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(54) **Titre : UTILISATION D'UN REACTIF POUR LA LYSE D'ERYTHROCYTES ET METHODES ET TROUSSES ASSOCIEES**
(54) **Title: USE OF A REAGENT FOR THE LYSIS OF ERYTHROCYTES AS WELL AS METHODS AND KITS RELATING THERETO**

(57) **Abrégé/Abstract:**
The present disclosure relates to use of a reagent for the lysis of erythrocytes, a method of lysing erythrocytes and a kit comprising the reagent.

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Les corrections suivantes sont faites en raison de l'article 107 des *Règles sur les brevets* et le brevet doit être lu tel que corrigé.

The following corrections are made pursuant to section 107 of the *Patent Rules* and the patent should read as corrected.

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The inventor FROEHNER, Stefanie should be read as being inventor in the patent.

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(54) **Title**: USE OF A REAGENT FOR THE LYSIS OF ERYTHROCYTES AS WELL AS METHODS AND KITS RELATING THERETO

(57) **Abstract**: The present disclosure relates to use of a reagent for the lysis of erythrocytes, a method of lysing erythrocytes and a kit comprising the reagent.

Use of a reagent for the lysis of erythrocytes as well as methods and kits relating thereto

The present disclosure relates to use of a reagent for the lysis of erythrocytes, a method of lysing erythrocytes and a kit comprising the reagent.

Methods of diagnosis and analysis usually aim at being minimal invasive, technically robust and universally applicable. Blood analysis has been used as a diagnostic and analytic tool for many years, as blood is obtainable without difficulty and the analysis of its components is usually relatively easy and automatable.

Blood is made up of several different kinds of cells and other compounds, including various salts and certain proteins. In vertebrates, blood is essentially composed of blood cells suspended in blood plasma. Plasma, which constitutes 55% of blood fluid, contains dissipated proteins, glucose, mineral ions, hormones, carbon dioxide (plasma being the main medium for excretory product transportation), and blood cells themselves. The blood cells are mainly red blood cells (also called RBCs or erythrocytes), white blood cells (also called WBCs or leukocytes) and platelets (thrombocytes). The most abundant cells in vertebrate blood are red blood cells. Humans have about 4 to 6 million erythrocytes per microliter of blood, whereas there are about 4,000–11,000 white blood cells and about 150,000–400,000 platelets in each microliter of human blood. Erythrocytes are mainly responsible for the transport of respiratory gases. Leukocytes are cells of the immune system and found throughout the body, including the blood and lymphatic system. Thrombocytes circulate in the blood of mammals and are involved in hemostasis, leading to the formation of blood clots.

In order to analyze components, particularly cellular components, of blood other than erythrocytes, it is desirable to remove erythrocytes, which is not easy due to their high number. For this, hemolysis, i.e. lysis or rupture of erythrocytes, has been used.

Many methods and protocols for erythrocyte lysis have been developed, some of which are detailed in the following:

Ammonium chloride was described as a penetrating salt enabling lysis of erythrocytes in whole blood. The classical ammonium chloride lysis buffer contains 150 mM NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA. Erythrocytes can be depleted quantitatively using this buffer, but around 30 % or more leukocytes also become lost. (see e.g. Meryman H. Red Cell Structure and Function 1969; 352-367, Sass M. Am J Physiol. 1979;236(5):C238-43, Claus R. et al. Folia Hematol. 1985; 5: 683-688, Terstappen et al. J Immun Methods. 1989: 103-112).

US 7,678,583 B2 discloses a method to lyse erythrocytes quantitatively using a buffer with the core components piperidine or pyrrolidine hydrochlorid, potassium hydrogen carbonate and carbonic anhydrase. This lysis buffer composition is described to result in a higher leukocyte discovery rate with a quantitative erythrocyte depletion compared to the lysis procedure with ammonium chloride.

US 5,840,515 describes a method for isolating and differenting leukocytes in a blood sample by lysis of erythrocytes with a solution whose osmolality and pH have been adjusted to maintain leukocyte integrity and containing saponin and inhibition of the lysis by diluting the sample with a solution having a substantially similar composition but not containing saponin. The reagent is composed of 0.1 to 2 g/l of saponin and having an osmolality between 200 and 400 milliosmoles and a pH between 6 and 8.

DE 102008032501 A1 relates to a general lysis reagent which can be used for nucleic acid analysis and which contains a non-ionic tenside and a polymer acting as thickening agent. To analyze nucleic acids in leukocytes, erythrocytes are depleted using the following lysis buffer composition: 320 mM Saccharose, 50 mM Tris/Cl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100.

US 5,155,044 discloses a lytic reagent system for selective chemical treatment of whole blood comprising an acidic aqueous solution consisting essentially of a diluent, a lytic

reagent selected from the group consisting of formic acid, acetic acid and their respective mixtures; the relative concentration of the lytic reagent in said acidic aqueous solution being sufficient to effect partitioning of a whole blood sample into a lysed red cell fraction and an essentially intact leukocyte fraction in such a state as to allow differential analysis of at least five subpopulations of such leukocytes; and a clarification effective amount of saponin in the range of from about 0.05 to about 0.2 percent.

All of these described erythrocytes lysis protocols and also protocol variations beyond of the cited ones above (hypotonic lysis, detergent-dependent lysis, ammonium-based lysis, acetic acid-based lysis) have been tested and have the disadvantage that at least 20-30 % of the leukocytes are lost during the erythrocyte lysis procedure (see Example 7). Therefore, these procedures are not appropriate for a quantitative erythrocyte depletion procedure combined with a high leukocyte recovery rate, e.g. of at least 90 %.

Accordingly, it was an object of the present disclosure to provide means and methods for quantitative erythrocyte depletion combined with a high viable leukocyte recovery rate. Preferably, the means and methods should not influence leukocyte morphology. Additionally, reactions having a negative influence on cell viability or on experiments following erythrocyte lysis (e.g. PCR or FACS or enzymatic reactions) should be avoided.

Surprisingly, the object was solved by a new reagent to be used in the lysis of erythrocytes, the reagent being an aqueous solution comprising or consisting of HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NH_4^+/NH_3, a chelating agent and optionally CO_3^{2-}/CO_3^-, wherein the final concentration during lysis of erythrocytes is in the range of

- from 2.5 mmol/l to 12 mmol/l HEPES,
- from 60 mmol/l to 120 mmol/l NH_4^+/NH_3,
- from 0.04 mmol/l to 0.8 mmol/l chelating agent, and
- from 0.15 mmol/l to 0.8 mmol/l CO_3^{2-}/CO_3^-, if present.

The newly developed lysis buffer is based on ammonium chloride as main lysis component. But in comparison to the established ammonium chloride buffers, concentration of ammonium chloride is lower in the developed buffer and other buffer

components, such as HEPES, a chelating agent and $KHCO_3$, are added to the reagent mixture. This buffer composition enables quantitative erythrocyte depletion with a high leukocyte recovery rate, preferably of at least 90 % (see Example 4) in contrast to established methods, in which the leukocyte recovery rate was considerably lower (see Examples 1 and 2). Additionally, components suspected of having adverse effects on cell viability or experiments following erythrocyte lysis are not present.

In comparison to the generally used ammonium chloride lysis protocols, the developed means and methods allow to maintain a physiologically stable pH value between 6.8 and 7.4 during the lysis procedure (see Example 6). When using the common ammonium chloride lysis buffer procedures a pH value around 8.0 is measured. Under such conditions also non-erythrocyte blood cells die due to the non-physiological pH value.

However, leukocyte isolation from whole blood enabled by an erythrocyte depletion procedure is an important step to study multiple physiological and pathophysiological blood cell and blood linked phenomena, e.g. immunological evaluations, such as determination of inflammatory and immune state; oncological approaches, such as investigation of different types of leukemia or detection of circulating tumor cells in blood; cardiovascular approaches, such as investigation of circulating endothelial cells in blood; non clinical safety approaches; or flow cytometry approaches, such as those based on every physiological and pathophysiological applications to study nucleated cells in blood (leukocytes and others).

Accordingly, in a first aspect, the present disclosure relates to the use of a reagent for the lysis of erythrocytes, the reagent being an aqueous solution comprising or consisting of HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NH_4^+/NH_3, a chelating agent and optionally CO_3^{2-}/CO_3^-, wherein the final concentration during lysis of erythrocytes is in the range of

- from 2.5 mmol/l to 12 mmol/l HEPES,
- from 60 mmol/l to 120 mmol/l NH_4^+/NH_3,
- from 0.04 mmol/l to 0.8 mmol/l chelating agent, and
- from 0.15 mmol/l to 0.8 mmol/l CO_3^{2-}/CO_3^-, if present.

As detailed above, the reagent may be used for the lysis of erythrocytes. In accordance with the present disclosure, the reagent is an aqueous solution, i.e. a solution based on water. The reagent comprises or consists of the components listed above in aqueous solution, which means that the aqueous solution consists of these components only (consisting of) or encompasses also at least one further component (comprising).

In a specific embodiment as presented herein, the reagent does not comprise one or more or all of the following agents: piperidine or salt thereof, pyrrolidine or salt thereof, carbonic anhydrase, saponin, a tenside, and a polymer acting as thickening agent.

During lysis, the final concentration of the components listed above is as defined above. For use in lysis, a ready-made reagent may be used which is mixed with a source comprising erythrocytes. In this case, the dilution of the reagent has to be considered when preparing the ready-made reagent. If e.g. blood or a blood product is used a source, the reagent may be added to thereto (or vice versa), thus diluting the reagent. Typical dilutions may be from 1:10 to 10:1 (source : reagent), thus requiring before dilution a 1.1-fold to 11-fold stock reagent, respectively. X-fold stock reagent means that the concentrations in the components of the reagent before dilution are increased by factor X relative to the final concentrations during lysis as indicated herein.

Red blood cell's lysis is usually achieved by a mechanism found on the osmotic balance disturbance. Erythrocytes normally are shaped as biconcave disks. In hypotonic environment, spherisation of the cells and subsequent increase in volume can be observed. When membrane tension exceeds a critical value, membrane ruptures, thus lysing erythrocytes. Accordingly, lysis in the present context refers to the breaking down of a cell by osmotic mechanisms that compromise its integrity.

One component of the reagent is HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), which is a zwitterionic organic chemical buffering agent. HEPES is widely used in biology, biochemistry, such as in cell culture, largely because it is better at maintaining physiological pH despite changes in carbon dioxide concentration when compared to other buffers. The dissociation of water decreases with falling temperature, but the dissociation constants (pK) of many other buffers do not change much with temperature. HEPES is like

water in that its dissociation decreases as the temperature decreases. This makes HEPES a more effective buffering agent for maintaining enzyme structure and function at low temperatures. A buffer is most effective when the pH is equal to the pKa of that buffer, and most efficient when in the range of one pH unit above and below that value. HEPES is commonly used to maintain pH levels in cell media. In comparison to the inorganic sodium bicarbonate buffer system, HEPES is more suitable for buffering in the physiological pH range of 7.2-7.6. HEPES is a "Good" buffer, containing both positive and negative ionizable groups, where the secondary and tertiary amine groups provide the positive charge and the negative charges are offered by the sulfonic and carboxylic acid groups. Usually, HEPES is added to media at concentrations of 15 mM to 25 mM, however, in the present disclosure the concentration of HEPES is lower.

Another component of the reagent is an ammonia (NH_3) / ammonium (NH_4^+) buffer. The final concentration of ammonia + ammonium is in the above range of from 60 mmol/l to 120 mmol/l. Usually, the buffer is prepared of ammonium chloride and ammonia, wherein the ratio of both components varies depending on the intended pH. The pKa of the buffer system is 9.25.

Additionally, the reagent comprises a chelating agent. A chelating agent is an agent complexing ions, which reduces their concentrations. Usually, the ions are metal ions, such as Ca, Mg, Fe, Zn and Cu, but non-metal ions, such as P, may be also complexed. By forming stable water soluble complexes with multivalent (metal) ions, chelating agents prevent undesired interaction by blocking normal reactivity of (metal) ions. Examples of suitable chelating agents include EGTA (ethylene glycol tetraacetic acid), BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) DTPA (diethylene triamine pentaacetic acid), EDTA (ethylenediamine tetraacetate) and NTA (N,N-bis(carboxy-methyl)glycine), wherein the reagent comprising EDTA as a chelating agent represents a particularly advantageous embodiment. EDTA is an example of a very common chelating agent which has nitrogen atoms and short chain carboxylic groups and is used as an anticoagulant.

Optionally, another component of the reagent is a carbonate (CO_3^{2-}) / hydrogen carbonate (HCO_3^-) buffer. The final concentration of carbonate + hydrogen carbonate is in the above

range of from 0.15 mmol/l to 0.8 mmol/, if present. Usually, the buffer is prepared by dissolving salts of carbonate and hydrogen carbonate, such as potassium or sodium salts, in water, wherein the ratio of carbonate / hydrogen carbonate varies depending on the pH. The pKa of bicarbonate is 6.1, yielding the best buffering capacity at a pH of 5.1-7.1.

In a further specific embodiment, one or more components as defined above are present in a final concentration during lysis in the range of

- from 3 mmol/l to 11 mmol/l HEPES, preferably from 3 mmol/l to 10 mmol/l HEPES, more preferably from 3.5 mmol/l to 4.5 mmol/l HEPES,
- from 70 mmol/l to 100 mmol/l NH_4^+/NH_3, preferably from 75 mmol/l to 85 mmol/l NH_4^+/NH_3,
- from 0.05 mmol/l to 0.5 mmol/l chelating agent, preferably from 0.06 mmol/l to 0.2 mmol/l chelating agent, more preferably from 0.07 mmol/l to 0.1 mmol/l chelating agent, and/or
- from 0.3 mmol/l to 0.6 mmol/l CO_3^{2-}/CO_3^-, preferably from 0.3 mmol/l to 0.5 mmol/l CO_3^{2-}/CO_3^-, more preferably from 0.35 mmol/l to 0.45 mmol/l CO_3^{2-}/CO_3^-, if present.

In a particular embodiment, the components as defined above are present in a final concentration during lysis in the range of from 3 mmol/l to 10 mmol/l HEPES, of from 70 mmol/l to 100 mmol/l NH_4^+/NH_3, of from 0.05 mmol/l to 0.5 mmol/l chelating agent and of from 0.3 mmol/l to 0.6 mmol/l CO_3^{2-}/CO_3^-.

In yet another particular embodiment, the components as defined above advantageously are present in a final concentration during lysis in the range of from 3 mmol/l to 10 mmol/l HEPES, of from 75 mmol/l to 85 mmol/l NH_4^+/NH_3, of from 0.06 mmol/l to 0.2 mmol/l chelating agent and of from 0.3 mmol/l to 0.5 mmol/l CO_3^{2-}/CO_3^-, particularly wherein the chelating agent is ethylene diamine tetraacetic acid (EDTA).

In yet another particular embodiment and with particular advantage, the components as defined above are present in a final concentration during lysis in the range of from 3.5 mmol/l to 4.5 mmol/l HEPES, of from 75 mmol/l to 85 mmol/l NH_4^+/NH_3, of from 0.07

mmol/l to 0.1 mmol/l chelating agent and of from 0.35 mmol/l to 0.45 mmol/l CO_3^{2-}/CO_3^-, particularly wherein the chelating agent is ethylene diamine tetraacetic acid (EDTA).

In yet a further specific embodiment, the pH of the reagent is in the range of from 6.4 to 7.7, in a particular embodiment advantageouslythe pH of the reagent is in the range of from 6.7 to 7.4, in yet another particular embodiment and with particular advantage the pH of the reagent is in the range of from 6.8 to 7.3. In chemistry, pH is a measure of the activity of the (solvated) hydrogen ion. Pure water has a pH very close to 7 at 25°C. Solutions with a pH less than 7 are said to be acidic and solutions with a pH greater than 7 are basic or alkaline. The pH of blood is usually slightly basic with a value in the range of from pH 7.35 to pH 7.45. In a further specific embodiment the pH is maintained in the range of from 6.4 to 7.7, particularly 6.7 to 7.4, more specifically 6.8 to 7.3 during lysis of erythrocytes, which is advantageous in order to maintain viability of non-erythrocyte cells.

Accordingly, the components as defined above are present in a final concentration during lysis in the range of from 3 mmol/l to 10 mmol/l HEPES, of from 70 mmol/l to 100 mmol/l NH_4^+/NH_3, of from 0.05 mmol/l to 0.5 mmol/l chelating agent and of from 0.3 mmol/l to 0.6 mmol/l CO_3^{2-}/CO_3^- and the pH of the reagent is in the range of from 6.4 to 7.7, preferably 6.7 to 7.4, more preferably 6.8 to 7.3.

Still more specifically, the components as defined above are advantageously present in a final concentration during lysis in the range of from 3 mmol/l to 10 mmol/l HEPES, of from 75 mmol/l to 85 mmol/l NH_4^+/NH_3, of from 0.06 mmol/l to 0.2 mmol/l chelating agent and of from 0.3 mmol/l to 0.5 mmol/l CO_3^{2-}/CO_3^- and the pH of the reagent is in the range of from 6.4 to 7.7, particularly 6.7 to 7.4, more specifically 6.8 to 7.3, particularly wherein the chelating agent is ethylene diamine tetraacetic acid (EDTA).

In a highly specific embodiment and with particular advantage, the components as defined above are present in a final concentration during lysis in the range of from 3.5 mmol/l to 4.5 mmol/l HEPES, of from 75 mmol/l to 85 mmol/l NH_4^+/NH_3, of from 0.07 mmol/l to 0.1 mmol/l chelating agent and of from 0.35 mmol/l to 0.45 mmol/l CO_3^{2-}/CO_3^- and the pH of the reagent is in the range of from 6.4 to 7.7, particularly 6.7 to 7.4, more

specifically 6.8 to 7.3, particularly wherein the chelating agent is ethylene diamine tetraacetic acid (EDTA).

Inyet further a specific embodiment, the reagent is used in the isolation of cells other than erythrocytes (also referred to as non-erythrocyte cells) from a sample comprising erythrocytes, particularly from a blood sample or blood product.

For providing a blood sample, blood needs to be taken from a subject. Particularly for mammals, this may be conveniently performed by taking venous blood from the subject. Venous blood may be obtained by venipuncture from the mammal, wherein usually only a small sample, e.g. 3 ml to 10 ml sample, of blood is adequate for the use in the present disclosure. Blood is most commonly obtained from the median cubital vein, on the anterior forearm (the side within the fold of the elbow). This vein lies close to the surface of the skin, and there is not a large nerve supply. Most blood collection in the industrialized countries is done with an evacuated tube system consisting of a plastic hub, a hypodermic needle, and a vacuum tube. However, blood may also be obtained by any other method known to the skilled person.

After isolation of the blood, blood may be processed, e.g. by adding an anti-coagulant. After having been obtained and optionally further processed, the blood may be immediately used for analysis or stored as known to the person skilled in the art. The feature “blood product” in the present disclosure refers to a product derived from blood, wherein blood has been processed to obtain the blood product. Examples include blood with additives (such as heparin), packed red blood cells, erythrocyte concentrates, thrombocyte concentrates, granulocyte concentrates, blood stem cell preparations, etc.

In a particular embodiment, blood is isolated as follows: Blood is taken from a subject and collected in container intended for blood collection. Those containers are commercially available and may be used in the method as presented herein. Usually, they comprise an anti-coagulant such as EDTA. An exemplary container is a routine EDTA Vacutainer tubes (BD Biosciences, Heidelberg, Germany). In order stabilize cell membranes of WBCs, suitable agents known to the skilled person may be added. Furthermore, a buffer solution

adapted for stabilisation at neutral conditions may be present. Blood samples may be gently inverted. Thereafter, the sample may be immediately used or stored until used.

Alternatives sources for mixtures of cells including red blood cells include biopsy samples, bone marrow, urine, stool, or body fluids with red blood cells as a "contaminant".

In a specific embodiment, the erythrocytes or the sample containing erythrocytes are obtained from a mammal, particularly from a mammalian domestic animal, such as cat, dog, rabbit, or guinea pig, or farm animal, such as cow, horse, goat, sheep, swine or camel. In a very specific embodiment, the erythrocytes or the sample containing erythrocytes are obtained from a human.

As detailed above, the reagent may be used in the detection, concentration or isolation of cells other than erythrocytes from a sample comprising erythrocytes. The sample may be any suitable sample, but a blood sample or a sample derived from blood, e.g. a processed blood sample or a blood product, is a specific embodiment which can be used with particular advantage. The reagent may be used to detect, concentrate or isolate cells other than erythrocytes from a mixture of cells comprising erythrocytes. The reagent is used for the lysis of erythrocytes, thus increasing the percentage of other cells in the sample. Accordingly, cells other than erythrocytes are concentrated by the lysis. Further steps of detection, concentration or isolation of cells of interest may be combined with the lysis, including centrifugation such as differential centrifugation or gradient centrifugation, labeling of cells of interest and subsequent detection or separation of the same, cell sorting, e.g. by FACS and so on. Suitable methods for detection, concentration or isolation of cells of interest are well known to the skilled person.

Evidently, the type of the cells of interest depends from the source chosen (blood etc., see above) and the intended application or technical field. In general, the cell of interest might be any cell present in the source or sample chosen. In a specific embodiment, the cells other than erythrocytes are leukocytes, B cells, T cells, eosinophils, circulating endothelial cells, or cancer cells such as circulating tumor cells, particularly circulating tumor cells or circulating tumor microemboli, which are of particular relevance for diagnostic purposes.

The present disclosure is particularly helpful for the detection or isolation of rare cells, particularly wherein in the population the ratio of rare cells to total cells is at most 5%, preferably at most 1%, especially at most 0.1%, such as at most 0.01 %. The method is particularly useful with rare cells, as the method increases the percentage of these cells considerably, which eases their detection or isolation. Rare cells may be in particular circulating tumor cells (CTC) and circulating tumor microemboli (CTM) in a patient's blood. The technical challenge in this field consists of finding 'rare' tumor cells (just a few CTCs mixed with the approximately 10 million leukocytes and 5 billion erythrocytes in 1 ml of blood) and being able to distinguish them from other cells, particularly epithelial non-tumor cells and leukocytes. However, these cells may be detected long before the tumor itself is detectable by standard means (and therefore a first diagnostic tool), which is evidently highly advantageous in the treatment of the cancerous diseases.

Evidently, the present disclosure is of particular interest for the isolation or detection of cells indicative of a particular state, such as a disease. Accordingly, the cells to be detected or isolated may be e.g. cardiovascular cells or vascular cells or vascular cells released by an inflammatory process, stem cells (e.g. cancerous stem cells), cells indicative of a minimal residual disease, cancer cells (e.g. leukemia cells) or bacterial cells, e.g. indicative of an infection. In this context, the method may be used for genotyping, diagnosis, prognosis, monitoring treatment etc.

Cancer cells are characterized by particular markers. Examples which may be mentioned are: especially oncogenes and tumor suppressor genes such as p53, genes of the ras family erb-B2, c-myc, mdm2, c-fos, DPC4, FAP, nm23, RET, WT1, and the like, LOHs, for example with regard to p53, DCC, APC, Rb and the like and also BRCA1 and BRCA2 in hereditary tumors, microsatellite instability of MSH2, MLH1, WT1 and the like; also tumorous RNAs such as CEA, cytokeratins, e. g. CK20, BCL-2, MUC1, in particular tumor-specific splice variants hereof, MAGE3, Muc18, tyrosinase, PSA,PSM, BA46, Mage-1 and the like, or else morphogenic RNAs such as maspin, hCG, GIP, motilin, hTG, SCCA-1, AR, ER, PR, various hormones and the like ; -- furthermore, especially RNAs and proteins which affect the metastasizing profile, i. e. the expression of molecules involved in angiogenesis, motility, adhesion and matrix degradation such as bFGF, bFGF-R, VEGF, VEGF-Rs, such as VEGF-R1 or VEGF-R2, E-cadherin, integrins, selectins,

MMPs, TIMPs, SF, SF-R and the like, the cell cycle profile or proliferation profile, such as cyclins (e. g. expression ratio of cyclins D, E and B), Ki67, p120, p21, PCNA and the like, or the apoptosis profile, such as FAS (L+R), TNF (L+R), perforin, granzyme B, BAX, bcl-2, caspase 3 and the like. Accordingly, erythrocytes may be removed from a sample in order to increase concentration of other cells and allow for the detection of the above markers.

In a second aspect, the present disclosure relates to a method of lysing erythrocytes, the method comprising

a) providing a sample comprising erythrocytes;
b) incubating the sample with the reagent as defined in any of claims 1 to 5, thereby lysing erythrocytes; and
c) optionally removing erythrocyte debris.

With respect to the terms used in the second aspect of the present disclosure it is referred to the terms, examples and specific embodiments used in the first aspect of the present disclosure, which are also applicable to the second aspect of the present disclosure.

As a first step of the method as presented herein a sample comprising erythrocytes is provided. Details on a suitable sample are given above. The sample may be contained in a vessel, wherein the vessel is a tube, such as a centrifuge tube or spin tube, syringes, cartridge, chamber, multiple-well plate, or test tube, or combinations thereof. The sample may be pre-treated in order to support lysis or erythrocytes or detecting or isolation or other cells. The size / volume of the sample may vary and may be chosen depending from the method to be carried out. If e.g. the method is used for the isolation of cells other than erythrocytes, the sample size will depend from the frequency of these cells.

In a second step the sample is incubated with the reagent as defined above, thereby lysing erythrocytes. For this, the sample may be added to the reagent or the reagent may be added to the sample. The result of contacting is an aqueous solution. The contacting is for a time and under conditions suitable for allowing the lysis of the erythrocytes.

Suitable conditions include appropriate temperature and solution to avoid e.g. death of cells other than erythrocytes or denaturation of proteins of interest, as far as present and required. Suitable conditions will depend from the particular design of the method and sample chosen and the skilled person will be able to select the same based on his general knowledge. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 30 hours. However, the incubation time will depend upon the method design, volume of solution, concentrations and the like. Usually, the methods will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 20° C to 40° C or 22°C to 37°C. During contacting, the mixture of reagent and sample may be agitated, vortexed or shaken or may be left to stand.

As a third and optional step, the erythrocyte debris may be removed, i.e. separated from the remainder, e.g. cells of interest. Typically, cellular debris is removed by techniques involving differences physical characteristics of debris and remainder, such as sedimentation time and size. Typical methods include centrifugation and filtration. Methods for removal erythrocyte debris are well known in the art.

In a particular embodiment as presented herein, the second and/or third step may be repeated. Accordingly, the method may comprise one or more (e.g. two or three) lysis steps (i.e. step b)) and/or one or more (e.g. two or three) washing steps (i.e. step c)). In a very specific embodiment the method comprise twice step b) and one, two or three times step c).

In yet another specific embodiment, the sample is a blood sample or a sample comprising erythrocytes and other cells, particularly white blood cells and/or circulating tumor cells (see also above details).

In yet another specific embodiment, the method as presented herein further comprises

d) detecting or isolating cells other than erythrocytes from a sample comprising erythrocytes, particularly from a blood sample, as described above.

For further details on detecting or isolating please see above.

Preferably, the cells other than erythrocytes are white blood cells or circulating tumor cells, particularly circulating tumor cells (see also above details).

Preferably, the incubating of step b) is for at most 30 min, preferably at most 20 min, more preferably for at most 10 min, especially at room temperature.

In a third aspect, the present disclosure relates to a kit for the isolation of white blood cells from a sample comprising erythrocytes, comprising

- a reagent for lysis of erythrocytes as defined above in the context of the uses and methods as disclosed herein; and
- a reagent for removing erythrocyte debris; and
- optionally, instructions for carrying out any of the methods as disclosed herein.

With respect to the terms used in the third aspect of the present disclosure it is referred to the terms, examples and specific embodiments used in the first and second aspect of the present disclosure, which are also applicable to the third aspect of the present disclosure.

In a specific embodiment, the reagent for removing erythrocyte debris is phosphate-buffered saline (PBS) comprising a chelating agent, especially in the range of from 0.1 mmol/l to 0.5 mmol/l, preferably in the range of from 0.2 mmol/l to 0.4 mmol/l, more preferably from 0.25 to 0.35 mmol/l, and/or especially wherein the chelating agent is EDTA.

PBS is a buffer solution commonly used in the biological, biochemical and medical field. It is a water-based salt solution comprising sodium chloride, sodium phosphate, and, in some formulations, potassium chloride and potassium phosphate. The buffer's phosphate groups help to maintain a constant pH. The osmolarity and ion concentrations of the solution usually match those of the human body (isotonic). PBS with EDTA is also used to disengage attached and clumped cells. Divalent metals such as zinc can be added to support precipitation. There are many different preparations of PBS. Some formulations do not contain potassium, while others contain calcium or magnesium. Generally, PBS comprises the following constituents (mmol/l): NaCl (137), KCl (2.7), Na_2HPO_4 (10), KH_2PO_4 (2.0). The pH is usually about 7.4. If used with cells, the solution can be

dispensed into aliquots and sterilized by autoclaving (20 min, 121°C, liquid cycle). Sterilization may not be necessary depending on its use. PBS can be stored at room temperature. However, concentrated stock solutions may precipitate when cooled and should be kept at room temperature until precipitate has completely dissolved before use. In the context of the present disclosure PBS comprises (mmol/l) e.g. NaCl (138), KCl (2.7), Na_2HPO_4 (8), KH_2PO_4 (1.5) and has a pH of 7.0 to 7.6, preferably 7.2 to 7.4.

The disclosure is not limited to the particular methodology, protocols, and reagents described herein because they may vary. Further, the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present disclosure. As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Similarly, the words "comprise", "contain" and "encompass" are to be interpreted inclusively rather than exclusively.

Unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the disclosure. Although any methods and materials similar or equivalent to those described herein can be used in the practice as presented herein, the specific methods, and materials are described herein.

The disclosure is further illustrated by the following examples, although it will be understood that the examples are included merely for purposes of illustration and are not intended to limit the scope of the disclosure unless otherwise specifically indicated.

EXAMPLES

In order to test the various reagents (referred to as lysis buffer) in the lysis of erythrocytes, while maintaining other cells, the following protocol was used:

Unless otherwise noted, one part of whole blood is mixed with parts of the lysis buffer (as defined below), incubated for 10 min at room temperature and centrifugated for 15 min at 300

x g. The supernatant is discarded and cell pellet is resuspended in four parts of the lysis buffer and centrifugated for 15 min at 300 x g. Supernatant is discarded and cell pellet is resuspended in four parts of PBS containing 0.3 mM EDTA and centrifugated for 15 min at 300 x g. The supernatant is discarded and cell pellet is resuspended in a distinct amount of PBS containing 0.3 mM EDTA.

Example 1: Erythrocyte Lysis with Conventional Products

In a first test, the suitability of conventional products was tested according to the above protocol. The following conventional products were used EasySep® Red Blood Cell Lysis Buffer (StemCell Technologies, Cat. No. 20110), HetaSep® (StemCell Technologies, Cat. No. 07806), Stromatolyser NR Lyse (Sysmex, Cat. No. SNR-200, SNR-210A).

The results are shown in following table 1:

Table 1: Effect of Conventional Products for Erythrocyte Lysis on Recovery Rate of White Blood Cells (WBCs)

Easysep **1 portion blood + 2 portions lysis buffer, 2 x lysis (10 min), 2 x washing steps**				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD
6300	5600	88.89		
	5700	90.48		
	4900	77.78	85.71	6.92
HetaSep **5 portions blood + 1 portion lysis buffer, 1 x lysis (11 min), 2 x washing steps**				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD
6500	3200	49.23		
	2900	44.62		
	3200	49.23	47.69	2.66
Stromatolyser (Sysmex) **1 portion blood + 2 portions lysis buffer, 1 x lysis (ca. 30 sec), 2 x washing steps**				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD
6500	500	7.69		
	600	9.23	8.46	1.09
Stromatolyser (Sysmex) **1 portion blood + 5 portions lysis buffer, 1 x lysis (ca. 30 sec), 2 x washing steps**				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD
6500	4000	61.54		
	4200	64.62	63.08	2.18

The above results show that with conventional products a high percentage of white blood cells (WBC) is lost during the lysis of erythrocytes, namely up to more than 50 %.

Example 2: Erythrocyte Lysis in reagent without NH_4Cl

In a second test, the suitability of reagents without NH_4Cl was tested according to the above protocol. The results are shown in following table 2:

Table 2: Effect of reagent without NH_4Cl on Recovery of White Blood Cells (WBCs)

Lysis buffer: 0.225 % NaCl, 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min), 2 x washing steps				
WBCS/µl blood	WBCS/µl	%	Mean %	SD
5900	3900	66.10		
	3600	61.02		
	3400	57.63	61.58	4.27
Lysis buffer: 0.225 % NaCl, 1 portion blood + 9 portions lysis buffer, 2 x lysis (10 min), 2 x washing steps				
WBCS/µl blood	WBCS/µl	%	Mean %	SD
5900	3000	50.85		
	2900	49.15		
	2600	44.07	48.02	3.53
Lysis buffer: 0.3 % HAc, 45 mM Na_2CO_3 1 portion blood + 10.5 portions lysis buffer, 2 x washing steps				
WBCS/µl blood	WBCS/µl	%	Mean %	SD
5300	4500	84.91		
	4400	83.02		
	4000	75.47	81.13	4.99
9000	7400	82.22		
	7600	84.44		
	4000	44.44	70.37	22.48

The above results show that buffers without NH_4Cl are not suitable for the intended use, as a considerable portion of white blood cells (WBC) is lost during the lysis of erythrocytes.

Example 3: Erythrocyte Lysis in different reagents with NH_4Cl

In a third test, the suitability of different reagents with NH_4Cl was tested according to the above protocol. The results are shown in following table 3:

Table 3: Effect of different reagents with NH_4Cl on Recovery of White Blood Cells (WBCs)

Lysis buffer: 150 mM NH_4Cl + 10 mM NaAc, pH 5 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min), 2 x washing steps				
WBCS/µl Blood	WBCS/µl	%	Mean %	SD
11400	10500	92.11		
	9500	83.33		
	9600	84.21	86.55	4.83
Lysis buffer: 75 mM NH_4Cl + 0.45 % NaCl 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min), 2 x washing steps				
WBCS/µl Blood	WBCS/µl	%	Mean %	SD
11400	6700	58.77		
	7600	66.67		

	6400	56.14	60.53	5.48
Lysis buffer: 150 mM NH_4Cl + 10 mM Tris pH 7.5 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min), 2 x washing steps				
WBCS/µl Blood	WBCS/µl	%	Mean %	SD
5300	4900	92.45		
	4100	77.36		
	4600	86.79	85.53	7.63
Lysis buffer: 150 mM NH_4Cl + 5 mM Hepes 1 portion blood + 6 portions lysis buffer, 1 x lysis (10 min), 2 x washing steps				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
9000	8400	93.33		
	7900	87.78		
	7900	87.78	89.63	3.21
8400	7600	90.48		
	7100	84.52		
	7100	84.52	86.51	3.44
Lysis buffer: 80 mM NH_4Cl + 10 mM Tris pH 7.5 1 portion blood + 5 portions lysis buffer, 1 x lysis (10 min)				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
10700	8700	81.31		
	9200	85.98	83.64	3.30
5100	4300	84.31		
	4400	86.27	85.29	1.39
5500	4100	74.55		
	4600	83.64	79.09	6.43
7100	5400	76.06		
	5800	81.69	78.87	3.98
Lysis buffer: 80 mM NH_4Cl + 10 mM Tris pH 7.5 +0.1 mM EDTA 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min)				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
10700	9200	85.98		
	8800	82.24	84.11	2.64
5100	4600	90.20		
	4600	90.20	90.20	0.00
3200	2600	81.25		
	2700	84.38	82.81	2.21
7400	6200	83.78		
	7000	94.59	89.19	7.64

The above results show that buffers with NH_4Cl and HEPES are suitable for the intended use, as only a low number of white blood cells (WBC) is lost during the lysis of erythrocytes. Additionally, EDTA seems to increase WBCs recovery.

Example 4: Erythrocyte Lysis in different reagents with NH_4Cl and HEPES

In a forth test, the suitability of different reagents with NH_4Cl and HEPES was tested according to the above protocol. The results are shown in following table 4:

Table 4: Effect of different reagents with NH_4Cl and HEPES on Recovery of White Blood Cells (WBCs)

Lysis buffer: 80 mM NH_4Cl + 5 mM HEPES 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min)				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
4700	4200	89.36		
	3600	76.60	82.98	9.03
5300	4900	92.45		
	3800	71.70	82.08	14.68
6900	5900	85.51		
	5600	81.16	83.33	3.07
5700	5100	89.47		
	4700	82.46	85.96	4.96
4200	3400	80.95		
	3400	80.95	80.95	0.00
6200	4900	79.03		
	5000	80.65	79.84	1.14
5700	4800	84.21		
	5100	89.47	86.84	3.72
Lysis buffer: 80 mM NH_4Cl + 10 mM HEPES 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min)				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
8800	7600	86.36		
	7900	89.77	88.07	2.41
4700	3700	78.72		
	3800	80.85	79.79	1.50
7100	6200	87.32		
	6500	91.55	89.44	2.99
5800	5200	89.66		
	5300	91.38	90.52	1.22
5000	4100	82.00		
	4500	90.00	86.00	5.66
5700	5000	87.72		
	5700	100.00	93.86	8.68
5600	5000	89.29		
	4900	87.50	88.39	1.26
6100	5100	83.61		
	5400	88.52	86.07	3.48
5500	4800	87.27		
	4900	89.09	88.18	1.29
7100	5500	77.46		
Lysis buffer: 80 mM NH_4Cl + 5 mM HEPES + 0.5 mM $KHCO_3$ + 0.1 mM EDTA 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min)				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
5600	5000	89.29		
	5200	92.86	91.07	2.53
6100	5500	90.16		
	5200	85.25	87.70	3.48

3200	2800	87.50		
	2800	87.50	87.50	0.00
7400	6800	91.89		
	6700	90.54	91.22	0.96
6700	5600	83.58		
	5900	88.06		
	5900	88.06		
	5800	86.57	86.57	2.11
5600	4600	82.14		
	4600	82.14		
	4500	80.36		
	4700	83.93	82.14	1.46
6600	5500	83.33		
	5700	86.36		
	5500	83.33		
	5800	87.88	85.23	2.27
3800	3100	81.58		
	3000	78.95		
	3100	81.58		
	3100	81.58	80.92	1.32
6800	6400	94.12		
	6000	88.24		
	6400	94.12		
	6200	91.18	91.91	2.82
4700	4400	93.62		
	4400	93.62		
	4200	89.36		
	4300	91.49	92.02	2.04
7300	6500	89.04		
	7000	95.89		
	6700	91.78		
	6900	94.52	92.81	3.04
7200	6800	94.44		
	6500	90.28		
	6200	86.11		
	6600	91.67	90.63	3.47
6900	6700	97.10		
	6700	97.10		
	6300	91.30		
	6400	92.75	94.57	2.99
5000	4300	86.00		
	4600	92.00		
	4500	90.00		
	4800	96.00	91.00	4.16
6800	6000	88.24		
	6100	89.71		
	5700	83.82		
	6500	95.59	89.34	4.86
4800	4300	89.58		
	4500	93.75		
	4400	91.67		

	4400	91.67	91.67	1.70
4300	3900	90.70		
	4000	93.02		
	3900	90.70		
	3900	90.70	91.28	1.16
6900	6200	89.86		
	6500	94.20		
	6500	94.20		
	6500	94.20	93.12	2.17
5900	5100	86.44		
	5200	88.14		
	5400	91.53		
	5300	89.83	88.98	2.19
5800	4800	82.76		
	5500	94.83		
	5400	93.10		
	5400	93.10	90.95	5.52
12900	11800	91.47		
	12000	93.02		
	12400	96.12		
	12500	96.90	94.38	2.56
7400	6500	87.84		
	7400	100.00		
	6800	91.89		
	6700	90.54	92.57	5.23
6600	5700	86.36		
	5600	84.85		
	6100	92.42	87.88	4.01
6300	5300	84.13		
	5800	92.06		
	5400	85.71		
	5800	92.06	88.49	4.17
7000	6000	85.71		
	6100	87.14		
	6000	85.71		
	6100	87.14	86.43	0.82
4600	3800	82.61		
	4000	86.96		
	4200	91.30		
	4100	89.13	87.50	3.71
5100	4500	88.24		
	4400	86.27		
	4300	84.31		
	4200	82.35	85.29	2.53
5600	4700	83.93		
	4600	82.14		
	4400	78.57	81.55	2.73
8500	7900	92.94		
	7600	89.41		
	7400	87.06	89.80	2.96
4500	3800	84.44		

	4300	95.56		
	3900	86.67	88.89	5.88
3400	3000	88.24		
	3000	88.24		
	2900	85.29	87.25	1.70
5400	4300	79.63		
	4700	87.04		
	4800	88.89	85.19	4.90
6200	5300	85.48		
	5700	91.94		
	5600	90.32	89.25	3.36
4600	3900	84.78		
	4400	95.65		
	4200	91.30	90.58	5.47
5900	5100	86.44		
	5200	88.14		
	5400	91.53	88.70	2.59
8900	7500	84.27		
	7600	85.39		
	7500	84.27	84.64	0.65
10000	9200	92.00		
	8900	89.00		
	9000	90.00	90.33	1.53
6500	6000	92.31		
	5600	86.15		
	5700	87.69	88.72	3.20
11500	11000	95.65		
	11100	96.52		
	11000	95.65	95.94	0.50
11100	10600	95.50		
	10300	92.79		
	11000	99.10	95.80	3.16
7500	7200	96.00		
	7100	94.67		
	7100	94.67	95.11	0.77
6000	5300	88.33		
	5500	91.67		
	5300	88.33	89.44	1.92
4300	4400	102.33		
	4100	95.35		
	4100	95.35	97.67	4.03
7700	6900	89.61		
	7800	101.30		
	7700	100.00	96.97	6.41
5200	4600	88.46		
	4600	88.46		
	4800	92.31	89.74	2.22
6400	6300	98.44		
	6100	95.31		
	5400	84.38	92.71	7.38
7000	6800	97.14		

	6500	92.86		
	6200	88.57	92.86	4.29
4800	4300	89.58		
	3800	79.17		
	3800	79.17	82.64	6.01
6400	6000	93.75		
	5600	87.50		
	5800	90.63	90.63	3.13
9900	9300	93.94		
	8600	86.87		
	8900	89.90	90.24	3.55
5500	4700	85.45		
	5200	94.55		
	4900	89.09	89.70	4.58
6400	5700	89.06		
	6000	93.75		
	5600	87.50	90.10	3.25
5000	4700	94.00		
	4400	88.00		
	4500	90.00	90.67	3.06
6300	5500	87.30		
	5500	87.30		
	5200	82.54	85.71	2.75
6700	6300	94.03		
	6500	97.01		
	6000	89.55	93.53	3.76
6100	5100	83.61		
	5400	88.52		
	5600	91.80	87.98	4.13
5100	4300	84.31		
	4200	82.35		
	4400	86.27	84.31	1.96
6900	6100	88.41		
	6800	98.55		
	6700	97.10	94.69	5.49
10300	10000	97.09		
	9700	94.17		
	10300	100.00	97.09	2.91
8100	7400	91.36		
	8200	101.23		
	8200	101.23	97.94	5.70
4900	4300	87.76		
	4300	87.76		
	4700	95.92	90.48	4.71
6200	5500	88.71		
	5500	88.71		
	5500	88.71	88.71	0.00
4800	4500	93.75		
	4400	91.67		
	4700	97.92	94.44	3.18
5800	5600	96.55		

	5700	98.28		
	5500	94.83	96.55	1.72
4200	4000	95.24		
	3800	90.48		
	4200	100.00	95.24	4.76
7900	7700	97.47		
	7700	97.47		
	7700	97.47	97.47	0.00
6800	6200	91.18		
	6000	88.24		
	6600	97.06	92.16	4.49
8200	7100	86.59		
	7400	90.24		
	7800	95.12	90.65	4.28
9900	8900	89.90		
	9600	96.97		
	9600	96.97	94.61	4.08
6100	5500	90.16		
	5200	85.25		
	5400	88.52	87.98	2.50
7500	6500	86.67		
	6200	82.67		
	6400	85.33	84.89	2.04
6800	6100	89.71		
	5900	86.76		
	5900	86.76	87.75	1.70
5700	4900	85.96		
	5000	87.72		
	4800	84.21	85.96	1.75
8200	7200	87.80		
	7400	90.24		
	7000	85.37	87.80	2.44
5700	4600	80.70		
	4900	85.96		
	4800	84.21	83.63	2.68
6700	6000	89.55		
	5500	82.09		
	5600	83.58	85.07	3.95
3400	2600	76.47		
	2900	85.29		
	2700	79.41	80.39	4.49
13000	13000	100.00		
	13600	104.62		
	14000	107.69	104.10	3.87
5400	4800	88.89		
	5000	92.59		
	5200	96.30	92.59	3.70
8500	7900	92.94		
	7800	91.76		
	8500	100.00	94.90	4.45
6300	5300	84.13		

	5700	90.48		
	5200	82.54	85.71	4.20
6800	6400	94.12		
	5700	83.82		
	6300	92.65	90.20	5.57
5700	5400	94.74		
	5400	94.74		
	5400	94.74	94.74	0.00
9400	8500	90.43		
	8800	93.62		
	8700	92.55	92.20	1.63
5000	4000	80.00		
	4000	80.00		
	4500	90.00	83.33	5.77
6100	5500	90.16		
	5400	88.52		
	5600	91.80	90.16	1.64
4900	4400	89.80		
	4300	87.76		
	4000	81.63	86.39	4.25
13300	11600	87.22		
	12400	93.23		
	12600	94.74	91.73	3.98
4200	4100	97.62		
	3800	90.48		
	4000	95.24	94.44	3.64
12800	11600	90.63		
	12500	97.66		
	12200	95.31	94.53	3.58
6400	5900	92.19		
	6100	95.31		
	6300	98.44	95.31	3.13
4700	4300	91.49		
	4100	87.23		
	4300	91.49	90.07	2.46
6500	6200	95.38		
	6100	93.85		
	6700	103.08	97.44	4.95
5000	4600	92.00		
	5100	102.00		
	5000	100.00	98.00	5.29
6800	6400	94.12		
	6500	95.59		
	6500	95.59	95.10	0.85
7800	7700	98.72		
	7200	92.31		
	7700	98.72	96.58	3.70
5500	5500	100.00		
	5500	100.00		
	5700	103.64	101.21	2.10
9300	9300	100.00		

	9600	103.23		
	9500	102.15	101.79	1.64
6300	5400	85.71		
	5900	93.65		
	6300	100.00	93.12	7.16
6500	5500	84.62		
	6000	92.31		
	6800	104.62	93.85	10.09
6200	5900	95.16		
	6000	96.77		
	6300	101.61	97.85	3.36
4200	3800	90.48		
	3800	90.48		
	3800	90.48	90.48	0.00
7400	6900	93.24		
	7300	98.65		
	7200	97.30	96.40	2.81
4900	4700	95.92		
	4400	89.80		
	4600	93.88	93.20	3.12
8400	7400	88.10		
	7800	92.86		
	8300	98.81	93.25	5.37
5800	5200	89.66		
	5400	93.10		
	5700	98.28	93.68	4.34
7800	6700	85.90		
	6700	85.90		
	7100	91.03	87.61	2.96
3900	3300	84.62		
	3500	89.74		
	3500	89.74	88.03	2.96
5200	4200	80.77		
	4800	92.31		
	5100	98.08	90.38	8.81
6600	5900	89.39		
	6000	90.91		
	6200	93.94	91.41	2.31
5400	5000	92.59		
	4900	90.74		
	4800	88.89	90.74	1.85
4400	3200	72.73		
	3600	81.82		
	3200	72.73	75.76	5.25
5700	5200	91.23		
	5300	92.98		
	4800	84.21	89.47	4.64
5800	4400	75.86		
	4800	82.76		
	5000	86.21	81.61	5.27
7700	7400	96.10		

	7500	97.40		
	7600	98.70	97.40	1.30
6300	5800	92.06		
	5400	85.71		
	5800	92.06	89.95	3.67
6100	6200	101.64		
	6700	109.84		
	6500	106.56	106.01	4.13
5900	4500	76.27		
	4900	83.05		
	5500	93.22	84.18	8.53
6700	5900	88.06		
	5800	86.57		
	6000	89.55	88.06	1.49
6800	6000	88.24		
	6100	89.71		
	6600	97.06	91.67	4.73
8300	8300	100.00		
	7500	90.36		
	7300	87.95	92.77	6.38
4300	3500	81.40		
	3500	81.40		
	3700	86.05	82.95	2.69
Lysis buffer: 80 mM NH_4Cl (based on RBCL buffer*) + 5 mM HEPES 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min)				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
6300	5500	87.30		
	5400	85.71	86.51	1.12
9100	8600	94.51		
	8500	93.41	93.96	0.78
6200	5500	88.71		
	5200	83.87	86.29	3.42
5700	5100	89.47		
	5200	91.23	90.35	1.24
Lysis buffer: 80 mM NH_4Cl (based on RBCL buffer*) + 10 mM HEPES 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min)				
WBCs/µl Blood	WBCs/µl	%	Mean %	SD %
6300	5800	92.06		
	5600	88.89	90.48	2.24
9100	8500	93.41		
	8400	92.31	92.86	0.78
8800	8300	94.32		
	8500	96.59	95.45	1.61
4700	4400	93.62		
	4200	89.36	91.49	3.01

*RBCL buffer- Red Blood Cell Lysis buffer (Roche Applied Science, Cat. No. 11814389001): 150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM

Again, the above results show that buffers with NH_4Cl and HEPES are suitable for the intended use, as only a low number of white blood cells (WBC) is lost during the lysis of erythrocytes. Additionally, EDTA and $KHCO_3$ increase WBCs recovery. In the absence of these, 10 mM HEPES is superior to 5 mM HEPES.

For lysis buffer consisting of 80 mM NH_4Cl + 5 mM HEPES + 0.5 mM KHCO3 + 0.1 mM EDTA (used with 1 portion blood + 5 portions lysis buffer, 2 x lysis (10 min)), 122 samples were tested. 90.77 ± 3.37 % oft he WBC present before lysis (100.00 ± 3.38 %) were recovered after lysis.

Example 5: Erythrocyte Lysis in different reagents with NH_4Cl and HEPES

In a fifth test, the suitability of a reagent with 80 mM NH4Cl+ 10 mM Hepes + 0.1 mM EDTA was tested according to the above protocol. In addition to recovery of WBCs and RBCs, the viability of recovered WBCs was measured by Trypanblue exclusion test. Therefore the WBCs have been stained with Trypanblue (Sigma, Cat. No. T8154-20ML), (Dilution WBC suspension : Trypanblue = 1:2) and stained cells (dead cells) have been counted using the C-Chip counting chamber (Biochrom, Cat. No. P DHC-N01). The results are shown in following table 5:

Table 5: Effect of reagent on Recovery and Viability of White Blood Cells (WBCs) and Red Blood Cells (RBCs)

WBCs/ µl Blood	WBCs/µl	%	Mean %	SD %	non viable cells (%)	RBC/ µl Blood	RBC/µl
7100	6200	87.32			330	$4.49x10^6$	$0.04x10^6$
	6500	91.55	89.44	2.99	(5.32)		$0.04x10^6$
5800	5200	89.66			335	$5.45x10^6$	$0.04x10^6$
	5300	91.38	90.52	1.22	(6.44)		$0.03x10^6$
5000	4100	82.00			160	$4.70x10^6$	$0.02x10^6$
	4500	90.00	86.00	5.66	(3.9)		$0.03x10^6$
5700	5000	87.72			485	$4.63x10^6$	$0.03x10^6$
	5700	100.00	93.86	8.68	(9.7)		$0.04x10^6$
8800	8300	94.32			115	$4.97x10^6$	$0.03x10^6$
	8500	96.59	95.45	1.61	(1.39)		$0.02x10^6$
4700	4400	93.62			145	$4.87x10^6$	$0.03x10^6$
	4200	89.36	91.49	3.01	(3.3)		$0.02x10^6$

The above results show that while the number of RBCs is decreased drastically, the number and viability of WBCs is maintained at a very high level, proofing the suitability of the reagent for the specific lysis of erythrocytes.

Example 6: Erythrocyte Lysis in different reagents with NH_4Cl and HEPES

In a sixth test, the suitability of different reagents with NH_4Cl and HEPES was tested according to the above protocol. The results are shown in following table 6:

Table 6: Effect of reagent composition on Recovery of White Blood Cells (WBCs) and pH value after lysis

		80 mM NH_4Cl + 10 mM Hepes + 0.1 mM EDTA		80 mM NH_4Cl + 5 mM Hepes + 0.1 mM EDTA		100 mM NH_4Cl + 10 mM Hepes + 0.1 mM EDTA		100 mM NH_4Cl + 5 mM Hepes + 0.1 mM EDTA	
Sample 1		w/o $KHCO_3$		0.5 mM $KHCO_3$		w/o $KHCO_3$		0.5 mM $KHCO_3$	
WBC / µl	6400	4700	4800	4900	5000	4900	5300	4800	5000
% recovery WBCs		73,44	75,00	76,56	78,13	76,56	82,81	75,00	78,13
1.Lysis pH super-natant		7,20	7,20	7,18	7,18	7,26	7,26	7,22	7,25
2.Lysis pH super-natant		7,01	7,02	7,09	7,09	6,99	7,00	7,08	7,06

		120 mM NH_4Cl + 10 mM Hepes + 0.1 mM EDTA		120 mM NH_4Cl + 5 mM Hepes + 0.1 mM EDTA		100 mM NH_4Cl + 10 mM Hepes + 0.1 mM EDTA		100 mM NH_4Cl + 5 mM Hepes + 0.1 mM EDTA	
Sample 2		w/o $KHCO_3$		0.5 mM $KHCO_3$		w/o $KHCO_3$		0.5 mM $KHCO_3$	
WBC / µl	6200	5700	6300	5800	5600	5700	5400	5400	5400
% recovery WBCs		91,94	101,61	93,55	90,32	91,94	87,10	87,10	87,10
1.Lysis pH super-natant		7,21	7,19	7,23	7,22	7,20	7,21	7,19	7,20
2.Lysis pH super-natant		6,98	6,98	7,03	7,03	6,99	6,99	7,06	7,06

		80 mM NH_4Cl + 10 mM Hepes + 0.1 mM EDTA		80 mM NH_4Cl + 5 mM Hepes + 0.1 mM EDTA		100 mM NH_4Cl + 10 mM Hepes + 0.1 mM EDTA		100 mM NH_4Cl + 5 mM Hepes + 0.1 mM EDTA	
Sample 3		w/o $KHCO_3$		0.5 mM $KHCO_3$		w/o $KHCO_3$		0.5 mM $KHCO_3$	
WBC /	4800	4500	4800	4700	3900	4700	4800	4500	4400

µl									
% recovery WBCs		93,75	100,00	97,92	81,25	97,92	100,00	93,75	91,67
1.Lysis pH super-natant		7,20	7,20	7,20	7,20	7,17	7,17	7,20	7,20
2.Lysis pH super-natant		7,00	7,00	7,08	7,09	6,99	6,99	7,07	7,07

Sample 3		120 mM NH_4Cl + 10 mM Hepes + 0.1 mM EDTA w/o $KHCO_3$		120 mM NH_4Cl + 5 mM Hepes + 0.1 mM EDTA 0.5 mM $KHCO_3$	
WBC / µl	4800	5700	6300	5800	5600
% recovery WBCs		91,94	101,61	93,55	90,32
1.Lysis pH super-natant		7,20	7,20	7,23	7,23
2.Lysis pH super-natant		6,98	6,99	7,04	7,05

Various buffers with 80 to 120 mmol NH_4Cl + 5 mM to 10 mmol/l HEPES + 0.1 mM EDTA +0 to 0.5 mM $KHCO_3$ were tested for their effectiveness. The above results show that any of the buffers tested was suitable in maintaining the number of WBCs at a very high level, proofing the suitability of the reagent for the specific lysis of erythrocytes. Additionally, the pH values in the supernatant after the 1st and 2nd lysis were in the range of from 6.7 to 7.7, particularly of from 6.9 to 7.3.

Example 7: Erythrocyte Lysis in different reagents with different lysis components

In a seventh test, the effectiveness of existing erythrocyte lysis protocols was tested as indicated. The results are shown in following table 7:

Table 7: Effect of conventional erythrocyte lysis protocols on Recovery of White Blood Cells

	Method	Testing conditions	Number of experiments	WBC recovery rate (Mean) [%]	Remarks
1	Ammonium Chlorid based method	NH_4Cl (RCLB*); Dilution Blood: Lysis buffer = 1:2	10	63,2	according to manufacturer`s protocol
2	Ammonium Chlorid based method	NH_4Cl (RCLB*); Dilution Blood: Lysis buffer = 1:5	14	71,0	according to manufacturer`s protocol
3	Ammonium Chlorid based method	NH_4Cl (150 mM), 10 mM Tris pH 7,5; Dilution Blood: Lysis buffer = 1:5	1	35,9	according to RCLB protocol
4	Ammonium Chlorid based method	NH_4Cl (150 mM), 5 mM Hepes; Dilution Blood: Lysis buffer = 1:6	1	35,5	according to RCLB protocol
5	Ammonium Chlorid based method	NH_4Cl (75 mM)/ NaCl (0,45 %); Dilution Blood: Lysis buffer = 1:5	1	57,0	according to RCLB protocol
6	Ammonium Chlorid based method	NH_4Cl (RCLB*); Dilution Blood: Lysis buffer = 1:1	1	30,0	according to manufacturer`s protocol
7	Ammonium Chlorid based method	NH_4Cl (RCLB*); Dilution Blood: Lysis buffer = 1:2; Washing of cells: 2 x RPMI	2	55,5	according to manufacturer`s protocol
8	Acetic acid based lysis	Acetic acid (1%), Na_2CO_3, 4 ml blood; Dilution Blood: Acetic acid: Na_2CO_3 = 1:3:7,5	4	64,2	protocol according to US 5,155,044
9	Acetic acid based lysis	Acetic acid (1%), Na_2CO_3, 5 ml blood; Dilution Blood: Acetic acid: Na_2CO_3 = 1:3:7,5	2	70,0	protocol according to US 5,155,044 (with modifications)
10	Acetic acid based lysis	Acetic acid (3%), Na_2CO_3; 5 ml blood; Dilution Blood: Acetic acid: Na_2CO_3 : 1:4:8	1	30,6	protocol according to US 5,155,044 (with modifications)
11	Acetic acid based lysis	Acetic acid (3%), Na_2CO_3; 5 ml blood; Dilution Blood: Acetic acid: Na_2CO_3 : 1:3:6	1	52,8	protocol according to US 5,155,044 (with modifications)
12	NaCl based lysis	NaCl (0,225%); 2 x lysis (1:5)	2	52,5	according to RCLB protocol
13	NaCl based lysis	NaCl (0,225%); 2 x lysis (1:9)	1	60,0	according to RCLB protocol
14	Saccharose/ Triton based lysis	Saccharose (320 mM); Tris (50 mM pH 7,5); $MgCl_2$ (5 mM); Triton (1 %)	1	complete lysis of all cells	protocol according to DE 102008032501

15	Saccharose/ Triton based lysis	Saccharose (320 mM); Tris (12 mM pH 7,5); $MgCl_2$ (5 mM); Triton (1 %)	1	complete lysis of all cells	protocol according to DE 102008032501
16	NaCl based lysis	Tris (10 mM pH 7,5); NaCl (12,45 mM); $MgCl_2$ (0,5 mM)	1	24,2	according to RCLB protocol
17	Piperidin-Hydrochlorid based lysis	Piperidin-Hydrochlorid (0,17 M); $KHCO_3$ (2,5 mM), Hepes (5mM) pH7,5; EDTA (0,1 mM); Washing of cells 2 x with PBS	4	63,0	protocol according to US 7,678,583 B2
18	Piperidin-Hydrochlorid based lysis	Piperidin-Hydrochlorid (0,17 M); $KHCO_3$ (2,5 mM), Hepes (5mM) pH7,5; EDTA (0,1 mM); Washing of cells 2 x with Hepes	1	0,0	protocol according to US 7,678,583 B2
19	Piperidin-Hydrochlorid based lysis	Piperidin-Hydrochlorid (0,128 M); $KHCO_3$ (2,5 mM), Hepes (5mM) pH7,5; EDTA (0,1 mM); Washing of cells 2 x with PBS	1	66,0	protocol according to US 7,678,583 B2
20	Piperidin-Hydrochlorid based lysis	Piperidin-Hydrochlorid (0,085 M); $KHCO_3$ (2,5 mM), Hepes (5mM) pH7,5; EDTA (0,1 mM); Washing of cells 2 x with PBS	1	43,3	protocol according to US 7,678,583 B2
21	Piperidin-Hydrochlorid based lysis	Piperidin-Hydrochlorid (0,17 M); $KHCO_3$ (2,5 mM), Hepes (5mM) pH7,5; EDTA (0,1 mM); Washing of cells 2 x with 0,9% NaCl	1	64,1	protocol according to US 7,678,583 B2
22	NaCl based lysis	Tris (10 mM pH 7,5); NaCl (12,45 mM); $MgCl_2$ (0,5 mM)	1	37,8	according to RCLB protocol
23	Ammonium Chlorid based method	NH_4Cl (150 mM)/ Na-acetat (10mM); Dilution Blood: Lyis buffer = 1:5; pH 5	8	66,7	according to RCLB protocol
24	Saponin based method	0,02 % Saponin in PBS + 1 % BSA; Dilution Blood:Saponin = 1:1	2	40,1	protocol according to US 5,840,515 (with modifications)
24	Saponin based method	0,04 % Saponin in PBS + 1 % BSA; Dilution Blood:Saponin = 1:1	2	35,3	protocol according to US 5,840,515 (with modifications)

* RCLB:Red Blood Cell Lysis buffer (Roche Applied Science, Cat. No. 11814389001): 150 mM NH4Cl, 1 mM KHCO3, 0.1 mM

The above results show that none of the buffers tested was suitable in maintaining the number of WBCs at a high level, proofing the advantageous effect associated with the reagent as presented herein

Claims

1. Use of a reagent for the lysis of erythrocytes, the reagent being an aqueous solution comprising or consisting of HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NH_4^+/NH_3, a chelating agent and optionally CO_3^{2-}/CO_3^-, wherein the final concentration during lysis of erythrocytes is in the range of
   - from 2.5 mmol/l to 12 mmol/l HEPES,
   - from 60 mmol/l to 120 mmol/l NH_4^+/NH_3,
   - from 0.04 mmol/l to 0.8 mmol/l chelating agent, and
   - from 0.15 mmol/l to 0.8 mmol/l CO_3^{2-}/CO_3^-, if present.

2. The use of claim 1, wherein the final concentration during lysis is in the range of
   - from 3 mmol/l to 11 mmol/l HEPES,
   - from 70 mmol/l to 100 mmol/l NH_4^+/NH_3,
   - from 0.05 mmol/l to 0.5 mmol/l chelating agent, and/or
   - from 0.3 mmol/l to 0.6 mmol/l CO_3^{2-}/CO_3^-, if present.

3. The use of claim 1 or 2, wherein the chelating agent is ethylene diamine tetraacetic acid (EDTA).

4. The use of any one of claims 1 to 3, wherein the pH of the reagent is in the range of from 6.4 to 7.7.

5. The use of any one of claims 1 to 4, wherein the reagent is used in the detection, concentration or isolation of cells other than erythrocytes from a sample comprising erythrocytes.

6. The use of claim 5, wherein cells other than erythrocytes are leukocytes, circulating endothelial cells, or circulating tumor cells.

7. A method of lysing erythrocytes, the method comprising
   a) providing a sample comprising erythrocytes;
   b) incubating the sample with the reagent as defined in any one of claims 1 to 4, thereby lysing erythrocytes; and
   c) optionally removing erythrocyte debris.

8. The method of claim 7, wherein the sample is a blood sample or a sample comprising erythrocytes and other cells.

9. The method of claim 7 or 8, wherein the method further comprises

d) detecting or isolating cells other than erythrocytes from a sample comprising erythrocytes.

10. The method of claim 9, wherein cells other than erythrocytes are white blood cells or circulating tumor cells, particularly circulating tumor cells.

11. The method of any one of claims 7 to 10, wherein the incubating of step b) is for at most 30 min.

12. The method of claim 11, wherein the incubating of step b) is at room temperature.

13. A kit for the isolation of white blood cells from a sample comprising erythrocytes, comprising

- a reagent for lysis of erythrocytes as defined in claims 1 to 4; and
- a reagent for removing erythrocyte debris; and
- optionally, instructions for carrying out the method of any one of claims 7 to 12.

14. The kit of claim 13, wherein the reagent for removing erythrocyte debris is phosphate-buffered saline (PBS) comprising a chelating agent.