example, chromic salts, for example, chromic chloride $CrCl_3$, and aluminium compounds, for example aluminium chloride $AlCl_3$.

The heavy metal compound can be dissolved in the appropriate concentration in an aqueous solution which is buffered to a pH of 6.5 to 7.5, preferably 6.7 to 7.4, more preferably around 7.2, and aged for a period of time sufficient to allow any precipitate to form. Suitable buffered aqueous solutions include, for example, phosphate buffered saline solutions.

Preferably, however, the heavy metal compound is stored as a relatively concentrated solution, for example, as a 1% w/v solution and is diluted to the

desired concentration level with an appropriate aqueous buffer solution before use. With the preferred heavy metal compounds, it is found that the concentrated solutions are stable, but that on dilution a precipitate may form as the pH rises. Sufficient time should be allowed for the solution to age and precipitation to occur before the solution is used.

The diluted aqueous solution of the heavy metal compound is therefore allowed to stand, preferably for at least one week, more preferably from one week to one month, before use. The reason why the performance of the solution improves with standing is not understood, but may be due to the formation of hydrated metal hydroxy ionic species in the solution. It has been observed with some buffered solutions of chromium compounds, for example, that the freshly made solutions change from green to purple over a 24 hour period, indicating the presence of charged complex ions, together with the formation of a precipitate which may be a chromium hydroxy polymeric species. This is preferably filtered off from the solution before use. The formation of a precipitate will, of course, lower the concentration of heavy metal ions in the solution, and if this should occur, an analysis of the solution should be carried out to determine whether the concentration of the heavy metal compound is still within the preferred range.

The aged solution of heavy metal compound retains its effectiveness over considerable periods of time, but

is preferably discarded after 12 months and more preferably after 6 months.

The aqueous solution of the heavy metal compound is preferably added to the whole blood preparation as an isotonic solution in which the optimum final concentration of the heavy metal compound is preferably less than 1% w/v, more preferably from 0.005% to 0.75% w/v, still more preferably 0.01% to 0.5% w/v and most preferably from 0.05% to 0.25% w/v, for example, 0.1% w/v. The pH of the aqueous solution is preferably from 6.5 to 7.5, preferably 6.7 to 7.4, before addition. The whole blood preparation is preferably exposed to the heavy metal compound for an incubation period of from 5 minutes to 18 hours, for example, about 60 minutes.

The incubation temperature is preferably from 0°C to 8°C, more preferably from 2°C to 6°C, for example about 4°C.

After the first incubation period, the whole blood is treated with an aqueous solution of paraformaldehyde, which can, for example, be at a preferred final concentration of up to 1% w/v, preferably from 0.1% to 0.5% w/v, for example 0.35% w/v. When making up the paraformaldehyde solution it is preferable to keep the temperature below 60°C, more preferably below 50°C, in order to avoid the reversion of paraformaldehyde to formaldehyde and evolution of formic acid. The age of the paraformaldehyde solution has also been found to be important, and it is preferably greater than one week

old, and no older than one month, preferably no older than two weeks.

In a preferred aspect of the invention, it has been found that very good results are obtained by mixing the paraformaldehyde, preferably in aqueous solution, with an aged aqueous solution of a heavy metal compound.

The aged aqueous solution of a heavy metal compound may be any of those previously described, and the metal compound may be present in the mixed solution in an amount of from 0.01% to 0.5% w/v. Preferably the ratio of heavy metal compound to paraformaldehyde in the solution is in the range of from 5:1 to 1:50, for example, around 1:3.5.

Preferably the aqueous paraformaldehyde solution has a pH of from 6.5 to 7.5, more preferably 6.7 to 7.4, and may comprise a suitable isotonic buffer. A preferred solution can be, for example, a 0.85% phosphate buffered saline solution.

A preferred mixed paraformaldehyde/heavy metal compound solution can be made, for example, by mixing a 0.7% w/v solution of paraformaldehyde with an equal volume of a 0.2% w/v solution of the heavy metal compound.

Exposure to the paraformaldehyde or mixed solution is preferably from 6 hours to 24 hours at a temperature range of from 0 to 8°C, preferably from 2 to 6°C, for example, about 4°C.

The interval between the sequential treatment stages 1) and 2) is preferably at least 30 minutes, more preferably at least one hour, still more preferably at least 12 hours, for example 24 hours.

After washing in isotonic phosphate buffered saline solution the plasma, if previously removed, can be added back to the stabilised whole blood preparation. This plasma can, but need not necessarily be, that obtained from the original donation. Bacterial growth inhibitors and antibiotics for example gentamycin are preferably also added to the final preparation. The preparation is then retained at between 0°C and 8°C for from 1 to 5 days before use.

All the above procedures are preferably carried out under sterile conditions and preferably the entire procedure is carried out in the venesection pack in which the donated blood is collected.

In the commercial manufacture of a stabilised whole blood preparation according to the invention, it is possible and may be preferable to pool a number of units of donated blood from different sources and to stabilise the resultant pool.

In another aspect of the invention, it is possible to add further cell lines to the whole blood preparation, either before or after the stabilisation of the whole blood preparation. For example, it is possible to add additional CD34+ cells in the form of cell line KG1 or as human umbilical cord blood (which is rich in CD34+ cells)

to normal whole blood to provide a quality control for CD34+ cell measurements.

The stabilisation process can be applied to both normal and leukaemic cells providing a known normal control and an abnormal (leukaemic) control. It has been found that with normal cells, using chromium chloride as the metal compound usually provides the best results. However with leukaemic cells, aluminium chloride often gives the best results.

The invention provides a method of stabilising whole human blood preparations without the necessity to lyse the red blood cells (erythrocytes) and stabilise the leucocytes separately.

The stabilised whole blood preparations of the invention, can provide an excellent stable quality control and reference material, which can be used in leucocyte immunophenotyping by both UV microscopy and flow cytometry. The preparation is of value in the quality control of the whole blood lysing procedure without any excess contamination from debris. The stabilised sample can comprise all of the normal peripheral blood leucocytes (granulocytes, monocytes and lymphocytes) or subsets thereof. The procedure can, under optimum conditions, retain the leucocyte antigenic profiles ensuring phenotyping and quality control of the procedure. The values for the common antigenic determinants can be determined and can be stable for more than 300 days. The investigator can also successfully

derive values for antigens that may be of specific interest. Furthermore, the preparation can be used to quality control the differential obtained from the flow cytometer. This is a parameter that is used for the monitoring of anti-viral therapy in HIV infected individuals.

New methods for phenotyping blood specimens are being developed which do not require the sample to be treated with a lysing agent after staining. These techniques are termed no-wash, no-lyse. The whole blood preparations of this invention can be used to provide quality control for these techniques and can also be used on flow cytometers that analyse no-wash, no-lyse procedures.

In general the stabilised whole blood preparations of the invention can be used in quality control of UV microscopy and flow cytometric immunophenotyping techniques, both of the whole blood lysis and whole blood non-lysis techniques.

Investigations with preferred embodiments of the stabilised whole blood preparations of the invention show that they are also suitable for use in immunocytochemical analysis using techniques such as the alkaline phosphatase anti-alkaline phosphatase immunocytochemical technique (APAAP) and can for example be used to determine the antigenic profile of leucocytes on peripheral blood smears. They can be used as a day to day reference materials or in external (inter-laboratory)

quality control. Multiple smears can be made in advance and then stored at -20°C until use. The smears can be stained on a daily basis, or used as controls when staining other smears.

The stabilised whole blood preparations of the invention may also find application as standard reference materials for use in enzyme linked immunosorbent assay techniques (ELISA) and in immunoradiometric assay techniques.

Further uses of the stabilised whole blood preparations of the invention in the laboratory may include as a quality control and calibrant for haematology analysers, as a quality control material for monitoring iron deficiency by the zinc protoporphyrin technique (ZPP), and as a quality control material in the red cell folate technique. Finally, on a broader basis, the stabilised whole blood preparations of the invention may find application in the quality control of blood glucose level tests, thereby enabling diabetic patients to carry out this technique in their own homes.

The invention is illustrated by the following Examples:

EXAMPLE 1

A stabilised whole blood preparation is made up as described below and its ageing characteristics compared with a similar untreated whole blood sample.

Preparation

1. Venesect a unit of 500ml of blood into a sterile venesection pack containing 600mg of ethylenediaminetetraacetic acid (disodium or tri potassium salt) dissolved in 50ml of phosphate buffered saline solution (PBS).
2. Centrifuge the unit at 800g for 1 hour. Remove and retain the plasma.
3. Wash the remaining cells twice with sterile phosphate buffered saline solution (PBS)
4. Remove the supernatant PBS.
5. Add 300ml of filtered 0.1% aged (> 1 month) Chromium Chloride hexahydrate (pH 6.7) in PBS and incubate for 1 hour in the dark at 4°C mixing occasionally.
6. Centrifuge for 45 minutes at 800g and decant supernatant.
7. Resuspend in 300ml aged (>1 month) and filtered 0.1% Chromium Chloride hexahydrate pH 6.7 in 0.35% paraformaldehyde in PBS. Incubate at 4°C in the dark for 16 - 22 hours.
8. Centrifuge at 800g for 45 minutes and decant supernatant.
9. Wash twice by centrifuging at 800g with PBS. Decant final supernatant.
10. Resuspend the stabilised whole blood in the plasma detailed in step 2. Add broad range antibiotics such as gentamycin, ciprofloxacin.

12. Place at 4°C for 2 - 3 days until use.

Phosphate Buffered Saline pH 7.2

7.83g/1 Sodium Chloride

0.36g/1 disodium EDTA

0.28g/1 Potassium Chloride

0.26 g/1 Potassium dihydrogen phosphate (monobasic)

2.35g/1 disodium hydrogen phosphate (dibasic)

The comparative results are illustrated in the accompanying Drawings in which:

Figure 1 shows the flow cytometric characteristics of "fresh" blood after staining for the antigens CD3, CD4, CD8, and CD20. The staining was carried out employing the whole blood lysis technique and the positive levels related to the negative controls (a) Forward & side scatter (FSC & SSC) characteristics, (b) CD3 PE & CD20 FITC, (c) CD3 FITC & CD4 PE, (d) CD3 FITC & CD8 PE.

Figure 2 shows the effect of ageing on the flow cytometric FSC, and SSC of "fresh" unstabilised blood over a period of 8 days (a) Day 1, (b) Day 2 (c) Day 3, (d) Day 8.

Figures 3a and 3b show the effect of ageing on the expression of the antigens described in Figure 1b, c & d over a 10 day period 3a (i) (ii) (iii) Day 1, and 3b (i) (ii) (iii) Day 10.

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Figures 4a and 4b show the stability of FSC, SSC and the negative control characteristics, as determined by flow cytometry, upon the stabilised whole blood preparation over a period of 60 days, 4a day 3, 4b day 60.

Figures 5a and 5b show the stability of the antigens CD3, CD4, CD8, CD19, and CD16 + CD56, as measured by flow cytometry, over a 57 day period 5a Day 3, 5b Day 60.

Figure 6 shows the effect of storage on the flow cytometric leucocyte differential on "fresh" blood over a period of 8 days. Further analysis after day 8 was not possible due to marked deterioration of the sample. Analysis used a FACScan flow cytometer.

Figure 7 shows the stability of the flow cytometric differential of the stabilised blood preparation over a 25 day period. Analysis used a FACScan flow cytometer.

Figure 8 shows the stability in zinc protoporphyrin (ZPP) level in a sample of the stabilised whole blood preparation over a period of 36 days measured in μmoles ZPP/mole of haemoglobin.

Figure 9 shows the stability of the total white cell count and total lymphocyte count over a 117 day period for a sample of the stabilised whole blood preparations measured using a Toa (Sysmex) NE8000 haematology analyser.

Finally, Figure 10 shows the measurement of red cell folate for samples of the stabilised whole blood

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preparation and fresh stored whole blood over a 50-day period.

The flow cytometric profile in Figure 1 shows the normal position of the lymphocytes, monocytes and granulocytes after lysis of the red cells. The antigen staining characteristics are also shown in Figure 1b, c, d, & e. As the sample increases with age (Figure 2) the flow cytometric and antigen expression characteristics alter. At day 8 the amount of debris makes the analysis unsatisfactory. The fluorescence labelling characteristics have also deteriorated as shown by Figure 3.

Figure 4 displays the forward and side scatter characteristics together with the negative control of the stabilised sample at day 3 and day 60. The individual populations are retained in their respective positions immediately post stabilisation. After 60 days preservation the forward and side scatter flow cytometric characteristics remain intact (Figure 4b). In addition the antigen expression characteristics remain unaltered over this period (Figure 5). The same machine settings were retained throughout.

The stabilised blood preparation can be used to monitor the leucocyte differential. Using a specific combination of antibodies directed against the CD14 and CD45 antigens a three-population differential can be generated. Figure 7 shows the stability of this parameter over 25 days, whereas Figure 6 shows the

instability of the "fresh" whole blood over an 8 day period. Analysis of the latter after this time was aborted due to contamination with excessive debris.

As shown in Figure 8, ZPP levels in the stabilised whole blood preparation remain substantially constant, with only a very slight increase after 36 days. Similarly, as shown in figure 9, the total white cell count and total lymphocyte count are also scarcely affected by time over a 117 day period.

Figure 10 shows the very substantial improvement obtained in the stability of red cell folate using a stabilised whole blood preparation according to the invention.

EXAMPLE 2

A stabilised whole blood preparation is made up as described in Example 1, except that the reactions with chromium chloride hexahydrate and with the mixture of chromium chloride hexahydrate and paraformaldehyde are carried out in phosphate buffered saline solution at a pH of 7.2.

Figure 11 shows the stability of the FSC, SSC (Figure 11a) and negative control (Figure 11b) characteristics, as determined by flow cytometry upon the stabilised whole blood preparation, at 11 days post stabilisation.

Figure 12 (a), (b) and (c) shows the stability of the antigens CD3, CD4, CD8, and CD19 as measured by flow cytometry at 11 days post stabilisation.

Figure 13 (a) and (b) shows the stability of the FSC, SSC and negative control characteristics as determined by flow cytometry, upon the stabilised whole blood preparation, at 29 days post stabilisation.

Figure 14 (a), (b) and (c) shows the stability of the antigens CD3, CD4, CD8 and CD19 as measured by flow cytometry at 29 days post stabilisation.

EXAMPLE 3

A stabilised whole blood preparation is made up as described in Example 1, except that aluminium chloride hexahydrate is substituted for the chromium chloride hexahydrate, and the reactions with the aluminium chloride and the mixture of aluminium chloride and paraformaldehyde are carried out at pH 7.2.

Figure 15 (a) and (b) shows the stability of FSC, SSC and negative control characteristics, as determined by flow cytometry upon the stabilised whole blood preparation at 11 days post stabilisation.

Figure 16 (a), (b) and (c) show the stability of the antigens CD3, CD4, CD8 and CD19 as measured by flow cytometry at 11 days post stabilisation.

Figure 17 (a) and (b) shows the stability of FSC, SSC and negative control characteristics as determined by

flow cytometry upon the stabilised whole blood preparation at 29 days post stabilisation.

Figure 18 (a), (b) and (c) shows the stability of the antigens CD3, CD4, CD8 and CD19 as determined by flow cytometry at 29 days post stabilisation.

The results show very good stabilisation of the whole blood preparation, with very little debris even after 29 days and good retention of antigenic characteristics.

The reader's attention is directed to all papers and documents which are filed concurrently with this specification and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

All the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps or any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Each feature disclosed in this specification (including any accompanying claims, abstract and drawings), may be replaced by alternative features serving the same, equivalent or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated otherwise, each feature disclosed is one example

only of a generic series of equivalent or similar features.

The invention is not restricted to the details of the foregoing embodiment(s). This invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

CLAIMS

1. A method of treating a suspension of cells capable of lysis, to produced a stabilised cell suspension still capable of lysis, which comprises:

treating the cell suspension, in an aqueous medium maintained at a pH of from 6.5 to 7.5, sequentially with,

1) an aged aqueous solution of a heavy metal compound, the pH of the solution being from 6.5 to 7.5, and the solution having been maintained at a pH of from 6.5 to 7.5 for a period of time sufficient to allow any precipitate to be formed, and

2) an aqueous solution of paraformaldehyde, the pH of the solution being from 6.5 to 7.5.

2. A method according to claim 1, in which the cell suspension is a whole blood preparation.

3. A method according to claim 1 or 2, in which the heavy metal is aluminium or a transition metal.

4. A method according to any of the preceding claims, in which the heavy metal compound is a metal salt.

5. A method according to claim 4, in which the metal salt is chromic chloride.

6. A method according to claim 4, in which the metal salt is aluminum chloride.

7. A method according to any of the preceding claims, in which the aqueous solution of a heavy metal compound has a pH of from 6.7 to 7.4.

8. A method according to any of the preceding claims, in which the heavy metal compound is stored as a relatively concentrated solution and is diluted with an aqueous buffer solution before use.

9. A method according to any of the preceding claims, in which the aqueous solution of a heavy metal compound is aged by being allowed to stand for at least one week before use.

10. A method according to any of the preceding claims, in which the aqueous solution of the heavy metal compound is added to the cell suspension as a 0.01% to 0.5% w/v solution.

11. A method according to any of the preceding claims, in which the cells are exposed to the aqueous solution of the heavy metal compound for a period of

from 5 minutes to 18 hours at a temperature of from 0°C to 8°C.

12. A method according to any of the preceding claims, in which the paraformaldehyde is added as a 0.1% to 0.5% w/v aqueous solution.

13. A method according to any of the preceding claims, in which the aqueous paraformaldehyde solution has a pH of from 6.7 to 7.4.

14. A method according to any of the preceding claims, in which the aqueous paraformaldehyde solution is mixed with an aged aqueous solution of a heavy metal compound having a pH in the range of from 6.5 to 7.5.

15. A method according to Claim 14, in which the ratio of heavy metal compound to paraformaldehyde is in the range of from 5:1 to 1:50.

16. A method according to any of the preceding claims, in which the exposure of the cell suspension to the paraformaldehyde solution or mixed solution is from 6 to 24 hours at a temperature of from 0°C to 8°C.

17. A method according to any of the preceding claims, in which steps 1) and 2) are carried out

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sequentially at a time interval of from 1 hour to 24 hours.

18. A method according to any of the preceding claims, in which a further cell line is added to a whole blood preparation, either before or after the stabilisation of the whole blood preparation.

19. A method according to Claim 18, in which the cell line comprises CD34+ cells.

20. A method according to any of the preceding claims substantially as described in Example 1.

21. A method according to any of Claims 1 to 19, substantially as described in Examples 2 and 3.

22. A method according to any of the preceding claims substantially as hereinbefore described.

23. A method according to claim 2, in which the whole blood preparation has been obtained by pooling samples from different blood donations.

24. A stabilised cell suspension prepared by a method according to any of the preceding claims.

25. A stabilised whole blood preparation substantially as described in the Examples.

26. A stabilised whole blood preparation substantially as hereinbefore described.

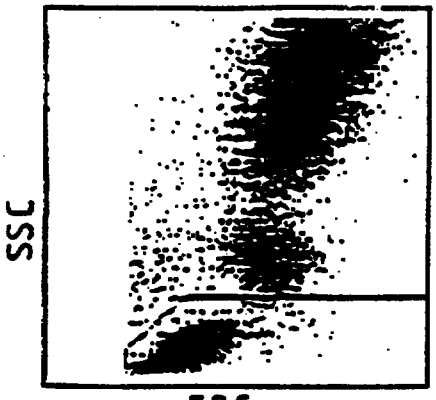
27. An unseparated stabilised whole blood preparation capable of lysis in which the stabilising agent comprises effective amounts of a heavy metal compound and paraformaldehyde.

28. A quality control material for use in leucocyte immunophenotyping which comprises a stabilised whole blood preparation according to claim 27.

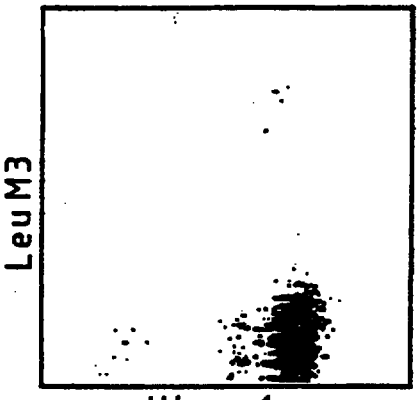
29. A method for the quality control of leucocyte immunophenotyping in which there is used as a quality control reference material a stabilised whole blood preparation according to claim 27.

30. A method for the quality control of any of zinc protoporphyrin, red cell folate, total white cell count and lymphocyte count blood monitoring techniques in which there is used as a quality control reference material a stabilised whole blood preparation according to Claim 27.

31. A method for monitoring blood sugar levels in the treatment of diabetes in which there is used as a calibration material a stabilised whole blood preparation according to Claim 27.

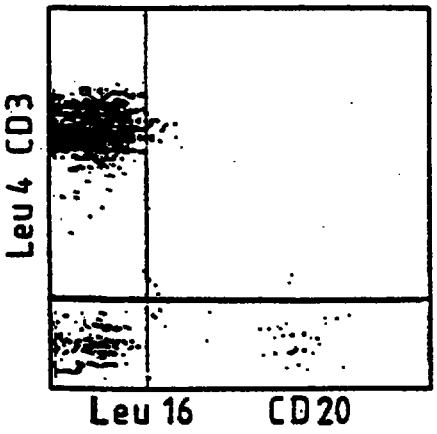


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Total Events : 15000



Quadrant correction : Yes

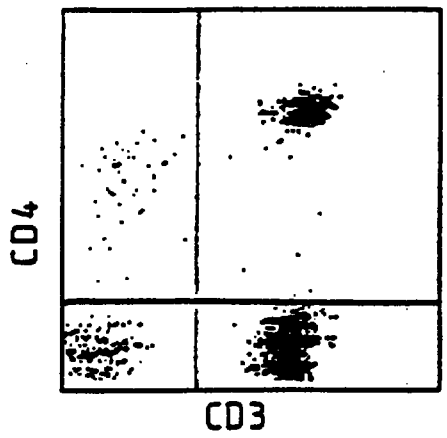
Fig.1a.



Gated Events : 2271
Total Events : 10000

Quad	Cell Type	Corr %L	Cells/ cu mm
1	CD3+/CD20-	89	1510
2	CD3+/CD20+	2	30
3	CD3-/CD20-	7	120
4	CD3-/CD20+	2	30

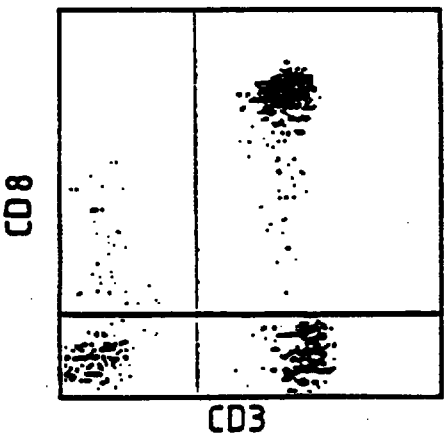
Fig.1b.



Quad	Cell Type	Corr %L	Cells/ cu mm
1	CD4+/CD3-	2	30
2	CD3+/CD4+	18	310
3	Negative	8	140
4	CD3+/CD4-	71	1210

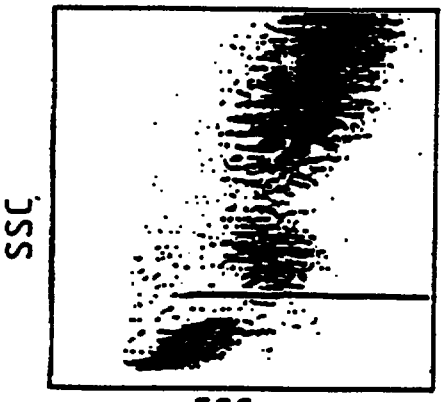
Gated Events : 2396
Total Events : 10000

Fig.1c.



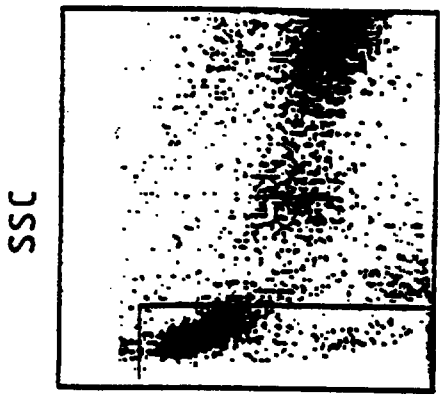
Quad	Cell Type	Corr %L	Cells/ cu mm
1	CD8+/CD3-	3	50
2	CD8+/CD3+	71	1210
3	Negative	7	120
4	CD8-/CD3+	19	320

Fig.1d.



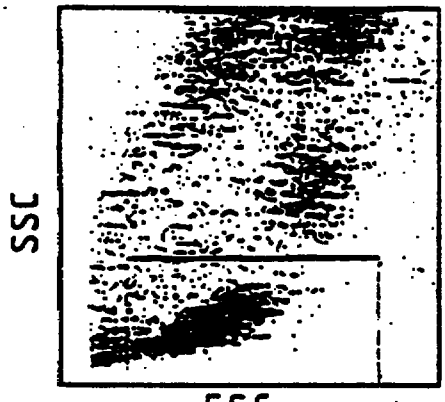
Gated Events : 3646
Total Events : 15000

Fig.2a.



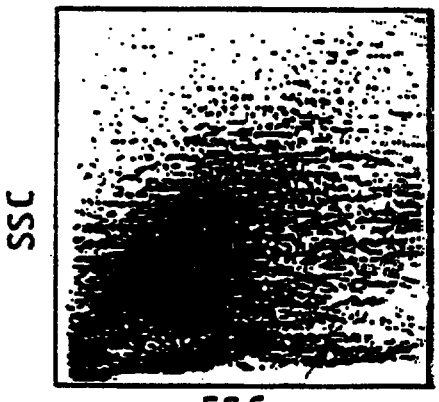
Gated Events : 2409
Total Events : 15000

Fig.2b.



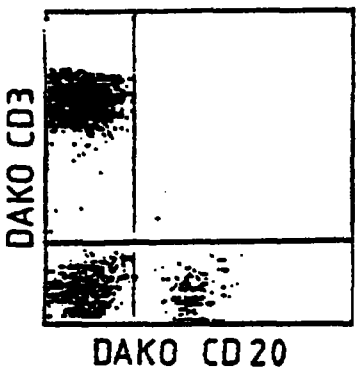
Gate Events : 3149
Total Events : 15000

Fig.2c.



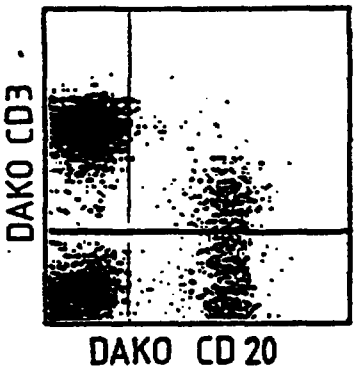
Gated Events : 7289
Total Events : 50000

Fig.2d.



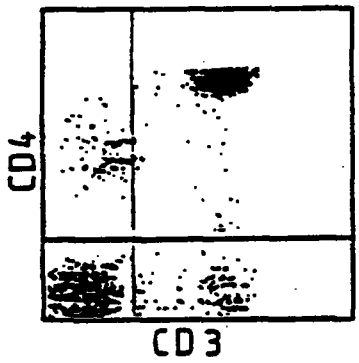
Quad	Cell Type	Corr %L
1	T LYMPHS	65
2	Non-spec	0
3	Negative	23
4	B LYMPHS	12

Fig.3a(i)



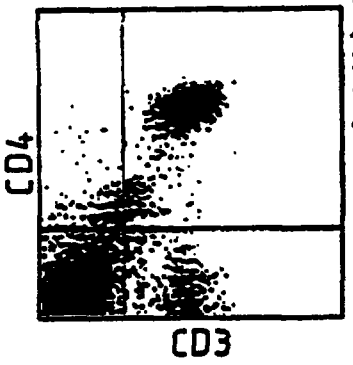
Quad	Cell Type	%L
1	T LYMPHS	41
2	Non-spec	6
3	Negative	40
4	B LYMPHS	13

Fig.3b(i)



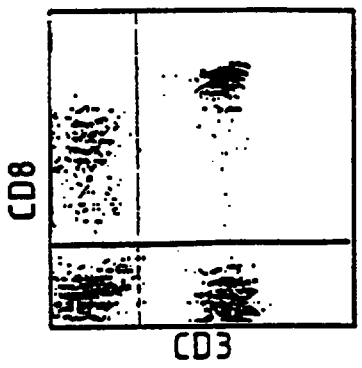
Quad	Cell Type	Corr %L
1	CD4+/CD3-	5
2	CD3+/CD4+	42
3	Negative	31
4	CD3+/CD4-	22

Fig.3a(ii)



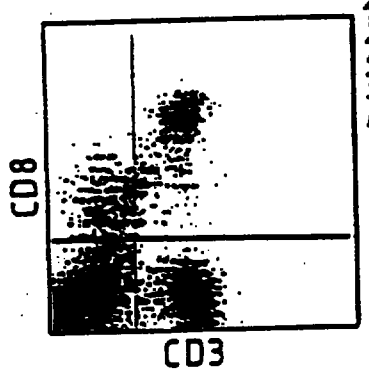
Quad	Cell Type	%L
1	CD4+/CD3-	4
2	CD3+/CD4+	31
3	Negative	53
4	CD3+/CD4-	12

Fig.3b(ii)



Quad	Cell Type	Corr %L
1	CD8+/CD3-	13
2	CD8+/CD3+	22
3	Negative	18
4	CD8-/CD3+	41

Fig.3a(iii)



Quad	Cell Type	%L
1	CD8+/CD3-	9
2	CD8+/CD3+	13
3	Negative	48
4	CD8-/CD3+	30

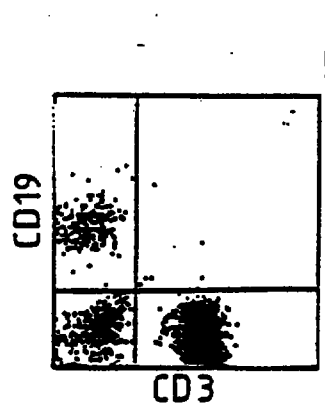
Fig.3b(iii)

Quad
1
2
3
4
Cell Type
NSS PE
NSS++
Unstained
NSS FITC
Corr %L
1
0
99
0
Cells/ cu mm
10
0
1140
0
Gamma-2
Gamma-1
SSC
FSC
Gated Events : 3579
Total Events : 15000

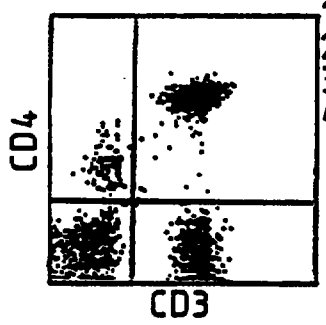
Fig.4a

Quad
1
2
3
4
Cell Type
NSS PE
NSS++
Unstained
NSS FITC
%L
0
0
99
0
Cells/ cu mm
0
0
70
0
Gamma-2
Gamma-1
SSC
FSC
Gated Events: 4051
Total Events : 15000

Fig.4b

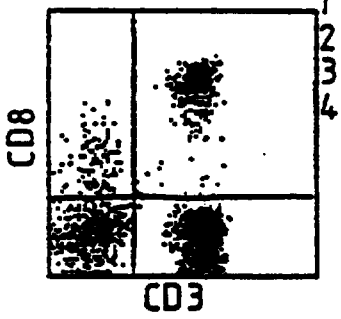


Quad	Cell Type	Corr L
1	CD3-/CD19+	11
2	CD3+/CD19+	0
3	CD3-/CD19-	15
4	CD3+/CD19-	73

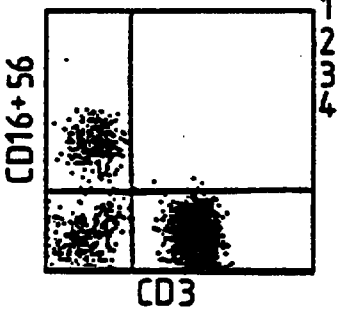


Quad	Cell Type	Corr %L
1	CD3-/CD4+	4
2	CD3+/CD4+	49
3	CD3-/CD4-	23
4	CD3+/CD4-	24

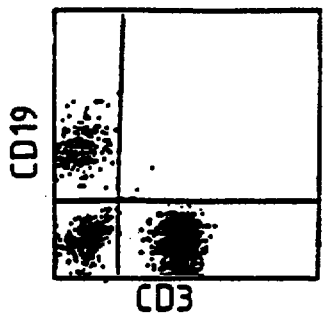
Fig.5a



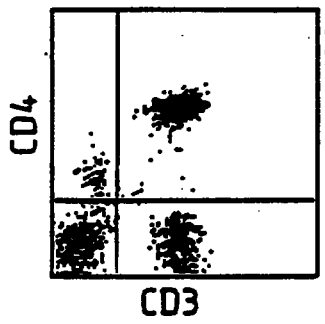
Quad	Cell Type	Corr %L
1	CD3-/CD8+	5
2	CD3+/CD8+	23
3	CD3-/CD8-	20
4	CD3+/CD8-	51



Quad	Cell Type	Corr %L
1	CD3-16_56+	15
2	CD3+16_56+	0
3	CD3-16_56-	10
4	CD3+16_56-	75

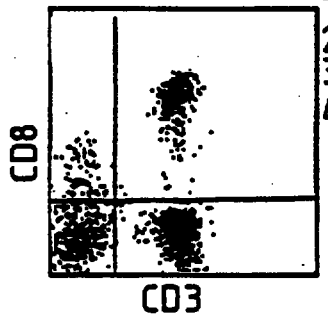


Quad	Cell Type	Corr %L
1	CD3-/CD19+	12
2	CD3+/CD19+	0
3	CD3-/CD19-	16
4	CD3+/CD19-	72

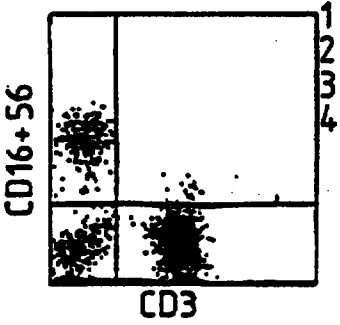


Quad	Cell Type	Corr %L
1	CD3-/CD4+	3
2	CD3+/CD4+	48
3	CD3-/CD4-	25
4	CD3+/CD4-	24

Fig.5b



Quad	Cell Type	Corr %L
1	CD3-/CD8+	5
2	CD3+/CD8+	23
3	CD3-/CD8-	23
4	CD3+/CD8-	49



Quad	Cell Type	Corr %L
1	CD3-16_56+	15
2	CD3+16_56+	2
3	CD3-16_56-	13
4	CD3+16_56-	70

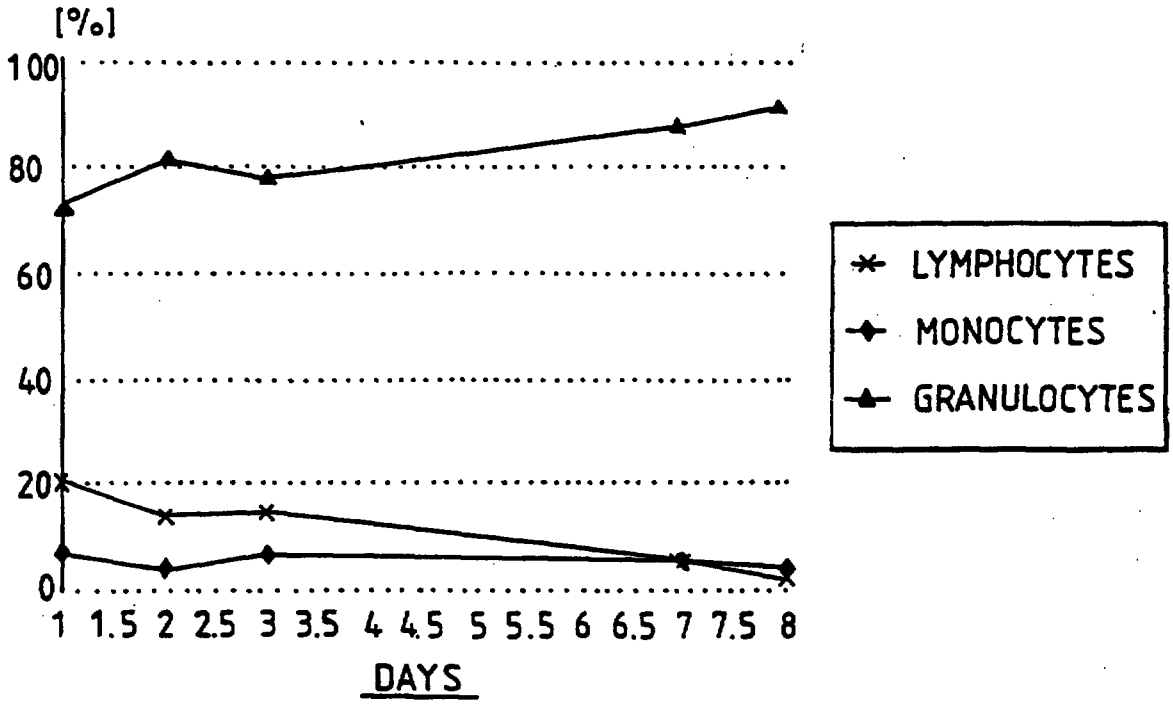

WHOLE BLOOD
[%]
100
80
60
40
20
0
1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8
DAYS
LYMPHOCYTES
MONOCYTES
GRANULOCYTES

Fig.6.

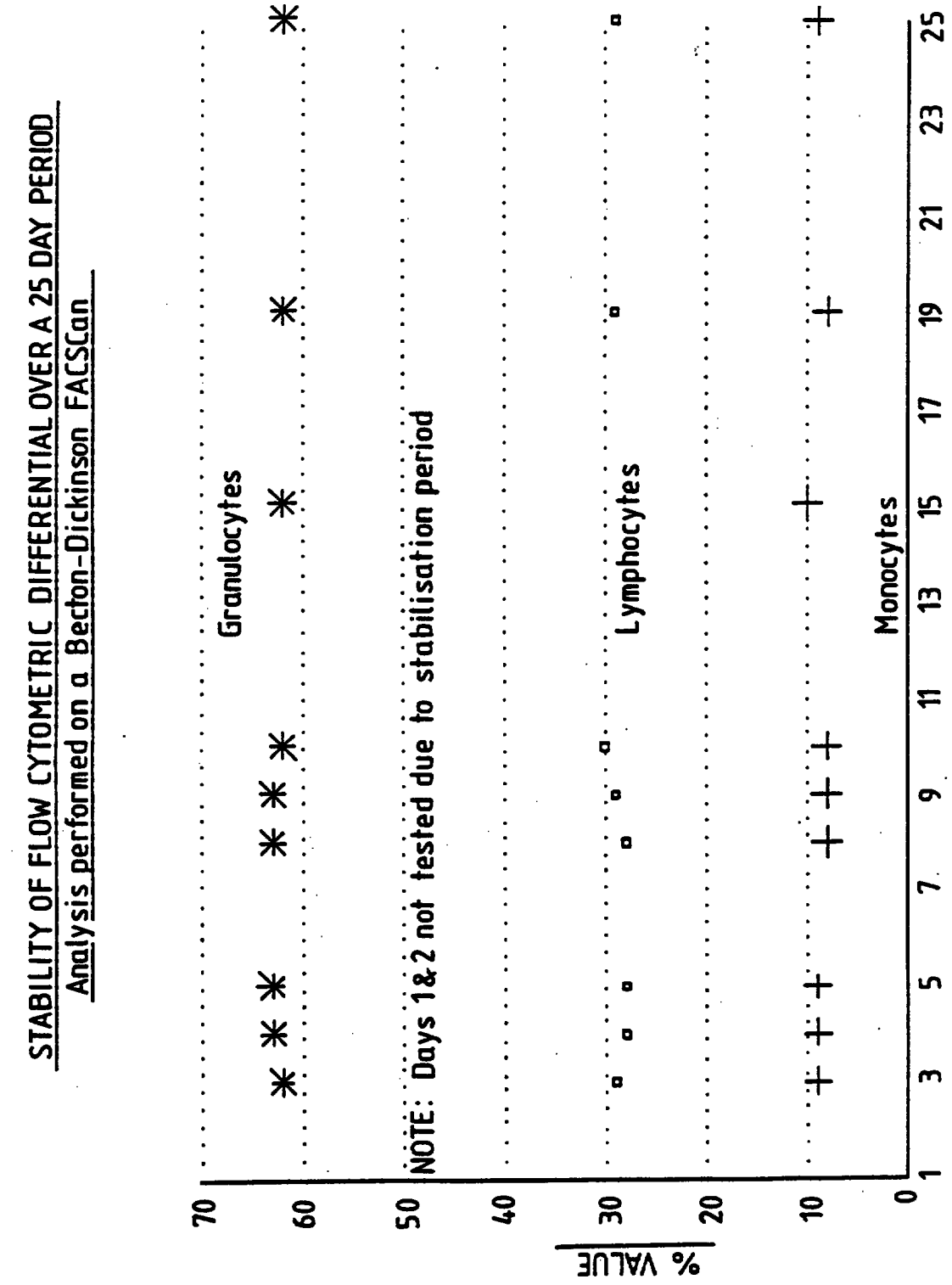

STABILITY OF FLOW CYTOMETRIC DIFFERENTIAL OVER A 25 DAY PERIOD
Analysis performed on a Becton-Dickinson FACSCan
Granulocytes
Lymphocytes
Monocytes
NOTE: Days 1&2 not tested due to stabilisation period
% VALUE
DAYS
70
60
50
40
30
20
10
0
1
3
5
7
9
11
13
15
17
19
21
23
25

Fig. 7.

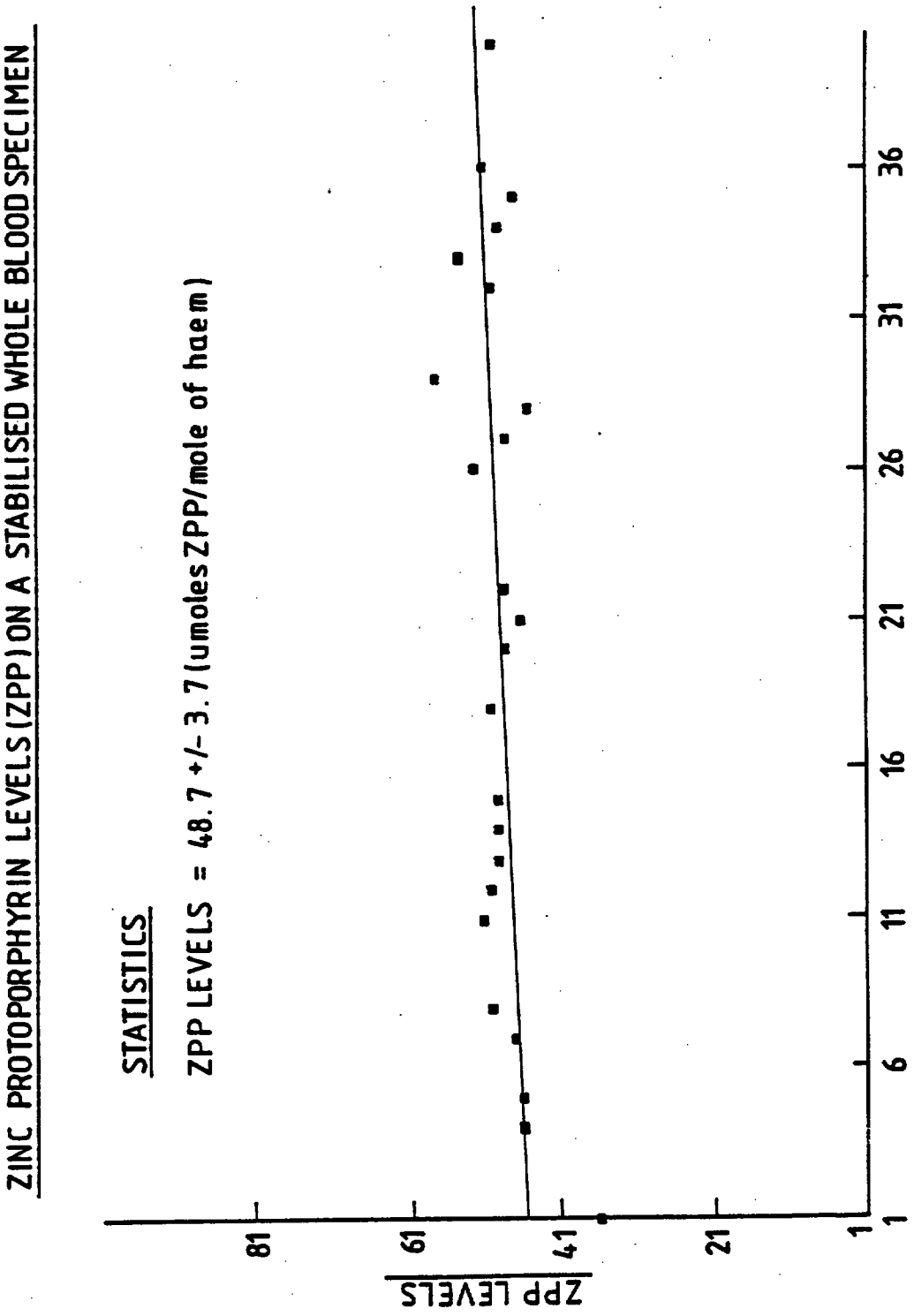

ZINC PROTOPORPHYRIN LEVELS (ZPP) ON A STABILISED WHOLE BLOOD SPECIMEN
STATISTICS
ZPP LEVELS = 48.7 +/- 3.7 (umoles ZPP/mole of haem)
ZPP LEVELS
81
61
41
21
1
1
6
11
16
21
26
31
36
DAYS

Fig.8

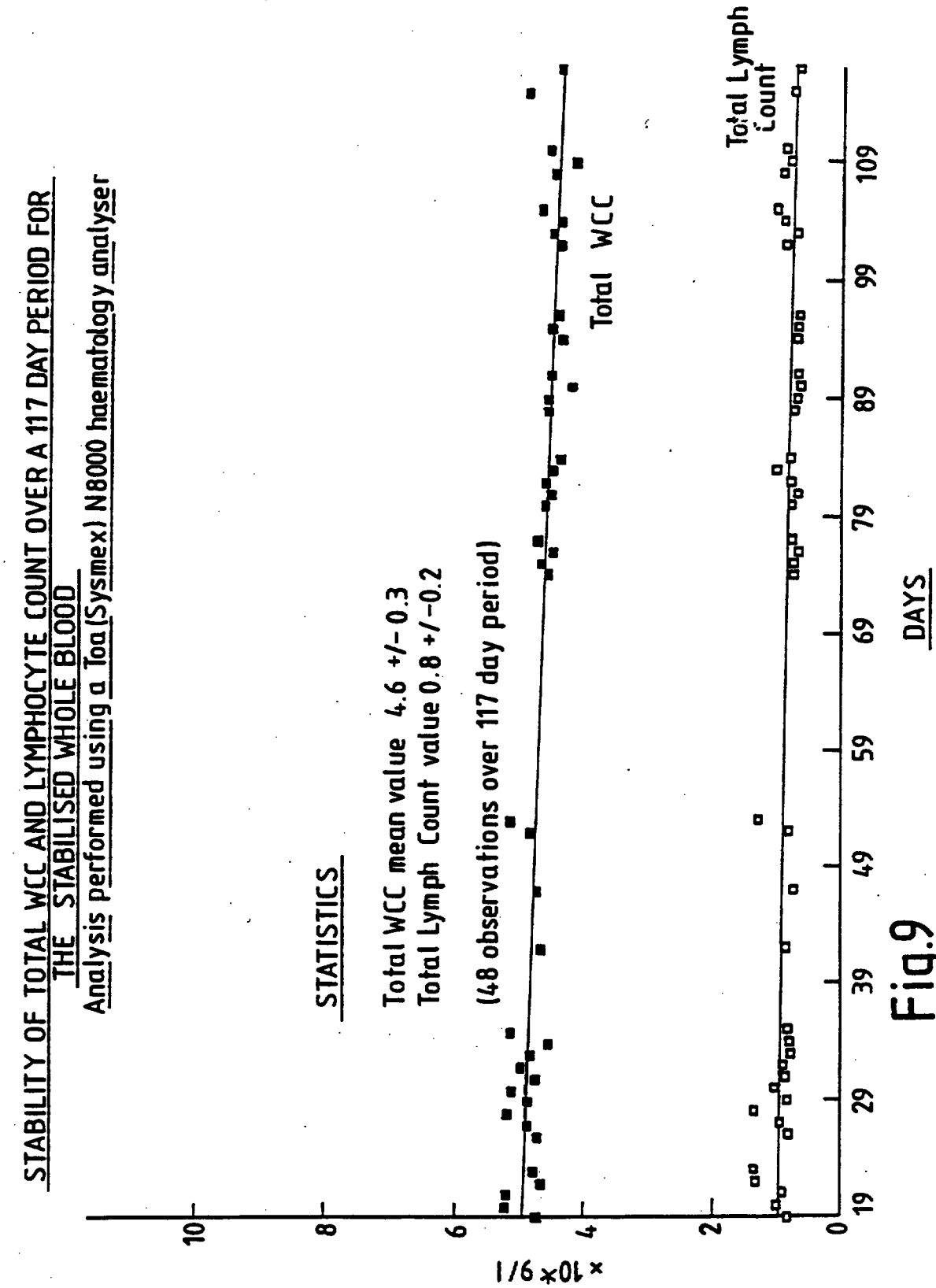

STABILITY OF TOTAL WCC AND LYMPHOCYTE COUNT OVER A 117 DAY PERIOD FOR THE STABILISED WHOLE BLOOD
Analysis performed using a Toa (Sysmex) N8000 haematology analyser
STATISTICS
Total WCC mean value 4.6 +/- 0.3
Total Lymph Count value 0.8 +/-0.2
(48 observations over 117 day period)
Total WCC
Total Lymph Count
x 10x 9/l
0
2
4
6
8
10
19
29
39
49
59
69
79
89
99
109
DAYS

Fig.9

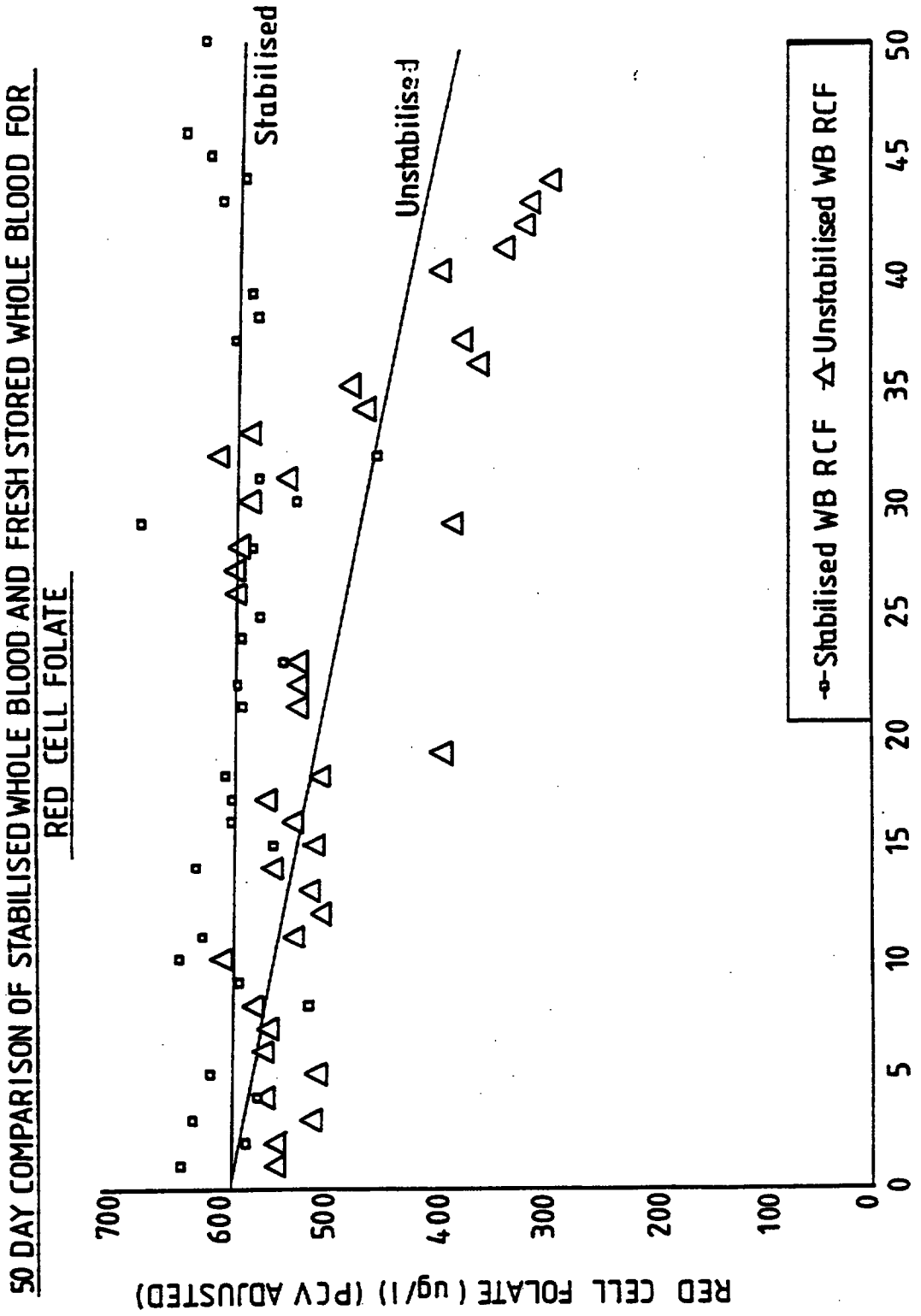

50 DAY COMPARISON OF STABILISED WHOLE BLOOD AND FRESH STORED WHOLE BLOOD FOR RED CELL FOLATE
Stabilised
Unstabilised
RED CELL FOLATE (ug/l) (PCV ADJUSTED)
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
45
50
DAYS
-o-Stabilised WB RCF -Δ-Unstabilised WB RCF

Fig.10.

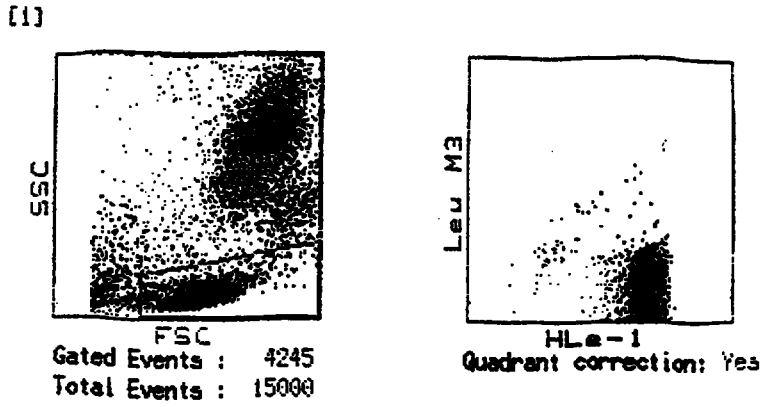
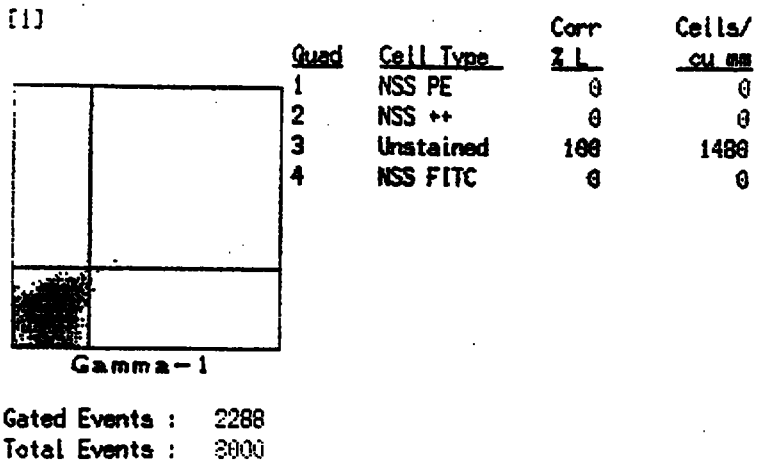
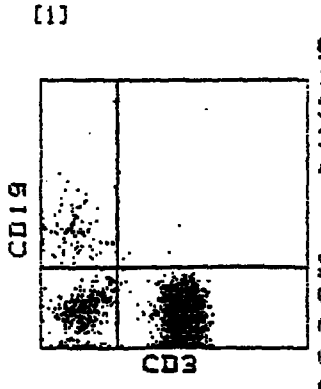


FIGURE 11a



Quad	Cell Type	Corr % L	Cells/ cu mm
1	NSS PE	0	0
2	NSS ++	0	0
3	Unstained	100	1400
4	NSS FITC	0	0

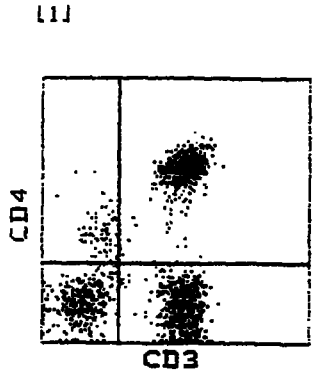
FIGURE 11b



Quad	Cell Type	Corr % L	Cells/ cu mm
1	CD3-/CD19+	5	70
2	CD3+/CD19+	0	0
3	CD3-/CD19-	13	190
4	CD3+/CD19-	82	1210

Subset Label	Corr % L
Q1 CD3/CD19	5 ok
Q2 CD3/CD19	0 ok
Q3 CD3/CD19	13 ok
Q4 CD3/CD19	82 ok

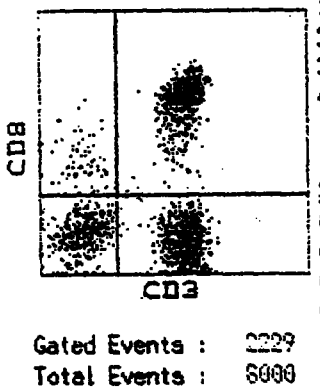
FIGURE 12a



Quad	Cell Type	Corr % L	Cells/ cu mm
1	CD3-/CD4+	3	40
2	CD3+/CD4+	44	650
3	CD3-/CD4-	14	210
4	CD3+/CD4-	39	580

Subset Label	Corr % L
Q1 CD3/CD4	3 ok
Q2 CD3/CD4	44 ok
Q3 CD3/CD4	14 ok
Q4 CD3/CD4	39 ok

FIGURE 12b



Quad	Cell Type	Corr % L	Cells/ cu mm
1	CD3-/CD8+	4	60
2	CD3+/CD8+	38	560
3	CD3-/CD8-	15	220
4	CD3+/CD8-	43	640

Subset Label	Corr % L
Q1 CD3/CD8	4 ok
Q2 CD3/CD8	38 ok
Q3 CD3/CD8	15 ok
Q4 CD3/CD8	43 ok

FIGURE 12c

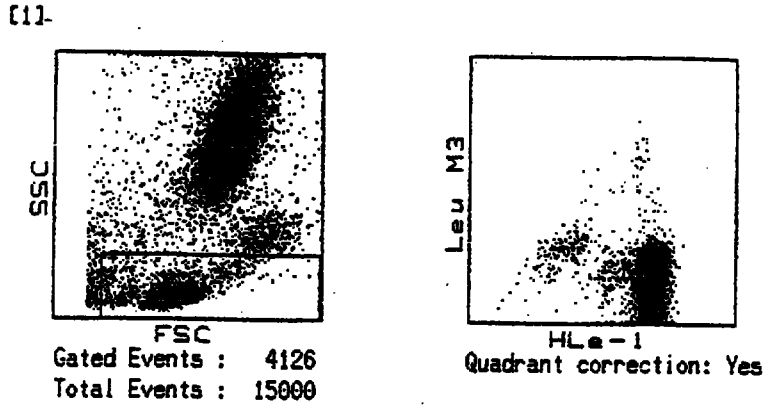
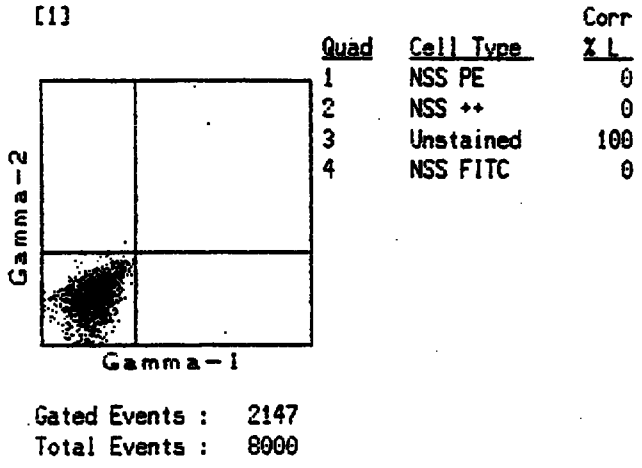
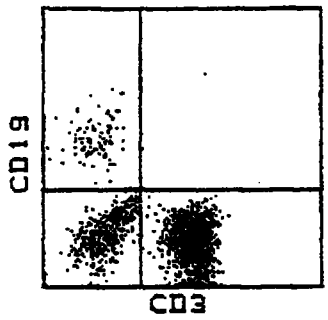


FIGURE 13a



Quad	Cell Type	Corr % L
1	NSS PE	0
2	NSS ++	0
3	Unstained	100
4	NSS FITC	0

FIGURE 13b

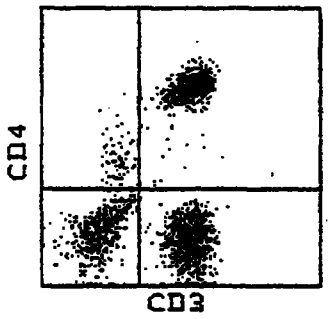


Quad	Cell Type	Corr % L
1	CD3-/CD19+	5
2	CD3+/CD19+	0
3	CD3-/CD19-	14
4	CD3+/CD19-	81

Subset Label	Corr % L	
Q1 CD3/CD19	5	ok
Q2 CD3/CD19	0	ok
Q3 CD3/CD19	14	ok
Q4 CD3/CD19	81	ok

Gated Events : 2257
Total Events : 8000

FIGURE 14a

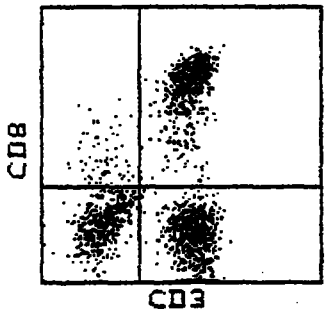


Quad	Cell Type	Corr % L
1	CD3-/CD4+	3
2	CD3+/CD4+	42
3	CD3-/CD4-	16
4	CD3+/CD4-	39

Subset Label	Corr % L	
Q1 CD3/CD4	3	ok
Q2 CD3/CD4	42	ok
Q3 CD3/CD4	16	ok
Q4 CD3/CD4	39	ok

Gated Events : 2211

FIGURE 14b



Quad	Cell Type	Corr % L
1	CD3-/CD8+	4
2	CD3+/CD8+	39
3	CD3-/CD8-	15
4	CD3+/CD8-	43

Subset Label	Corr % L	
Q1 CD3/CD8	4	ok
Q2 CD3/CD8	39	ok
Q3 CD3/CD8	15	ok
Q4 CD3/CD8	43	ok

Gated Events : 2085
Total Events : 8000

FIGURE 14c

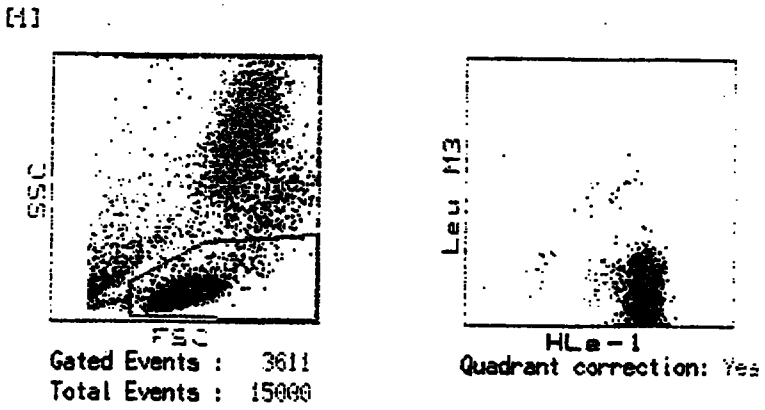
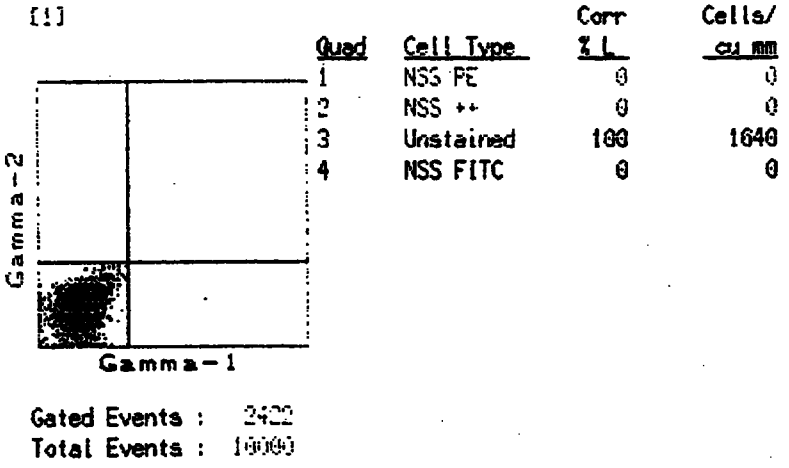


FIGURE 15a



Quad	Cell Type	Corr % L	Cells/ cu mm
1	NSS PE	0	0
2	NSS ++	0	0
3	Unstained	100	1640
4	NSS FITC	0	0

FIGURE 15b

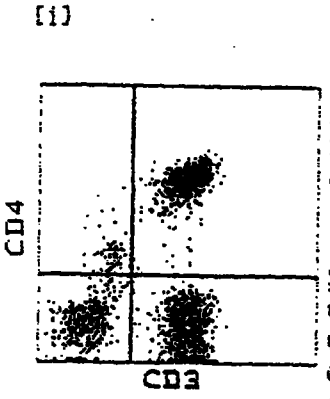


Quad	Cell Type	Corr % L	Ce..s/ cu mm
1	CD3-/CD19+	4	70
2	CD3+/CD19+	0	0
3	CD3-/CD19-	14	230
4	CD3+/CD19-	82	1340

Subset Label	Corr % L	
Q1 CD3/CD19	4	ok
Q2 CD3/CD19	0	ok
Q3 CD3/CD19	14	ok
Q4 CD3/CD19	82	ok

Gated Events : 2430
Total Events : 10000

FIGURE 16a

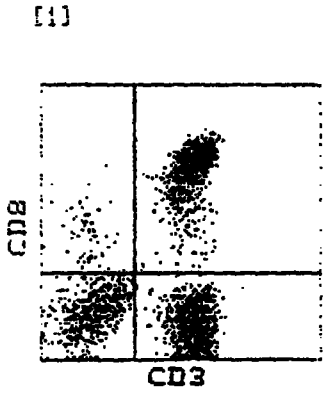


Quad	Cell Type	Corr % L	Cells/ cu mm
1	CD3-/CD4+	3	50
2	CD3+/CD4+	43	710
3	CD3-/CD4-	14	230
4	CD3+/CD4-	40	660

Subset Label	Corr % L	
Q1 CD3/CD4	3	ok
Q2 CD3/CD4	43	ok
Q3 CD3/CD4	14	ok
Q4 CD3/CD4	40	ok

Gated Events : 2359
Total Events : 10000

FIGURE 16b



Quad	Cell Type	Corr % L	Cells/ cu mm
1	CD3-/CD8+	3	50
2	CD3+/CD8+	40	660
3	CD3-/CD8-	17	280
4	CD3+/CD8-	40	660

Subset Label	Corr % L	
Q1 CD3/CD8	3	ok
Q2 CD3/CD8	40	ok
Q3 CD3/CD8	17	ok
Q4 CD3/CD8	40	ok

Gated Events : 2509
Total Events : 10000

FIGURE 16c

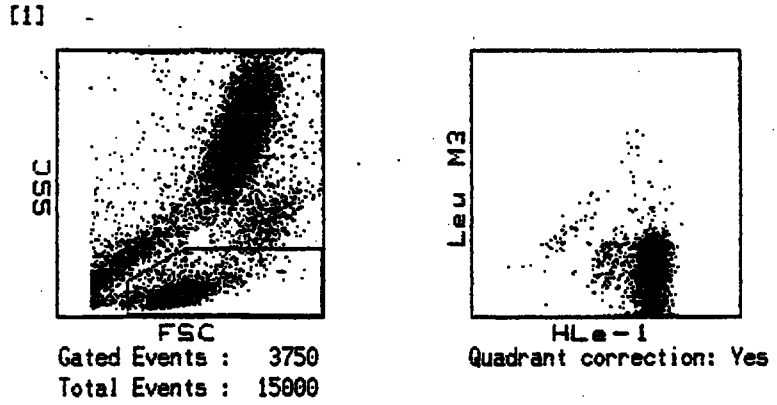
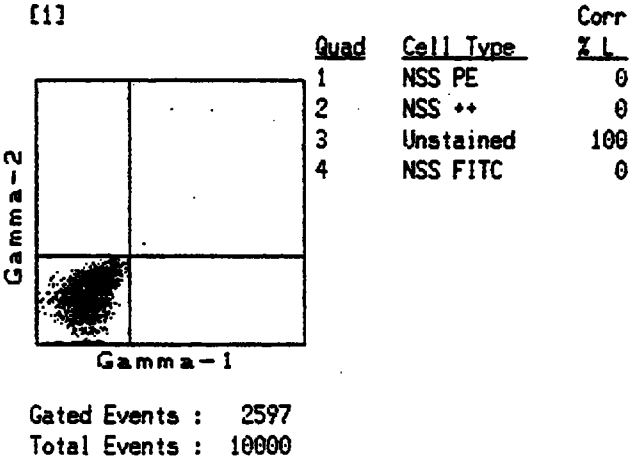
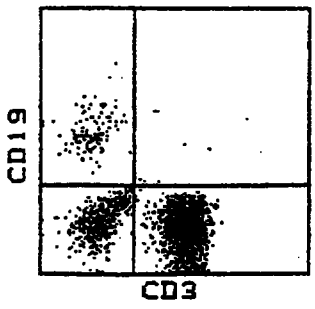


FIGURE 17a



Quad	Cell Type	Corr % L
1	NSS PE	0
2	NSS ++	0
3	Unstained	100
4	NSS FITC	0

FIGURE 17b



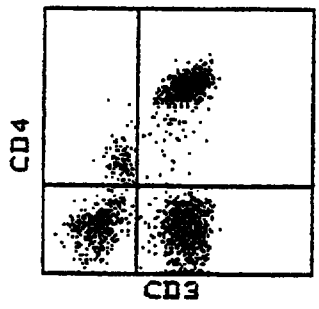
Quad	Cell Type	Corr % L
1	CD3-/CD19+	5
2	CD3+/CD19+	0
3	CD3-/CD19-	14
4	CD3+/CD19-	81

Subset Label	Corr % L
Q1 CD3/CD19	5 ok
Q2 CD3/CD19	0 ok
Q3 CD3/CD19	14 ok
Q4 CD3/CD19	81 ok

Gated Events : 2661
Total Events : 10000

FIGURE 18a

[1]



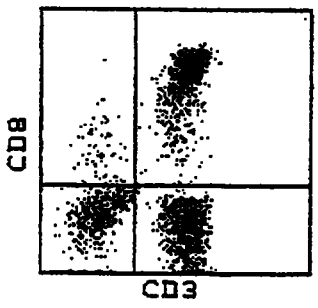
Quad	Cell Type	Corr % L
1	CD3-/CD4+	5
2	CD3+/CD4+	43
3	CD3-/CD4-	13
4	CD3+/CD4-	39

Subset Label	Corr % L
Q1 CD3/CD4	5 ok
Q2 CD3/CD4	43 ok
Q3 CD3/CD4	13 ok
Q4 CD3/CD4	39 ok

Gated Events : 2507
Total Events : 10000

FIGURE 18b

[1]



Quad	Cell Type	Corr % L
1	CD3-/CD8+	3
2	CD3+/CD8+	39
3	CD3-/CD8-	15
4	CD3+/CD8-	43

Subset Label	Corr % L
Q1 CD3/CD8	3 ok
Q2 CD3/CD8	39 ok
Q3 CD3/CD8	15 ok
Q4 CD3/CD8	43 ok

Gated Events : 2475
Total Events : 10000

FIGURE 18c

In International Application No. WO91/17436, the entire disclosure of which is incorporated herein by reference, there is described a blood diluent and lysing agent for differential determination of white blood cells (leucocytes) in which the stabilising agent is diazolidinyl urea. Such leucocyte preparations have not been suggested as flow cytometric preparations possibly because they have insufficient stability and lack certain specific antigenic activity for those routine quality control procedures which need to assess results from a large number of laboratories.

International Application No. WO92/19951 discloses the use of diazolidinyl urea, imidazolidinyl urea, dimethylol-5,5-dimethylhydantoin, dimethylol urea, 2-bromo-2-nitropropane-1,3-diol and quaternary adamantane as tissue fixatives which are free of aldehydes. The formulations may contain inter alia mordants such as zinc, calcium, barium and chromium salts. It is not suggested, however, that any of these salts have stabilising properties.

GB1563839 provides a process for preparing a stabilised preparation of erythrocytes, wherein a suspension of erythrocytes in an aqueous isotonic solution is reacted with, simultaneously or successively in any sequence, an aliphatic aldehyde and a water-soluble salt of 2- or 3- valent chromium. In the examples, the erythrocytes are treated with formaldehyde

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and glutaraldehyde, followed by an aqueous solution of chromium III chloride.

It has been found that cell suspensions containing erythrocytes treated in this way are no longer suitable for use in a whole blood lysing technique, and thus the process of GB1563839 cannot be used to prepare stabilised whole blood samples, for example, for flow cytometry quality control.

GB2001757 describes a method of preparing a stable suspension of blood platelets which comprises adding chromium ions to a platelet-rich plasma and adding at least one organic aldehyde to the chromium-platelet-rich plasma mixture. In the examples, the platelet-rich plasma is treated with solutions of potassium dichromate and glutaraldehyde. This process, if used on leucocyte cells, substantially increases autofluorescence and renders the suspension unsuitable for use in flow cytometry.

The entire disclosures of the above-mentioned patent and applications are incorporated herein by reference.

It will be appreciated from the above, that there is a need for an improved method for stabilising cell suspensions, particularly of whole blood and blood products, giving a cell preparation that is still capable of lysis, for a variety of quality control, monitoring and calibration applications.

In UK Patent Application No. GB2279653 it is disclosed that certain heavy metal

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compound, particularly a heavy metal salt, and paraformaldehyde, and a method of stabilising a whole blood preparation capable of lysis by adding effective amounts of the compound and paraformaldehyde thereto.

DETAILED DESCRIPTION OF THE INVENTION

In UK Patent Application No. GB2279653, leucocyte preparations separated from lysed whole blood are stabilised by the addition of a stabilising agent, and then added back to leucocyte depleted whole blood (red blood cells) to from a stabilised whole blood preparation. The present invention provides an alternative method for the preparation of stabilised whole blood in which the lysing and separating steps can be omitted.

The invention will now be particularly described with reference to the manufacture of stabilised whole blood preparations for use in laboratory quality control procedures, although it will be appreciated that the invention is not limited thereto and that, for example, the stabilised whole blood preparations may find other uses, and that the stabilisation procedures described may find application in the manufacture of stabilised cell suspensions from sources other than blood, for example microbial cells, plant cells and similar materials derived from living tissue.

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In this specification, a suspension of cells capable of lysis is defined as: a suspension of cells, such as, for example, whole human blood, in which specific cell populations can be selectively lysed using erythrocyte lysing reagents. Lysis comprises rupture of the cell membrane and leakage of the cell contents. Lysing reagents and conditions suitable for the selective lysis of the erythrocyte component of whole human blood include, for example, treatment in aqueous solution with an ammonium salt, for example, ammonium chloride, or a quaternary ammonium salt and urea, or a substituted urea, or urea derivative, as described in UK Patent Application No. GB2279653.

Whole blood preparations in this specification include preparations containing substantially all the components of fresh blood, and preparations containing substantially all the components of fresh blood other than plasma.

The process by which the stabilisation of the whole blood is achieved uses a series of stages incorporating the addition of both organic and inorganic compounds. The initial process of drawing a unit of blood is well documented and can be any of those used by those employed in the art of venesection. Anticoagulated blood is preferably used, and several suitable anticoagulants are commercially available. However, the use of a potassium EDTA salt is preferred.

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Figures 4a and 4b show the stability of FSC, SSC and the negative control characteristics, as determined by flow cytometry, upon the stabilised whole blood preparation over a period of 60 days, 4a day 3, 4b day 60.

Figures 5a and 5b show the stability of the antigens CD3, CD4, CD8, CD19, and CD16 + CD56, as measured by flow cytometry, over a 57 day period 5a Day 3, 5b Day 60.

Figure 6 shows the effect of storage on the flow cytometric leucocyte differential on "fresh" blood over a period of 8 days. Further analysis after day 8 was not possible due to marked deterioration of the sample. Analysis used a FACScan flow cytometer.

Figure 7 shows the stability of the flow cytometric differential of the stabilised blood preparation over a 25 day period. Analysis used a FACScan (trademark) flow cytometer.

Figure 8 shows the stability in zinc protoporphyrin (ZPP) level in a sample of the stabilised whole blood preparation over a period of 36 days measured in μmoles ZPP/mole of haemoglobin.

Figure 9 shows the stability of the total white cell count and total lymphocyte count over a 117 day period for a sample of the stabilised whole blood preparations measured using a Toa (Sysmex) NE8000 (trademark) haematology analyser.

Finally, Figure 10 shows the measurement of red cell folate for [illegible] stabilised whole blood

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from 5 minutes to 18 hours at a temperature of from 0°C to 8°C.

12. A method according to any of the preceding claims, in which the paraformaldehyde is added as a 0.1% to 0.5% w/v aqueous solution.

13. A method according to any of the preceding claims, in which the aqueous paraformaldehyde solution has a pH of from 6.7 to 7.4.

14. A method according to Claim 1, in which the aqueous paraformaldehyde solution is mixed with an aged aqueous solution of a heavy metal compound having a pH in the range of from 6.5 to 7.5.

15. A method according to Claim 14, in which the ratio of heavy metal compound to paraformaldehyde is in the range of from 5:1 to 1:50.

16. A method according to any of the preceding claims, in which the exposure of the cell suspension to the paraformaldehyde solution or mixed solution is from 6 to 24 hours at a temperature of from 0°C to 8°C.

17. A method according to any of the preceding claims, in which steps 1) and 2) are carried out

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(57) Abstract

Stabilised cell suspensions are prepared by treating the cells with 1) an aqueous solution of a heavy metal compound, and paraformaldehyde. The stabilised cell suspensions can be stabilised whole blood preparations for use in quality control procedures.

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