

The present invention concerns a method of detecting antigens or antibodies using carrier-bound antibodies as well as inert particles, such as polyacrylamide gel particles.

5 Carrier-bound antigens and antibodies are routinely utilized for a multitude of analytical determinations. In this connection, a distinction is generally made between two principles:

a) The antigen or antibody is bound to a solid
10 carrier, such as small glass or plastic test tubes, glass or plastic beads, glass or plastic plates, paper, etc., and the liquid containing the antibody or antigen to be identified is added. If, after a certain reaction time, the latter react with the bound antigen or antibody, they
15 are bound to the carrier and can, at least by means of a further reaction, be made measurable. As a rule, this takes place by labelling the antibody or antigen with radioactive, fluorescent, or enzymatically active tagging substances. A drawback of this method is that it is
20 trouble-prone. Since washing is necessary after each reaction, insufficient washing or insufficient removal of the washing solution can falsify the results. Moreover, this method is hardly applicable to natural, carrier-bound antigens or erythrocytes, leukocytes, platelets, and other
25 natural cells because of their size.

b) Another principal method consists in binding the antigen or antibody to small particles such as latex or erythrocytes. After a certain reaction time with the
antibody or antigen to be identified, the evaluation takes
30 place on the basis of the agglutination pattern. Unlike the method described under a) above, antigens and antibodies on erythrocytes, leukocytes, or platelets can be identified by this method. Drawbacks of this method are that, especially in a weak concentration, it is hard
35 to differentiate between agglutinated and non-agglutinated

(free) particles. Moreover, free particles can easily be adsorbed on the reaction vessel or the agglutination pattern mechanically destroyed. A further source of error resides in the possible adhesion of free particles to one another, thus simulating an agglutination. All these eventualities can lead to a false evaluation.

European Published Application No. 0 039 195 describes an antibody detection method wherein erythrocytes in negatively charged form are used in an isotonic solution of low ionic strength. A polymer in solution is added as an aid to agglutination. The agglutination is observed visually, preferably under a microscope. The agglutinate can be dissociated again by adding a solution of citrate and sugar.

It is known moreover from European Published Application 0 194 212 that in the antigen test erythrocytes, which are loaded with antibodies in a separate reaction, can be separated from free erythrocytes by centrifugation with a second antibody in a gel. Surprisingly it was discovered that a second antibody is not necessary if the size of the gel particles is between the size of the erythrocytes and of the erythrocyte aggregates and the first antibody is added to the gel. In this case the aggregates form in passage through the gel and are pushed upward by the gel particles which are comparatively smaller. In the case of weak antigens or antibodies, the test succeeds only if their reactivity is strengthened, whether by exposing covered antigens by means of enzymes, decreasing the ionic strength in the buffer or by negative charging of the erythrocytes with cations.

In the case of extremely weak antigens or antibodies, it is imperative that the force of gravity takes effect precisely on the bottom of all reaction vessels so that a decision as to positive or negative can be made.

With conventional centrifuges this has not been the case so far; these centrifuges, however, can be re-equipped slightly by changing their fixture.

It is the object of this invention to provide an improved method of detecting antibodies or antigens which is simpler than the prior art methods, free of their drawbacks, and which permits a simple and reliable reading.

It was found that the above object can be achieved through the features defined in the characterizing part of patent claim 1.

The subject matter of the present invention is thus the method defined in patent claim 1 and the kit, defined in claims 16 to 22, to carry out the method.

The chemical composition of the inert particles used in the method is not critical. The term "inert" is intended to mean that the particles must not enter into any unspecific reactions with the antigens or antibodies. Preferably, such inert porous particles as are available in commerce for liquid or gas chromatography (e.g. by Merck, LKB, Pharmacia and Biorad) will be used. Examples are "Sephadex^R", "Sephacryl^R" or "Bio-Gel^R". These are products on the basis of cross-linked polymers, such as agarose, polyacrylamide, polydextran, or styrene-divinylbenzene polymers. Porous glass or silica gel also enters into consideration. The particle size is preferably from 10-200 μm . Those skilled in the art can determine by means of simple preliminary tests whether the particles are usable for a certain method of determination. As in the case of the above-mentioned inert particles, the type of carrier for the antigen or antibody is also not critical. For visual or optical-automatic measuring methods, it should be coloured by nature (erythrocytes). However, the carriers may also be tagged by means of a stain (e.g., latex, polymerized agarose).

Other marking systems also enter into consideration, such as isotope, fluorescent, or enzyme labelling. These naturally require an appropriate measuring method. Moreover, the carriers are preferably likewise in particle form, the antigens or antibodies being bound to their surfaces.

The antigens and antibodies are preferably bound to these particles by chemical bonding, the type of bonding not being critical. Certain antigens, such as those of erythrocytes and platelets, already exist bound to these carriers. Leukocytes and platelets may be stained by known methods, if need be.

In the case of leukocytes or platelets, the inert particles may alternatively be stained. In that event, the reading zone is whitish, and the inert particles are coloured.

According to the method of the present invention, it is possible to determine the free antigens or antibodies, certain carrier-bound antibodies or antigens, respectively, having to be given. Conversely, the carrier-bound antigens or antibodies may also be determined, the free antigens or antibodies, having to be given. The reaction vessels used for carrying out the methods are, as a rule, small test tubes or microtitre plates of glass or plastic. The material is not critical.

For carrying out the method of the present invention, small tubes or microreaction vessels are preferably used. Any desired number of these may, for example, be arranged side by side on a card. There may be an additional vessel, e.g., for preparing a test dilution or test suspension. The vessels and cards may be of plastic, such as PVC/PVDC, PET or polystyrene. The vessels may, for instance, be made by the blister method, by a welding process, or by gluing. The vessels may contain the inert particles or industrially prepared reaction solutions.

Since the invented method preferably includes a centrifuging step, special, suitable centrifuge heads must be used for the tubes or the cards with the containers. With one advantageous centrifuge head, at least 12 cards having, 5 for example, six reaction vessels can be centrifuged simultaneously. In this case, 72 tests can be carried out parallel to one another.

Although sedimentation can be brought about by allowing the vessels to stand and taking advantage of the 10 force of gravity, it is more advantageous to use the centrifuging method since the desired sedimentation can be achieved after only a short time. The optimum conditions (centrifuging time and number of g) must be ascertained for each analysis system since the specific gravity, size, 15 shape, deformability, and stability of the carrier-bound antigen-antibody complex, of the non-complexed, carrier-bound antibodies and antigens, and of the inert particles have an influence which is difficult to calculate.

Certain aspects of the invention will now be 20 described in detail with reference to the accompanying drawings, in which:

Fig. 1 shows the patterns arising with positive, weakly positive and negative reactions;

Fig. 2 is a side elevation and a front 25 elevation of a typical microtube suitable for affixing to a card;

Fig. 3a + 3b are a side and a front elevation, respectively, of a card with six small test tubes as in Figure 2;

30 **Fig. 4** shows the test procedure schematically;

Fig. 5, 6 + 7 show test cards for typical routine clinical tests.

Theoretically, the method according to the present 35 invention can be used to test a sample of liquid for an

antigen or an antibody which is specific to a known antibody or antigen, respectively. Either the known antigen or antibody, or the unknown antibody or antigen, must be bound to a carrier, e.g., an erythrocyte. The method of detection is based upon the realization that carrier-bound antigens and antibodies have different centrifugation properties from carrier-bound antigen-antibody complexes. If an antigen-antibody complex on a carrier is centrifuged together with a suspended, inert carrier material, the carrier-bound complex lies on the inert particles. If no reaction has taken place, only a free, carrier-bound antigen or antibody is in the test tube with the inert, suspended material. After centrifuging, this antigen or antibody is situated beneath the layer of inert particles. In this way, the positive or negative antibody reaction can plainly be read visually. It is also possible to automate this reaction. Weakly positive reactions may also occur, in which case the carrier-bound antigen-antibody complex is situated within the layer of inert particles.

A pattern of a positive reaction is shown in Fig. 1a. If the reaction is only weakly positive, i.e., if only a few antigen-antibody complexes are formed, the antibody can be detected in the upper part of the inert particles in the centrifuge glass (Fig. 1b). If, on the contrary, there is no antigen-antibody complex but only a carrier-bound, free antigen or antibody, the latter is deposited beneath the inert particles after centrifuging. This is shown in Fig. 1c.

As already mentioned, although various types of reaction vessels may be used, one such as is depicted in Fig. 2 is preferred. A side view is shown on the left, and a front view on the right. This small tube is covered, thus making it possible for certain industrially manufactured reagents to be supplied directly in the small

test tube. The test tube is suitable for affixing to a card, which may hold several tubes, e.g., as shown in Fig. 3, where there are six small test tubes. Fig. 3a is a side view of the test card, while Fig. 3b is a front view.

5 By means of this arrangement, a direct comparison of parallel tests is made possible.

The test card may be made in various ways. For example, small tubes may be glued to a card or may form an integral part of the card, in the manner of blister
10 packaging. A mixture of inert particles and antibodies or antigens may be hermetically enclosed in these tubes in a predetermined quantity by the manufacturer, in which case the tubes may be sealed by means of a welded-on film. Test kits manufactured in this way are easily handled and
15 may be used in an automatic analysis method. In this case, the pipetting of specimens, identification, automatic reading, evaluation, print-out, etc., is controlled by means of electric < sic. electronic > data-processing. A further advantage is that only very small
20 specimens are necessary. For instance, with 10-50 ul of blood, all clinically relevant antigens can be detected with small amounts of reagents. Micro-batches are particularly important in the case of substances which cannot be prepared synthetically and are available only in
25 limited quantities; e.g., with 1 ml of the Rh antibody C, 20 antigen determinations can be carried out by a conventional method, whereas 1000 determinations are possible with the amount described. If the test system is
30 appropriately prepared, the determinations are so easy to carry out, and the results so plainly readable that the test can even be carried out by auxiliary medical personnel.

Since no special laboratories are necessary, the interval between diagnosis and the start of therapy can be
35 considerably reduced.

The invention will now be explained in more detail by means of the following examples.

Examples

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Blood Group Antigens (ABO System)

Example 1: A-Antigen

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a) Preparation of inert particles suspension

5 ml of Sephacryl, S200 Gel (Pharmacia) are washed twice in saline solution. After centrifuging (5 min., 1250 g), the supernatant is discarded, and the sediment is filled up to 4.5 ml with isotonic imidazol buffer (0.014 mol/l of imidazol 0.85% NaCl) pH 7.6.

b) Addition of antibody

0.5 ml of anti-A is added to 4.5 ml of the above suspension. The suspension is mixed well and is ready for use in this form.

c) Preparation of reaction vessels

The above antibody suspension is placed in polyethylene microtubes (ET-29MM, Milian SA), 100 ul in each tube. The inert particles settle to the bottom of the tube within a few minutes.

d) Test procedure

20 ul of an approximately 4% erythrocyte suspension of the unknown blood specimen in isotonic imidazol buffer, pH 7.0 (one part blood to nine parts buffer) is placed in the reaction vessel filled with the antibody suspension and centrifuged for 10 min. at approximately 100 g (Centrifuge Heraeus Digifuge GL 122 089, 800 rpm).

e) Evaluation

If the tested blood belongs to blood sample <sic. group> A (A_1 or A_2), the antigen-antibody complex lies on top of the inert particles (Fig. 1a). If it belongs to the rare A subgroups A_3 or A_x , the complex is distributed among the inert particles (Fig. 1b). If the blood belongs to groups B or O, no agglutination can take place, and the erythrocytes collect at the bottom of the tube beneath the inert particles after centrifugation (Table 1).

Table 1

Blood Group	$A_1/A_2/A_1B/A_2B$	$A_3/A_3B/A_x$ A int.	B/O
Test	positive	weakly positive	negative
	(Fig. 1a)	(Fig. 1b)	(Fig. 1c)

Completely analogously to Example 1, the B antigen is determined with anti-B instead of anti-A. The H antigen can be detected with H lectin. A_1 is distinguished from A_2 by means of A_1 lectin. Example 1 admits of a multitude of variations of the inert particles, the buffer used, the reaction vessels, the centrifugation time, and the number of g. The origin of the antibodies, whether human, animal, or vegetable, whether polyclonal or monoclonal, is unimportant, provided the variations do not change the reaction image (Fig. 1).

II. Rh-System Blood Groups (D, Du, C, E, c, e, Cw)

Example 2: E Antigen

The preparation of the reaction vessels (steps a - c) is as in Example 1. Instead of anti-A, anti-E is used, for example.

d) Test procedure

Step d) of Example 1 is modified in that an enzyme solution (DiaBrom, DiaMed AG) is used instead of the imidazol buffer. It is known that concealed Rh antigens can be exposed thereby. 50 ul of blood is suspended in 0.5 ml of enzyme solution. After approximately 5 min., 20 ul of this suspension is placed in the reaction vessel prepared according to Example 1, steps a) - c) and centrifuged for 10 min. at 100 g.

10 e) Evaluation

If the blood specimen contains antigen E, the antigen-antibody complex rests on top of the inert particles (Table 2). If antigen E is not present, the erythrocytes collect under the inert particles.

Table 2

	E antigen present	E antigen absent
Test	postive (Fig. 1a)	negative (fig. 1c)

The other Rh antigens can be determined completely analogously by means of the appropriate antisera. The clinically important D-variant Du can be differentiated by varying the concentration of anti-D.

25 II. <sic. III> Antibodies

Example 3: Reverse Serum Test

Preparation of the reaction vessels (steps a) - c) takes place as in Example 1, except that imidazol buffer is substituted for the antibody.

d) Test procedure

Since antibodies are to be identified in this example, erythrocytes with known antigens are used, e.g., A, B, and O test erythrocytes. These are suspended in the known LISS solution (50 ul of blood and 2.0 ml of LISS). 50 ul of serum or plasma of the unknown blood specimen are placed in each of three identically filled reaction vessels.

100 ul of the A-cell suspension in LISS is added to the tube 1, 100 ul of the B-cell suspension to tube 2, and 100 ul of the O-cell suspension to tube 3. As in Examples 1 and 2, the suspensions are centrifuged for 10 min. at 100 g.

15 e) Evaluation

If the unknown specimen contains anti-A, tube 1 is positive, tubes 2 and 3 negative (Table 3).

Table 3

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	A cells	B cells	O cells
Test	positive	negative	negative
	(Fig. 1a)	(Fig. 1c)	(Fig. 1c)

If the specimen contains anti-B, tube 2 is positive, tubes 1 and 2 <sic. 3> negative. If tube 3 is positive, and tubes 1 and 2 positive or negative, the specimen contains antibodies which are not anti-A or anti-B, but directed to other antigens which lie on the O cells. In this event, further investigation is necessary.

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Example 4: Antibody Screening Procedure (Coombs Test)

The preparation of the reaction vessels is as in Examples 1 - 3, except that Coombs serum (Diamed AG) is added to the inert particles suspension. As is well

known, Coombs serum consists of anti-human-IgG and anti-complement C3 (C3b + C3d), as well as anti-IgM and anti-IgA, and is used for detecting and identifying, in serum from patients, antibodies directed against erythrocyte antigens. It is expedient first to carry out an antibody screening procedure in which O erythrocytes having a known antigen for clinically relevant antibodies are used. If the result is positive, identification by means of a cell panel takes place.

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Test procedure

50 ul of serum or plasma from the patient is placed in each well or funnel of one or more identical reaction vessels filled with Coombs serum suspension. 100 ul of the O-cell suspension (50 ul of blood in 2.0 ml of LISS) is added. The mixture is incubated for 10 to 20 min. at 37° C, at room temperature, or at 2 - 8° C, depending upon the antibody sought, and centrifuged for 10 min. at 100 g. Reading takes place as in the previous examples.

If the specimen contains an antibody against one or more erythrocyte antigens, the positive pattern of Fig. 1a appears. With weaker antibodies, the pattern is as in Fig. 1b. Analogous to the above test procedure, the antibody discovered may be identified by means of a cell panel containing various antigens (e.g., commercial products Ortho Dade, DiaMed).

IV. Blood Typing on a Card

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Example 5: Blood Groups A R1 R2 (CCDee) Kell negative, Controls negative

Typing can be done individually in small tubes, on microtitre plates, or on cards. In this example, blood

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typing on cards designed for determining a patient's blood group is described. The preparation of the reaction vessels takes place as described above. The cards are shown in Fig. 5 - 7.

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V. Determination of Free Antigens or Antibodies Not Belonging to Blood-Group Systems

The reactions in Examples 1 - 5, in which the antigen
10 is bound to the erythrocytes by its nature, can be applied to free antigens or antibodies by binding the respective antibodies or antigens to fixed erythrocytes or other particles by known methods.

15 Example 6: Rheumatoid Factor Test

a) Preparation of erythrocytes:

5 ml of goat's blood in 0.011 mol citrate buffer, pH 6, is washed three times in saline solution. The sediment
20 is suspended with 5 ml of saline solution, mixed with 0.5 ml of 30% glutaraldehyde solution (Merck), and allowed to react for 24 hrs. at room temperature with stirring. The sediment is washed three times in saline solution, mixed with 0.5 ml of rabbit IgG (10 mg/ml), and incubated for 24
25 hrs at room temperature with stirring. After washing three times in saline solution, an approximately 40% suspension of the loaded erythrocytes in imidazol buffer is prepared.

30 Test procedure

The reaction vessels are prepared as in Example 3. An approximately 4% erythrocyte suspension is prepared with imidazol buffer. As in Example 4, 50 ul of serum
35 from the patient is placed in the well of the test tube,

and 20 ul of the 4% erythrocyte suspension is added.
After about 10 min. incubation at room temperature,
centrifuging takes place as usual. If the serum contains
rheumatoid factors, the reaction pattern of Fig. 1a or b
5 appears. If ascertainment of the titre is desired, the test
is repeated with serum diluted accordingly (0.9% NaCl).

The test described is of course not limited to the
rhematoid factors; for example, hepatitis antigens could
be detected after coupling of HBsAg antibodies. By
10 coupling inactivated HIV virus or synthetically produced
proteins thereof to the erythrocytes, HIV antibodies can
likewise be easily detected.

Based upon these examples, it is immediately apparent
that other proteins, viruses, or bacteria, or antibodies
15 thereof, can easily be determined by the same method.

Patent Claims

1. A method for the detection of antibodies and/or antigens by optically making visible carrier-bound antigen
5 complexes with antibodies in a aqueous medium in a reaction vessel, wherein a solution containing antibodies or antigens is brought into contact with carrier-bound antigens or antibodies, a slurry or a suspension of inert
10 particles being added to the reaction vessel prior to this step, and the mixture subsequently being exposed to sedimentation of the gravitation, wherein if an antigen-antibody complex is formed, in a strongly positive reaction it lies on the sediment of the inert particles and in a weakly positive reaction it is present within the inert
15 particles, and in the absence of an antigen-antibody complex, i.e., in a negative reaction it lies beneath the sedimented inert particles, characterized in that the entire method is carried out in a single micro reaction vessel according to Fig. 2 or 3.

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2. The method of claim 1, characterized in that for optical detection the carrier to which the antigen or the antibody is bound is naturally coloured or tagged.

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3. The method of claim 2, characterized in that the carrier is tagged by colour, isotope, fluorescence or enzyme.

4. The method of claim 2 or 3, characterized in that
30 the carrier consists of erythrocytes, leukocytes, or platelets.

5. The method of claim 4, characterized in that the carrier is pretreated with proteolytic enzyme to intensify
35 the reaction.



6. The method of claim 5, characterized in that the enzyme is papain, ficin or bromelin.

5 7. The method of one of claims 1 to 3, characterized in that the carrier to which the antigen or antibody is bound, is made of latex or polymerized agarose.

10 8. The method of one of claims 1 to 7, characterized in that the inert particles are globules of porous polyacrylamide gel, of cross-linked dextran, or of silica gel with a particle size of 10 to 200 um.

15 9. The method of one of claims 1 - 8, characterized in that the sample mixture is exposed to gravitation by subjecting it to centrifugation.

20 10. The method of claim 9, characterized in that a centrifuge head causing the force of gravity to take effect precisely on the bottom of the reaction vessel is used for the centrifugation.

25 11. The method of one of claims 1 - 8, characterized in that it is carried out in an isotonic buffer at a pH of 4 to 8.

12. The method of claim 11, characterized in that the reaction is carried out in a buffer of low ionic strength.

30 13. The method of one of claims 1 - 12, characterized in that it is used to type blood groups.

14. The method of one of claims 1 - 12, characterized in that the carrier-bound antibodies are directed against
35 proteins, viruses, bacteria or coat proteins.



15. The method of claim 11, characterized in that the carrier-bound antigens are components of body fluids, e.g. blood, serum or plasma components.

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16. A test kit for detecting antigens or antibodies according to the method of one of claims 1 - 15, characterized in that it contains at least one micro reaction vessel according to Fig. 2 or 3, inert particles and a
10 carrier-bound antibody or antigen per reaction vessel.

17. A test kit for detecting carrier-bound antigens or antibodies according to the method of one of claims 1 - 15, characterized in that it contains at least one micro
15 reaction vessel according to Fig. 2 or 3, inert particles and one antibody or antigen per reaction vessel.

18. The test kit of claim 16 or 17 for simultaneously carrying out several tests, characterized in that it comprises several micro reaction vessels according to Fig. 2
20 or 3, said vessels being plainly grouped and interconnected.

19. The test kit of claim 18, characterized in that
25 the micro reaction vessels are disposed parallel next to each other on a card.

20. The test kit of claim 19, characterized in that it also comprises a centrifuge head designed to accept
30 several cards, the cards used being pivotably disposed in planes which contain the axis of rotation of the centrifuge as a common element, and when the centrifuge is operated, the cards are directed so that the axes of the micro reaction vessels are brought to a right angle
35 relative to the pivot axis of the centrifuge so that the



centrifugal force takes effect precisely on the bottom of the micro reaction vessels.

21. The test kit of one of claims 16 - 20, characterized in that the components to be used for the test are separately packaged for insertion in the appropriate micro reaction vessel at the time of testing together with the sample.

22. The test kit of one of claims 16 - 20, characterized in that the micro reaction vessels are closed by means of lids, and contain components for carrying out the method.

