Title: IMMUNOMAGNETIC COMPLEX AND ITS USE IN RED BLOOD CELL GROUPING/PHENOTYPING

Abstract: The present invention relates to a reagent for RBC grouping/phenotyping comprising a suspension of magnetic particles or beads coated with an antiglobulin saturated with RBC anti-antibodies, and a kit or device containing the above. The present invention also relates to an RBC grouping/phenotyping method using such a suspension of magnetic particles or beads according to the present invention.
IMMUNOMAGNETIC COMPLEX AND ITS USE IN RED BLOOD CELL GROUPING/PHENOTYPING

The present invention relates to a reagent for red blood cell grouping/phenotyping comprising a suspension of magnetic particles or beads coated with an antiglobulin saturated with an anti-antigen red blood cell (RBC) antibody and a kit or device containing it. The present invention also relates to an RBC grouping/phenotyping method using such a suspension of magnetic particles or beads according to the present invention.

In transfusion clinical practice, RBC phenotyping, the screening and identification of blood group antigens on the surface of blood cells (with the exception of the ABO system in which a search is also carried out for the presence of the corresponding regular antibodies), is applied to both recipient and donor.

With regard to the recipient and donor, three levels of RBC phenotype exist, making RBC concentrates compatible in risk situations, available to the recipient:
- determination of ABO and Rhesus standard grouping (presence or absence of D antigen);
- determination of the Rhesus Kell phenotype (presence or absence of C, E, c, e and K antigens); and
- determination of the extended (or enlarged) phenotype i.e. the presence of antigens from the Duffy system (Fya, Fyb), Kidd system (Jka, Jkb) and MNSs system (S and s antigens);
- other antigens that may be investigated depending on the type of risk and/or irregular antibody detected in the recipient's serum.

The techniques habitually used in phenotyping generally consist of using test serums containing the appropriate antibodies to look for the presence or absence of the relevant antigen. The antibodies contained in these test serums should preferably be agglutinins (IgM or IgA) to obtain total or partial agglutination of the RBCs to be phenotyped when the latters carry the antigen corresponding to the antibody present in the test serum. However, it is possible to use non-agglutinin test antibodies (IgG type). In this case, agglutination is produced by an anti-human immunoglobulin that
is visible, notably after the centrifugation and resuspension of the packed red blood cells ("indirect Coombs technique"). The test is called "indirect Coombs" because the first stage consists of incubating the red blood cells with the test IgG at 37°C. After incubation, the cells are washed by centrifugation to eliminate excess non-fixed antibodies and allow for maximum fixation of anti-human globulin (AHG). Fixation of AHG (anti-human globulin) to the test antibodies allows the red blood cells to agglutinate by forming a macromolecular complex. This agglutination is promoted after centrifugation and resuspension of the packed red blood cells.

Among the variations to the techniques used for phenotyping or for irregular antibody screening (IAS) are the methods developed generally to enable detection, in a sample, of an analyte capable of binding to a cell by using magnetic particles, notably to suppress centrifugation, an operation required in techniques based on agglutination such as the antiglobulin technique (indirect Coombs test by agglutination or by immuno-adherence on a solid phase) for IAS. It is also required for phenotyping or, in the same way as for IAS, when it is necessary to wash sensitized red blood cells to remove the non-specific antibodies capable of recognizing the anti-immunoglobulin used in the next step.

The centrifugation step is always difficult to implement when it is planned to fully automate a process due mainly to cost, the size and handling of centrifuges etc.

Magnetic particles have been used for many years in the detection of ligand-receptor or antibody-antigen complexes. Examples include the processes described in the following patent documents:

- document WO 92/17781 which describes a method of determining the presence of a ligand in a sample in which magnetic latex particles are incubated, which may be of different colors, coated with a substance such as an antibody capable of binding to the ligand. This is followed by application of a magnetic field to the incubation medium and finally observation of the presence or absence of agglutination; or

- document EP 0 426 170 which describes a method of determining the presence of a ligand in a sample by incubating magnetic gelatin particles sensitized with antigens or antibodies capable of binding to the ligand. Then by applying a magnetic field to the incubation medium and finally observation of the presence or absence of
agglutination, said method being characterized in that the sliding state of these particles can be observed after tilting the container, notably in the cupule of microplate with V-shaped base.

Such magnetic particles have already been used in immunohematology for phenotyping and/or IAS. Among the documents describing such applications are:
- document WO2005 121805 which describes a grouping/phenotyping and irregular agglutinin screening method using an aqueous ferrofluid solution;
- document EP 0 351 857 which describes an immunological assay method using magnetized markers such as antibodies or antigens fixed on magnetic latex beads;
- document EP 0 528 708 which describes a process of detection by immunoadherence of a biological substance likely to be present in a sample using magnetic latex beads; and
- patent document EP 0 230 768 which describes a process of co-aggregation method for magnetic particles capable of binding with a substance contained in a sample by means of polycationic or polyanionic compounds in the presence of a magnetic field.

Although the techniques using magnetic beads or a modified ferrofluid remove the need for centrifugation, they require the use of solid phases previously coated with blood cells used for tests or phenotyping, which lack stability or have to be prepared by the end user. Moreover, see document EP 0 230 768 on the use of certain magnetic compounds which lead to the non-specific co-aggregation of red blood cells after application of the magnetic field, permanently removing the possibility of reading a specific agglutination of red blood cells in the presence of the RBC anti-antigen antibody, a reference technique in the blood transfusion field.

What is now required is a fast, simple process that can be implemented on a practical and available support such as a microplate, a process in which the centrifugation step is replaced by the application of a magnetic field. The process could then be fully automated and the grouping/phenotyping of red blood cells could be done by reading off a specific agglutination by means of IgG test antibodies (non-agglutinating but widely available and providing widely-diverse specificities), without interfering significantly with the final image. This method could also be applicable for Simonin testing (indirect testing) in ABO phenotyping. Such a method would be more advantageous if the magnetic compound used did not cause the non-
specific aggregation of red blood cells and, for phenotyping, avoided the need for agglutinating serums which are less common and, therefore, more expensive.

This is the aim of the present invention.

The inventors have shown that RBCs put in contact with a diluted, aqueous suspension of magnetic particles (magnetic microparticles or beads) coated with antiglobulin such as anti-human globulin (AHG), the latter having been previously saturated with an anti-antigen antibody of the RBC group/phenotype, were capable of agglutinating specifically after being subjected to a magnetic field, whatever the agglutinating nature (IgG or IgM) of the RBC group/phenotype anti-antigen antibodies used.

Moreover, the reagent allows for grouping or phenotyping without centrifugation and, consequently, the complete automation of the process.

Finally, the inventors have shown that such a ready-to-use reagent was stable and could be stored before use. The inventors have also shown, unexpectedly, that the presence of these magnetic beads or particles in the diluted suspension did not interfere significantly with the specific agglutination. In fact, the agglutination could be easily seen by the naked eye or by any automated reading system capable of detecting the presence or absence of RBC agglutinates.

Finally, for phenotyping, such a reagent allows for the use of RBC group/phenotype anti-antigen antibodies of the IgG type, leading to a reading by specific agglutination.

The term "grouping" is used for the ABO blood group system; the term "phenotyping" refers to other blood group systems.

The present invention achieves RBC grouping/phenotyping based on test IgG without the need for washing step to eliminate excess specific IgG after incubation at 37°C or centrifugation to stimulate the agglutination of the complex formed in the presence of RBCs.

The invention relates to a method used for blood grouping and phenotyping by means of a ready-to-use reagent in a kit.

The method uses magnetic particles coated in anti-human globulin, which has itself been previously saturated in IgG specific to the antigen being sought in the red blood cell (cf. figure 1). This makes it possible to carry out an indirect Coombs test.
without washing or centrifugation, since, on the one hand, the specific IgG is already fixed on the anti-globulin and does not require washing and, on the other hand, since after fixation of the red blood cells to the immunomagnetic complex, the application of a magnetic field will draw the red blood cells to the bottom of the well and enhance their agglutination. Agitation after the magnetization phase resuspends the packed red blood cells and reveals the agglutinate.

The process may allow for the grouping and phenotyping of all the antigens present on the surface of the red blood cells if there is an antibody available.

It is also possible to perform this type of test using IgM, thereby strengthening their agglutinating power. An anti-IgM anti-human globulin has to be fixed on the magnetic bead and saturated with an IgM specific to the antigen to be revealed.

It is also possible to mix two types of beads in the same ready-to-use reagent, each one coupled to an anti-globulin and a specific antibody, and by this combination improve the detection of a variant or weak antigen.

This is particularly so in the search for a weak D, where mixtures of anti-D clones are often used to maximize recognition of all the weak and variant D antigens. It then becomes easy to perform specific agglutinations and phenotype an individual's red blood cells completely, using any type of antibody regardless of its isotype.

The macromolecular, immunomagnetic complex then becomes capable of agglutinating red blood cells presenting the required antigen, after application of a magnetic field. The centrifugation stage normally used is then replaced by a magnetization step to enhance agglutination.

Thus the aim of the present invention is a suspension of magnetic particles or magnetic beads, characterized in that the said magnetic particles are coated on their surface with an antiglobulin complex, preferably an anti-human globulin (AHG)/antibody and in which complex, said antibody is specifically directed against an RBC group/phenotype antigen (called RBC group/phenotype anti-antigen antibody).

It is evident that, if the antiglobulin (AG) is an anti-species AG of a given species, the said RBC group/phenotype anti-antigen antibody will have as its origin the species being targeted by the said AG. For example, and preferably, when the AG
is an AHG, the said antibody in the complex is an RBC group/phenotype anti-antigen antibody of human origin.

Nevertheless, the AG could be of the AG murine type, in which case the antibody is an antibody of murine origin. The same applies to rats or any other mammals.

In the preferred embodiment of the invention, the AHG may be replaced by an A protein or G protein, or by any protein or polypeptide capable of binding an antibody specifically by its Fc fragment, preferably a bifunctional polypeptide (two antibodies can be bound by such a bifunctional polypeptide).

In the present invention, the term "magnetic particle" or "magnetic bead" refers to the same object viz. a spherical magnetic bead with a diameter of between 0.5 μm minimum and 10 μm maximum (by opposition to ferrofluid which does not lie within the scope of this definition).

Among the suppliers whose magnetic particles can be used for the purposes of this invention is Ademtech (33600 Pessac, France), a company selling magnetic beads with a diameter of approximately 100 to 500 nm that can be functionalized by an acid or amine function. It also sells the protocols and reagents used to achieve the required grafting of these functions. The magnetic particles consist of more than 50% of a core made of ferromagnetic compound (iron oxide type), the core then being coated with polystyrene. Another supplier is Bioclon Inc. (San Diego, CA, USA), which has an entire catalogue of suitable functionalized magnetic beads. A third potential supplier is Dynal (Invitrogen, USA) with its very wide range of Dynabeads™ notably micromagnetic beads activated with streptavidin, Tosyl or a carboxylic function. Merck-Chimie SAS (Fontenay Sous Bois, France) has a range of magnetic microparticles (ESTAPOR™) in various sizes from 200 nm to 1.5 μm made with polystyrene or divinylbenzene and including up to more than 50% of ferrite. These microparticles may or may not be functionalized, e.g. with a carboxylic function, amine or Tosyl. The particles are prepared by a process involving the polymerization of styrene in the presence of the ferromagnetic compound. Another company is JSR Micro (Japan), which markets magnetic particles of various sizes from 1 μm to 3 μm activated with streptavidin or having carboxylic or Tosyl functions. JSR's magnetic beads consist of a central particle coated with a magnetic
substance, which is itself coated with a monomer to encapsulate the magnetic substance. Among the available beads, the 1 µm hydrophobic beads are preferred. They have an iron content of approximately 48% and a carboxylic function rate of approximately 15 nmol per mg beads. The beads are characterized in that they enable coupling by physical adsorption or by chemical coupling.

The magnetic beads suitable for use in this invention are superparamagnetic, with a magnetic particle size of between 200 nm and 1.5 µm. They are preferably hydrophobics and functionalized by carboxylic groups or Tosyl. The mean iron content in magnetic beads is approximately 45% for carboxylic beads and approximately 30% for Tosyl beads.

Preferably, the suspension of magnetic particles according to the invention is characterized in that the said AHG is selected from among AHGs of the anti-IgG or anti-IgM type.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said RBC group/phenotype anti-antigen antibody is of the IgG type when AHG is of the anti-IgG type and the said RBC group/phenotype anti-antigen antibody is of the IgM type when AHG is of the anti-IgM type.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said AG is saturated with RBC group/phenotype anti-antigen antibodies.

In the present description, the term "specific blood group anti-antigen antibody" refers to polyclonal, monoclonal or recombinant antibodies with a known specificity, capable of recognizing and binding to the RBC carrying the antigen that it is targeting.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said RBC group/phenotype anti-antigen antibody is selected from among the polyclonal or monoclonal (preferably monoclonal) anti-A, anti-B, anti-D, anti-C, anti-E, anti-c, anti-e, anti-K, anti-Fya, anti-Fyb, anti-Jka, anti-Jkb anti-S and anti-s antibodies or specifically directed against any other RBC group/phenotype antigen for which testing is performed to determine its presence on the surface of an RBC.
Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said magnetic particles are selected from among superparamagnetic particles.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said magnetic particles are selected from magnetic particles with a diameter of between 0.75 \( \mu \text{m} \) and 5 \( \mu \text{m} \), and preferably between 1 \( \mu \text{m} \) and 3 \( \mu \text{m} \) and between 1 \( \mu \text{m} \) and 2 \( \mu \text{m} \).

The immunodiagnostic, cell capture or cell sorting techniques using magnetic particles have been widely covered in publications and are well known to those skilled in the art.

Among the techniques are the ones that use functionalization of the magnetic particle to obtain a reactive surface function capable of reacting, in appropriate conditions and with the appropriate reagents, with the antigen, antibody or, generally, with any protein or its derivatives (e.g. glycoproteins or their fragments) that are to be grafted onto the particle in a covalent manner, in particular an acid, amine, alcohol, tosyl, epoxy or aldehyde function to name but the commonest.

Even if less preferred, there are also techniques that use passive adsorption of the antigen that is to be fixed to the particle, notably by adequate processing to produce beads charged positively or negatively depending on the antigens and the conditions under which the passive absorption is realized.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said magnetic particles have been previously functionalized with a group selected from among the carboxylic, amine, alcohol or tosyl groups.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said magnetic particles have been previously functionalized with a group selected from among the carboxylic, amine, alcohol or tosyl groups and have a functionalization rate of between 20 \( \mu \text{eq/gram beads} \) and 350 \( \mu \text{eq/gram beads} \), preferably between 20 \( \mu \text{eq/gram beads} \) and 80 \( \mu \text{eq/gram beads} \).

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said magnetic particles have a mean iron content of between 30\% and 50\%, preferably between 30\% and 40\%.
Preferably again, the suspension of magnetic particles according to the invention is characterized in that the concentration in coated AHG on the said magnetic particles lies between 10 µg/mg particles and 70 µg/mg particles, preferably between 20 µg/mg ± 5 µg/mg particles for anti-IgM AHG and 50 µg/mg ± 10 µg/mg particles for anti-IgG AHG.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said magnetic particles are suspended in an aqueous solution containing a surfactant, preferably at a particle concentration of between 0.1% and 2.5% (w/v), preferably between 0.2% and 1.5% (w/v), preferably again between 0.25% and 1.0% (w/v), preferably again at 0.5% ± 0.25% (w/v), 0.3% also being preferred.

Suspension of magnetic particles, characterized in that the said surfactant is selected from among non-ionic surfactants or detergents, preferably chosen from among non-ionic hydrophilic detergents such as Tween 20, 40 or 80, poloxamers such as Synperonic PE/F68® or F127.

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said surfactant is at a concentration of between 0.1% and 2.5% (m/v), preferably between 0.25% and 1%, preferably at 0.5% ± 0.15% (m/v), again preferably at 0.75% ± 0.25% (m/v).

Preferably again, the suspension of magnetic particles according to the invention is characterized in that the said magnetic particles are suspended in an aqueous buffer solution containing bovine serum albumin (BSA) at a concentration of between 0.05% and 0.75%, preferably between 0.1% and 0.5% (w/v), preferably 0.5% (w/v), preferably in a physiological buffer such as phosphate buffered saline 0.3 M, pH 7.4.

Surprisingly, the inventors have shown that such a buffer molarity (approximately twice the molarity of the standard PBS) would provide significantly better results especially in terms of the storage of these suspensions of magnetic particles coated with AG and, if need be, antibodies (ready-to-use suspension as a grouping/phenotyping reagent).

Preferably again, this suspension of magnetic particles according to the invention is characterized in that the said magnetic particles are suspended at 0.1% ±
0.25% (w/v) in a phosphate buffer saline 0.3 M, pH 7.4 (PBS) containing bovine serum albumin (BSA) at a concentration of 0.1% (w/v).

In certain applications, especially when there is a need to improve the sensitivity or reaction speed (without increasing the test's non-specificity), a low ionic strength buffer, or "LISS" ("Low Ionic Strength Solution"), can be used.

Those skilled in the art will understand the terms "buffer" or "saline solution" to refer to the buffer commonly used in cell biology, notably in immuno-hematology, to avoid lysis of red blood cells. Such buffers or solutions will, for example, be buffers with a physiological pH of between 6.8 and 7.5 and a molarity adjusted to bring it close to the molarity of an NaCl solution at 9 permil000 (close to 0.15 M of NaCl). One such solution, out of many, is the phosphate buffer saline (PBS) with pH 7 - 7.4 which is well known to those skilled in the art.

The composition of LISS buffers will not be described here since these buffers are well-known in immuno-hematology to enhance agglutination. These buffers are notably available from the suppliers of reagents for immuno-hematology (one example from among several is the LISS buffer with the following composition: 16 g/l glycine, 0.03 M NaCl and 0.015 M phosphate with pH 6.7).

Under another aspect, the present invention includes an RBC grouping/phenotyping kit comprising a suspension of magnetic particles according to the invention in which the said RBC group/phenotype anti-antigen antibody is directed specifically against a given antigen.

Preferably, the said RBC grouping/phenotyping kit comprises at least two distinct suspensions of magnetic particles according to the invention, in which each of the said RBC grouping/phenotyping anti-antigen antibodies is directed specifically against a different given antigen.

Preferably, the said RBC grouping/phenotyping kit comprises a mixture of at least two suspensions of magnetic particles according to the invention in which each of the said RBC grouping/phenotyping anti-antigen antibodies is directed specifically against a different given antigen, preferably each is directed against an antigen selected from among antigens of the weak D, partial D and/or D variant types.

Under another aspect, the present invention relates to a method for grouping/phenotyping of RBC from a biological sample containing RBCs for which
the group and/or phenotype has to be determined, and characterized in that it includes the following steps:

a) a step in which the suspension of RBCs to be grouped or phenotyped is put in contact, in a container, with a suspension of magnetic particles according to the invention or with a mixture of at least two of the said suspensions of magnetic particles with distinct specific antibodies (in particular, for certain phenotypes comprising D-variants or a weak D-antigen);

b) an incubation step with, if need be, a prior mixture of the suspension of particles and RBCs (this mixture can be made by stirring the container);

c) application of a magnetic field to the said container using a magnet located outside and under the said container so that the said magnetic particles are drawn to it, if need be with the RBCs bound to the said magnetic particles;

d) the re-suspension of the packed red blood cells obtained during step c) by stirring;

e) the reading, by the naked eye and/or by any other appropriate reading system, of the presence or absence of agglutinates in the container, the presence of agglutinates being indicative of the presence of the antigen against which is specifically directed the RBC group/phenotype anti-antigen antibody, present on the magnetic particle.

It is evident that, in this method for determining the presence of a blood group antigen by means of an RBC agglutination reaction according to the invention, it is also preferable for step c), application of a magnetic field to the said container, to be performed using a magnet outside and underneath the container so that the magnetic particles are drawn vertically down to the base of the said container.

In the preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that in step d) can, if necessary, be followed by a further stirring step to collect any small agglutinates that may have formed by low intensity reactions and, in doing so, to form larger agglutinates.

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that the said suspension of RBCs to be grouped/phenotyped in step a) is a suspension of between 0.20% and 0.1% (v/v) in an aqueous solution, preferably between 0.3% and 0.75% (v/v), preferably 0.5%.
In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that the said aqueous solution is a Low Ionic Strength Solution buffer.

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that in step b), incubation, is performed for a period of between 10 and 35 minutes, preferably between 15 and 30 minutes, preferably for 20 minutes, preferably for 10 minutes, preferably for 4 minutes to 6 minutes, to the said container, preferably at room temperature.

In the method according to the invention, the application of the magnetic field will preferably be obtained by a permanent magnet located outside and underneath the reaction container(s).

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, in step c), the said magnet is a permanent magnet with magnitude of between 10,000 and 14,000 gauss, preferably 12,000 gauss, and that the magnetic field is applied for between 2.5 minutes and 10 minutes, preferably for 4 minutes to 6 minutes, to the said container, preferably at room temperature.

In the method according to the invention, the application of the magnetic field will preferably be obtained by a permanent magnet located outside and underneath the reaction container(s).

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, in step d), stirring, is performed for a period of between 30 seconds and 2 minutes 30 seconds, preferably for 1 minute to 2 minutes.

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, at step a), the said suspension of RBCs taken from RBCs contained in the sample is taken from packed red blood cells obtained after sedimentation of a sample of total blood from an individual.

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, at step a), the said container is a microplate, preferably with round-bottomed wells.
In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, at step b), stirring is carried out by a microplate stirrer at a speed of between 500 and 1,200 rpm, preferably between 900 and 950 rpm.

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, at step d), stirring is carried out in 2 stages using a microplate stirrer, an initial stirring for 10 to 15 seconds at a speed of between 900 and 1,200 rpm, preferably at a speed of 900 rpm, and a second stirring for a period of between 1 minute 10 seconds and 1 minute 45 seconds, preferably 1 minute 30 seconds at a speed of 700 rpm.

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, at the end of step d), stirring is performed again for a period of between 10 seconds and 1 minute, preferably at a speed of between 300 and 500 rpm, preferably 45 seconds at a speed of 450 rpm.

In an equally preferred embodiment the invention relates to a method for a RBC grouping/phenotyping method according to the present invention characterized in that, at step a), 35 µl to 45 µl RBC suspension at 0.5%, preferably 40 ul, is brought into contact in the said well with 10 µl particle suspension at 0.3% (w/v).

Under another aspect, the present invention comprises a device or apparatus for RBC grouping/phenotyping characterized in that it comprises a suspension of magnetic particles according to the present invention.

Preferably, the said device or kit for the determination, by RBC agglutination, of the presence of a blood group antigen on the surface of an RBC from a sample is characterized in that it comprises:

a) a container containing a suspension of magnetic particles as defined in the present invention;

b) at least one magnet or set of magnets that can be installed outside and underneath the said container(s);

c) a stirring system for the said container(s); and, if need be,

d) a reader capable of assessing the presence of RBC agglutinates in each of the containers.
The invention also relates to a device according to the present invention, characterized in that the said reaction container is a microplate, preferably with round or V-shaped wells.

Preferably again, the method according to the invention is characterized in that the said aqueous solution of magnetic particles is previously diluted in a buffer or saline solution. For the method according to the invention for the determination, by RBC agglutination, of the presence of a blood group antigen or a blood group anti-antigen antibody, the reaction container is preferably a microplate, preferably with round or V-shaped wells.

The following examples and figures are designed to illustrate the invention without limiting its scope in any way whatsoever.

**Figure captions:**

**Figure 1:** Diagrammatic representation of an agglutinating immunomagnetic macromolecular complex.

From centre to exterior: Magnetic particle (or bead), anti-IgG (or anti-IgM) anti-human globulin (AHG) and, on the periphery, RBC group/phenotype anti-antigen antibodies.

**Figures 2A-2B:** Reaction/result obtained for a positive sample (Figure 2A) and negative sample (Figure 2B).

**Figure 3:** Reactions/Results obtained with positive samples (weak D, type 1, 2 and 3).

**Figure 4:** Reactions obtained with positive samples (partial D, type VI and other types of partial Ds).

**Figure 5:** Reactions/Results obtained with negative and positive samples (D positive and partial D type VI).

**Figure 6:** Coupling of magnetic beads to the anti-IgG or anti-IgM AHG.

**Figure 7:** Secondary coupling of anti-globulin with IgG anti-RBC antibodies using non-purified monoclonal antibody concentrates.

**Figure 8:** Principle of RBC phenotyping by agglutination using an immunomagnetic macromolecular complex of the IgG type.
**Figure 9:** Secondary coupling of the anti-globulin with IgM anti-RBC antibodies using non-purified monoclonal antibody concentrates.

**Figure 10:** Principle of RBC phenotyping by agglutination using an immunomagnetic macromolecular complex of the IgM type.

**EXAMPLE 1: Formation of the immunomagnetic macromolecular complex**

The immunomagnetic macromolecular complex (see figure 1) is produced in 2 steps. A) Step 1, or primary coupling:

The first step, primary coupling, consists of fixing anti-IgG or anti-IgM anti-human globulin on magnetic beads containing functional groups. These functional groups may be of different types such as amine function (-NH2), alcohol function (-OH), carboxylic function (-COOH) or Tosyl function. All these functions are well-known to those skilled in the art.

The links established between the antibody and the magnetic bead may be electrostatic, hydrophobic or covalent. The covalent fixation of an antibody on magnetic beads is achieved by using functional groups on the surface of the beads.

The magnetic beads used are superparamagnetic with a size of 1 to 3 µm. They are hydrophobic and functionalized by carboxylic or Tosyl groups depending on the antibodies to be coupled. Among the possible beads are magnetic beads from several different suppliers e.g. ESTAPOR, Dynal or JSR Micro. Preferably the magnetic beads used will come from ESTAPOR, have a size of 1 to 1.2µm and are hydrophobic or will be 1.5µm beads from JSR Micro. The chemical functions capable of fixing the IgG or IgM AHG may be of the Tosyl or carboxylic type, the NH2 type or the alcohol type, with a preference for Tosyl functions for anti-IgG AHG and carboxylic functions for anti-IgM AHG.

The mean iron rate of magnetic beads is approximately 45% for carboxylic beads and approximately 30% for Tosyl beads. The functionalization rate in functional groups is variable. For Tosyl-type beads, the number of functions varies from 40 to 80 µeq/gram beads with a preference for a number of Tosyl functions of 75 µeq/g, while for beads with carboxylic groups, the number of functions can vary from 20 µeq/ gram beads to 350 µeq/g, with a preference for a carboxylic function quantity of 20 µeq/g beads.
The anti-IgG or anti-IgM anti-human globulins coupled to the functionalized magnetic beads are both of isotype IgG.

For the coupling of anti-IgG antiglobulins, it is preferable to use Tosyl beads of 1 to 1.2 μη from ESTAPOR, obtained under reference R01-24 and including a quantity of Tosyl functions of 75μeq/grams beads, or Tosyl beads of 1.5 μη from JSR obtained under the reference MS150/Tosyl.

For the coupling of anti-IgM antiglobulins, it is preferable to use 1 μη carboxylic beads from ESTAPOR obtained under the reference: EMI-100/40. They include a quantity of carboxylic functions of 20 μeq per gram of beads.

The concentration of AHGs coupled to magnetic beads can vary from 20 μg/mg beads to 50 μg/mg beads, depending on the antiglobulins. Anti-IgG anti-human globulin is used at a concentration of 50 μg/mg beads, while anti-IgM anti-human globulin is coupled at a concentration of 20 μg per mg of beads.

B) Step 2, or secondary coupling:

The second stage in the formation of the immunomagnetic complex, or secondary coupling, consists of coupling the antibodies specific to the RBC antigens onto AHG using a supernatant concentrate of a culture of hybridomas that secrete monoclonal antibodies. This coupling is achieved by incubating the beads coupled to the AHG with a concentrate of monoclonal antibodies. The process leads to immunopurification of the concentrate containing various proteins present during the culture of the hybridome that secretes the specific antibody.

After this secondary coupling, AHG is totally saturated by the specific IgG or IgM antibody (depending on the AHG coupled). It then constitutes a ready-to-use, macromolecular, immunomagnetic reagent that can be used for the grouping and phenotyping of RBC antigens, depending on the specific antibodies used (anti-A, anti-B, anti-D, anti-Fya, anti-Fyb, anti-Jka, anti-Jkb, etc.).

Various hybridomas culture concentrates containing monoclonal antibodies specific to the red blood cells can be coupled with AHGs. It is also possible to produce immunomagnetic complexes of other types by coupling an anti-species anti-globulin capable of recognizing the second antibody from another species specific of a RBC antigen (e.g. produced in mouse). Thus, for example, the anti-globulin will be mouse anti-IgG or anti-IgM antiglobulin and the blood group/phenotype anti-antigen
antibody will be an IgG or IgM produced by the mouse. Secondary coupling concentration depends on the quantity of antibodies present in the culture concentrate and on the type of antibody.

This concentration can vary from 120 μg total protein per mg beads to 3,000 μg total protein per mg beads.

The final solution, or storage buffer containing the beads coupled with AHG and the specific antibody of interest, consists of a PBS containing the BSA at 0.1% (m/v) and a non-ionic detergent of the Synperonic PE/F68 type. Synperonic PE/F68 is a hydrophilic non-ionic surfactant also known as poloxamer Synperonic F68 obtained from Sigma Aldrich under reference: 81 112. This three-block polymer consists of a central hydrophobic part comprising blocks of polyoxypropylene surrounded by 2 hydrophilic polyoxyethylene blocks.

The use of detergent is necessary when using nanoparticles, to avoid their spontaneous aggregation in particular physical and chemical conditions of pH, ionic strength and temperature. It has notably been demonstrated that changes to the surface of the particles by the adsorption of amphiphilic non-ionic macromolecules such as poloxamers help the non-aggregation of particles in solution.

When particles are placed in solution in a buffer with a high ionic strength, such as PBS, the nanoparticles tend to lose their colloidal stability and to aggregate. The addition of a surfactant retains the colloidal stability and avoids the aggregation of particles in a buffer with high ionic strength.

There are several types of non-ionic surfactants such as Tween 20, 60 and 80 or Synperonic PE/F68 or F127. In this invention we prefer to use Synperonic PE/F68. To have an effect on the colloidal stability of the beads coupled with the various antibodies, the Synperonic concentration must be greater than 0.1% and less than 2.5%, cell viability threshold in the presence of this concentration of surfactant, with a preference for a concentration of 1% (m/v).

The final concentration of beads coupled with the antibodies of interest depends on the antibody used and can vary from 0.1% (m/v) to 0.5% in the storage buffer described above, i.e. PBS 0.1% BSA +1% Synperonic PE/68.
EXAMPLE II: Determination of blood grouping or phenotyping

The presence of an antigen of interest on an individual's red blood cells is determined from a blood sample collected on tube with anticoagulant of EDTA or citrate type. A globular suspension of between 0.3% and 1%, preferably 0.5%, is carried out in a tube or in the round-bottomed well of a microplate by diluting 10 µl packed red blood cells in 2 ml low ionic strength buffer, in particular a LISS buffer. A total of 10 µl of the solution of beads coupled with antibodies (AHG + antibody specific to the antigen to be detected) are deposited in the round-bottomed well, then 40 µl of the 0.5% RBC suspension is added. The compounds are homogenize by placing the microplate on an automatic microplate stirrer. The stirring speed can vary from 900 rpm to 1,200 rpm for 10 seconds, preferably 1,000 rpm.

The microplate is then incubated for between 15 and 30 minutes, preferably 20 minutes at 37°C, to enable the antibodies to bind to the antigens present on the red blood cells. At the end of incubation, the microplate is placed on a plate containing 96 battery magnets that adjust perfectly beneath each well of the microplate. Under the effect of the magnetic field, the beads draw the RBCs down to the bottom of the well and enhance their agglutination if the antibodies have bound to the red blood cells.

Magnetization can last from between 2 and 10 minutes, preferably 4 minutes at room temperature.

The microplate is then placed on a stirrer to suspend non-agglutinated red blood cells i.e. RBCs that are negative for the screened antigen. The sequence of agitations allows both the resuspension of non-agglutinated red blood cells and the visualization of RBC agglutinates as follows: the first, fairly strong stirring unsticks the packed red blood cells off the bottom of the wells after magnetization. This stirring can vary from 900 to 1,200 rpm for 10 seconds, preferably 900 rpm. The second stirring resuspends the RBCs that are negative for the screened antigen, and therefore non-agglutinated, to a suspension. The speed of this stirring can vary from 650 rpm to 900 rpm, preferably 700 rpm for 1 minute 30 seconds. At the end of stirring, the non-agglutinated blood cells are dispersed, forming a homogeneous suspension, while the RBCs that strongly express the screened antigen form a compact agglutinate. For less strongly expressed antigens, the agglutinates formed are smaller and more dispersed at the end of stirring, requiring a "re-collection" stirring
that will assemble all the small, dispersed agglutinates to form a complete, clearly-defined agglutinate at the bottom of the well. The re-collection stirring is carried out at low speed i.e. at between 300 and 500 rpm, preferably 450 rpm for at least 10 seconds but less than 1 minute, preferably 45 seconds. The re-collection stirring has no effect on the negative RBCs or the strong agglutinates. The microplate can then be read off by the naked eye or by an automatic reader equipped with a camera.

**EXAMPLE III: Producing an immunomagnetic macromolecular complex of the IgG anti-D type**

To produce this complex, various anti-D specifics of certain variants of the D antigen can be used.

Anti-Ds that recognize particular variants of D antigens used in this test:

- Anti-D of the IgG type, clone HM16 (Diagast)
- Anti-D of the IgG type, clone P3X35 (Diagast)
- Anti-D of the IgG type, clone P3X290 (Diagast)
- Anti-D of the IgG type, clone ESDI (Alba Biosciences)
- Anti-D of the IgM type, clone P3X61 (Diagast)
- Anti-D of the IgM type, clone P3X2123B10 (Diagast)

Each of the anti-Ds used is known to recognize certain types of D antigen variants. Table 1 below describes the D antigen variants recognized by each of the anti-Ds used.

**Table 1**

| ANTI-D Clone | D-fab+ Type 1, 2 a/b | DOL | DHL | DHLa | DIVa | DIVb | DIVc | DVI | DVB | DBF | DCS | DRF | DBE | DAR | DHK | DAH | RAH | HMi |
|--------------|---------------------|-----|-----|------|------|------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| HM16 (IgG)  | + +               |     |     |      |      |      |      |     |     |     |     |     |     |     |     |     |     |
| P3X35 (IgG) | + +               |     |     |      |      |      |      |     |     |     |     |     |     |     |     |     |     |
| P3X290 (IgG)| i i i i i i a     | i   |     |      |      |      |      |     |     |     |     |     |     |     |     |     |     |
| ESDI (IgG)  | + +               |     |     |      |      |      |      |     |     |     |     |     |     |     |     |     |     |
| P3X61 (IgM) | + + + + + i i     |     | i i |      |      |      |      |     |     |     |     |     |     |     |     |     |     |
| P3X2123B10 (IgM)| + + + + + i i a |     |     |      |      |      |      |     |     |     |     |     |     |     |     |     |     |
A) Primary coupling of anti-IgG anti-human globulin onto the Tosyl beads

a. Preparation of buffers

Sodium Phosphate buffer 0.1 M, pH 7.4

- Weigh 2.62 g NaH2P04 x H20
- Weigh 14.42 g Na2H P04 x 2H20
- Dissolve in distilled water then adjust the volume to 1 liter

Ammonium Sulfate Buffer (NH4)2S04, 3M

- Weigh 39.64 g of (NH4)2S04
- Dissolve in 100 ml Sodium phosphate buffer
- Adjust the pH with NaOH or HCl

PBS pH 7.4 + 0.5% BSA (w/v) (Saturation buffer) ("PBS" for phosphate buffered saline)
- Add 1.53 g NaCl to 80 ml phosphate buffer saline 0.02M, pH 7.4

PBS pH 7.4 + 0.1% BSA (w/v)
- Add 1.53 g NaCl to 80 ml phosphate buffer saline 0.02M, pH 7.4

b. Reagents and material

- Beads: Tosyl magnetic beads (reference: R01-24), 10% (1mg beads = 10µl)

AHG antibody: purified anti IgG AHG; initial concentration: 1.8 mg/ml

Buffers: PBS pH 7.4; PBS 0.3 M, pH 7.4; Ammonium Sulfate Buffer (NH4)2S04, 3M; PBS pH 7.4 + 0.5% BSA; PBS pH 7.4 + 0.1% BSA

- Material: Eppendorf tubes, adjustable pipettes and corresponding tips, agitation wheel, magnet and glass test tubes

c. Primary coupling protocol
The following protocol is carried out in quadruplicate to conduct the secondary coupling with the four IgG anti-D: HM16, P3x35, P3x290 and ESDI. To do this, 4x15 mg beads (i.e. 4 x 150µl) are coupled with AHG in the following ratio: 50 µg AHG per 1 mg beads.

5 Coupling:
- Put 150 µl Tosyl beads at 10%, i.e. 15 mg beads in an Eppendorf® tube
- Wash the beads over a magnet:
- Place Eppendorf® tube containing 150 µl beads on a magnet
- Aspirate the supernatant when the beads are decanted
- Add 1000 µl Sodium phosphate buffer
- Resuspend the beads into suspension in the Sodium phosphate buffer
- Place the Eppendorf® tube over the magnet
- Aspirate the supernatant
- Resuspend the beads with 416 µl antibodies (i.e. 750µg)
- Mix thoroughly by vortexing
- Add 200µl buffer (NH4)2SO4, 3M
- Mix thoroughly by vortexing
- Place the Eppendorf® tube on the stirring wheel for 24 hours at room temperature.

20 Washing and saturation of coupled beads:
- Place the tubes over the magnet and decant the beads
- Aspirate the supernatant
- Resuspend the beads in 1 ml saturation buffer (PBS + 0.5% BSA pH 7.4)
- Place the tubes over the magnet and decant the beads

25 - Aspirate the supernatant
- Resuspend the beads in 1 ml saturation buffer (PBS + 0.5% BSA pH 7.4)
- Incubation of beads on the stirring wheel for 2 hours at room temperature
- Place the tubes over the magnet and decant the beads
- Aspirate the supernatant and replace it with 1 ml PBS + 0.1% BSA

30 - Resuspend the beads completely by vortexing for 5 to 10 seconds
- Place the tube over the magnet and decant the beads
- Aspirate the supernatant
- Resuspend in 1.5 ml PBS + 0.1% BSA final concentration of beads 1%.

B) Secondary coupling of the specific antibody (anti-D) and the anti-IgG anti-human globulin

a. Preparation of buffers

PBS pH 7.4 + 0.1% BSA (m/v) + 0.5% Synperonic PE/F68®. (m/v) (Synperonic PE/F68®. is the brand name of an ethylene oxide and propylene oxide copolymer belonging to the ICI Group)
- Add 1.53g NaCl to 80 ml Sodium phosphate buffer 0.02M, pH 7.4
- Weigh 0.1 g BSA and 0.5 g Synperonic PE/F68
- Add to the phosphate buffer prepared above
- Mix
- Adjust the volume to 100 ml Sodium phosphate buffer 0.02M pH 7.4

PBS pH 7.4 + 0.1% BSA (m/v) + 1% Synperonic PE/F68® (m/v)
- Add 1.53g NaCl to 80 ml Sodium phosphate buffer 0.02M, pH 7.4
- Weigh 0.1 g BSA and 1 g Synperonic PE/F68
- Add them to the phosphate buffer prepared above
- Mix
- Adjust the volume to 100 ml Sodium Phosphate buffer 0.02M pH 7.4.

b. Reagents and material

- Beads: Magnetic beads coupled to anti-IgG AHG at 1% (1 mg beads per 100 µl)
- Antibodies:
  HM16 Anti-D monoclonal, IgG type, culture concentrate;
  P3x35 Anti-D monoclonal, IgG type, culture concentrate;
  P3x290 Anti-D monoclonal, IgG type, culture concentrate.
- ESDI Anti-D monoclonal, IgG type, culture concentrate
- Buffers: PBS + BSA 0.1%; PBS + 0.1% BSA + 0.5% Synperonic® F68
- Material: Adjustable pipettes and corresponding tips, glass test tubes, Eppendorf® tubes and magnet.

c. Preparation of IgG anti-D antibodies for coupling

The non-purified anti-D antibody concentrates are each coupled to 15 mg Tosyl beads coupled to AHG in a ratio of 120 µg antibodies per mg beads.
  Initial concentration: 11.5 g/L total protein
  Quantity to be added: 1800 µg i.e. a volume of 155 µl

- P3X35: anti-D monoclonal, IgG type, from culture concentrate. Non-purified antibody
  Initial concentration: 10.5 g/L total protein
  Quantity to be added: 1800 µg i.e. a volume of 170 µl

- P3X290: anti-D monoclonal, IgG type, from culture concentrate. Non-purified antibody
  Initial concentration: 14 g/L total protein
  Quantity to be added: 1800 µg i.e. a volume of 130 µl

- ESDI: anti-D monoclonal, IgG type, from culture concentrate.
  Initial concentration: 16.7 g/L total protein
  Quantity to be added: 1800 µg i.e. a volume of 108 µl

d. Protocol
   - 1.5 ml beads coupled with AHG (15 mg beads per secondary coupling) are incubated directly with the anti-D antibody concentrates, in the volumes described above.

- Place the Eppendorf® tubes on the stirring wheel for 2 hours at room temperature.
  - At the end of secondary coupling, the Eppendorf® tubes are placed over a magnet, the beads are decanted and the supernate is aspirated.
  - The beads are then washed once with 1 ml PBS 0.1% BSA then twice with PBS 0.1% BSA + 0.5% Synperonic F68.
  - The beads are resuspended with 1.5 ml PBS, 0.1% BSA + 1% Synperonic PE/F68, and stored at 4°C.

C) Dilution of the immuno-magnetic reagent in its final buffer.

After coupling, each immuno-magnetic complex is diluted at 0.3% in PBS 0.1% BSA + 1% Synperonic PE/F68.
EXAMPLE IV: Producing an IgM-type immunomagnetic macromolecular complex

A) Primary coupling of anti-IgM anti-human globulin with carboxylic beads (COOH)

a. Reagents and material

Table 2

<table>
<thead>
<tr>
<th>BEADS</th>
<th>Carboxylic magnetic beads at 10% (1mg beads = 10µl) reference: EM1-100/40</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANTIBODIES</td>
<td>anti IgM AHG anti-IgM AHG (purified) Initial concentration=1.1 mg/ml</td>
</tr>
<tr>
<td>BUFFERS</td>
<td>( \text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4 ) 10 mM pH=6 ( \text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4 ) 20 mM pH=7.5</td>
</tr>
<tr>
<td>COUPLING AGENT</td>
<td>Solution of EDC (18 mg/ml)+NHS (6 mg/ml) in ( \text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4 ) 10 mM pH=6</td>
</tr>
<tr>
<td>MATERIAL</td>
<td>Eppendorf tubes Adjustable pipettes and corresponding tips Agitation wheel Magnet Glass test tubes</td>
</tr>
</tbody>
</table>

b. Protocol

The following protocol is conducted in duplicate to carry out secondary coupling with both IgM: P3x61 and P3x21223B10. To do this, 2 x 15 mg beads (i.e. 2 x 150 µl) are coupled with anti-IgM AHG in a ratio of 20 µg AHG to 1 mg beads.

Coupling:
- Put 150 µl carboxylic magnetic beads at 10%, i.e. 15 mg beads in an Eppendorf® tube

Wash the beads over a magnet:
- Place the Eppendorf® tube containing 150µl beads over the magnet
- Aspirate the supernatant when the beads are decanted
- Add 1000 µl buffer: Na2HP04/ NaH2P04 10 mM pH 6
- Resuspend the beads by vortexing

Repeat the washing step with Na2HP04/ NaH2P04 10 mM pH 6
- Transfer the beads into a 5 ml glass tube
- Aspirate all the supernatant
- Add 2.5 ml coupling solution (EDC+NHS in Na2HP04/NaH2P04 10 mM pH 6)
- Resuspend the beads by vortexing
- Place the tube on a stirring wheel for 15 minutes at room temperature
- At the end of incubation, place the tube over a magnet and decant the beads
- Aspirate the supernatant
- Resuspend the beads with 1 ml HCl 2 mM buffer
- Place the tube over a magnet and decant the beads
- Aspirate the supernatant
- Resuspend the beads in 890 µl Na2HP04/NaH2P04 20 mM pH 7.5
- Immediately add the anti-IgM AHG to be coupled: i.e. 300 µg AHG per 15 mg beads, i.e. 272 µg AHG
- Vortexing
- Place the tube on a stirring for 2 hours at room temperature
- Wash the beads twice with the NaHP04/NaH2P04 20 mM pH 7.5
- Resuspend the beads in 1.5 ml Na2HP04/NaH2P04 20 mM pH 7.5

Washing and saturation of the coupled beads:
- Place the tubes over the magnet and decant the beads
- Aspirate the supernatant
- Resuspend the beads in 1 ml saturation buffer PBS + 0.5% BSA pH 7.4
- Place the tubes over the magnet and decant the beads
- Aspirate the supernatant
- Resuspend the beads in 1 ml saturation buffer PBS + 0.5% BSA pH 7.4
- Incubate the beads over the stirring wheel for 2 hours at room temperature
- Place the tubes over the magnet and decant the beads
- Aspirate the supernatant and replace it with 1 ml PBS + 0.1% BSA
- Resuspend the beads completely by vortexing for 10 seconds
- Replace the tube over a magnet and decant the beads
- Aspirate the supernatant
- Resuspend in 1.5 ml PBS + 0.1% BSA, the final concentration of beads is 1%
B/ Secondary coupling of specific antibody (anti-D) and anti-IgM anti-human globulin

a. Reagents and material

Table 3

<table>
<thead>
<tr>
<th>BEADS</th>
<th>Magnetic beads coupled with anti-IgM AHG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td>P3x61 Anti-D monoclonal, IgM type, culture concentrate.</td>
</tr>
<tr>
<td>Buffers</td>
<td>PBS + 0.1% BSA</td>
</tr>
<tr>
<td>Material</td>
<td>Adjustable pipettes and corresponding tips</td>
</tr>
</tbody>
</table>

b. Preparation of antibodies to be coupled

The non-purified anti-D antibody concentrates are each coupled to 15 mg carboxylic magnetic beads coupled to anti-IgM AHG in the following ratio:

- 320 µg antibody/mg beads for antibody P3x61
  Quantity to be added: 4800 µg i.e. a volume of 457 µl

- 2000 µg antibody /mg beads for antibody P3x21223B10
  Quantity to be added: 30 000 µg i.e. a volume of 1.2 ml

c. Protocol

- 1.5 ml magnetic beads coupled to anti-IgM AHG (15 mg beads per secondary coupling) are incubated directly with the anti-D antibody concentrates at the volumes described above.

- The Eppendorf® tubes are placed on the stirring wheel for 2 hours at room temperature.

- At the end of secondary coupling, the Eppendorf tubes are placed over a magnet, the beads are decanted and the supernate is aspirate.

- The beads are washed once with 1 ml PBS 0.1% BSA then twice with PBS 0.1% BSA + 1% Synperonic®PE/F68.
The beads are resuspended with 1.5 ml PBS 0.1% BSA + 1% Synperonic PE/F68, for storage at 4°C.

C) Dilution of the immunomagnetic reagent in its final buffer:

After coupling, each immunomagnetic complex is diluted to the precise concentration i.e. 0.3% for anti-D P3X61 and 0.1% for anti-D P3X2123B10 in a PBS 0.1% BSA + 1% Synperonic PE/F68 buffer.

EXAMPLE V: Use of the various immunomagnetic complexes in a blood phenotyping test

Performing a test to detect weak and/or truncated isoforms of the RhD antigen.

a. Reagents and material

<table>
<thead>
<tr>
<th>BEADS at 0.3% in PBS + 0.1% BSA + 0.5% Synperonic PE/F68</th>
<th>magnetic beads + AHG+ HM16</th>
<th>magnetic beads + AHG + P3x35</th>
<th>magnetic beads + AHG+ P3x290</th>
<th>magnetic beads + AHG + ESD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUFFERS</td>
<td>PBS + 0.1% BSA + 0.5% Synperonic F68</td>
<td>LISS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MATERIAL</td>
<td>Adjustable pipettes and corresponding tips</td>
<td>Glass test tubes</td>
<td>Eppendorf tubes</td>
<td>Magnet</td>
</tr>
<tr>
<td>MATERIAL</td>
<td>Greiner microplate with 96 round-bottomed wells, irradiated</td>
<td>Freelys Nano</td>
<td>Reader</td>
<td></td>
</tr>
<tr>
<td>SAMPLES</td>
<td>Whole blood collected on anticoagulant (EDTA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>Hema (IQC) Tube A⁺ (positive control) Tube AB⁻ (negative control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

b. Preparation of 0.5% RBC suspensions

- Take 5μl packed red blood cells from a tube of whole blood collected over an anticoagulant
Dilute the packed red blood cells in a tube containing 1 ml Liss
Homogenize the suspension by stirring
c.
Test
For each sample and each type of bead

Preparation
- Shake the containers of coupled beads before using them
- In a round-bottomed well of a 96-well microplate, deposit 10 µl beads coupled with an anti-D
- Add 40µl 10.5% RBC suspension
- Stir the plate for 10 seconds at 900 rpm to homogenize the beads and the RBC suspension
- Incubate the plate for 20 minutes at 37°C
- Place the microplate on a plate containing 96 battery magnets in direct contact with each well of the microplate, for 4 minutes at room temperature
- Place the microplate on an automatic stirrer and stir for 10 seconds at 900 rpm Then 1 minute 30 seconds at 700 rpm and, finally, 45 seconds at 450 rpm
- Visualize the microplate by naked eyes to determine the positive and negative reactions or place the microplate on an automatic reader.

The positive reactions are characterized by the presence of an agglutinate at the base of the well; negative reactions produce a homogeneous RBC suspension.

EXAMPLE VI: Phenotyping of weakened or variant Rhesus D

EFS donors (Etablissements Francais du Sang, French blood transfusion service) with a weak or variant Rhesus D phenotype confirmed by genotyping have been tested with various immunomagnetic complexes that are specific for the D antigen.

Several red blood cells from donors known to have variants of the D antigen or a weak D antigen have been phenotyped with the various anti-D immunomagnetic complexes.

Likewise, patients with no D antigen (RhD negative) have been tested with the same immunomagnetic complexes to verify the specificity of the test.

a. Red blood cells with a weak D antigen (weak D)
3 donors expressing weak D Type 1 (Dw1)
7 donors expressing weak D Type 2 (Dw2)
2 donors expressing weak D Type 3 (Dw3)

(see Figure 3)

Each anti-D complex recognizes the various weak D antigens but the intensity of the reaction depends on each clone. For example, the complex coupled with the anti-D P3X290 produces slightly weaker reactions for weak D Type 2 antigens than for weak D Type 1 and Type 3 antigens.

However, all the IgG-type anti-D antigens coupled in the immunomagnetic complex are capable of agglutinating the red blood cells that express the weak D antigen. The reactions obtained are strong, even with antigens known to be very weak such as weak D Type 2 antigens (Dw2).

b. Red blood cells expressing a partial D antigen

4 donors expressing D VI antigen (DVI)
1 donor expressing D HMI antigen (DHMI)
1 donor expressing DFR antigen (DFR)
1 donor expressing DOL antigen (DOL)
1 donor expressing DNB antigen (DNB)

(see figure 4)

Each anti-D complex recognizes the variants of the D antigen differently. Recognition depends on the epitopic specificity of each clone (see epitopic chart of the various clones used).

Thus the immunomagnetic complexes coupled with anti-D HMI 6 and anti-D P3X61 do not recognize the DVI type partial D antigen.

However, it is observed that all the IgG anti-Ds coupled in the immunomagnetic complex are capable of agglutinating the red blood cells expressing variants of the D antigen. The intensity of the reactions depends on the clone and its ability to recognize the variant. The reactions are as expected.

Epitopic chart of the anti-D clones used (see Table 5)
Table 5

<table>
<thead>
<tr>
<th>ANTI-D</th>
<th>Donor</th>
<th>Dkb</th>
<th>Dkb</th>
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<th>Dkb</th>
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<tbody>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>FOSD (f)</td>
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<td>+</td>
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<tr>
<td>POS (f)</td>
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</table>

Specificity of the test

Negative donors sampled on EDTA anticoagulant were tested with the same immunomagnetic complexes to check the specificity of the test.

The expected negative reactions were negative while the positive controls (samples D positive and DVI partial positive) showed strong positive reactions (see Figure 5).
CLAIMS

1. A suspension of magnetic particles, characterized in that the said magnetic particles are coated on the surface with an anti-globulin (AG)/antibody complex wherein the said antibody is directed specifically against an antigen of the RBC group/phenotype (referred to as "antibody with an anti-antigen of the RBC group/phenotype")

2. A suspension of magnetic particles according to claim 1, characterized in that the said AG is anti-human globulin (AHG) and the said antibody anti-antigen of the RBC group/phenotype is an antibody of human origin.

3. A suspension of magnetic particles according to claim 1 or 2, characterized in that the said AG is an anti-IgG or anti-IgM anti-globulin.

4. A suspension of magnetic particles according to claim 3, characterized in that the said antibody anti-antigen of the RBC group/phenotype is of the IgG type when the AG is an anti-IgG and the antibody anti-antigen of the RBC group/phenotype is of the IgM type when the AG is anti-IgM.

5. A suspension of magnetic particles according to one of the claims 1 to 4, characterized in that the said AG is saturated with anti-antigen antibodies of the RBC group/phenotype.

6. A suspension of magnetic particles according to one of the claims 1 to 5, characterized in that the said antibody anti-antigen of the RBC group/phenotype is selected from among the polyclonal or monoclonal antibodies, preferably monoclonal, anti-A, anti-B, anti-D, anti-C, anti-E, anti-c, anti-e, anti-K, anti-Fya, anti-Fyb, anti-Jka, anti-Jkb anti-S and anti-s or antibodies specifically directed against any other RBC group/phenotype antigen whose presence on the surface of a red blood cell is being determined.

7. A suspension of magnetic particles according to one of the claims 1 to 6, characterized in that the said magnetic particles are magnetic particles selected from among superparamagnetic particles.

8. A suspension of magnetic particles according to one of the claims 1 to 7, characterized in that the said magnetic particles are selected from among the magnetic particles.
particles with a diameter of between 0.75 µm and 5 µm, preferably between 1 µm and 3 µm and between 1 µm and 2 µm.

9. A suspension of magnetic particles according to one of the claims 1 to 8, characterized in that the said magnetic particles are magnetic particles that have been previously functionalized with a group selected from among the carboxylic, amine, alcohol or tosyl groups.

10. A suspension of magnetic particles according to claim 9, characterized in that the said magnetic particles are magnetic particles that have been previously functionalized with a group selected from among the carboxylic, amine, alcohol or tosyl groups with a functionalization level of between 20 µeq/gram beads and 350 µeq/gram beads, preferably between 20 µeq/gram beads and 80 µeq/gram beads.

11. A suspension of magnetic particles according to one of the claims 1 to 10, characterized in that the said magnetic particles are magnetic particles with a mean iron content of between 30% and 40%.

12. A suspension of magnetic particles according to one of the claims 1 to 11, characterized in that the concentration of AHG coating the said magnetic particles lies between 10 µg/mg particles and 70 µg/mg particles, preferably between 20 µg/mg ± 5 µg/mg particles for anti-IgM AHG and 50 µg/mg ± 10 µg/mg particles for anti-IgG AHG.

13. A suspension of magnetic particles according to one of the claims 1 to 12, characterized in that the said magnetic particles are suspended in an aqueous solution comprising a surfactant, preferably at a particle concentration of between 0.1% and 2.5% (w/v), preferably between 0.25% and 1.5% (w/v), preferably again between 0.5% ± 0.25% (w/v), 0.3% also being preferred.

14. A suspension of magnetic particles according to claim 13, characterized in that the said surfactant is selected from non-ionic surfactants or detergents, preferably from among non-ionic, hydrophilic detergents such as Tween®, 20, 40 or 80, or poloxamers such as Synperonic®. PE/F68 or F127, preferably at a concentration of between 0.1% and 2.5% (w/v), preferably at 0.75% ± 0.25% (w/v).

15. A suspension of magnetic particles according to one of the claims 1 to 14, characterized in that the said magnetic particles are suspended in an aqueous buffer
solution comprising bovine serum albumin (BSA) at a concentration of between 0.05% and 0.75%, preferably between 0.1% and 0.5% (w/v), preferably 0.5% (w/v).

16. A suspension of magnetic particles according to one of the claims 1 to 15, characterized in that the said magnetic particles are suspended in a saline buffer, preferably a phosphate buffered saline at 0.3 M, pH 7.4.

17. A suspension of magnetic particles according to one of the claims 1 to 16, characterized in that the said magnetic particles are suspended at 0.1% ± 0.25% (w/v) in a phosphate buffered saline at 0.3 M, pH 7.4 (PBS) containing bovine albumin serum (BSA) at a concentration of 0.1% (w/v) and Synperonic PE/F68 at a concentration of 1%.

18. An RBC grouping/phenotyping kit comprising a suspension of magnetic particles according to one of the claims 1 to 17 in which the said RBC group/phenotype anti-antigen antibody is directed specifically against a given antigen.

19. An RBC grouping/phenotyping kit comprising at least two separate suspensions of magnetic particles according to one of the claims 1 to 17 in which each of the said RBC group/phenotype anti-antigen antibodies is directed specifically against a different given antigen.

20. An RBC grouping/phenotyping kit comprising a mixture of at least two suspensions of magnetic particles according to one of the claims 1 to 17 in which each of the said RBC group/phenotype anti-antigen antibodies is directed specifically against a different given antigen, preferably each of them targeting an antigen selected from among weak D antigens, partial D antigens and/or D antigen variants.

21. A device or equipment for RBC grouping/phenotyping characterized in that it includes a suspension of magnetic particles according to one of the claims 1 to 17.

22. An RBC grouping/phenotyping process using a biological sample containing RBCs to be grouped or phenotyped, characterized in that the method includes the following steps:

   a) one step in which the suspension of RBCs to be grouped/phenotyped is put in contact, in a container, with a suspension of magnetic particles according to one of
the claims 1 to 17, or with a mixture of at least two of the said suspensions of magnetic particles;

b) an incubation step, if necessary, with stirring;

c) the application of a magnetic field to the container by means of a magnet placed outside and underneath the said container to attract the said magnetic particles with, if need be, the red blood cells fixed on the said magnetic particles;

d) the resuspension of the packed red blood cells obtained in Step c) by stirring;

e) reading with the naked eye and/or by any other appropriate reading system of the presence or absence of agglutinates in the container, the presence of agglutinates being indicative of the presence of the antigen against which is specifically directed the RBC group/phenotype anti-antigen antibody present on the magnetic particle.

23. An RBC grouping/phenotyping method according to claim 22, characterized in that step d) may be followed, if necessary, with further stirring to re-collect the small agglutinates that may form with low intensity reactions and, in doing so, form larger agglutinates.

24. A method according to one of the claims 22 or 23, characterized in that the said suspension of RBCs being grouped/phenotyped in step a) is a suspension of between 0.20% and 0.75% (v/v) in an aqueous solution, preferably between 0.3% and 0.5 (v/v).

25. A method according to one of the claims 22 to 24, characterized in that the said aqueous solution is a low-strength ionic buffer.

26. A method according to one of the claims 22 to 25, characterized in that step b) (incubation) is carried out for a period of between 10 and 35 minutes, preferably between 15 and 30 minutes, preferably for 20 minutes, preferably at a temperature of between 20°C and 40°C, preferably between 30°C and 37°C, preferably at 37°C.

27. A method according to one of the claims 22 to 26, characterized in that in step c), the said magnet is a permanent magnet with a magnitude of between 10,000 and 14,000 gauss, preferably 12,000 gauss. The magnetic field is applied for 2.5 to 10
minutes, preferably 4 to 6 minutes, to the said container, preferably at room temperature.

28. A method according to one of the claims 22 to 26, characterized in that step d) (stirring) is carried out for a period of between 30 seconds and 2 minutes 30 seconds, preferably for 1 to 2 minutes.

29. A method according to one of the claims 22 to 27, characterized in that in step a), the said suspension of RBCs obtained from RBCs contained in the sample is performed from packed red blood cells obtained after sedimentation of a sample of whole blood from one individual.

30. A method according to one of the claims 22 to 28, characterized in that in step a), the said container is a microplate, preferably with round-bottomed wells.

31. A method according to claim 30, characterized in that in step b), stirring is carried out using a microplate stirrer at a speed of between 500 and 900 rpm, preferably between 700 and 750 rpm.

32. A method according to claim 30 or 31, characterized in that in step d), stirring is carried out in 2 steps using a microplate stirrer, the first being stirring for 10 to 15 seconds at a speed of between 900 and 1,200 rpm, preferably a speed of 900 rpm, and the second being stirring for a period of between 1 minute 10 seconds and 1 minute 45 seconds, preferably 1 minute 30 seconds at a speed of 700 rpm.

33. A method according to one of the claims 22 to 28, characterized in that at the end of step d), further stirring is performed for a period of between 10 and 45 seconds, preferably at a speed of between 300 and 500 rpm, preferably 15 seconds at a speed of 450 rpm.

34. A method according to one of the claims 30 to 33, characterized in that in step a) the following are brought into contact in the said well: 35 µl to 45 µl RBC suspension at 0.5%, preferably 40 µl with 10 µl of the said suspension of particles at 0.3% (w/v).

35. A device or kit for the determination, by RBC agglutination, of the presence of an antigen from the blood group on the surface of an RBC from a sample, characterized in that it includes:

a) a container containing a suspension of magnetic particles as defined in Claims 1 to 17;
b) at least one magnet or set of magnets that can be positioned outside and underneath the said container(s);

c) a stirring system for the said container(s); and where appropriate,

d) a reader capable of assessing the presence of RBC agglutinates in each of the containers.

36. A device according to claim 35, characterized in that the said reaction container is a microplate, preferably with rounded or V-shaped bottom wells.
FIGURE 3

FIGURE 4
Anti-D HM16
Anti-D P3x35
Anti-D P3X290
Anti-D ESD1
Anti-D P3x61
Anti-D
P3X21223B10

FIGURE 5
Magnetic beads coupled to anti-IgG AHG

Contaminants
Ex: BSA

Non-purified monoclonal antibody concentrates (IgG)

Immuno-magnetic macromolecular complex of IgG type

FIGURE 7
Reagent containing the immuno-magnetic complex anti-antigen red blood cell antibody

Red blood cells bearing antigen recognized by the immuno-magnetic complex

Specific agglutination of red blood cells

FIGURE 8
Magnetic beads coupled to anti-IgM AHG → Non-purified monoclonal antibody concentrates (IgM) → Immuno-magnetic macromolecular complex of IgM type
Reagent containing the immuno-magnetic complex anti-antigen red blood cell antibody

Red blood cells bearing antigen recognized by the immuno-magnetic complex

Specific agglutination of red blood cells

FIGURE 10