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Preparation and application methods

WORLD HEALTH ORGANIZATION
Regional Office for the Eastern Mediterranean

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Blood Grouping Reagents

Preparation and application methods

by

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Foreword

Increased efforts are being made to develop blood transfusion services in the WHO Eastern Mediterranean Region (WHO/EMR) in order to attain self-reliance in the supply of blood, blood components and blood products, based on voluntary, regular, non-remunerated blood donation.

Self-reliance should also be extended to include local production of basic reagents. Among important reagents required for blood transfusion services, and which could be prepared locally at a country level, are those used in routine grouping and cross-matching of blood, and in routine screening and identification of antibodies.

Dr Fereydoun A. Ala, Director of the West Midlands Regional Blood Transfusion Centre, Birmingham, UK, a consultant with deep rooted interest in WHO/EMR, collaborated with WHO/EMRO in organizing a series of successful workshops focusing on essential areas in transfusion medicine. One of these workshops dealt with blood group reagents used in blood transfusion, and took place at the Regional Training Centre on Blood Transfusion, Amman, Jordan.

The authors, among other WHO consultants, used the material included in this manual to conduct the aforementioned workshop. The manual provides excellent basic information on preparation and application methods of blood group reagents. It could be used to train nationals from countries of the EMR , as well as many other countries from other Regions, in the preparation and use of blood group reagents.

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Documentation

Documentation plays an important part in any procedure. It often contains information that can be crucial to the final interpretation of results. Documentation is the information link between patient and sample.The following protocol is intended to identify positively the investigation request and information with the patient's sample.

1. Check that the sample is from the same patient as stated on request form.

2. Check sample has sufficient clearly labelled patient identification information; for example:
   - full name
   - date of birth
   - hospital
   - hospital reference number
   - sample collection date
   - phlebotomist's initials

3. Check sample against request form.

4. Check request form has been completed and signed by medical officer.

5. Investigate any discrepancy between request form and sample.

6. Inform senior member of staff of any discrepancies between request form and sample details or inadequately labelled samples.

7. Search records for any previous test information that may be available.

8. Record any previous test information on request form.

9. Enter request details into day book; for example:
   - hospital code
   - patient's surname
   - patient's forename
   - hospital reference number

Agglutination reactions

It is important to define a standard method of interpreting the strength of agglutination reactions or quantifying the degree of haemolysis.The following descriptions are the standard definitions used within the West Midlands Regional Blood Transfusion Centre, Birmingham, United Kingdom.

Agglutination

The strength of agglutination reactions should be recorded as follows :-

4 (++++)	One complete mass of agglutination, readily visible on the slide before microscopic examination.
3 (+++)	Large separate masses of agglutination, readily visible on the slide before microscopic examination.
2 (++)	Smaller agglutinates, still readily visible on the slide before microscopic examination.
1 (+)	A granular appearance, just visible on the slide microscopically. The microscopic examination shows big clumps of more than 20 cells.
Bracket (+)	Smaller clumps of 12-20 cells, only detectable microscopically.
Weak (w)	Weak microscopic reactions with uniform distribution of small clumps of 4-6 cells.
Negative	All cells free and evenly distributed.
Mixed field	Agglutinates in a field of free cells.

Haemolysis

Haemolysis is one of the most spectacular manifestations of antibody/antigen reactions. In human blood group serology it is associated with particular blood group systems such as ABO, Lewis, Vel and P and is often indicative of any antibody pertaining to one of the previously mentioned systems.These antibodies bind complement components and activate the complement cascade leading to lysis of the red cells and the release of haemoglobin in the test system.

4^h	Supernatant red coloured and quite transparent after shaking.
3^{+h}	Supernatant red coloured but not quite transparent after shaking.
3^h	Only a few cells intact and settled, supernatant red coloured.
2^h	Approximately half of the cells intact and settled, supernatant strongly reddish.
1^h	Most cells intact and settled, supernatant reddish.
$(+)^h$	Nearly all cells intact and settled, supernatant reddish.
w^h	As far as detectable, all cells intact and settled; faint reddish colouring of the supernatant.
Negative	All cells intact and settled, no colouring of the supernatant.

Reading test results

This describes the technique for removing the sedimented cell button from test-tubes and transferring it to a glass slide for examination, after the completion of the test incubation period.

1. Select a wide bore Pasteur pipette.
2. Hold the pipette bulb between thumb and first finger.
3. Rinse the Pasteur pipette three times in saline.
4. Apply slight pressure to expel some air from the pipette bulb.
5. Insert the pipette into a 6 x 50 mm test-tube with the tip placed above the cell button.
6. Gently release the thumb pressure on the pipette bulb.
7. Allow the cell button to be drawn into the pipette.
8. Dispense the cell button onto a glass microscope slide. No more than four results should be placed on each slide.
9. In between removing the cell button from each test-tube, rinse the Pasteur pipette three times in saline.
10. Rock the slide very gently to allow the aspirated cells or agglutinates to disperse evenly on the slide. Do not rock too hard as this will break up weak agglutinates and can produce false negative results.
11. Examine the slide microscopically using the low power objective.
12. Record the results.

ABO blood grouping

Human red cells carry two main chemical determinants that allow the classification into four ABO blood groups: group O, group A, group B and group AB.

Human plasma contains antibodies to these determinants with antithetical specificity to the blood group of the red cells. These antibodies occur 'naturally'. They are complete (IgM) antibodies that induce direct agglutination of the red cells carrying the corresponding antigens. ABO agglutinins start to appear in infants three to four months after birth.

A and B antigens can be classified further into subgroups: A_1, A_2 and weak B.

This section describes the policy related to the ABO blood grouping of patient and donor samples by manual techniques.

1. When red cells are tested under optimum conditions with serum known to contain a specific antibody, and no interaction occurs between the cells and reagent antibody, it can be assumed that the cells lack the antigen against which the antibody is directed.

2. Conversely, any interaction implies that the cells possess that antigen.

3. Standard test protocol for ABO grouping:
   - Patient/donor cells should be tested with: anti-A, anti-B, anti-AB and anti-A_1 (where indicated).
   - Patient/donor serum or plasma should be tested with: A_1, A_2, B and O reagent red cells.
   - Auto-control: patient/donor's own cells tested with patient/donor's own serum.

4. Both cell and serum (forward and reverse) groupings are determined on samples from adults and children over four months old.

5. Cell grouping only is performed on samples from children less than four months old.

6. Babies should not be sub-typed by normal methods until they are more than four months old.

7. The finding of anti-A or anti-B in the serum, in the presence of the corresponding antigen on the red cells, is an abnormal occurrence and must be investigated.

8. All anomalous results must be repeated.

9. Positive sample identification must be established prior to commencement of testing.

10. All reactions must be graded.

11. All results must be recorded.

ABO manual grouping

This describes the test protocol for performing manual ABO grouping.

1. Confirm positive identification of patient sample by checking on both sample and request form:
   - name
   - address
   - date of birth
   - reference number
2. Confirm that details on both sample and request form are identical.
3. Centrifuge sample at 1000 *g* for three minutes.
4. Label appropriate test-tubes with patient's name or reference number.
5. Transfer separated serum and red cells into appropriately labelled test-tubes.
6. Wash red cells three times in isotonic saline.

This protocol should precede the following four methods of ABO grouping.

Test-tube group

1. Prepare a 3% suspension of thrice washed red cells in isotonic saline.
2. Place one volume of each reagent antisera i.e. anti-A, anti-B, anti-AB, in a 6 x 50 mm test-tube.
3. Add one volume of patient's or donor's 3% cell suspension.
4. Take a cell free sample of patient's or donor's serum.
5. Place one volume of serum in five 6 x 50 mm test-tubes.
6. Add one volume of 3% reagent red cell suspension of A_1, A_2, B, O and patient's or donor's own cells respectively.
7. Control each reagent antibody by testing against known A_1, A_2, B, and O cells.
8. Mix thoroughly and incubate at room temperature (limit 18-22°C) for one hour.

9. Read reactions microscopically.

10. Record results and interpret.

Test-tube group: immediate spin

1. Place two volumes of anti-A, anti-B, and anti-AB, respectively, in three labelled 12 x 75 mm test-tubes.

2. Add one volume of 3% suspension of patient's or donor's cells to each test-tube.

3. Mix thoroughly and centrifuge for one minute at 200 *g*.

4. Gently shake test-tubes and observe macroscopically for agglutination.

5. Read negative results microscopically.

6. Record results and interpret.

Tile group

1. Prepare a 10% suspension of washed cells in saline or a 40% suspension of red cells in their own serum or plasma.

2. Place one volume of anti-A, anti-B, and anti-AB on a white ceramic tile.

3. Add one volume of patient's or donor's cell suspension.

4. Mix cells and sera over an area approximately 15 mm in diameter.

5. Maintain tile at room temperature (18-22°C).

6. Read the reactions after two minutes using an illuminated reader.

7. Record results and interpret.

Saline room temperature technique

1. Add one volume of reagent antiserum or patient's serum to a 6 x 50 mm test-tube.

2. Add one volume of 3% saline cell suspension to the test-tube containing the reagent antiserum or patient's serum.

3. Mix the test-tube thoroughly.

4. Record time on the test test-tube rack.

5. Incubate rack at room temperature for one hour.

6. Read tests microscopically.

Interpret reactions as follows:

Reagent	Patient 1	Patient 2	Patient 3	Patient 4
anti-A	+	-	+	-
anti-B	-	+	+	-
anti-AB	+	+	+	-
own control	-	-	-	-
A_1	-	+	-	+
A_2	-	+	-	+
B	+	-	-	+
O	-	-	-	-
Group	A	B	AB	O

A sub-grouping

1. Place two volumes of anti-A_1 (Dolichos biflorus) into a labelled 12 x 75 mm test-tube.

2. Add one volume of 3% suspension of patient's or donor's cells.

3. Control antisera by testing against known A_1 and A_2 cells.

4. Mix thoroughly and centrifuge for one minute at 200 *g*.

5. Gently shake test-tubes and observe macroscopically for agglutination.

6. Read negative results microscopically.

7. Records results and interpret.

Interpret reactions as follows:

Anti-A_1 (Dolichos biflorus)	Interpretation
+	A_1, A_1B
-	A_2, A_2B

Rh D typing

The Rh system is composed of a series of antigens and their corresponding antibodies which together form a highly complex blood group system.

The methods of detection are basically the same for all of the Rh antigens and antibodies. In this instance, the techniques will be described only for the detection of the D antigen, thus dividing individuals into being Rh positive or Rh negative. For 'D typing' the reagent must be of the specificity anti-D, of high titre and avidity, and free of any ABO antibodies so that it is suitable for testing blood of all ABO groups. Reagent antibody combinations of either anti-D+C or anti-D+C+E are not suitable.

The standard D typing reagent antisera can be divided into two distinct types:-

- Saline anti-D
- Incomplete anti-D

Saline anti-D occurs as a 'complete' IgM antibody which can agglutinate cells containing the corresponding antigen in a saline medium.

Incomplete anti-D occurs as an IgG antibody which will sensitize (or coat) the red cells in saline without demonstrating agglutination. In order for agglutination to form, a potentiating agent is required. The potentiating source can be in the form of a high protein medium such as 20% or 30% bovine albumin or an enzyme preparation such as 1% papain.

Rh D typing controls

It is essential that controls are incorporated into all test procedures. The controls used for D typing are:-

- D positive cell control
- D negative cell control
- AB serum control with cells under test

D positive cell control. The cells selected should have the least expression of the D antigen. Cells that have the R_1r (CcDee) phenotype have a weaker expression of D than R_2r (ccDEe) cells, therefore group O R_1r should be selected. This control must be set up in exactly the same way as the patient's/donor's Rh D typing and, unless it gives an acceptable positive reaction, all the tests should be repeated.

D negative cell control. In this instance known Rh negative cells are tested against the standard Rh typing reagent. If the anti-D has had anti-A and/or anti-B absorbed out of it, it is advisable to include a group of ABRh negative cells to confirm the absence of ABO antibodies.

AB serum control. In this control the standard anti-D typing reagent has been replaced

by inert AB serum. If this test is positive, then positive results obtained by the standard anti-D cannot be accepted with 100% confidence. The cells in question should be investigated further e.g. direct antiglobulin test.

Anti-D typing reagents

Where possible it is standard procedure to perform all red cell antigen typing using two different reagent antisera of the same specificity. Any discrepancy between the results of the two reagents necessitates a full investigation of reagents and test cells.

Interpretation of results

Rh D typing is always performed with two anti-D reagents. A negative result with both test sera will mean that the individual is probably D-negative. A positive result with both reagent antibodies will mean that the sample is Rh positive (D+). Samples which give doubtful reactions with one or both antisera should be re-tested with a panel of anti-D reagents if possible. A negative result with one reagent and a positive result with the other also necessitates re-testing with a selected panel of anti-D reagents.

In cases of acquired haemolytic anaemia or haemolytic disease of the newborn, the cells may be coated and therefore give positive reactions. This type of false positive results can be detected by the inclusion of the AB serum control as previously mentioned.

Cell	Anti-D	Anti-D	AB	Interpretation
1	4	4	-	Rh (D) positive
2	-	-	-	Rh (D) negative
3	weak	1	-	repeat tests
4	4	-	-	repeat tests
5	4	4	3	check DAT

Reagent red cell panel

When an unexpected antibody has been detected in a patient's serum sample, it must be identified to determine its clinical significance. Blood group antibodies are not all equally dangerous in transfusion therapy or in pregnancy. Antibody identification is accomplished by testing the serum sample against a panel of red cells having different antigen characteristics, observing the presence or absence of haemolysis or agglutination, and comparing the pattern of reactivity with the antigen profile of the cells.

Selection of reagent red cell panels

The antigen make-up of reagent red cell panels should allow for the resolution of commonly encountered antibody problems. It should be noted that the antibody specificities encountered will depend on the population being studied (e.g. healthy blood donors versus multiply transfused patients, or Caucasians versus Blacks, Orientals, or Indians) as well as the method of testing. For example, anti-Le^a and anti-Le^b occur very frequently in obstetric patients. Anti-Fy^a is an example of an antibody not frequently encountered in the Black population; however, it may be commonly found in other multiply transfused patients. The following list of antibodies are those most frequently encountered (the antibodies are grouped in the approximate order of frequency found – the group at the top being the most common and the group at the bottom rare):

1. Anti-D with or without anti-C or anti-E
2. Anti-Le^a and anti-Le^b – alone or together
3. Anti-K; anti-E; anti-P_1
4. Anti-c; anti-cE; anti-Fy^a; anti-M
5. Anti-Jk^a; anti-s
6. Anti-Ce; anti-e; anti-Jk^b; anti-N; anti-s; anti-Fy^b

Most samples of anti-P_1 and many anti-Le^a, anti-Le^b and anti-M will not react if room temperature testing is omitted.

A review of the antibody specificities most frequently encountered in your region will help to define better your needs prior to selecting a reagent red blood cell panel for routine use.For example, if multiple antibodies such as anti-D and anti-Fy^a are common, could each antibody be clearly identified with cells of the panel under consideration? Could many other underlying antibodies be excluded without extensive problem solving measures? Well-prepared panels will help to distinguish various specificities; however, it may not be possible to accomplish this in every circumstance.

The standard reagent red cell panel has been designed to resolve most antibody identification problems in the initial testing if possible.The antigenic profile is based, as near as

possible, on a structure that will cope with the antibody specificities most frequently encountered routinely.

The sequence of the Rh genotype of the 10 cells does not vary.This allows the investigator to recognize the patterns of commonly encountered Rh antibody specificities without delay. For example, anti-D and anti-C can be recognised by the pattern given with the first five cells and the first six cells, respectively.

No single panel will be able to solve all antibody problems, particularly when a serum contains multiple antibodies. Additional cell panels may have to be tested in order to obtain a 95% confidence level in antibody identification. Once the routine panel has been tested and exclusions have been made based on the reactivity observed, additional cells can be selected to make identification of antibodies easier and less time-consuming.

The standard reagent red cell panel consists of a series of red blood cells in 3% suspensions from 10 selected group O individuals and a pool of washed group O red blood cells from umbilical cords of newborns.

Antigen characteristics routinely provided on a standard reagent red cell panel

1. Consistent pattern of Rh phenotype on adult cells include the following:-

Cell 1	C^wDe/CDe	$R_1^wR_1$
Cell 2	CDe/CDe	R_1R_1
Cell 3	cDE/cDE	R_2R_2
Cell 4	cDE/cDE	R_2R_2
Cell 5	cDe/cde	Ror
Cell 6	Cde/cde	r"r
Cell 7	cdE/cde	r"r
Cells 8,9,10	cde/cde	rr
Cell 11	Cord	

2. Antigen make up of cde/cde (rr) cells will exclude common antibodies in the presence of anti-D.

3. At least one of each:

   - D positive, Fy^a negative, K negative cell
   - D positive, C negative, K negative cell
   - c negative, Fy^a positive, K negative cell
   - c positive, Fy^a negative, K negative cell

4. A minimum of:

   - three K positive cells that include the following:
     - D negative, Fy^a negative
     - D negative, C negative
     - c negative, Fy^a negative
   - two Le^a negative Le^b negative (Le a-b-) cells of which one is:
     - D positive and one D negative
   - three P_1 negative cells

5. The strength of the P_1 antigen is graded on the antigen profile print out.

6. At least one of each:

   - Lu^a positive
   - s negative

Antigen characteristics provided as often as possible

1. Three P_1 negative cells that include the following:

   - one that is Le^a positive
   - one that is Le^b positive
   - one that is M positive

2. Two M negative cells that include the following:

   - one that is Le^a positive
   - one that is Le^b positive

3. c negative cells possessing the most common other antigens.

4. At least one of:

   - Kp^a positive
   - Co^b positive

Reagent red cells for umbilical cords of newborns

Cells from umbilical cords of newborns are very valuable in aiding the resolution of antibody investigations and must be considered as an essential component of any reagent cell panel. This is based on some pertinent facts regarding umbilical cord cells:

- Some blood group antigens are at a different stage of transience e.g. i- I.
- Some blood group antigens are not well developed on umbilical cords e.g. Lutheran, Lewis and Sd^a.

Therefore the reactions of an antisera against umbilical cord cells can provide relevant information regarding the antibody's specificity.

1. The source of umbilical cord cells is organised by requesting the maternity hospital to collect fresh samples of newborn cord cells from group O babies for reagent use.

2. The samples are examined for date of bleeding and condition of the red cells. Samples that are more than four days old or that appear in poor condition are discarded.

3. Samples that are considered as possible reagent cells are:

   - ABO grouped by the tile method.
   - Checked as being direct antiglobulin test (DAT) negative.

   Samples that are not group O or are DAT positive are discarded.

4. Suitable samples are divided into two groups:

   - Clotted samples.
   - Anticoagulated samples.

5. The clotted samples are treated as follows:
   - Tip the clot into a wire strainer placed over a 250 mL glass beaker.
   - Using a pestle, grind the clot against the wire mesh of the strainer and collect the free cells into the glass beaker.
   - Occasionally use a little saline to wash the red cells into the beaker.
   - Do this for each clot, so producing a pool of umbilical cord cells.
   - Discard the clot debris for each sample.
   - Transfer the pooled cells to a Universal container for washing.
6. Anticoagulated samples are treated as follows:
   - Transfer the samples into Universal containers.
   - Centrifuge the Universal containers at 2000 rpm for four minutes.
   - Remove the supernatant and replace by 0.85% saline.
7. The anticoagulated samples and cells from the clotted samples are now washed in saline until the supernatant is clear.
8. A small sample from each batch of cells is taken off and made up to a 3% cell suspension.
9. A direct antiglobulin test is carried out on each sample.
10. A test-tube ABO grouping is carried out on each sample.
11. Providing that all criteria are satisfied the umbilical cord cell pools are labelled with batch number and date and are either stored in preservative for current reagent panel use or held frozen for future use.
12. The cells are issued as part of the routine reagent red cell panel as a 3% suspension, in preservative, of both enzyme-treated and normal cells.

Additional reagent red cells

There are additional reagent red cells to the standard routine panel which can either be obtained from fresh samples or may have been held in frozen storage.

1. A_1 and A_2; the subgroup of these cells must be verified by manual testing with anti-A_1, Dolichos biflorus.
2. A_1B rr, A_1B R_1R_1 and A_1B R_2R_2; these cells are supplied to check the Rh typing reagents for the presence of ABO antibodies. Consequently they must all be Rh phenotyped, and of A_1B not A_2B: this should be confirmed by manual testing with anti-A_1 Dolichos biflorus.

3. Group O adult cells (if available):
   - R_2R_1
   - R_2R_2
   - Wr^a positive

Preservative used for reagent red cell panels

The cells used as reagent red cells are suspended in a preservative in order to prolong their condition and antigenic activity.

Materials

Tri-sodium citrates	Na3 $C_6 H_5 O_7 2H_2O$	(MW 294 10)
Sodium chloride	Na Cl	(MW 58.44)
Dextrose		
Chloramphenicol	BP $C_{11} H_{12} Cl_2 N_2 O_5$	(MW 323.13)
Neomycin sulfate		
Adenosine 5' triphosphate (ATP) disodium salt		
Inosine	$C_{10} H_{12} N_4 O_5$	(MW 268.23)
Sterile distilled water		

Solutions (to make two litres)

1. Tri-sodium citrate	16 g	(1.6%)
Sodium chloride	8.4 g	(0.84%)
Sterile distilled water	1000 mL	
2. Dextrose	41 g	(4.1%)
Sterile distilled water	1000 mL	

Procedure

1. Mix solutions 1 and 2 prior to use.

2. Add to the two litres of solution:

– Chloramphenicol BP	0.8 g	(0.4%)
– Neomycin sulfate	0.2 g	(0.01%)
– Adenosine tri-phosphate	0.8 g	(0.4%)
– Inosine	0.8 g	(0.4%)
– Gentomycin	0.1 g	(0.005%)

3. Add the preservative to the concentrated reagent red cells to give:

   - 20% stock cell suspension of reagent cells
   - 4% working suspension of reagent cells
   - 2% working suspension of enzyme treated reagent cells

   NOTE: Do not store the final preparation of preservative.

Reagent red cells used for the detection of red cell antibodies by the indirect antiglobulin technique

1. **Selection of indirect antiglobulin reagent red cells**

   The antigenic structure of the reagent red cells used for antibody detection by the indirect antiglobulin test should be especially selected for the detection of weak antibodies by presenting the antigens for clinically significant antibodies in the homozygous form.The cells from three donors should be selected to provide homozygous condition for the Duffy, Kidd and MNSs systems and also the Rh antigens. Additionally the antigens Cellano, Kp^b and Lu^b should be present in homozygous dose, while K, P_1, C^w, Xh^a, Le^a and Le^b in single dose.

2. **Antigen characteristics routinely provided on standard reagent red cell screening panel (IAT)**

   Rh phenotype of reagent red cell are as follows:

   - Cell 1 CDe/CDe R_1R_1
   - Cell 2 cDE/cDE R_2R_2
   - Cell 3 cde/cde rr

   Antigen structure of cde/cde (rr), cell 3, will allow detection of common antibodies in the presence of anti-D and will be as follows:

   - K positive
   - Fy^a positive
   - S positive
   - Jk^a positive

3. **Antigen characteristics provided as often as possible**

   The three cells should include the following red cell antigens:

   - Lu^a positive
   - s negative
   - C^w positive

Direct antiglobulin technique

This technique is used to determine whether or not a patient's red cells have become sensitized by antigen-antibody complexes which have formed *in vivo*. If *in vivo* complexes have formed, this will be demonstrated by the development of agglutination after the addition of an anti-human globulin serum.

1. Label a 12 x 75 mm test-tube with the patient's name.
2. Check that the red cells are not pre-agglutinated, as this can lead to a false positive test result.
3. Place some concentrated red cells in the test test-tube.
4. Top up with 0.85% saline to make approximately 5% cell suspension.
5. Mix by gentle inversion.
6. Add one drop of the patient's 5% cell suspension to the labelled 12 x 75 mm test test-tube.
7. Top up with 0.85% saline.
8. Mix by gentle inversion.
9. Centrifuge at approximately 1000 *g* for one minute.
10. Remove supernatant saline by aspiration.
11. Agitate gently and top up with saline.
12. Repeat washing cycle four times.
13. Add two drops anti-human globulin to washed cells.
14. Mix gently.
15. Centrifuge at 200 *g* for one minute.
16. Shake test-tube gently to dislodge cell button.
17. Observe macroscopically for agglutination.
18. Record strength of reactions.

19. Add one drop of sensitised red cells to all 'negative' test-tubes.
20. Mix gently.
21. Centrifuge at 200 *g* for one minute.
22. Shake test-tube gently to dislodge cell button.
23. Observe macroscopically for agglutination.
24. Check all results are 'positive'.
25. Report all negative results with sensitized cells to a senior member of staff.
26. Repeat 'negative' tests.

Indirect antiglobulin technique

The method can be divided into three stages:

- Sensitization of the red cells by the formation of antigen-antibody complexes.
- Removal of all 'free' human globulin, globulin not coated onto the red cells, by thorough washing of the red cells in saline.
- Detection of any antigen-antibody complexes formed, sensitization of cells, by the addition of an anti-human globulin serum.

If an antigen-antibody reaction has taken place, antibody globulin will be attached to the red cell surface and a 'positive' antiglobulin test result, agglutination, will be found.

1. Place two or three drops of patient's serum into a labelled 12 x 75 mm test test-tube.
2. Add one drop of reagent red cell suspension.
3. Mix gently.
4. Incubate in water bath at 37°C for required time (LISS cells: 20 minutes; saline cells: 45 minutes).
5. Top up with 0.85% phosphate buffered saline (PBS)[1].
6. Mix by gentle inversion.
7. Centrifuge at 1000 *g* for one minute.
8. Remove supernatant saline by aspiration.
9. Agitate gently and top up with saline.
10. Repeat washing cycle four times.
11. Add two drops of anti-human globulin to washed cells.
12. Mix gently.
13. Centrifuge at 200 *g* for one minute.
14. Shake test-tube gently to dislodge cell button.

[1] An automated cell washer can be used.

15. Observe macroscopically for agglutination.

16. Record strength of reactions.

17. Record results in work book.

Confirmation of negative test results

After reading the antiglobulin test phase it is recommended that sensitized control cells are added to those tests which give a 'negative' result. This is based on the principle that when anti-human globulin serum is added to a test-tube containing only washed cells (no antibody-antigen reaction) the anti-human globulin serum is still potent and capable of agglutinating the sensitized control cells. If the test has not been washed sufficiently, the antiglobulin reagent can be neutralized by traces of serum globulin and therefore will be unable to agglutinate the sensitized cells. Thus a 'negative' result will be found which necessitates repeating the test.

Procedure

1. Add one drop sensitized red cells to all 'negative' test-tubes.

2. Mix gently.

3. Centrifuge at 200 *g* for one minute.

4. Shake test-tube gently to dislodge cell button.

5. Observe macroscopically for agglutination.

6. Check all results are 'positive'.

7. Report all negative results with sensitized cells to a senior member of staff.

8. Repeat 'negative' tests.

Albumin

Albumin may enhance the agglutination of antibody coated red cells at 37°C. It disperses the charges around the red cells and allows them to come close enough together to be agglutinated by some Rh antibodies.This technique may be of some use for resolving multiple antibody problems.

Advantages

Identification of strong IgG antibodies at 37°C.

Disadvantages

1. Enhances reactions of cold agglutinins.
2. Only of limited value because many Rh antibodies are not strong enough to be detected at 37°C.
3. Albumin suppresses haemolysis at 37°C.
4. Less sensitive than LISS or enzymes for identifying weak antibodies.

Albumin displacement technique

1. Add one volume of patient's serum to a 6 x 50 mm test-tube.
2. Add one volume of 3% saline cell suspension to the test-tube containing patient's serum.
3. Mix the test-tube thoroughly
4. Record time on rack.
5. Incubate rack at 37°C for one hour.
6. Check red cells have sedimented to bottom of test-tube.
7. Add one volume 30% albumin to the 6 x 50 mm test-tube after one hour's incubation. Allow albumin to run down the inside of the test-tube. Do not mix.
8. Incubate test-tube at 37°C for an additional 15 minutes.
9. Read tests microscopically.

Low ionic strength salt (LISS) solutions

Low ionic strength salt solutions enhance the first stage of agglutination by increasing the antibody uptake onto red cells. Two basic kinds of low ionic strength preparations are available, the wash solution and the additive solutions.

The wash solutions consist of a 0.03 M NaCl solution with glycine added as a stabilizer.This preparation requires that all test cells are washed and resuspended in the solution.

The additive LISS preparations vary in their formulation. However, they usually contain high molecular weight substances such as gelatin, salt-poor albumin, or polybrene to maintain the ionic strength and prevent non-specific agglutination. This solution is added to the serum-cell mixture.

The primary use of LISS solutions is in antibody detection and enhancing weak reactions. LISS preparations are of limited use in resolving multiple antibody identification problems.

Advantages

- Decreased incubation time without loss of sensitivity aids in ruling out or confirming the specificity of IgG antibodies.
- May enhance weak reacting antibodies.

Disadvantages

- Occurrence of non-specific antibodies reactive only when tested with LISS suspended cells.
- LISS solutions may detect unwanted cold agglutinins.

Low ionic strength salt (LISS) technique

Preparation of LISS solutions

1. Prepare 0.15 M phosphate buffer by mixing together 11.3 mL of 0.15 KH_2PO_4 and 8.7 mL of 0.15 M Na_2PO_4.
2. To 20 mL of 0.15 M phosphate buffer add 1.75 g of NaCl and 18 g of glycine.
3. Add 980 mL of distilled water.
4. Adjust the pH to 6.7 with 1 M NaOH.
5. Add 0.5 g of sodium azide as a preservative.
6. Store reagents at 4°C.
7. pH 6.7 ± 0.2
8. Conductivity 3.7 ± 0.3 mS/cm
9. Osmolality 295 ± 5 mosmol/Kg

Test procedure

1. Wash cells to be tested three times with normal saline.
2. Decant saline completely to make a dry cell button.
3. Resuspend the cells to a 2% suspension with LISS solution.
4. Add two volumes of serum to a labelled set of clean test-tubes.
5. Add two volumes of LISS-suspended cells to the appropriate test-tube.
6. Mix and incubate for 20 minutes at 37°C.
7. Centrifuge, observe for haemolysis, and read for agglutination.
8. Wash cells four times with isotonic saline and convert test-tubes to an indirect antiglobulin test.

Precautions

1. Always use equal volumes of LISS and serum. Altering this ratio will affect the ionic strength of the test media and may decrease the sensitivity of the test procedure.

2. LISS solutions may enhance the reactivity of cold agglutinins and result in complement being bound to the cell membrane. Using anti-IgG in the indirect antiglobulin test will eliminate these non-specific reactions.

Proteolytic enzymes

The use of enzymes can be of great benefit when attempting to detect and identify multiple antibody specificities.

Proteolytic enzymes, such as papain, bromelin, trypsin, and ficin, cleave off the polypeptide chains on the red cell membrane which contain sialic acid residues.This reduces the red cell's negative surface charge, Zeta potential, thereby increasing the degree of agglutination by incomplete IgG antibodies. The removal of these chains also increases the antibody uptake because the antigenic determinant sites become more accessible to the antibody.

Some antigens are cleaved from the red cell membrane by enzyme treatment of the red cell. Consequently the following antibodies do not react with enzyme treated red cells: anti- M, -N, -Fy^a, -Fy^b, -S, -Rg^a, -Xg^a and Ch^a.

Advantages

- Aids in the identification of multiple antibodies when M, N, S or Duffy(Fy^a and Fy^b) antibodies are suspected.
- Enhances the lysis of cells by complement binding antibodies, particularly Lewis and Kidd blood group antibodies.
- Enhances the agglutination of red cells by incomplete IgG antibodies.

Disadvantages

- Sensitive technique in which over or under treatment of cells may lead to confusing results.
- Enzymes enhance the reactivity of cold autoagglutinins.
- Some patients have antibodies against enzyme treated cells.

Two stage enzyme technique

Papainisation of red cells

In the two stage enzyme technique, the reagent red cells are retreated with the enzyme[1], washed and then incubated with the patient's serum. This method is more sensitive than the one stage technique and gives more reliable and consistent results.

1. Place some concentrated red cells in a labelled 12 x 75 mm test test-tube.
2. Top up with 0.85% saline.
3. Mix by inversion.
4. Centrifuge at 1000 *g* for one minute.
5. Remove supernatant saline by aspiration.
6. Agitate gently and top up with saline.
7. Repeat washing cycle three times.
8. Dispense four volumes buffered saline into a separate 12 x 75 mm test test-tube. Total volume will depend on requirements.
9. Add one volume 1% papain to test-tube.
10. Stopper test-tube.
11. Mix by inversion.
12. Pipette equal volumes of packed washed red cells and papain/buffered mixture into labelled 12 x 75 mm test-tube.
13. Mix contents of the test-tube.
14. Incubate at 37°C for required time in water-bath[2].
15. Wash cells four times in saline.
16. Make 5% cell suspension ready for use.
17. Use cells as required.

[1] Avoid contact with skin or eyes as it is a strong irritant.

[2] Incubation period must be determined for each papain batch.

Enzyme layering technique

1. Wash cells **twice** in normal saline either manually or using an automatic cell washer.
2. Prepare a 2-3% cell suspension in normal saline.
3. Add **one** volume patient's serum to 6 x 50 mm test-tube.
4. Add **one** volume papain solution, allowing it to run down the side of the test-tube.
5. Add **one** volume patient's 3% red cell suspension, carefully allowing it to run down the side of the test-tube.
6. **Do not mix** the test-tube. Check that there are three layers (cells, papain, and serum)in the test-tube.
7. Incubate the rack at 37°C for one hour.
8. Read tests microscopically.

Compatibility testing

The primary purpose of the compatibility test is to prevent a transfusion reaction. If the compatibility test fails to recognize antibodies in the serum of the recipient which would cause the rapid destruction of the red cells of the proposed donor, then a transfusion reaction will occur.

It is essential that the compatibility test should provide the ideal conditions suitable for the optimum reactivity of all clinically significant antibodies enabling them to be observed *in vitro*.

It is imperative that under normal circumstances, donor blood is tested against the intended recipient's serum prior to the transfusion. It is only in extenuating circumstances when request response time is of the essence, that a full compatibility test is not performed.

The compatibility test is performed to establish two major facts:

- To check the ABO blood group compatibility of both the donor and the recipient.
- To check that the recipient has no allo-antibodies that may give rise to a severe transfusion reaction.

The compatibility test procedure can be broadly divided into three main areas:

- Confirmation of the ABO and Rh groups of the patient which relates to the selection of suitable donor units.
- Compatibility testing of patient's serum with the proposed donor units to prevent a transfusion reaction from occurring.
- Screening the patient's serum for the presence of clinically significant antibodies.

The specimen from the patient should be a clotted blood sample less than 48 hours old, to ensure the presence of adequate levels of serum complement. Patients who have been transfused must have a fresh sample taken for crossmatching if 48 hours have elapsed since the administration of the transfusion. Crossmatched units of blood held for a patient who has been transfused must be re-crossmatched if more than 48 hours have elapsed since the transfusion.

Procedure

1. Centrifuge patient's sample for three minutes at 1000 *g*.

2. Label a 12 x 75 mm test-tube with patient's identification for patient's serum.

3. Separate serum into labelled 12 x 75 mm test-tube.

4. Perform 'rapid' ABO and Rh group.

Spin groups

1. Label four 12 x 75 mm test test-tubes with anti-A (a) ,anti-B (b), anti-AB (ab) and rapid anti-D respectively.

2. Add two volumes of each respective reagent antibody to the labelled test-tubes.

3. Add two volumes concentrated red cells to a 12 x 75 mm test-tube labelled with patient's name.

4. Add normal saline in order to prepare 10% cell suspension.

5. Add one volume of patient's cell suspension to test-tubes containing anti-A, anti-B, anti-AB and 'rapid' anti-D.

6. Mix thoroughly and centrifuge for one minute at 200 *g*.

7. Gently shake the test-tubes and observe macroscopically for agglutination.

8. Read negative results microscopically.

9. Record results and interpret.

10. Check ABO and Rh test results against groups written on request form.

11. Discard patient's 10% cell suspension.

Compatibility test[1]

1. Label compatibility test rack with patient's name.

2. Fill test rack with four columns of the appropriate number of 6 x 50 mm test test-tubes for saline room temperature, saline, albumin, and enzyme 37°C test methods.

3. Fill an additional column with 12 x 75 mm test-tubes for the indirect antiglobulin test method.

4. Label a 12 x 75 mm test-tube with "patient's name:DAT" and place in bottom right hand position of the rectangular section of the test rack.

5. Add two volumes concentrated red cells to a 12 x 75 mm test-tube labelled with patient's name.

[1] Refer to compatibility test rack diagram.

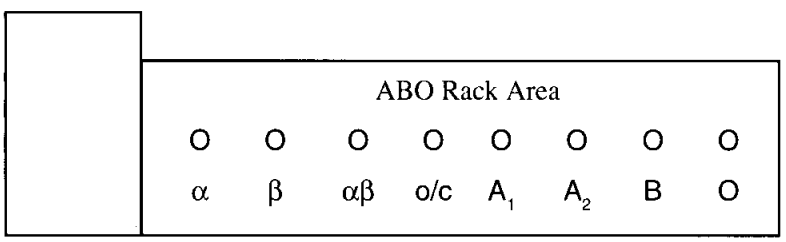
6. Wash donor's and patient's cells twice in normal saline.

7. Prepare a 3% suspension of patient's red cells.

8. Add two volumes patient's 3% cell suspension to 12 x 75 mm test-tube labelled "DAT" for direct antiglobulin test phase.

ABO group

1. Add one volume of each respective reagent antibody, anti-A(α), anti-B(β), anti-AB(αβ) to the first three 6 x 50 mm test-tubes in the ABO area of the test rack.



2. Add one volume of 3% suspension of patient's red cells to each test-tube.

3. Add one volume of 3% suspension of patient's cells to auto-agglutinin test-tube containing one volume of patient's serum.

4. Add one volume A_1, A_2, B and O cells to relevant test-tubes in ABO area of the test rack.

5. Add one volume of patient's serum to the test-tubes containing A_1, A_2, B and O cells.

Rh group

1. Add one volume of each respective reagent antibody, anti-D (1), anti-D (2) and AB serum, to the 6 x 50 mm test-tubes in the Rh area of the test rack.

2. Add one volume of patient's 3% cell suspension to 6 x 50 mm test-tubes containing anti-D (1 and 2), and AB serum.

3. Label three 12 x 75 mm test-tubes for the antibody screening cells with "S/C 1", "S/C 2" and "S/C 3".

4. Label one 12 x 75 mm test-tube for each unit to be crossmatched with patient's surname and test sequence number in rack.

5. Add four volumes of the patient's serum to the 12 x 75 mm labelled test-tubes for screening cells, labelled "S/C 1", "S/C 2", "S/C 3", and donation units.

6. Add one volume patient's serum to columns 1,2,3 and 4 of 6 x 50 mm test-tubes for:

   - Screening cells 1, 2 and 3, for the saline room temperature, saline 37°C, albumin and enzyme test phases.

   - Each donation unit to be crossmatched at saline room temperature, saline 37°C, albumin, and enzyme test phases.

7. Add one volume of papain solution to each test-tube in column 4, containing patient's serum, for enzyme test phase. Allow the solution to run down the side of the test-tube.

8. Allow the cells to run down the side of the test-tube on top of the enzyme solution for all enzyme tests[1].

9. Add one volume of screening cell 1 to 6 x 50 mm test-tubes for room temperature, 37°C albumin, and enzyme test phases.

10. Add two volumes of screening cell 1 to 12 x 75 mm test-tube for indirect antiglobulin test phase.

11. Add one volume of screening cell 2 to 6 x 50 mm test-tubes for room temperature, 37°C albumin, and enzyme test phases.

12. Add two volumes of screening cell 2 to 12 x 75 mm test-tube for indirect antiglobulin test phase.

13. Add one volume of screening cell 3 to 6 x 50 mm test-tubes for room temperature, 37°C albumin, and enzyme test phases.

14. Add two volumes of screening cell 3 to 12 x 75 mm test-tube for indirect antiglobulin test phase.

15. Add one volume of each donation unit's 3% cell suspension to 6 x 50 mm test-tubes for room temperature, 37°C albumin and enzyme test phases.

16. Add two volumes of each donation unit's 3% cell suspension to 12 x 75 mm test-tube for indirect antiglobulin test phase.

[1] Enzyme layering technique

Crossmatch control

1. Label one 12 x 75 mm test-tube 'weak Rh' and add two volumes of weak anti-Rh control reagent.

2. Add two volumes of O Rh positive cells to 12 x 75 mm test-tube for indirect antiglobulin phase control test.

3. Place two 6 x 50 mm test-tubes in the test columns 3 and 4 for the albumin and enzyme methods.

4. Add one volume of weak anti-Rh control reagent to each test-tube.

5. Add one volume of O Rh positive cells to weak Rh albumin phase control test.

6. Add one volume of papain solution to weak Rh enzyme phase control test, allowing the solution to run down the side of the test-tube.

7. Add one volume of O Rh positive cells to weak Rh enzyme phase control test, allowing the cells to run down the side of the test-tube.

8. Mix all test-tubes thoroughly except those for enzyme test phase as this is an enzyme layering method.

9. Record time on compatibility rack.

10. Separate test rack into its two parts.

11. Incubate the room temperature test phase part on the bench for one hour.

12. Incubate the rectangular section of the rack at 37°C for one hour.

13. Check red cells have sedimented to bottom of test-tube in the albumin phase tests.

14. Add one volume 30% bovine albumin to test phase in column 3 and also anti-D Rh group and AB serum control after one hour of an incubation period.

15. Incubate the rack at 37°C for an additional 15 minutes.

16. Complete the washing of the indirect antiglobulin phase tests.

17. Read tests microscopically with the exception of the indirect antiglobulin test phase.

18. Record results and interpret.

CROSSMATCH RACK LAYOUT

SAL		SAL	ALB	PAP	IAT
RT		37°C			
		0	0		0 *1
0		0	0	0	0 *2
0		0	0	0	0 *3
0		0	0	0	0 *4
0		0	0	0	
....		.....	.....	.....	.....
0		0	0	0	0 *5
0		0	0	0	0 *6
		D1	D2	AB	DAT
		0	0	0	0 *7
0	0	0	0	0	0 *8

SAL = Saline ALB = Albumin PAP = Papain

IAT = Indirect Antiglobulin Test DAT = Direct Agglutination Test

*1 Weak anti-Rh Vs R1r *2 Screening cell 3

*3 Screening cell 2 *4 Auto control

*5 Unit 2 *6 Unit 1

*7 Rhesus type *8 ABO group

0 = 6 x 50 mm glass precipitin test-tubes.

0 = 12 x 75 mm plastic/glass test-tubes.

The diagram above shows the rack layout for compatibility testing two units of blood. If further units are required, the necessary test-tubes should be inserted between unit 2 and the auto control (area shown by dotted line).

Determination of secretor status

Collection and preparation of saliva

1. Rinse mouth with distilled water.
2. Collect saliva in either a glass boiling test-tube or glass Universal container.
3. Boil container for 10 minutes to destroy enzyme activity.
4. Cool container.
5. Centrifuge for five minutes at 1000 *g* using a swing out centrifuge head.
6. Harvest supernatant.
7. Aliquot in 0.5 mL amounts into plastic 12 x 75 mm stoppered test-tubes.
8. Store at -30°C until required.

Secretor status determination

1. Dilute saliva 1:1 with saline.
2. Dilute anti-A, anti-B and anti-H in saline to a titration end-point of 1:32 to 1:64.
3. Label six 12x75mm test-tubes as follows:

1	anti-A + saliva	2	anti-A + saline
3	anti-B + saliva	4	anti-B + saline
5	anti-H + saliva	6	anti-H + saline

4. Add two drops anti-A each to test-tubes 1 and 2.
5. Add two drops anti-B each to test-tubes 3 and 4.
6. Add two drops anti-H each to test-tubes 5 and 6.

7. Add two drops diluted saliva each to test-tubes 1, 3, and 5.

8. Add two drops saline each to test-tubes 2, 4, and 6.

9. Mix thoroughly.

10. Incubate at room temperature for 30 minutes.

11. Label six 6 x 50 mm precipitin test-tubes 1-6.

12. Transfer one drop of the incubated antibody/saliva/saline mixtures to the appropriately numbered precipitin test-tubes.

13. Add one drop 3% suspension group A_2 cells to test-tubes 1 and 2.

14. Add one drop 3% suspension group B cells to test-tubes 3 and 4.

15. Add one drop 3% suspension group O cells to test-tubes 5 and 6.

16. Mix thoroughly.

17. Incubate at 18°C for 1-1½ hours.

18. Read the results microscopically.

Interpretation of results

Interpret reactions as follows:

Anti-A + saliva	negative	positive
Anti-B + saliva	negative	positive
Anti-H + saliva	negative	positive
Interpretation	secretor	non-secretor

Check that controls in test-tubes 2, 4 and 6 show strong positive reactions before accepting interpretation.

Titrations

On occasion it is necessary to try and quantify the antibody level present in the patient's serum sample. This section provides the rationale for the manual titration of serum antibodies.

1. The titre of an antibody is determined by testing a series of two-fold dilutions of the serum/plasma against selected red cell samples.

2. Results are expressed as the reciprocal of the highest dilution of serum that causes agglutination.

3. The larger the volume of the 'master dilutions', the greater the accuracy of the titration (the same size pipetting error becomes proportionally smaller the greater the volume of the whole).

4. Once the 'master dilutions' have been made, the titration should be tested as soon as possible as antibodies can be unstable in the diluted form.

5. If dilutions above 1:500 are required, prime dilutions with measured volumes should be made (0.1 mL serum made up to 50 mL using a volumetric flask gives a 1:500 dilution).

6. The volume of red cell suspension to which the diluted serum is added is not taken into account in expressing the final dilution.

7. The reactions must be graded. A system of scoring, in which the intensity of agglutination in each test-tube of the titration is taken into account, is used because of the difficulty of deciding the recise end point of a titre.

8. Titrations using a 'one stage enzyme' technique must be performed using 50% AB serum in saline as diluent, since the presence of an enzyme at high serum dilutions (where the protein concentration is low) may result in mechanical lysis.

9. Use of a 'dropping' method of making dilutions may be applied if the serum to be titrated is not available in good quantities. This ensures that exactly the correct amount of diluted serum is prepared and obviates wastage of serum.

10. In titrations with antenatal samples, care must be taken as any titration method must measure IgG and not IgM antibody since the former but not the latter can cross the placenta and enter the foetal circulation. In this respect antiglobulin titrations are better than albumin since they tend to measure IgG antibody while albumin methods measure both.

11. Automatic pipettes with disposable tips that can be changed after each dilution should be used to prevent 'carry over' of antibody which will result in higher titre values.

12. Incubation, temperature, and centrifugation times that are optimal for the antibody should be followed.

13. Concentration and age of the test red cells may affect the results.Therefore when several different samples of an antibody are to be compared all sera should be tested against red cells from the same donor. Preferably the cells should be fresh.

14. When testing a single serum sample against various red cell samples, the samples should be collected and stored in the same way and used at the same concentration in the tests.

15. Since titration results are not easily reproducible, comparisons are valid only when samples are tested concurrently. When sequential samples are tested for changing antibody titre, samples should be frozen for comparison with subsequent specimens. In comparative studies, a difference in titre of at least two dilutions can be considered significant.

Manual titrations

This protocol describes the specific procedure of performing manual titrations to measure antibody strength by testing serial dilutions of the antibody against the appropriate red cells.

1. Check sample identification.

2. Enter details on work sheet.

Master dilution

1. Calculate volume of diluted antibody required for test.

2. Label 10 12 x 75 mm test-tubes and place in a rack.

3. Place one volume of diluent (usually saline) in test-tubes numbered 2–10 using a graduated or automatic pipette.

4. Add one volume of serum to test-tubes 1 and 2 using the same pipette.

5. Mix the contents of the second test-tube and transfer one volume of the diluted serum to the third test-tube.

6. Mix the contents of the third test-tube.

7. Transfer one volume of the mixture to the fourth test-tube, and so on until the last test-tube is reached.

8. Test-tubes now contain 'master dilutions' of the serum.

Test

1. Transfer one volume of each dilution into the appropriate test-tubes of a series of 10 7 x 50 mm test-tubes (or more), starting with the highest dilution of serum first.

2. Add one volume of cell suspension[1] to each series of dilutions.

3. Mix thoroughly and incubate the tests at the appropriate temperature for the required time.

[1] Cell suspension is dependent on test method.

4. Read and grade reactions.

5. Record results and interpret.

6. Record result as titre and score value.

If results are to be displayed graphically, 'percentage' dilution gives a better 'spread' of results. Dilutions can be made up as shown below, giving a total volume of 250 ml.

Percentage concentration	Volume(µl)	
	Sample/Reagent	Diluent
100	250	0
90	225	25
80	200	50
70	175	75
60	150	100
50	125	125
40	100	150
30	75	175
20	50	200
10	25	225

Materials

Racks

6 x 50 mm precipitin test-tubes

12 x 75 mm test-tubes

Diluent

Automatic or graduated pipette

Scoring agglutination reactions

A useful way of reporting titration results is to assign a numerical value to each positive reaction in the titration, the value being based on the strength of agglutination in each test-tube. To determine the titration score of an antibody the scores assigned to each positive reaction are totalled.

Strength of reaction	Score value
4	12
3+	11
3	10
2+	9
2	8
1+	7
1	5
(+)	3
weak	2
negative	0

Test cells	Dilutions										
	N	2	4	8	16	32	64	128	256	512	Score
R_2R_2	4	4	4	3	2+	2	1	(+)	w	-	73
R_2r	4	3	3	2	1	1	-	-	-	-	50
r"r	4	4	3	2	1	1	w	-	-	-	54

Red cell antigen typing

Red cell antigen typing is basically a straightforward procedure but its simplicity can be misleading. Although generally three reagents are used (cells, serum, and suspending fluids), they are variable by nature and tend to change in character as a result of storage, age, bacterial contamination, genotype of the cells etc. Therefore, all tests must be rigidly controlled to ensure that neither false positive nor false negative results are obtained due to faults with any of the reagents. Some broad general principles applicable to all control tests can be given.

Control cells

1. All controls must be done at the same time and under the same conditions as the tests, and read immediately before them.

2. Positive controls ensure that the technique is suitable, that the serum is active, and that sufficient time has been allowed for the reaction to take place. Selected weak reacting cells are used to indicate that the serum is sufficiently potent. If strong reacting cells were used for the control, agglutination would occur with a serum which might nevertheless fail to agglutinate only weak reacting cells.

3. Negative controls indicate that the serum remains specific and the technique does not cause false positives.

4. The absorption control is to make sure that any serum which has had its anti-A or anti-B agglutinins absorbed to make it specific, remains so. Anti-A and anti-B sometimes reappear during storage.

5. The auto-agglutinin control is performed to detect false positive agglutination. An auto-agglutinin control must always be included irrespective of whether a red cell antigen is being determined, an antibody is being identified, a compatibility test is being performed, or a crossmatching difficulty is being resolved. The technique applied to the auto-agglutinin control must be identical to that applied to the cells or serum under test.

Control cells are usually provided by either the use of current reagent red cell panels or from cells stored in a cryopreservative solution.

Control cells for the various blood group systems

Antisera	Positive	Negative
Anti-A	A_1 & A_2	B & O
Anti-B	B	A & O
Anti-(A+B)	A_2 & B	O
Anti-A_1	A_1	A_2
Anti-M	MM & MN	NN
Anti-N	NN & MN	MM
Anti-S	Ss	ss
Anti-s	Ss	SS
Anti-K	Kk	kk
Anti-k	Kk	KK
Anti-Fy^a	Fy(a+b+)	Fy(a-b+)
Anti-Fy^b	Fy(a+b+)	Fy(a+b-)
Anti-Lu^a	Lu(a+b+)	Lu(a-b+)
Anti-Lu^b	Lu(a+b+)	Lu(a+b-)
Anti-Jk^a	Jk(a+b+)	Jk(a-b+)
Anti-Jk^b	Jk(a+b+)	Jk(a+b-)
Anti-P_1	P_1(4+) & P_1 wk	P_1 negative
Anti-Le^a	Le(a+b-)	Le(a-b+) & Le(a-b-)
Anti-Le^b	Le(a-b+)	Le(a+b-) & Le(a-b-)
Anti-D	$R_o r$	r'r
Anti-C	r'r	R_2R_2
Anti-E	r"r	R_1R_1
Anti-c	r'r	R_1R_1
Anti-e	r"r	R_2R_2
Anti-C^w	$R_1{}^w r$	rr
Anti-C+D	$R_1 r$	r"r
Anti-CDE	R_1R_2	r"r

Red cell antigen typing

When red cells are tested under optimum conditions with serum known to contain a specific antibody, and no interaction occurs between the cells and antiserum, it can be assumed that the cells lack the antigen against which the antibody is directed. Conversely, any interaction implies that the cells possess that antigen.

Test method

1. Wash cells twice in normal saline either manually or using an automatic cell washer.
2. Prepare a 2-3% cell suspension in normal saline.
3. Select a vial of antisera of the required specificity.

Indirect antiglobulin technique

1. Label 12 x 75 mm test-tube for each donation unit with:
   - donation number
   - test number
   - antisera specificity
2. Label 12 x 75 mm test-tube with 'positive' control and specificity.
3. Label 12 x 75 mm test-tube with 'negative' control and specificity.
4. Add volume of antisera as specified on antisera label or manufacturer's instruction insert.
5. Add the volume of cells as specified on antisera label or manufacturer's instruction insert.
6. Add appropriate cells to positive and negative control.
7. Mix all tests thoroughly.
8. Incubate test-tubes at 37°C for one hour or at manufacturer's recommended temperature and time.
9. Wash cells four times in normal saline.

10. Add two volumes AHG (antihuman globulin) to each test-tube.

11. Mix and centrifuge at 200g for one minute.

12. Read reactions macroscopically.

13. Record results on worksheet.

Albumin displacement technique

1. Label rack with test information:
   - patient's name
   - antisera specificity
2. Add one volume of antisera to 6 x 50 mm precipitin test-tubes for each typing and for positive and negative controls.
3. Label rack with test sequence number and positive and negative controls.
4. Add one volume of each donation cell suspension to the appropriate test-tube.
5. Add one volume of positive and negative cell suspension to the appropriate test-tube.
6. Mix all tests thoroughly.
7. Incubate test-tubes at 37°C for one hour.
8. Check red cells have sedimented to bottom of test-tube.
9. Add one volume 30% albumin to each test-tube.
10. Incubate test-tubes at 37°C for an additional 15 minutes.
11. Read reactions microscopically.
12. Record results on work sheet.

Saline spin method

1. Label 12 x 75 mm test-tube for each donation unit with:
   - donation number

- test number
- antisera specificity

2. Label 12 x 75 mm test-tube with 'positive' control and specificity.

3. Label 12 x 75 mm test-tube with 'negative' control and specificity.

4. Add volume of antisera as specified on antisera label or manufacturer's instruction insert.

5. Add volume of cells as specified on antisera label or manufacturer's instruction insert.

6. Add appropriate cells to positive and negative control.

7. Mix all tests thoroughly.

8. Incubate test-tubes at optimum conditions as recommended by reagent manufacturer.

9. Centrifuge at 200 *g* for one minute.

10. Read reactions microscopically.

11. Record results and interpret.

Microplate technique

ABO and Rh grouping

Microplates have been used for many years in hospital blood banks and blood transfusion centres using techniques ranging from fully automated to semi-automated test systems.

ABO grouping cells

1. Select suitable group A_1 and B cells for use as the standard reverse grouping cells.
2. Label two 12 x 75 mm test-tubes with A_1 and B.
3. Place a few drops of the concentrated red cells into the corresponding labelled test-tube.
4. Wash the cells four times in saline.
5. Add saline to each test-tube after the final wash to make a 2% cell suspension.
6. Check the concentration using a densimoneter. The red cell concentration should be standardised where possible using a calibrated instrument.

Sample testing

1. Centrifuge the samples at 1000 *g* for three minutes.
2. Sort samples into numerical order in racks of 10.
3. Place two 12 x 75 mm test-tubes in front of each sample.
4. Separate the plasma and some red cells into one test-tube respectively.
5. Add 1 mL of bromelin solution to the test-tube for the red cells.
6. Select the appropriate number of microtitre U-well plates required.
7. Add two drops (60-70µL) of plasma to each of wells G and H in the appropriate column.
8. Add the concentrated red cells to the test-tube containing the bromelin mixture.
9. Adjust the concentration to 2% cell suspension using a densimoneter.
10. Add one drop (30-35µL) of this suspension to wells A to F in the appropriate column.

11. Add one drop (30-35mL) of both the 2% group A_1 and B cells to plasma group rows G and H respectively.

12. Agitate the microplates on the plate shaker for five seconds.

13. Incubate at room temperature for 15 minutes to obtain optimum results.

14. Centrifuge the plates by taking the RCF up to 200 *g* and switching off.

15. Agitate the microplates on the plate shaker for a minimum period necessary to disperse the negative controls and cell reference rows.

16. Read the plates after mixing either visually or using an automated plate reader.

Microplate ABO test format

Reagents		1	2	3	4	5	6	7	8	9	10	11	12
Anti-CDE	A	0	0	0	0	0	0	0	0	0	0	0	0
Anti-D	B	0	0	0	0	0	0	0	0	0	0	0	0
Anti-AB	C	0	0	0	0	0	0	0	0	0	0	0	0
Anti-B	D	0	0	0	0	0	0	0	0	0	0	0	0
Anti-A	E	0	0	0	0	0	0	0	0	0	0	0	0
Cell ref	F	0	0	0	0	0	0	0	0	0	0	0	0
A_1 cells	G	0	0	0	0	0	0	0	0	0	0	0	0
B cells	H	0	0	0	0	0	0	0	0	0	0	0	0

Reuse of microplates

Microplates, although considered to be disposable items, can be reused several times. The best way to recycle the plates is as follows:

1. Soak the plates in laboratory detergent after use.
2. Rinse plates and place in an ultrasonic bath.
3. Rinse plates in distilled water.
4. Submerge plates in a bath of 0.02% Tween "20" then dry at 37°C.
5. Check individual plates for damage and discard unsuitable plates.

'Streaking' microplate method

Preparation of test cells (approximately 1.5%)

1. Label the required number of 12 x 75 mm test-tubes for samples.
2. Place one drop of the concentrated red cells into the corresponding labelled test-tube. Ensure that the Pasteur pipette is washed thoroughly between samples.
3. Wash the cells once in saline.
4. Add 2 mL of saline to each test-tube.

Setting up the tests

1. Select the appropriate number of microtitre V-well plates required. Each plate will accommodate 94 tests plus two controls.
2. Label each microtitre plate accordingly.
3. Place one volume of each reagent antibody into the appropriate number of wells of a labelled microtitre plate.
4. Place reagent antibody into each of the two control wells of each plate.
5. Add one volume of 1% bromelin to each well containing antiserum.
6. Add one volume of test cell suspension to each well containing reagent antibody and bromelin.

7. Add one volume of 1.5% positive and negative cells to the control wells.

8. Mix the contents of each well thoroughly using the microplate shaker.

Incubation of the test

1. Incubate each plate at 37°C for 50 minutes.

2. Transfer the plate to 20°C for 10 minutes.

Developing the test

1. Remove the plate to a bench surface and tilt to an angle of 70° to enable streaking of the cells in the negative reactions. Allow the cells to streak for about one minute.

2. Reverse the plate to cause the cells to streak in the opposite direction. Allow the cells to streak for about one minute.

3. Reverse the plate twice more and allow the cells to streak for approximately one minute at each stage.

Reading and interpretation of the results

1. Reading is most easily accomplished by holding the plate in an inclined position against a white background.

2. Reading of the results may require some familiarity with the technique. Tests exhibiting dubious reactions usually benefit from reversing the tilt in the opposite direction. Viewing of the tests from beneath the wells rather than from above can also enable better viewing of the reaction.

3. Positive reactions appear either as a consolidated lump of red cells which usually slides to the lowest part of the well during the tilting operation or as a flat carpet of agglutinated red cells covering the area of the bottom of the well. The agglutinated carpet of red cells will often overlap or tear during the tilting operation and will not exhibit the streaking pattern of a negative result.

4. Negative reactions demonstrate that the red cells which are unagglutinated will streak readily in a thin line upon tilting the plate.

Cold antibodies

These antibodies react best at room temperature and below, while 37°C incubation and in the indirect antiglobulin phase tests give negative or weakly positive reactions. Some cold-agglutinating antibodies, notably anti-I, -IH, -P_1 and the Lewis antibodies, bind complement following the antigen-antibody interaction. Subsequent 37°C incubation may cause the antibody to elute from the cell, but the attached complement remains and produces a positive reaction when the polyspecific anti-human serum is added. An antibody which displays these characteristics in the antibody screening test or in incompatible crossmatches should be incubated at 18°C then 4°C with specially selected cells.

Procedure

1. Label test-tubes with the number of the panel cell being tested. Include an autologous control cell, a group O cord cell and ABO compatible A_1, A_2 and B cells. Begin with a standard panel of group O cells, and prepare serum and cell suspensions as described for saline (room temperature) phase of antibody screening test. Include autologous cell control.

2. Add two drops of the serum to each test-tube.

3. Add one drop of the appropriate cell suspension to the appropriate test-tube.

4. Mix and centrifuge at 200 *g* for one minute.

5. Observe for haemolysis and examine for agglutination.

6. Incubate at room temperature (22°C) for 30 minutes.

7. Centrifuge, observe test-tubes for the presence of haemolysis, resuspend cells and observe for agglutination.

8. Record results.

9. Incubate test-tubes at 10-18°C for 30 minutes.

10. Centrifuge at 200 *g* for one minute and observe for haemolysis.

11. Return the test-tubes, after centrifugation, to 18°C for one or two minutes.

12. Read for agglutination (one or two test-tubes at a time).

13. Incubate test-tubes at 4°C for 30 to 60 minutes if necessary, and repeat step 9.

Conclusions

1. **Some cells in panel positive; auto control negative**. The antibodies most often identified by such results are anti-M, anti-N, -P_1, Lewis antibodies.

2. **All cells in panel positive; auto control negative or only weakly positive**. The antibody may be allo anti-I (rare) or anti-IH. Anti-IH occurs in individuals of groups A_1, A_1B, and (less often) B. The auto control is usually negative.

3. **All cells in panel positive; auto control positive**. A cold reacting auto-antibody, usually anti-I, may be present. Since cold auto-antibodies may mask the simultaneous presence of an allo-antibody, the serum should be auto-absorbed, and the antibody detection and/or identification procedures repeated on the absorbed serum.

4. **All group O cells negative; auto control negative; A_1 cells positive**. Anti-A_1, which occurs in a small number of A_2 and up to 25% of A_2B individuals.

Auto-absorption of cold auto-antibodies

Before meaningful antibody screening tests and compatibility studies can be done, interfering cold auto-antibodies must be removed. This can be done with the patient's untreated or enzyme-treated cells. Enzyme pre-treatment of cells results in greater antibody removal, and is more efficient on very high-titre auto-antibodies. Untreated auto-absorption is faster and easier. One or two auto-absorptions are usually satisfactory for the removal of most cold auto-agglutinins.

Procedure

1. To one volume of washed, packed autologous red cells add one volume of enzyme solution. Any enzyme may be used, usually a 1% concentration of the ficin papain or trypsin.

2. Mix and incubate at 37°C for 15-30 minutes.

3. Wash cells several times in isotonic saline to remove the enzyme solution.

4. Add one volume of patient's serum to one volume of washed packed enzyme-treated cells.

5. Mix and incubate at 4°C for 30-60 minutes.

6. Centrifuge at 1000 *g* for three minutes and harvest serum.

7. Test patient's auto-absorbed serum against autologous cells to determine if cold auto-agglutinin has been completely removed. If not, repeat absorption with fresh enzyme-treated cells.

Auto-absorption

For auto-absorption of patient's serum or plasma which contain antibodies against papainised red cells follow the steps :

1. Place some concentrated red cells in labelled 12 x 75 mm test-tube.

2. Top up with 0.85% saline.

3. Mix by gentle inversion.

4. Centrifuge at 1000 *g* for one minute.

5. Remove supernatant saline by aspiration.

6. Agitate gently and top up with saline.

7. Repeat washing cycle three times.

8. Dispense four volumes phosphate buffered saline (pH 5.4) to a separate 12 x 75 mm test-tube.

9. Add one volume 1% papain to test-tube.

10. Stopper test-tube.

11. Mix by inversion.

12. Pipette equal volumes of packed washed red cells and papain/buffered mixture into labelled 12 x 75 mm test-tube.

13. Mix gently by inversion.

14. Incubate at 37°C for required time in water-bath[1].

15. Wash cells four times in saline.

16. Remove small volume of cells for use as auto-control in the papainised panel tests.

17. Add an equal volume of patient's serum or plasma to remainder of papainised cells.

18. Mix gently by inversion.

19. Incubate at room temperature for one hour, mixing every 15 minutes gently by inversion.

20. Centrifuge at 200 *g* for five minutes.

21. Pipette supernatant serum or plasma into labelled 12 x 75 mm test-tube.

22. Repeat absorption if test results indicate that all activity against papainised cells has not been completely removed.

[1] Incubation period must be determined for each papain batch.

Manual polybrene technique

Polybrene disperses the net negative charge of red cells and causes the aggregation of normal red cells. The reduction of the intracellular distances between cells allows antigen-antibody linking to occur between antigen positive cells. The polybrene-induced aggregation is reversible by adding nacitrate; however, agglutination resulting from antigen-antibody reactions will remain.

Immediate spin phase

1. Place two volumes test serum or plasma in labelled 12 x 75 mm glass test-tube.
2. Add one volume 3% red cell suspension.
3. Add 12 volumes low ionic medium.
4. Mix thoroughly.
5. Incubate at room temperature for one minute.
6. Add two drops 0.05% polybrene.
7. Mix thoroughly.
8. Incubate at room temperature for 15 seconds.
9. Centrifuge at 1000 *g* for 15 seconds.
10. Decant clear supernatant.
11. Add two drops re-suspension medium.
12. Tilt test-tube slightly and gently rock back and forth to re-suspend cells. This should take 10-20 seconds
13. Examine the tests for agglutination.
14. Read tests within three minutes as agglutination may disperse.

Antiglobulin test

1. Add one additional drop of re-suspension medium.
2. Mix thoroughly to neutralise any residual polybrene activity.
3. Top up test-tubes with 10 mmol tri-sodium citrate in saline.
4. Mix gently by inversion.
5. Centrifuge at 1000 *g* for one minute.
6. Decant supernatant.
7. Repeat washing cycle twice more.
8. Add two drops polyspecific anti-human globulin to washed cells.
9. Mix gently.
10. Centrifuge at 200 *g* for one minute.
11. Shake test-tube gently to dislodge cell button.
12. Observe macroscopically for agglutination.
13. Record strength of reactions.
14. Record results in work book.

Materials

Low ionic medium (LIM):

5% dextrose solution prepared in sterile distilled water

2gm per litre EDTA

Adjust pH to 6.4 using 3N NaOH

Polybrene

Stock solution: 10%solution W/V in 0.85%saline

Working solution: 0.05% solution W/V in 0.85%saline, prepared by diluting stock solution 1: 200

Re-suspension medium

Tri-sodium citrate solution:

tri-sodium citrate 58.8g

distilled water 1 litre

Resuspension medium

0.2M tri-sodium citrate 60 mL

5% dextrose 40 mL

IAT wash solution (10mM tri-sodium citrate)

0.2M tri-sodium citrate 50 mL

0.85% saline 950 mL

Glass test-tubes:

All tests must be carried out using 12 x 75 mm glass test-tubes.

Elution procedure

Once an antibody has attached to red cells, either *in vivo* or *in vitro*, it can be recovered from those cells by the process known as 'elution'.The recovered antibody is called an 'eluate'.

Antibody elution is a valuable serological tool and can be applied to:

- Identification of antibodies from sera containing mixtures of antibodies.
- Confirmation of antibody specificity.
- Confirmation of the presence of weak antigens on red cell samples.
- Identification of antibodies causing haemolytic disease of the newborn.
- Identification of antibodies that have caused transfusion reactions.
- Investigation of antibodies of patients with acquired haemolytic anaemia.
- Preparation of serological typing reagents.

Elution of an antibody requires that the antigen-antibody complexes be broken to effect the release of the antibody molecules.This often involves the destruction of the red cells and a resultant release of haemoglobin into the eluate.

Some workers have found that elution of an antibody into saline results in an unstable eluate unless the eluate is to be tested on the same day as prepared. However, if the eluate is to be stored before testing, then elution into AB serum or 6% bovine albumin in saline is preferred. Since the object of elution is to recover only that antibody attached to cells, very thorough washing of the cells prior to elution is essential. A useful control to show that all free antibodies have been removed by washing is to test the supernatant saline from the last wash in parallel with the eluate.The 'wash saline' must, of course, be shown to be devoid of antibody activity before it can be assumed that any activity in the eluate has been recovered from the cells.

Rubins ether elution technique

Materials

Diethyl ether (analytical grade)

12 x 75 mm glass test-tubes

Saline (0.85% NaCl)

Procedure

1. Wash cells from which eluate has to be made, six times in large volumes of saline.

2. Add an equal volume of saline to the washed cells and mix.

3. Add analytical grade diethyl ether equal to the total volume of cells plus saline.

4. Stopper and mix by inversion. Carefully remove stopper to release volatile ether.

5. Incubate at 37°C for 30 minutes with a stopper loosely in place (or vented stopper - see notes).

6. Centrifuge at 1000 *g* for six minutes.

7. Test-tube will contain three layers:
   - top layer clear ether
   - middle layer red cell stroma
   - bottom layer haemoglobin stained eluate

8. Aspirate and discard the top layer of ether.

9. By carefully tilting the test-tube to an angle of approximately 45°, the layer of stroma will float up and allow access to the haemoglobin stained eluate, which can then be aspirated. Ensure that the air is expelled from the pipette prior to placing the tip in the eluate.

10. Incubate the eluate in an unstoppered test-tube at 37°C for 15 minutes or longer, to drive off residual ether.

11. Test eluate as required.

Note

1. If the eluate is to be stored for any length of time, it is recommended that either inert AB serum or 6% bovine albumin be used in step 2 in place of saline.
2. During the incubation stage the test-tube should be stoppered to prevent the evaporation of the ether. However, if the test-tube is tightly stoppered expansion of the ether can result in the test-tube exploding. A convenient solution to this problem is to incubate the eluate in a tightly stoppered test-tube with a narrow gauge syringe needle stuck through the stopper. The pressure caused by the expanding ether is allowed to escape but not enough ether is lost to interfere with the elution procedure.
3. Only glass equipment must be used for this technique as ether is a solvent for plastic test-tubes.
4. The diethyl ether should be stored in a special cabinet for hazardous chemicals.
5. Retain the denatured stroma layer for possible further testing.

Landsteiner and Miller heat elution

This technique is used to remove antibody from sensitized cells. The heat elution is especially useful in removing ABO antibodies and Ss antibodies.

Procedure

1. Wash antibody-coated red cells four times with normal saline.
2. Add half the volume of normal saline to one volume packed cells.
3. Agitate the suspension continuously in a water-bath at 56°C for 10 minutes.
4. Centrifuge for three minutes at 1000 *g*. Pre-heated centrifuge cups are recommended.
5. Remove the supernatant, which contains the eluted antibody, as quickly as possible.
6. Test eluate by appropriate techniques.

Lui–Easy freeze elution

This technique is used to remove antibody from sensitized cells.The Lui-Easy freeze elution is useful when only small quantities of cells are available.This technique is best for ABO antibodies.

Procedure

1. Add 1-2 drops of 0.9% NaCL to 6-8 drops of washed, packed red cells.
2. Mix and stopper.
3. Coat sides of test-tube by rotation. Place test-tube at -60 to -30°C for 10 minutes.
3. Thaw rapidly (under running tap water).
4. Centrifuge the haemolyzed cells.
5. Test clear haemolyzate against A, B, and O cells by routine techniques.

Neutralization of anti-P_1 antibodies using hydatid cyst fluid (HCF)

1. Place 11 volumes patient's serum in labelled 12 x 75 mm test-tube.
2. Add one volume hydatid cyst fluid.
3. Mix thoroughly.
4. Incubate at room temperature for 20 minutes to enable neutralisation to take place.
5. Test serum/HCF mixture against routine cell panels using same test criteria that demonstrated initial anti-P_1 reactions.
6. Negative test reactions indicate inhibition of anti-P_1 in test serum.
7. Positive test reactions indicate that either the antibody is not anti-P_1 or another antibody is present in addition to anti-P_1.

Materials

The following steps show how to deal with 'in-house' freeze-dried material.

1. Score round top of vial with glass file.
2. Snap off top carefully.
3. Add 0.5 mL distilled water using graduated pipette.
4. Allow hydatid cyst fluid to dissolve completely.
5. Transfer to 12 x 75 mm test-tube using clean Pasteur pipette.
6. Store at -30°C until required.

Destruction of IgM antibodies by 2-mercaptoethanol (2-ME) reduction

The activity of IgM antibodies is destroyed by the addition of 2-mercaptoethanol. This procedure may help in determining the immunoglobulin class of an antibody by selectively destroying antibodies of the IgM class in mixtures of IgG and IgM.

Method

1. Label two sets of test-tubes, "Test" and "Control".
2. Place 0.5 mL patient's serum into both 12 x 75 mm test-tubes.
3. Add 0.5 mL 0.1M 2-mercaptoethanol (2-ME) to test-tube marked "Test".
4. Add 0.5 mL 0.85% saline to test-tube marked "Control".
5. Mix well.
6. Incubate both test-tubes at 37°C for two hours in water bath.
7. Test the 2-ME treated serum by saline or albumin techniques.
8. Test the dilution control in parallel.

Interpretation

Test	Control	Interpretation
-	+	Antibody belongs to IgM class of immunoglobulins.
-	-	Test invalid, antibody has most likely been diluted out.
Weak positive	Strong positive	Serum contains mixture of IgM and IgG antibodies or the IgM immunoglobulin has not been completely destroyed.
+	+	Antibody was not a pure IgM or 2-mercaptoethanol was not working.

Materials

Prepare 0.1M 2-mercaptoethanol in saline by adding

- 2-mercaptoethanol 0.56 mL
- 0.85% saline 99.44 mL

Precautions

This should be prepared in a fume cupboard as 2-ME has a very foul smell and should be used with caution. Do not mouth pipette. The solution is light sensitive. Keep out of direct sunlight in a well-stoppered brown glass bottle. There is little loss of reactivity over two to three weeks, if kept at 4°C.

Note

1. A pure IgM antibody (anti-H or anti-I) and a weak IgG antibody should be used as controls. The 2-mercaptoethanol is considered to be working if it completely inactivates the IgM antibody but has no effect on the IgG antibody.
2. It is not necessary to remove the 2-mercaptoethanol by dialysis after treatment of the serum at 37°C. At the concentration used, the 2-mercaptoethanol does not interfere with agglutination.

Preparation of anti-A_1 from Dolichos biflorus

This method describes the preparation of anti-A_1 from the seeds of Dolichos biflorus by a simple extraction method.

1. Fill a 250 mL beaker with the seeds of Dolichos biflorus and transfer the seeds to the cup of a blender. Switch on the blender and grind the seeds until the particles are about the size of coarse sand. If a blender is not available, the seeds may be ground using a pestle and mortar.

2. Transfer the ground seeds to a suitably large container and add 1000 mL of phosphate buffered saline (pH 7.2).

3. Incubate the extract at room temperature overnight, mixing occasionally.

4. Transfer the supernatant fluid to centrifuge bottles and centrifuge hard for approximately five minutes to obtain a clear supernatant.

5. Filter the supernatant through sequential filters down to 0.2µM and determine the activity of the extract as shown below.

6. Add one drop of a 3-5% suspension of reagent A_1, A_2, A_1B, A_2B, B and O red cells to appropriately labelled 75 x 12 mm plastic test-test-tubes.

7. Add one drop of extract to each test-tube.

8. Mix and centrifuge at 1000 *g* for 15 seconds.

9. Gently dislodge the button of cells and observe for agglutination. Record the results.

10. The lectin should agglutinate A_1 and A_1B red cells, but not A_2, A_2B, O or B red cells. If the extract is found to react with cells other than A_1 and A_1B, carefully dilute the extract with saline until it is specific for A_1 and A_1B cells. The best way to do this is to make serial dilutions of a small amount of the extract and repeat steps 6-9 for each dilution.

11. Dilute the bulk extract to the same dilution, adding sodium azide at a concentration of 1g/L.

12. The reagent may then be dispensed into suitably sized aliquots (e.g. 2 mL) into sterile glass dropper bottles which should be labelled accordingly.

13. The stability of the reagent should be monitored at three month intervals according to the

relevant standard operational procedure. An initial expiry of 12 months may be given if initial testing is satisfactory. Before the release of a new formulation, at least three months stability data should be collected, testing monthly.

Avidity (ABO)

1. Prepare a 10% cell suspension to be used by tile method, for each A_1 and A_1B test cell.
2. Place one volume of undiluted reagent on a ceramic tile.
3. Place one volume of red cell suspension in close proximity with, but not flooding into, the reagent.
4. Mix thoroughly, using the end of a test test-tube, over an area of approximately 15mm.
5. Start stopwatch simultaneously with commencement of mixing.
6. Rock the tile gently back and forth to encourage antigen-antibody reaction.
7. Record the time when agglutinates, approximately 1mm in size, are visible macroscopically.
8. Record the time in the reagent book.
9. Compare current time with previous results.
10. Inform the head of department of any discrepancy related to possible poor performance of the reagents.

Note

1. Bulk preparations which are not needed for immediate use may be frozen at -20°C or below.

ABO absorptions

In order to produce specific typing reagents for the determination of red cell antigens, it is desirable to remove unwanted antibodies from the plasma or serum. In the majority of cases these 'unwanted antibodies' belong to the ABO blood group system and their removal enables the reagents to be used against specimens from all ABO groups.

These antibodies are, of course, anti-A, anti-B and anti-A+B. Separation of antibody mixtures is obtained by absorbing the serum or plasma with red blood cells that are 'positive' for the antibody to be removed and 'negative' for the antibody that is to remain in the serum/plasma. Under certain circumstances, it is necessary to reverse the procedure and absorb the 'wanted' antibody onto the red cells and leave the 'unwanted' in the serum. The 'wanted' antibody is then recovered from the red cells by the method of elution.

Materials

Plasma or serum donations

Concentrated red cells

0.85% saline

600 mL centrifuge bottles

Refrigerated centrifuge

400 mL transfer packs

Stripper rollers for transfer line

Hand sealer clips

40% calcium chloride

20% EDTA

0% sodium azide

1% papain

Having decided what serum or plasma pack is to be absorbed, the first stage is to check the donation ABO group.

Selection of cells for ABO absorptions

Group A

Serum/plasma will contain anti-B. Therefore group B cells are required to remove it from the material. It should be remembered that group A_2 donations may also contain anti-A_1 and this must be investigated when checking final absorbed serum or plasma.

Group B

Serum/plasma will contain anti-A and for its removal group A_1 cells are required. It must be noted that if group A_2 cells are used in place of group A_1, then the material may not be completely absorbed and anti-A_1 may still be present in the reagent.

Group O

Serum/plasma will contain anti-A and anti-B and must be absorbed with A_1B cells or a mixture of A_1 and B cells.

Anti-A is usually stronger than anti-B and to ensure that it is removed in a single absorption, the volume of A_1 cells should equal the volume of serum.

Preparation of absorption cells

1. Centrifuge the donation bags at 5000 *g* for 15 minutes and remove the plasma.

2. Transfer each donation of red cells into a 600 mL centrifuge bottle which is then filled with saline.

3. Mix the bottles contents gently by inversion.

4. After carefully balancing the bottles in the centrifuge buckets the bottles are centrifuged for 15 minutes at 2500 *g*.

5. Remove the supernatant saline; refill the bottles with saline, mix, balance and centrifuge again at 2500 *g* for 15 minutes.

6. After washing the red cells three times, the bottles are refilled and given a final centrifugation of 2500 *g* for 30 minutes in order to pack the cells hard.This hard spin reduces the plasma dilution factor that is sometimes caused by excess saline being retained in the washed cells.

7. After carefully removing the supernatant saline, the plasma to be absorbed is added to the packed cells in the ratio of equal volume of plasma to equal volume of cells.

8. The bottles are mixed by inversion several times, then placed in the 4°C refrigerator overnight.

9. The following morning, the bottles are centrifuged at 2500 *g* for 30 minutes.

10. The supernatant plasma is harvested into a clean glass donation bottle using a vacuum pump. Care should be taken not to contaminate the plasma with red cells as this would necessitate recentrifuging the bottle.

11. The absorbed plasma is converted to serum by the addition of one volume of 40% calcium chloride per 100 mL plasma. The conversion of plasma to serum may be performed at an earlier stage prior to absorption when it is first collected from the donor.

12. Transfer the plasma (plus calcium chloride) to a 400 mL transfer pack by a gravity feed method. This is accomplished as follows:

    - Place end of inlet test-tube in bottom of donation bottle.
    - Allow transfer bag to hang well below the level of the plasma.
    - Using the stripper rollers, prime the inlet test-tube by pulling the rollers away from the bottle towards the transfer pack. This operation may have to be repeated several times to pull the plasma into the inlet tubing. Once the plasma has reached a certain point, it will flow automatically when the rollers are released.
    - Allow all the plasma to run into the transfer pack, after which seal end of inlet tubing using metal clips and sealer.

13. After the recalcification process has been completed add to the final product and mix:

    - 1 mL per 100 mL of serum of 20% EDTA.
    - Sodium azide to a final concentration of 0.1% as a preservative.

14. Samples as required are taken for ABO antibody(s) and quality control evaluation.

15. The pack and samples are labelled as follows:

    - Name
    - Reagent code
    - Specificity
    - Date
    - Absorbed
    - + EDTA
    - + Sodium azide

16. The pack of absorbed recalcified plasma is stored at -20°C until the evaluation tests have been completed.

Preparation for use (machine reagents)

Reagents that are being used by automated methods such as the Olympus PK7100, Groupamatic G2000, and Technicon 16C test systems are prepared in a slightly different manner. Automated test systems may detect low levels of 'unwanted' ABO antibodies that have not been observed by the manual quality control tests and so give anomalous test results. In order to obviate this problem the following procedure is introduced after stage 2:

1. Remove supernatant saline and estimate volume of packed red cells.
2. Add 1% papain to the packed red cells in the ratio of one volume of 1% papain to 30 volumes of packed cells.
3. Mix by inversion and incubate in a 37°C water bath for 10 minutes.
4. After the incubation period the procedure is continued from stage 2 as described for manual reagents.

Evaluation of absorbed reagents

Using the sample recalcified plasma/serum that was removed after the completion of the absorption/recalcification stage the following tests are carried out:

1. The sample is tested against group A_1, A_2, B, and O papainized cells at 4°C for the presence of ABO antibodies. Positive reactions necessitate the reagents being reabsorbed with the appropriate cell or cells.

2. The sample is tested against the complete standard investigation reagent red cell panel plus Wr^a positive and cord cells as follows:
   - 4°C saline and papainized cells
   - 37°C saline and papainized cells
   - Indirect antiglobulin tests; saline cells

3. The specificity of the antibody is checked against at least three heterozygous cells, where possible, using the recommended method.

4. On obtaining satisfactory results from the above investigations the reagent is used to test 50 random red cell samples.

5. Reagents that are intended for automated machine test systems have a 20 mL sample withdrawn for machine evaluation.

6. Only on completion of all tests with satisfactory test results is the reagent passed for general issue and entered on the current stock listings.

Conversion of plasma to serum

Plasmapheresis is a convenient and practical way of obtaining large volumes of plasma from subjects with good, usable antibodies. Blood collected into ACD, CPD or CPD-A_1 is prevented from clotting by the action of the citrate that chelates the calcium present in the blood and is essential for coagulation. Such CPD plasma can be converted into serum by the addition of an excess of calcium. This, in effect, completes the coagulation cycle by converting fibrinogen to fibrin (all the factors necessary for coagulation with the exception of calcium being present in fresh plasma). A fibrin clot forms (in the absence of red cells that play no part in the coagulation cycle) and retracts, leaving serum instead of plasma.

Materials

Calcium chloride $CaCl_2$ (MW 110.99)

Distilled water

400 mL transfer packs

Solutions

Calcium chloride 40gms

Distilled water 100mL

Procedure

1. Measure volume of plasma to be converted.
2. Add 1 mL of 40% (W/V) $CaCl_2$ and mix in a beaker for each 100 mL of plasma.
3. Transfer the contents of the beaker to a 400 mL transfer pack or packs by gravity feed method, and seal inlet test-tube.
4. Place the pack of plasma into a 30°C water bath. A firm clot should form within one hour.
5. Add an additional 1 mL $CaCl_2$ per 100 mL plasma if the contents of the pack do not form a fibrin clot.
6. Remove the pack and place in -20°C deep freeze overnight once the fibrin clot has formed.

7. Thaw the pack, after which the inlet test-tube is opened and the serum allowed to run into an additional pack or beaker.

8. When the serum initially flows, the fibrin clot within the bag may block the outlet part. This is overcome by folding the bag slightly and retaining the fibrin clot in the lower part of the pack.

9. The pack is carefully rolled up thus squeezing off the serum from the clot and releasing the serum trapped in the clot. This enables the maximum amount of serum to be harvested.

10. To the final product add:

    - 1 mL per 100mL of serum of 20% Na_4 EDTA
    - Sodium azide to a final concentration of 0.1% as a preservative

11. The pack and box are labelled with the following information:

    - Reagent specificity code e.g. D
    - Reagent number e.g. 2700
    - Date e.g. 14.03.93

12. A 5 mL sample is removed for quality control tests prior to issue. The pack is then stored at -20°C until required.

EDTA addition to reagents

After conversion of plasma to serum using calcium chloride a white precipitate of insoluble calcium salts often appears after prolonged periods of storage. In order to reduce this, EDTA solution is added to the serum to bind excess calcium ions.This is carried out after the final filtration to remove the clot.

Materials

Ethylene diaminetetra-acetic acid disodium salt $C_{10}H_{14}O_8N_2Na_22H_2O$ (MW372.24)

Solutions

Na_4 EDTA 20g

Distilled water 100 mL

Procedure

1. Measure volume of plasma.
2. Add 1mL of 20% Na_4 EDTA per 100 mL of serum.
3. Mix thoroughly and store serum.

Addition of preservative to serum

The most commonly used preservative in blood typing serum is sodium azide which has a bacteriostatic effect and, at the concentration used, does not interfere with serological reactions.

After plasma has been converted to serum, sodium azide to a final concentration of 0.1% should be added.

Materials

Sodium azide (NaN3) (MW 65.01)

Solutions

Sodium azide (NaN3) 10 g

Distilled water 100 mL

This solution should be prepared in a fume cupboard.

Procedure

1. Measure volume of plasma.
2. Add 1 mL of 10% sodium azide stock solution per 100 mL of serum/plasma. This gives a final concentration of 0.1%.
3. Mix thoroughly and store.

Precautions

- Sodium azide forms very sensitive explosive metallic salts.
- Contact with water or an acid liberates a highly poisonous gas.
- Avoid contact with skin, eyes and clothing.

Centrifugation

Although agglutination will occur after a set incubation period by sedimentation, centrifugation can be used to accelerate and enhance the agglutination reaction.

There are several points to consider in the use of 'spin' methods:

- Relative centrifugal force
- Centrifugation time
- Test suspension medium

Relative centrifugal force

Relative centrifugal force (RCF) is determined by speed and the radius of the rotor head from the centre spindle to the bottom of the centrifuge cup. The centrifugal force exerted on material in a centrifuge varies according to both the speed of rotation and the rotating radius 'r'. As the value of 'r' will vary with different rotor heads, centrifuge speeds quoted relate to the type of centrifuges used within the blood transfusion centre.

The equation for the calculation of RCF is as follows:

$RCF = 0.0000111 \times r \times n^2r$

n = revolutions per minute (rpm)

r = radius in cm

The RCF used to enhance agglutination should only be enough to produce a well defined cell button. When using a gentle rocking motion the cells should become dislodged.If considerable force is required to dislodge the cells then the RFC should be re-evaluated.

Relative centrifugal force (*g*)

Centrifuge Type	'High' Spin	'Low' Spin
Petalfuge	900 *g*	200 *g*
Sumamat	900 *g*	400 *g*
Sorval	1500 *g*	550 *g*
Diacent	21250 *g*	550 *g*

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