

TECHNICAL SERIES

Anti Human Globulin Reagent
Basic Concepts and Practice



...Setting trends

Anti Human Globulin Test – Historical Perspective

The first step towards a rational approach to blood transfusion became possible with Landsteiner's discovery in 1901 of the ABO blood group system. Nearly half a century later, the next advance occurred with the introduction of serological techniques permitting the detection of incomplete antibodies, which are nonagglutinating immunoglobulin G (IgG) antibodies that sensitize red blood cells. In 1945, Coombs et al. described the use of anti human globulin test for the detection of weak and non-agglutinating antibodies in serum. Further in 1946, Coombs et al. described the use of anti human globulin test to detect in vivo sensitization of red blood cells in babies suffering from haemolytic disease of the newborn. Although the test initially was of great value in investigating Rh₀ (D) haemolytic disease of the newborn, it was not long before its versatility for the detection of other IgG blood group antibodies became evident.

Although Coombs and his coworkers were instrumental in introducing the anti human globulin test to blood group serology, the principle of the test had in fact been described by Moreschi in 1908. Moreschi's studies involved the use of rabbit anti goat antiserum to agglutinate rabbit red blood cells that were sensitized with low non-agglutinating dose of goat anti rabbit red cell serum.

Later in 1957, Dacie et al. published data regarding the importance of anti human complement activity. Further in 1960 many reports were published indicating the need for anti human complement activity in anti human globulin serum to allow the detection of antibodies in the indirect test. Though many of the antibody specificities mentioned in these reports are now considered of little clinical significance, however one specificity that was consistently mentioned and is still considered to be clinically significant was Anti-Jk^a. Further it was also shown that the presence of anti human complement activity would enhance the reaction of some antibodies namely Anti-Fy^a and Anti-K.

Preliminary Notes-Antigen and antibody related to blood group serology

Antigens are foreign substances, which initiate an immune response in human body and react specifically with antibodies. Blood group antigens that are located on the surface of red blood cells when transferred to another individual are almost certain to carry antigens not possessed by the recipient. These foreign antigens then initiate an immune response and production of antibodies in vivo.

Antibodies are counter agents produced as a defense mechanism when foreign antigen enters human body. With respect to blood group serology it means that blood group antibodies are specific and are formed against red blood cell antigens not possessed by the recipient.

Antibody nomenclature with respect to blood group serology

Red blood cell antibodies may be naturally occurring or immune in nature. Naturally occurring antibodies are produced because of the exposure of human body to certain bacteria and plant substances whose antigenic structure resembles blood group antigens. Naturally occurring antibodies are most often IgM class of antibodies.

Immune antibodies (alloantibodies) are antibodies present in an individual due to transfusion or pregnancy. Immune antibodies most often are IgG class of antibodies, sometimes IgM or a mixture of IgM and IgG class of antibodies.

IgM class antibodies

IgM antibodies are pentamers in which five polypeptide sub units are held together by disulphide bonds and joined by J chain. IgM antibodies do not cross the placenta but are most effective in activating complement.

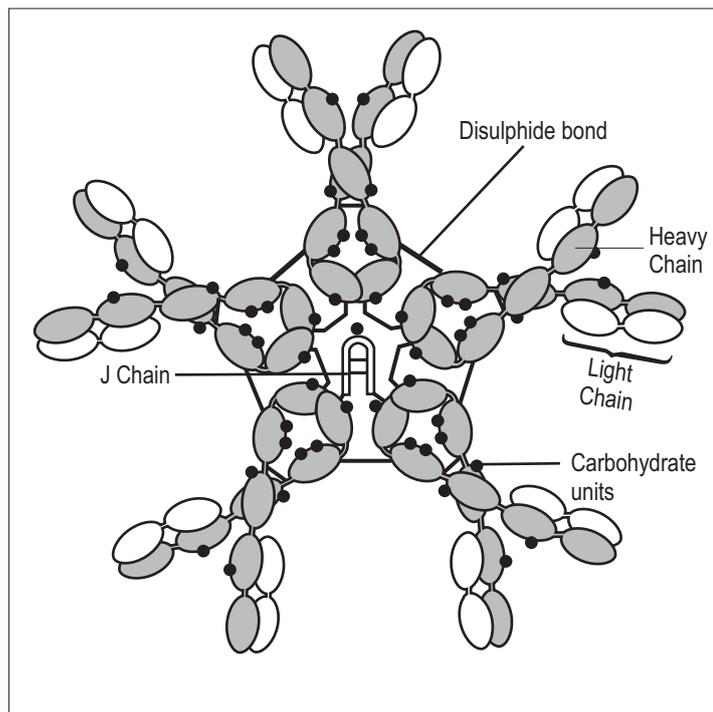


Fig1. Illustration of IgM molecule

Sialic acid present on the red cell membranes impart to the cells, a net negative charge. As a result mutual repulsive force exists between two red blood cells known as zeta potential and the red blood cells are separated by a distance. In blood group serology IgM antibodies are termed as complete antibodies because of their ability to directly bridge the distance between two red blood cells in spite of the zeta potential and bring about visible agglutination. IgM antibodies are able to bridge this distance and bring about visible agglutination.

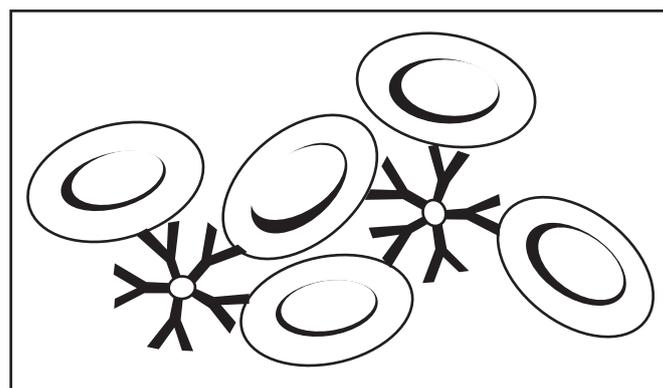


Fig.2 Illustration of complete antibodies

IgG class antibodies:

IgG antibodies are monomers with two heavy chains and two light chains. IgG is the only immunoglobulin that can cross the placental barrier, a fact which explains the role of IgG antibodies in haemolytic disease of the newborn. Four subclasses of IgG are present and they vary widely in their ability to activate complement. IgG3 molecules are highly effective in activating complement, IgG1 moderately activate complement, IgG2 slightly activate complement and IgG4 do not activate complement.

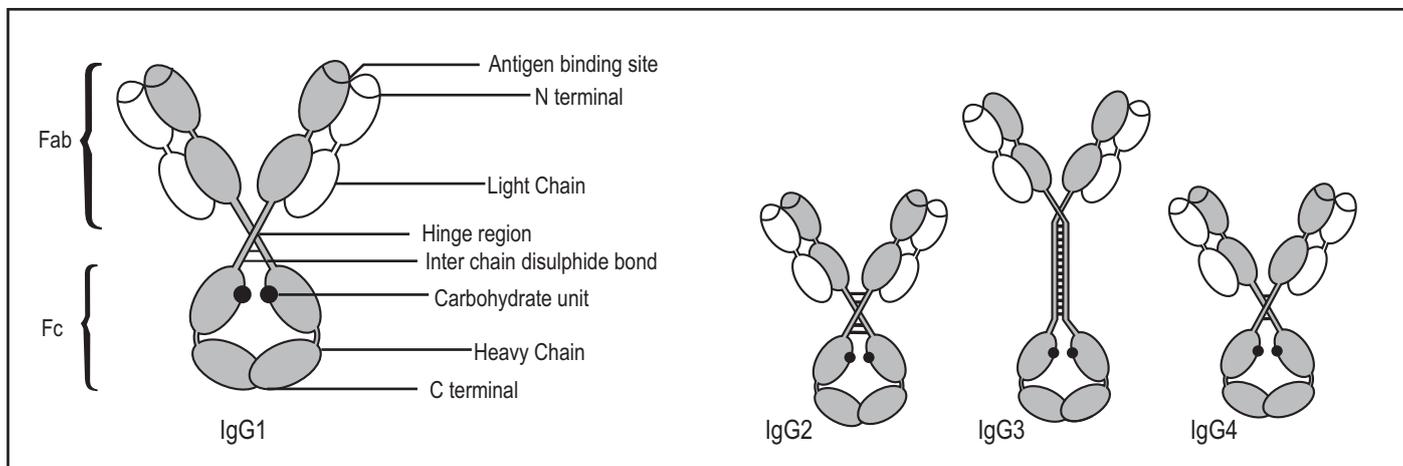


Fig. 3 Structure of four subclasses of IgG

IgG antibodies are also known as incomplete antibodies because they cannot bridge the distance between two red blood cells and bring about direct agglutination like the IgM immunoglobulins. Antibodies that do not agglutinate red blood cells directly are termed as incomplete antibodies or sensitizing antibodies and the red blood cells that have such antibodies on their surface are known as sensitized cells. Incomplete antibodies sensitized onto red blood cells can be agglutinated by anti human globulin reagent through the usage of enhancement medium to visualize agglutination.

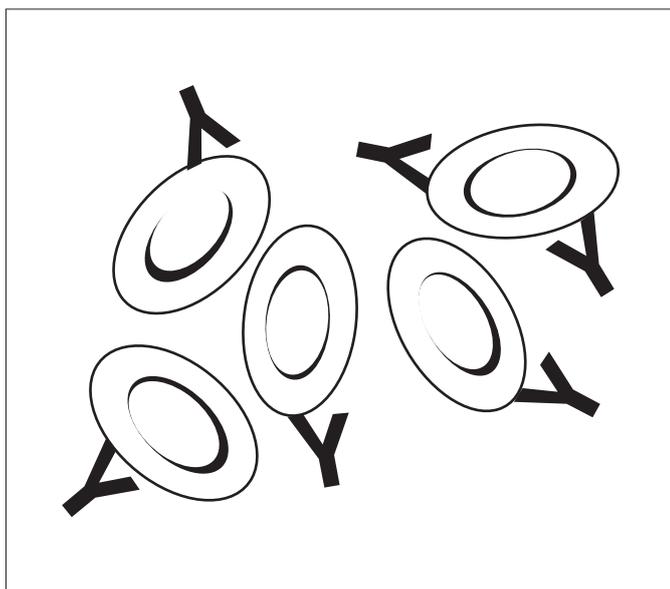


Fig. 4 Illustration of red blood cells sensitized with incomplete antibodies

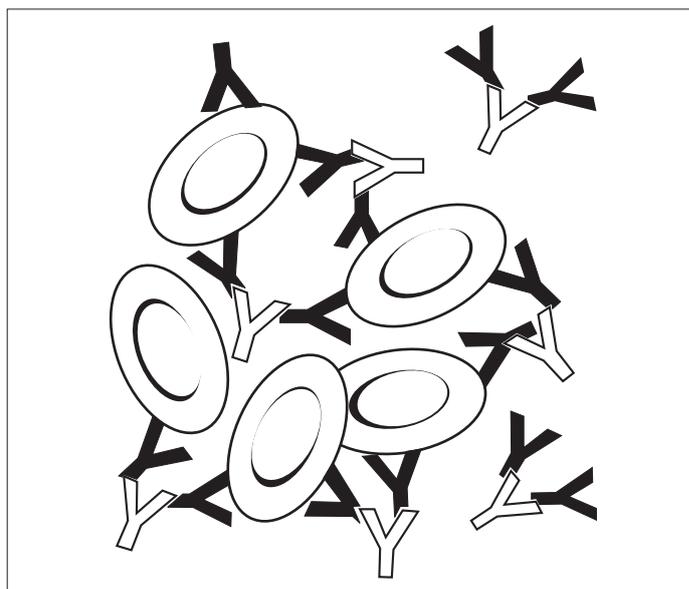


Fig. 5 Illustration of IgG sensitized red blood cells agglutinated by AHG reagent

Role of enhancement medium

An enhancement medium can either be a protein (e.g. Bovine serum albumin) or enzymes (e.g. papain, bromelain, ficin) or low ionic salt solution (LISS) or charged molecules (polybrene) that reduce zeta potential, enhance antibody uptake, enhancement in agglutination reactions.

In order to clearly understand antibody enhancement methods, it is important to consider the basic aspect of haemagglutination reactions. Agglutination is a reversible chemical reaction and occurs probably in two stages:

- Sensitization i.e. attachment of antibody to red blood cell membrane antigen.
- Formation of bridges between the sensitized red blood cells to form lattice (crosslinking) to visualize agglutination.

Thus various factors that affect these two stages can be manipulated to enhance agglutination.

Enhancement techniques

● Albumin additives

Bovine serum albumin, a dipolar protein has been used routinely for many years as an enhancement medium. The enhancement effect of albumin is attributed to its influence on the second stage of agglutination by reducing the net negative charge on the red blood cell membranes thereby potentiating agglutination. Bovine serum albumin is available as solutions of 22% or 30% concentration.

● Enzymes

Treating red blood cells with certain enzymes enhances agglutination of antibodies with red blood cell antigens. Most commonly used enzymes in immunohaematological procedures are papain, bromelain and ficin belong to thiol protease class of enzymes.

Proteolytic enzymes reduce the red blood cell surface charge by cleaving sialic acid molecules on red blood cell membrane, lowering the zeta potential and enhance agglutination. Enzyme treatment also causes spicule formation on the red blood cell membrane. This greatly increases the potential number of contact points for antibodies to react with corresponding antigens on red blood cell membrane.

The reaction of Anti-P, Lewis and Kidd blood group antibodies are stronger with enzyme treated red blood cells. Another important observation is that Lewis and Kidd blood group antibodies may lyse enzyme treated red blood cells.

● Positively charged molecules

In the presence of positively charged polymers such as hexadimethrine bromide (Polybrene[®]), protamine sulphate and poly-L-lysine, normal red blood cells exhibit spontaneous aggregation. Thus any red blood cells sensitized with antibody will exhibit enhanced agglutination.

The action of these charged molecules may be due to neutralization of negative charge contributed by sialic acid and release of water molecules that cover red blood cell membrane thereby enhancing agglutination.

Polybrene[®] generally is added to red blood cells that have been incubated with antibody at low ionic strength and low pH.

● Polyethylene Glycol (PEG)

PEG is a water-soluble polymer used as an additive to increase antibody uptake. Its action is to remove water molecule that takes up more space around the red blood cell membrane, thereby enhancing antibody uptake and reaction strength.

● LISS (Low Ionic Salt Solution) and LISS additives

LISS (approximately 0.03 M) greatly increases stage 1 uptake of antibody to red blood cells, as compared to normal saline (approximately 0.17M). To prevent lysis of red blood cells at such a low ionic strength, a non-ionic substance such as glycine is incorporated in LISS.

LISS additives may sometimes contain macromolecules (e.g. BSA) in addition to ionic salts and buffers. Addition of macromolecules to LISS reduces ionic strength with shortened incubation times.

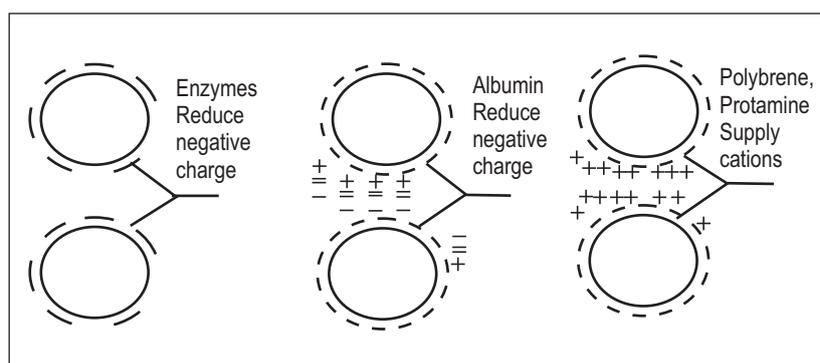


Fig. 6 Illustration of Antibody Enhancement

Complement

An observable phenomenon that may result from red blood cell antigen-antibody interaction is haemolysis. This requires co-operation of another defense mechanism of body known as the complement system. Complement is the name given to a set of approximately twenty serum proteins, which in response to antigenic stimulus react with one another in a sequential cascade.

The most important complement component is C3b, which can be, activated either by the classical or the alternate pathway. The antigen-antibody interactions activate the classical pathway that is of great significance in blood group serology.

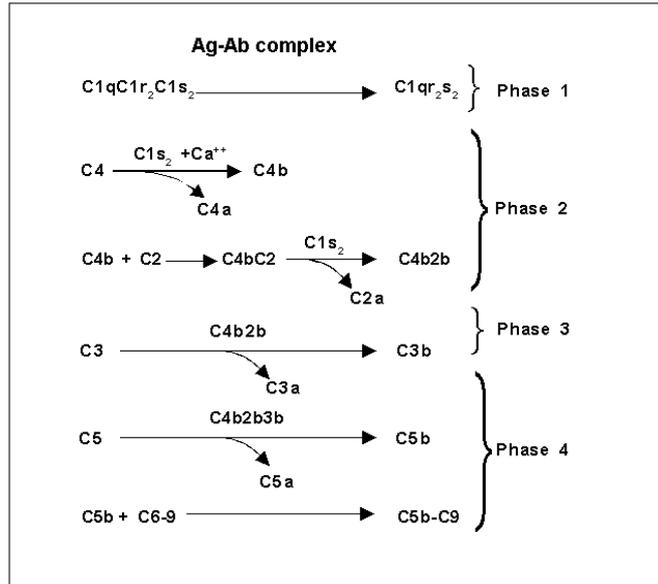


Fig. 7 Activation of Classical complement pathway

Immunoglobulin requirements for activation of classical pathway

The mode of activation of classical pathway is through binding of C1q to CH2 domain of IgM or to the CH2 domain of IgG1 or IgG3 antibodies. The C1q molecule has six Fc binding sites and in order to make a firm bond with antibody molecule, at least two of these sites must bind to antibody.

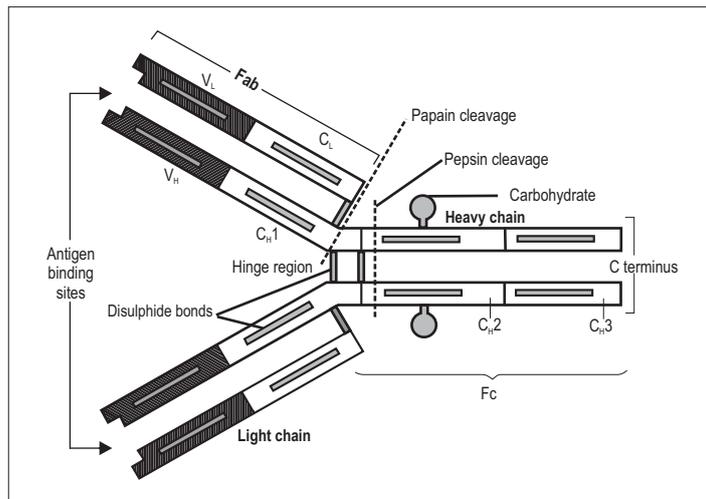


Fig.8 Illustration of immunoglobulin molecule

In case of IgM antibodies, when the molecule is in planar or star form, there is only a single binding site for C1q, as a result it cannot bind firmly and activate C1q. However when combined with antigen, IgM molecule usually assumes staple form with the F(ab) at right angle with Fc. The distortion produced by this movement exposes an additional C1q binding site. As a result IgM antibody combined with its corresponding antigen bind to two C1q sites on single IgM molecule.

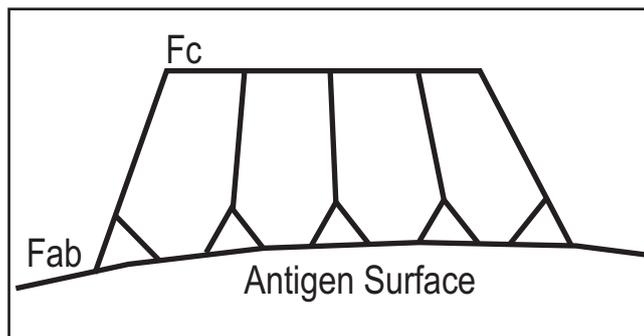


Fig 9. Illustration of staple form-IgM molecule

In case of IgG antibodies, two IgG antibodies must be present on antigen surface at a distance of 20-30 nm apart from each other. This is the maximum span of the C1q molecule. It is for this reason IgM is considered more effective in activating C1q than IgG molecule.

An important point to note is that all examples of immune Anti-D fail to activate complement. The reason being that the D antigenic sites are too far apart from each other on the red blood cell surface. As discussed two IgG molecules must be at a distance of 20-30 nm apart from each other so as to activate C1q. Since D antigenic sites are too far apart from each other, the bound IgG molecules cannot activate C1q.

Complement binding antibodies

Complement components may attach to red blood cells in vivo or in vitro via one of the probable mechanisms

- Complement binding alloantibody (blood group antibodies present in individual due to transfusion or pregnancy) specific for red blood cell antigen may cause sensitization of complement component on red blood cell surface due to transfusion.

Complement binding alloantibodies when present in low concentrations bring about the attachment of complement component upto the C3 stage, but produce little or no haemolysis. Further activation of C3 occurs only when complement is powerfully activated due to high concentration of antibodies, leading to the generation of large amount of C3b. Certain antibodies such as Anti-Jk^a are seldom lytic but invariably activate complement thereby leading to the deposition of complement components on the red blood cell surface.

Complement binding by human red cell Antibodies			
System	Antibody	Readily detectable haemolysis*	Positive antiglobulin test (anti-complement)
ABO	anti-A } anti-B } ant-A,B }	many	many
	anti-A ₁	none	very few
	anti-HI	none	few
	anti-H ⁱ	all	all
Lewis	anti-Le ^a	some	all
	anti-Le ^b	few	many
P	anti-P ₁	few	few
	ant-PP ₁ P ^k	all	all
	anti-P (auto-)	all	all
	anti-P (allo-)	many	all
Ii	anti-I } anti-i } auto-		
Sd	anti-Sd ^a	none	some
Rh	anti-D	none	none
	anti-c, E, etc.	none	none
K	anti-K	none	many
Fy	anti-Fy ^a	none	many
Jk	ant-Jk ^a	few	all
MNSs	anti-M, -N	none	none
	anti-S, -s	none	some
Lu	anti-Lu ^a , -Lu ^b	none	none
Di	anti-Di ^a	few	few-
Xg	anti-Xg ^a	none	many

- About 10-20% of patients with warm AIHA (Autoimmune haemolytic anaemia) demonstrate in vivo sensitization with complement component C3.
- In cold haemagglutinin disease, the cold reactive autoantibody can react with red blood cell antigens at temperature upto 32°C range (thermal amplitude 10-32°C). When the patient is exposed to cold temperature, red blood cell is sensitized with autoantibody, which activates complement. If the red blood cells escape haemolysis, they return to the central circulation where the temperature is 37°C and the autoantibody dissociates from the cells, leaving complement component C3d firmly bound to the red blood cell membrane.

Basic concepts of Anti human Globulin testing

Two major class of antibodies react with red blood cell antigens namely the complete antibodies and the incomplete antibodies.

Complete antibodies, or saline agglutinins will agglutinate red blood cells suspended in saline, which are usually IgM class of antibodies.

Incomplete antibodies do not react in saline but require special techniques to agglutinate red blood cells; which are IgG class of antibodies.

After combining with their corresponding antigen, blood group antibodies have the ability to activate complement. Thus sensitization of incomplete antibodies or complement component can occur either in vivo or in vitro. Anti human globulin reagent is used to detect IgG or complement sensitized red blood cells.

For detecting in vivo antibody or complement sensitization DAT (Direct Antiglobulin Test) is used whereas for detecting in vitro antibody or complement sensitization IAT (Indirect Antiglobulin Test) is used.

Anti Human Globulin Reagents

Polyspecific Anti human globulin (Coombs) Reagent:

The term 'polyspecific' reagent refers to reagent system containing blend of antibodies for more than one antigen i.e. blend of Anti human-IgG, Anti human C3b, Anti human C3d. Polyspecific reagent may be a blend of:

- Polyclonal Anti human-IgG, polyclonal Anti human C3b, polyclonal Anti human C3d.
- Polyclonal Anti human-IgG, monoclonal Anti human C3b, monoclonal Anti human C3d.
- Polyclonal Anti human IgG, monoclonal Anti human C3d.

ICSH Recommendations for Polyspecific Anti Human Globulin reagents

ICSH has recommended certain specifications, which a commercially available Anti human globulin reagent should comply:

- **Specificity:**
The reagent should only agglutinate red blood cells sensitized with antibodies and /or coated with significant levels of complement components.
- **Potency of Anti-human IgG antibodies:**
The potency of Anti-human IgG component should be evaluated by serological titration (chequer board titration).
- **Specificity and potency of Anti human complement antibodies:**
A polyspecific reagent should contain Anti-C3c and Anti-C3d at controlled levels to avoid false positive reactions or a suitable potent monoclonal Anti-C3d. It should contain little or no Anti-C4 activity. The assessment of these qualities requires red cells specifically coated with complement components for evaluating the serological potency.
- All the evaluations should be done in parallel with international reference reagents.

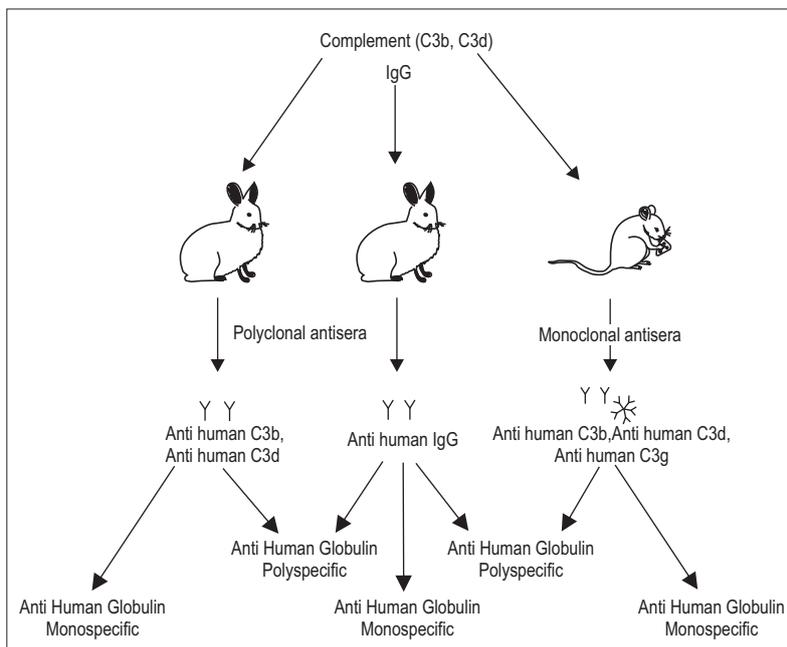


Fig. 10 Anti Human Globulin Manufacture

Polyclonal Anti human-IgG is usually prepared in rabbits, though when large volumes of antibody are required sheep or goats may be used. In production of polyclonal Anti human-IgG, animals are hyperimmunised to produce high titer, high avidity Anti human IgG class antibodies. After achieving the desired specificity and titer, the animals are bled for the production batch of reagent.

With monoclonal antibodies, the hybrid clones are screened for antibodies with the required specificity and affinity. The antibody secreting clones may then be propagated in culture or by inoculation into mice, in which case the antibody is collected in ascites. Monoclonal anti human complement may be either IgM or IgG class of antibodies.

Thus separate blends of Anti human-IgG and Anti human complement antibodies are made, and each pool is then adsorbed with A, B and O red blood cells to remove heteropsecific antibodies. The total antibody content of each pool is determined and then pools are analysed by titration method to calculate the optimum antibody dilution for Anti human IgG component and Anti human complement component.

Optimum concentration of Anti human IgG component is ascertained by chequer board titration method. In chequer board titration dilutions of Anti human IgG are reacted with various dilutions of Anti-D IgG sensitized cells. This is a critical step because excess Anti human IgG may lead to prozoning during test returning false negative results, correspondingly if Anti human IgG concentration is less than optimum it may lead to false negative results with weakly sensitized cells. The evaluation procedure should also involve parallel testing with international reference reagent (e.g. W.H.O. RIIM – Polyspecific Anti human globulin serum, available from Central Laboratory of Netherlands Red Cross Transfusion Service, Amsterdam).

Similarly optimum concentration of Anti human Complement is ascertained by sensitizing complement to O Rh₀ (D) positive red blood cells by the sucrose method, preparing various dilutions of complement sensitized cells. The optimum concentration of Anti human complement component is evaluated by reacting dilutions of Anti human complement with dilutions of cells sensitized with different levels of serially diluted complement in parallel with international reference reagent.

Once the required optimum performance characteristic for each component is ascertained, reagent blend of Anti human IgG and Anti human Complement antibodies is prepared and run as a system in parallel with the international reference reagent. Thus the final product is one that contains optimum concentration of Anti human IgG and Anti human Complement antibodies for broad spectrum reactivity.

Dilutions of IgG Sensitized Cells

	N		16		32		64		128		256		512		1024		2048		4096		
Dilutions of AHG	N	3.5	3.5	3.5	3.5	3.5	3.5	2.5	3.5	2.5	2.5	1.0	1.0	1.5	1.5	1.0	1.5	1.0	1.5	G ^r	2.0
1:2	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	2.0	2.0	2.0	2.0 ^s	1.5	1.0 ^s	1.5	1.0 ^s	2.0	2.0	1.0	1.0	
1:4	3.5	3.5	3.5	3.5	3.5	3.5	2.5	3.0	1.5	2.0	1.5	1.5	1.5	1.0 ^s	1.0 ^s	1.0	1.0 ^s	1.0	1.0 ^w	1.0 ^w	
1:8	3.5	3.5	3.5	3.5	3.0 ^s	3.0 ^s	2.0 ^w	3.0	1.5	1.5	1.0 ^s	1.5	1.0 ^s	1.5	1.0 ^s	1.0	1.0 ^w	1.0 ^s	1.0 ^w	1.0	
1:16	3.5	3.5	3.5	3.0 ^s	3.0 ^s	2.5	2.0	2.0	1.5	1.5	1.5	1.5	1.0 ^s	1.0 ^s	1.0 ^s	1.0 ^s	1.0 ^w	1.0	1.0 ^w	1.0	
1:32	3.5	3.5	2.0	3.0 ^s	3.0 ^s	2.5	2.0	2.0 ^w	2.0	1.0 ^s	1.5	1.0 ^s	1.0 ^s	1.0	1.0	1.0	1.0 ^w	0.5	1.0 ^w	Gr	
1:64	3.5	3.5	2.0 ^s	2.0	2.0	2.0	2.0	1.5	1.0 ^s	1.0 ^w	Gr	0.5	0.5	0.5	Gr	---					

W.H.O. R III M
 Commercially available Polyspecific AHG

Fig. 11 Chequer board titration results

Monospecific Coombs Reagent:

Monospecific reagents for use in anti human globulin test are prepared by similar procedure, however the reagent contains antibody of only one specificity.

- Monospecific Anti human IgG reagent contains only polyclonal Anti human IgG
- Monospecific Anti human C3b reagent contains only polyclonal Anti human C3b or monoclonal Anti human C3b.
- Monospecific Anti human C3d reagent contains only polyclonal Anti human C3d or monoclonal Anti human C3d.

Direct Anti Human Globulin Test (DAT)

DAT is used to detect in vivo sensitization of red blood cells with immunoglobulin, complement or both. A positive DAT, with or without shortened red blood cell survival, may result from:

- Autoantibodies to intrinsic red blood cell antigens.
- Alloantibodies in recipients circulation reacting with antigens on recently transfused donor red blood cells.
- Alloantibodies in donor plasma, plasma derivatives or blood fractions, which react with antigens on red blood cells of transfusion recipients.
- Alloantibodies in maternal circulation, which cross placenta and sensitize foetal red blood cells (HDN).
- Antibodies directed against certain drugs, which bind to red blood cell membranes (e.g. Penicillin)
- Adsorbed proteins, including immunoglobulins, which attach to abnormal membranes or red blood cells modified by therapy with certain drugs, notably those of cephalosporin group.
- Complement components or rarely IgG bound to red blood cells after administration of drugs such as quinidine and phenacetin may induce drug/anti drug interaction.
- Non red blood cell immunoglobulins associated with red blood cells in patients with hypergammaglobulinaemia or recipients with high dose of intravenous gammaglobulin.
- In-patients with organ transplantation, passenger lymphocytes of donor origin produce antibodies directed against ABO or other antigens on the recipient's cells, causing a positive DAT.
- Patients receiving ALG (antilymphocyte globulin) or ATG (antithymocyte globulin) of animal origin may develop a positive DAT within a few days, apparently related to high titer heterophile antibodies in these products and the presence of corresponding antibodies in animal derived AHG sera.

Major applications of DAT in blood group serology

Haemolytic disease of the newborn (HDN)

In HDN foetal red blood cells in vivo are sensitized with IgG alloantibody of maternal origin thereby demonstrating a positive DAT with cord red blood cells. The most commonly observed HDN is due to Rh_o (D) incompatibility between mother and foetus.

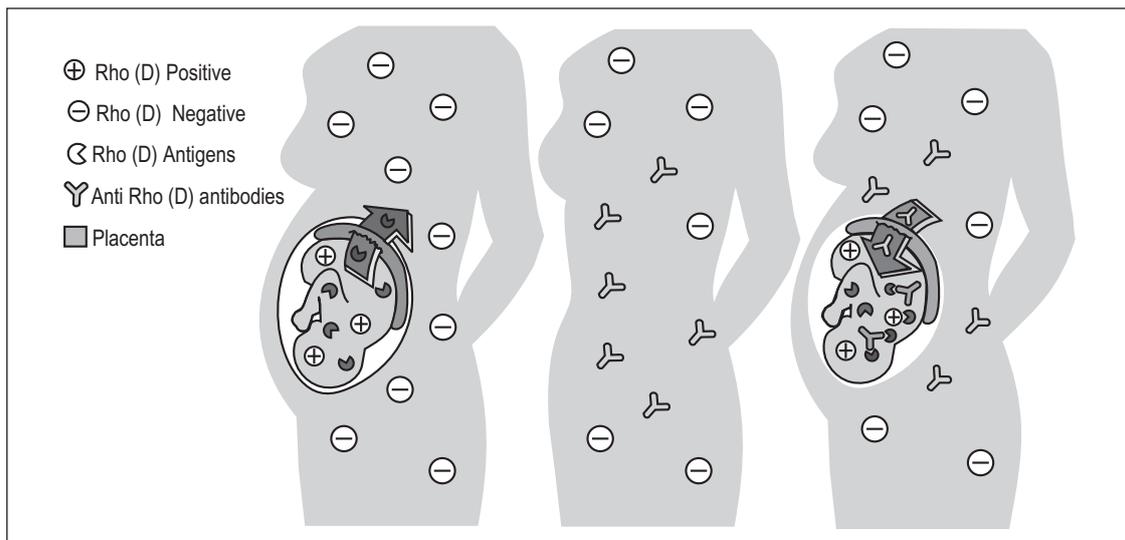


Fig. 12 Illustration of HDN

If the father is Rh_o (D) positive and the mother is Rh_o (D) negative and during first pregnancy their progeny inherits Rh_o (D) positive red blood cell antigens. During parturition, the foetal red blood cells can enter mother's circulation providing antigenic stimulus for the production of Anti-D antibodies. These Anti-D antibodies normally will not have any effect during the first Rh-incompatible pregnancy unless the mother has Anti-D antibodies by previous incompatible blood transfusions.

During subsequent pregnancy, for the same couple, if the foetus is Rh_o (D) positive again, the Anti-D antibodies will be activated along with the presence of Anti-D antibodies from the first pregnancy already in the circulation. Since the IgG antibodies cross the placental barrier, these circulating Anti-D will sensitize and destroy foetal Rh_o (D) positive cells. This process is demonstrated by a positive DAT on cord red blood cells.

Transfusion Reactions

A patient will demonstrate positive DAT, if serum contains antibodies against red blood cell antigens of donor red blood cells. Likewise antibody present in donor plasma may also react with recipient red blood cells thereby demonstrating positive DAT.

Other immune haemolytic diseases

A positive DAT may be observed due to acquired haemolytic anaemia probably because of autoantibodies directed against individuals own intrinsic red blood cell antigens.

Classification of Autoimmune Haemolytic Anaemia:

- Warm Autoimmune Haemolytic Anaemia (WAIHA)
 - Primary (idiopathic)
 - Secondary (to conditions such as lymphoma, SLE, carcinoma, drug therapy)
- Cold Agglutinin Syndrome (CAS)
 - Primary (idiopathic)
 - Secondary (to conditions such as lymphoma, mycoplasma pneumonia, infectious mononucleosis)
- Mixed type AIHA
 - Primary (idiopathic)
 - Secondary (to conditions such as SLE, lymphoma)
- Paroxysmal Cold Haemoglobinuria (PCH)
 - Primary (idiopathic)
 - Secondary (to conditions such as syphilis, viral infections)
- DAT Negative AIHA
 - Primary (idiopathic)
 - Secondary (to conditions such as lymphoma, SLE)

Drug induced haemolytic anaemia:

Also certain drugs namely penicillin, procainamide, cephalosporins may also be associated with immune red blood cell destruction thereby demonstrating a positive DAT.

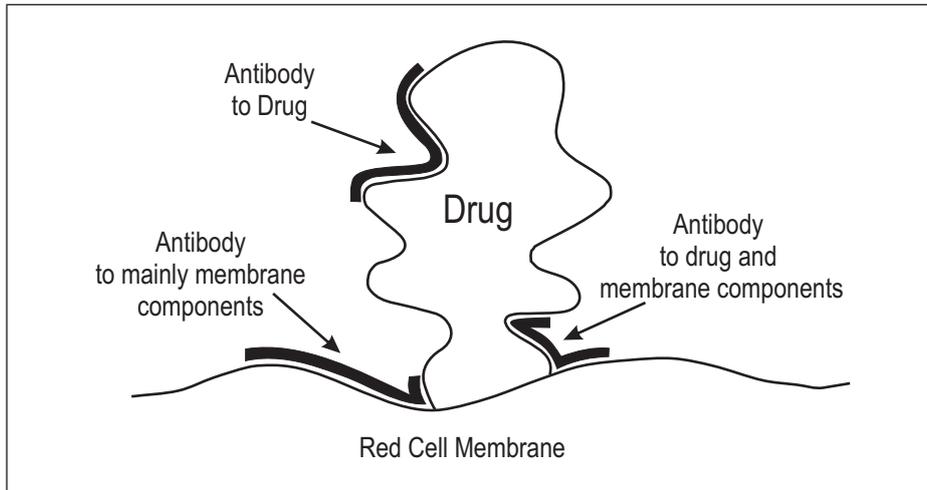


Fig. 13 Illustration of Drug induced antibody reactions

Importance of serological studies in DAT positive results

As per AABB technical manual, three investigation approaches are helpful in evaluation of positive DAT:

- Test the DAT positive red blood cells with Monospecific Anti human-IgG and Monospecific Anti-C3d reagents to characterize type of proteins sensitized with red blood cell membrane.
- Test serum/plasma to detect and identify clinically significant antibodies to red blood cell antigens.
- Test eluate prepared from sensitized red blood cells with a panel of reagent red blood cells to define whether the sensitized protein is immunoglobulin or complement component. Elution frees antibody from sensitized red blood cells and recovers antibody in usable form. When only complement is sensitized, eluates are frequently non-reactive.

Probable serological findings with DAT positive-AIHA/Drug induced HA:

Parameter	WAIHA	CAS	Mixed type AIHA	PCH	Drug induced AIHA
DAT positive result	IgG/ IgG + C3/ C3	Mostly C3	IgG +C3	Mostly C3	IgG/IgG + C3
Immunoglobulin Type	IgG (sometimes IgA or IgM rarely alone)	IgM	IgG, IgM	IgG	IgG
Eluate	IgG	Non reactive	IgG	Non reactive	IgG
Serum	<ul style="list-style-type: none"> • May React by IAT • May haemolyse enzyme treated red cells at 37°C • Mostly agglutinate enzyme treated red cells at 37 °C • May agglutinate untreated red cells at 20°C • Rarely agglutinate untreated Cells at 37 °C 	<ul style="list-style-type: none"> • IgM haem-agglutinating antibody reactive at 4 °C usually react at 30°C in albumin 	<ul style="list-style-type: none"> • IgG IAT reactive antibody • IgM haem agglutinating antibody usually react at 30 - 37°C in saline, also may react at 4°C saline 	<ul style="list-style-type: none"> • IgG biphasic haemolysin (Donath Landsteiner antibody) 	<ul style="list-style-type: none"> • IgG antibody similar to WAIHA
Specificity	Usually Rh Specificity	Usually Anti-I but can be Anti-i, rarely Anti-Pr	Usually specificity unclear, can be Anti-I, Anti-i or other cold agglutinin specificities	Anti-P (non-reactive with p and P ⁺ red cells)	Specificity often Rh related

Indirect Anti human Globulin Test (IAT)

In IAT procedures, serum or plasma is incubated with red blood cells, washed to remove unbound globulins. Agglutination that occurs after addition of Anti human Globulin reagent indicates reaction between antibody in the serum and antigen present on the red blood cell membrane.

Applications of IAT

IAT determines in vitro sensitization of red blood cells and is used in following situations:

- Detection of incomplete antibodies to potential donor red blood cells, pregnant women, blood donors.
- Identification of antibody specificity using a panel of red blood cells with known antigenic profile.
- Determination of red blood cell phenotype using known antisera (e.g. D^u testing)
- Titration of incomplete antibodies.

Probable sources of Error in Antihuman Globulin Testing

False Negative Results

- Neutralization of Antihuman Globulin Reagent
- Failure to wash cells adequately to remove all serum/plasma. Fill tube atleast three fourth full of saline for each wash.
 - If increased serum volumes are used, routine wash may be inadequate. Wash additional times more than three or four wash phases.
 - Contamination of Anti human globulin reagent by extraneous protein. Do not use finger or hand to cover tube. Contaminated droppers or wrong reagent dropper can neutralize entire vial of Anti human globulin reagent.
 - High concentration of IgG paraproteins in test serum (cryoglobulin). Wash additional times more than three or four wash phases.
- Interruption in testing
 - Bound IgG may dissociate from red blood cells or leave too little IgG to detect or may neutralize Anti human globulin reagent. Perform the test immediately.
 - Agglutination of IgG coated cells will weaken. Centrifuge and read immediately.
- Improper reagent storage
 - Anti human globulin reagent may loose reactivity if frozen. Reagent may become bacterially contaminated. Store at the recommended storage condition.
 - Excess heat or repeated freeze/thaw cycles may cause loss of reactivity of Anti Human globulin reagent. Replace the reagent back to the recommended storage condition.

- Improper procedure
 - Overcentrifugation may pack cells so tightly that agitation required to resuspend cells breaks up agglutinates. Undercentrifugation may not be optimal for agglutination. The optimum centrifugation speed should be ascertained for each centrifuge.
 - Failure to add test serum, enhancement medium or Anti human globulin reagent may lead to negative test result. Follow the manufacturers instructions meticulously.
 - Too heavy red cell concentration may mask weak agglutination. Too light suspension may be difficult to read.
 - Improper/insufficient serum:cell ratio
- Complement
 - Rare antibodies, notably Anti-Jk^a and Anti-Jk^b may only be detected when polyspecific Anti human globulin reagent is used and active complement is present.
- Saline
 - Low pH of saline solution can decrease sensitivity of Anti human globulin test. Optimal wash solution for most antibodies is pH 7.0-7.2. It has been observed that commercially available infusion saline/ saline stored in plastic containers can seriously compromise the sensitivity of anti human globulin test. Saline stored in plastic containers and further autoclaved leads to leaching of certain chemicals which shifts the pH to the acidic side and impacts the sensitivity of anti human globulin test. Preferably use phosphate buffered saline as wash solution or suspending medium.
 - Some antibodies may require saline to be at specific temperature to retain antibody on red blood cell. Use 37°C or 4°C saline.

False Positive Results

- Particles or Contaminants
 - Dust or dirt in glassware may cause clumping of cells. Fibrin or precipitates in test serum may similarly produce cell clumps that mimic agglutination
- Improper procedure
 - Overcentrifugation may pack cells so tightly that they do not easily disperse and appear positive
 - Centrifugation of test with polyethylene glycol or positively charged polymers prior to washing may create clumps that do not disperse.
- Cells with positive DAT result
 - Cells that are positive by DAT will also be positive in any indirect antiglobulin test. In such case antibodies should be eluted from the sensitized cells.
- Complement
 - Complement components, primarily C4, may bind to cells from clots or from CPDA-1 donor segments during storage at 4°C and occasionally at higher temperature. For DATs, use red blood cells anticoagulated with EDTA, ACD or CPD
 - Samples collected in scratched glass tubes can lead to spurious activation of complement.
 - Complement may attach to cells in specimens collected from infusion lines used to administer dextrose-containing solutions. Strongest reactions are seen when large bore needles are used or when sample volume is less than 0.5 ml.

Coombs Control Cells/Complement Coated Cells

Coombs control cells should be used routinely in direct and indirect Anti Human Globulin test. Coombs control reagent is Anti-D IgG sensitized, washed and made up to a 5% suspension. Coombs control cells are used for:

- Procedural validation of tests employing Coombs reagent. Coombs control cells are added after performing Anti Human Globulin test. To a negative result after addition of Coombs control cells, agglutination indicates that AHG was indeed added and that it has not been neutralized.
- Functional validation of Coombs reagent. The performance of Coombs reagent can be validated as a quality control measure on routine basis.

Similarly complement coated cells can also be prepared in vitro. Thus complement coated cells can also be used for functional validation of Coombs reagent.

Now with commercially available red blood cell stabilizing solution, the Coombs control cells and complement coated cells can be prepared in situ and stored in cell stabilizing solution for long term use and storage.

Appendix

Indirect Anti human Globulin Test for the detection of Red blood cell antibodies

● Saline Phase Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. Polyspecific AHG or Anti-IgG reagent.
3. Coombs control cells.
4. Donor cells/ reagent red blood cells

Procedure:

1. Add two drops of serum to properly labeled test tubes.
2. Add one drop of reagent red blood cells or donor red blood cells as a 2-5% saline suspension to each tube and mix.
3. Centrifuge and observe for haemolysis and agglutination. Grade and record the results.
4. Incubate at 37°C for 30-60 minutes.
5. Centrifuge for 15-20 seconds at approximately 900-1000 g and observe for haemolysis and agglutination. Grade and record the results.
6. Wash the red blood cells three or four times with saline and completely decant after the final wash.
7. Add AHG reagent to the dry cell button according to the manufacturers instructions. Mix well.
8. Centrifuge for 15-20 seconds at approximately 900-1000 g and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

● Albumin Phase Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. Bovine albumin (22% or 30%).
3. Polyspecific AHG or Anti-IgG reagent.
4. Coombs control cells.
5. Donor cells/ reagent red blood cells

Procedure:

1. Add 2 drops of serum to properly labeled tubes.
2. Add an equivalent volume of 22% or 30% bovine albumin (unless manufacturers directions state otherwise).
3. Add 1 drop of 2-5% saline suspended reagent or donor red blood cells to each tube and mix.
4. Incubate at 37°C for 15-30 minutes.
5. Centrifuge and observe for haemolysis and agglutination. Grade and record the results.
6. Wash the cells three or four times with saline and completely decant after final wash.
7. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
8. Centrifuge and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

● LISS Phase Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. LISS
3. Polyspecific AHG or Anti-IgG reagent.
4. Coombs control cells.
5. Donor cells/ reagent red blood cells

Procedure:

1. Wash reagent or donor red blood cells three times in normal saline and completely decant saline after last wash.
2. Resuspend the cells to a 2-5% suspension in LISS.
3. Add two drops of serum to properly labeled tube.
4. Add two drops of LISS suspended red blood cell suspension and incubate according to manufacturers direction. Typically this is 10-15 minutes at 37°C.
5. Centrifuge according to manufacturers direction. Typically this is 15-30 seconds at 900-1000g and observe for haemolysis and agglutination by gently resuspending the cell button. Grade and record results.

6. Wash the cells three or four times with saline and completely decant after final wash.
7. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
8. Centrifuge and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

● PEG Enhanced Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. PEG (20% in PBS)
3. Polyspecific AHG or Anti-IgG reagent.
4. Coombs control cells.
5. Donor cells/ reagent red blood cells

Procedure:

1. For each sample to tested, mix 2 drops of test serum, 4 drops of 20% PEG in PBS, and 1 drop of 2-5 % red blood cell suspension.
2. Incubate according to manufacturers directions. Typically this is 37°C for 15 minutes.
3. Do not centrifuge.
4. Wash the cells four times with saline and completely decant after the final wash.
5. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
6. Centrifuge and observe for reaction. Grade and record the results.
7. Confirm the validity of negative tests by adding Coombs control cells.

● LIM (Low Ionic Medium-Polybrene) Indirect Anti Human Globulin Test

Specimen:

Serum or plasma may be used. Preferably freshly collected serum should be used.

Reagents:

1. Normal saline
2. Low ionic Medium (LIM): To a 500 ml volumetric flask add 25 g of dextrose and 1 g of Na₂EDTA.2H₂O. Fill flask to 500 ml mark with distilled water.
3. Polybrene (Commercially available)
4. Resuspending medium: 0.2 M trisodium Citrate, 5% dextrose; Working solution made by mixing 60 ml of 0.2 M trisodium citrate with 40 ml of 5% dextrose.
5. Washing solution (for Antihuman Globulin Testing): 0.01M trisodium citrate
6. Polyspecific AHG or Anti-IgG reagent.
7. Coombs control cells.
8. Donor cells/ reagent red blood cells

Procedure:

1. Prepare 1% suspension of donor or reagent red blood cells in the serum used for testing.
2. Add 1.0 ml of LIM solution. Mix and incubate for 1 minute at room temperature.
3. Add 0.1 ml of 0.05% Polybrene to each tube and mix.
4. Centrifuge according to manufacturers directions. Typically this is 10 seconds at 900-1000 g and decant the supernatant fluid. Do not resuspend cell button.
5. Add 0.1 ml of resuspending solution. Shake tube gently and observe for persistent agglutination. If strength of agglutination is weak, examine the test and a known negative control macroscopically. Do not recentrifuge.
6. If desired, the Anti Human Globulin test may be performed as follows:
 - a) Add 0.05 ml of resuspending solution to each tube and mix well.
 - b) Wash the cells three times with 0.01 M trisodium citrate solution.
 - c) Add two drops of Anti-IgG to the dry button and mix.
 - d) Centrifuge for 15 seconds at 900-1000 g. Read and record the results.
 - e) Add one drop of Coombs control cells to each negative tube.

Interpretation of results for Anti Human Globulin Tests:

1. The presence of agglutination/haemolysis after incubation at 37°C constitutes a positive test.
2. The presence of agglutination after addition of AHG reagent constitutes a positive test.
3. Anti Human Globulin tests are considered negative when no agglutination is observed after initial centrifugation and positive result with Coombs control cells. If after addition of Coombs control cells a negative result is observed then the test is invalid and must be repeated.
4. For the LIP (Low Ionic Polybrene) procedure, agglutination that persists after addition of resuspending solution indicates a positive result.

Controls:

1. The procedure used for the detection of unexpected antibodies in pretransfusion testing should be checked daily with weak antibodies.
2. When LIP technique is used test an unknown serum against reagent red blood cells, an inert serum should be tested against a random cell sample for comparative purposes.

Notes:

1. The incubation time and volume and concentration of red cells incubated are those given in literature. In all cases, the manufacturers package insert should be strictly adhered to.
2. For the PEG procedure:
 - a) Omit centrifugation after 37°C incubation, as red blood cells will not resuspend readily.
 - b) Use Anti-IgG rather than polyspecific AHG to avoid unwanted positive reactions due to C3-binding antibodies.
3. LISS additive and PEG solutions are available from various commercial sources. Manufacturers instruction should be followed when using these reagents.

● Papain -One stage Enzyme technique/ Two stage Enzyme technique

Specimen:

Serum to be tested. Preferably freshly collected serum should be used.

Reagent:

1. Reagent red blood cells
2. Polyspecific AHG or Anti-IgG reagent.
3. Coombs control cells.
4. Donor cells/ reagent red blood cells

Procedure for One stage enzyme technique:

1. Add two drops of serum to an appropriately labeled tube.
2. Add two drops of 2-5% saline suspension of reagent red blood cells.
3. Add two drops of papain solution and mix well.
4. Incubate at 37°C for 30 minutes.
5. Centrifuge and gently resuspend the cells, observe for agglutination. Grade and record the results.
6. Wash the cells four times with saline and completely decant after the final wash.
7. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
8. Centrifuge and observe for reaction. Grade and record the results.
9. Confirm the validity of negative tests by adding Coombs control cells.

Procedure for Two stage enzyme technique:

1. Add one drop of washed packed cells and one drop of papain reagent to appropriately labeled tube
2. Incubate at 37°C for 30 minutes.
3. Wash the papain treated three times with isotonic saline and prepare 2-5% cell suspension.
4. To an appropriately labeled tube add one drop of papain treated red blood cell suspension and two drops of serum under test.
5. Mix well and incubate at 37°C for 30 minutes.
6. Centrifuge and gently resuspend the cells, observe for agglutination. Grade and record the results.
7. Wash the cells four times with saline and completely decant after the final wash.
8. Add AHG to dry cell button according to the manufacturers instruction. Mix well.
9. Centrifuge and observe for reaction. Grade and record the results.
10. Confirm the validity of negative tests by adding Coombs control cells.

Antibody Titration studies

● Antibody Titration for characterizing type of antibody in serum

Specimen:

Serum (antibody) to be titrated.

Reagents:

1. Red blood cells that express the antigen(s) corresponding to the antibody specificity(ies), in a 2-5% saline suspension. Uniformity of cell suspensions is very important to ensure comparability of results.
2. Normal saline. (Dilutions may be made with albumin if desired).

Procedure:

The master dilution technique for titration studies is as follows:

1. Label ten test tubes according to the serum dilution (e.g. 1 in 1, 1 in 2, etc.).
2. Deliver one volume of saline to all test tubes except the first.
3. Add an equal volume of serum to each of the first two tubes (undiluted and 1 in 2).
4. Using a clean pipette, mix the contents of the 1 in 2 dilution several times, and transfer one volume into the next tube (1 in 4 dilution).
5. Continue the same process for all dilutions, using a clean pipette to mix and transfer each dilution. Remove one volume of diluted serum from the final tube and save it for use if further dilutions are required.
6. Label ten 10 x 75 mm or 12 x 75 mm tubes for the appropriate dilutions.
7. Using separate pipettes for each dilution, transfer two drops of each diluted serum into the appropriately labeled tubes, and add one drop of red blood cell suspension.
8. Mix well and test by serologic technique appropriate to the antibody.
9. Examine test results macroscopically, grade and record the reactions. The prozone phenomenon may cause reactions to be weaker in the more concentrated serum preparations than in higher dilutions; to avoid misinterpretation of results, it may be preferable to examine first the tube containing the most dilute serum and proceed through the more concentrated samples to the undiluted specimen.

Interpretation:

1. Observe the highest dilution that produces 1+ macroscopic agglutination. The titer is the reciprocal of the dilution. If there is agglutination in the tube containing the most dilute serum, the endpoint has not been reached, and additional dilutions should be prepared and tested.
2. In comparative studies, a significant difference in titer is three or more dilutions. Variations in technique and inherent biologic variability can cause duplicate tests to give results that differ by one dilution in either direction.
3. Titer values alone can be misleading, without additional evaluation of strength of agglutination. The observed strength of agglutination can be assigned a number and the sum of these numbers for all tubes in a titration study represents the score. The arbitrarily assigned threshold for significance in comparing scores is a difference of 10 or more between different test samples.

Examples of Antibodies Titers, Endpoint and Scores

		Reciprocal of Serum Dilution										Titer	Score
		1	2	4	8	16	32	64	128	256	512		
Sample 1	Strength:	3+	3+	3+	2+	2+	2+	1+	±	±	0	64(256)	
	Score:	10	10	10	8	8	8	5	3	2	0	64	
Sample 2	Strength:	4+	4+	4+	3+	3+	2+	2+	1+	±	0	128(256)	
	Score:	12	12	12	10	10	8	8	5	3	0	80	
Sample 3	Strength:	1+	1+	1+	1+	±	±	±	±	±	0	8(256)	
	Score:	5	5	5	5	3	3	3	2	2	0	33	

Notes:

Titration is a semiquantitative technique and technical variables greatly affect the results. Hence care should be taken to achieve the most uniform possible practices.

1. Careful pipetting is essential. Pipettes with disposable tips that can be changed after each dilution is recommended.
2. Optimal time and temperature of incubation, time and force of centrifugation must be used consistently.
3. The age, phenotype and concentration of test red blood cells influence the results. When the titers of several antibody containing sera are to be compared, all should be tested against red blood cells (preferably freshly collected) from the same donor. If this is not possible, the tests should use a pool of reagent red blood cells from donors of the same phenotype. When a single serum is to be tested against different red blood cell samples, all samples should be collected and preserved in the same manner, and diluted to the same concentration before use.
4. Completely reproducible results are virtually impossible to achieve. Comparisons are valid only when specimens are tested concurrently. In prenatal testing of sequential serum samples to detect changing antibody activity, samples should be frozen for comparison with subsequent samples. Each new sample should be tested in parallel with the immediately preceding sample. In tests with a single serum against different red blood cell samples, material from the master dilution must be used for all tests.
5. Measurements are more accurate with large volumes than with small volumes, a master dilution technique gives more reliable results than individual dilutions for a single test. The volume needed for all planned tests should be calculated and an adequate quantity of each dilution prepared.

● Antibody Titration studies for early detection of Haemolytic disease of the newborn

Specimen:

Serum for titration (containing potentially significant unexpected antibodies to red blood cell antigens, 1 ml). If possible, test the current sample in parallel with the most recent previously submitted (preceding) sample from the current pregnancy.

Materials:

1. Anti Human IgG reagent
2. Dilute bovine albumin (approximately 6% w/v), optional: 22% (w/v) bovine albumin, 1 ml; isotonic saline, 3 ml.
3. Micropipettes or equivalent: 0.1-0.5 ml delivery, with disposable tips.
4. Red blood cells: Group O reagent RBCs with double dose expression of antigen to which the serum contains antibody (use R₂R₂ RBCs when titrating Anti-D); wash three times and dilute to a 2% suspension with isotonic saline.

Quality Control:

1. Test the preceding sample in parallel with the current sample.
2. Prepare dilutions using separate pipette for each tube. Failure to do so will result in falsely high titers due to carry-over.
3. Confirm all negative reactions with Coombs control cells.

Procedure:

1. Using 0.5 ml volumes, prepare serial dilutions of serum in saline or 6% albumin. The initial tube should contain undiluted serum and the doubling dilution range should be from 1 in 2 to 1 in 2048 (total of 12 tubes).
2. Place 0.1 ml of each dilution into appropriately labeled 10 or 12 x 75 mm test tubes.
3. Add 0.1 ml of RBC cell suspension to each dilution.
4. Gently agitate the contents of each tube; incubate at 37°C for 1 hour.

5. Wash the tubes four times with saline; completely decant the final wash supernatant.
6. To the dry RBC buttons thus obtained, add Anti-IgG according to the manufacturers direction.
7. Centrifuge as for haemagglutination tests.
8. Examine the results macroscopically; grade and record the reactions.
9. Add one drop of Coombs control cells to all negative tests; recentrifuge and examine the tests macroscopically for mixed field agglutination; repeat antibody detection tests when tests with Coombs control cells are nonreactive.

Results:

The titer is reported as the reciprocal of the highest dilution of serum at which 1+ agglutination is observed. A titer greater than or equal to 16 is considered significant and may warrant monitoring for HDN by cordocentesis, high resolution ultrasound, or examination of the amniotic fluid for bilirubin pigmentation.

Notes:

1. Titration studies should be performed upon initial detection of the antibody; save an aliquot of the serum (frozen at -20°C or colder) for comparative studies with the next submitted sample.
2. When the titer is less than 16 and the antibody specificity has been associated with HDN, it is recommended that repeat titration studies be performed every 2-4 weeks, beginning at 18 weeks gestation; save an aliquot of the serum (frozen at -20°C or colder) for comparative studies with the next submitted sample.
3. When the decision has been made to monitor the pregnancy by an invasive procedure such as amniocentesis, no further titrations are warranted.
4. Each institution should develop a policy to ensure some degree of uniformity in reporting and interpreting antibody titers.
5. For antibodies to low incidence antigens consider using paternal RBCs.
6. Do not use enhancement techniques (albumin, PEG, LISS) or enzyme treated RBCs, because elevated titers may be obtained.
7. LISS should not be used as diluent in titration studies; nonspecific uptake of globulins may occur in serum-LISS dilutions.
8. Failure to obtain the correct results may be caused by incorrect technique, notably; failure to use separate pipette tips for each dilution or failure to mix thawed frozen serum.

Use of Sulphydryl Reagents to distinguish between IgM and IgG antibodies

Specimen:

2 ml of serum to be tested

Reagents:

1. Phosphate buffered saline at pH 7.3
2. 0.01 M dithiothreitol (DTT) prepared by dissolving 0.154 g of DTT in 100 ml of pH 7.3 PBS. Store at $2-8^{\circ}\text{C}$.

Procedure:

1. Dispense 1 ml of serum into each of two test tubes.
2. To one tube, labeled as control, add 1 ml of pH 7.3 PBS.
3. To the other tube, labeled as test, add 1 ml of 0.01 M DTT.
4. Mix and incubate at 37°C for 30-60 minutes.
5. Test the antibody activity in each sample by titration against red blood cells of appropriate phenotype.

Effect of Dithiothreitol on Blood Group Antibodies

Test Sample	Dilution					Interpretation
	1/2	1/4	1/8	1/16	1/32	
Serum + DTT	3+	2+	2+	1+	0	IgG
Serum + PBS	3+	2+	2+	1+	0	
Serum + DTT	0	0	0	0	0	IgM
Serum + PBS	3+	2+	2+	1+	0	
Serum + DTT	2+	1+	0	0	0	IgG+IgM*
Serum + PBS	3+	2+	2+	1+	0	

* May also indicate only partial inactivation of IgM

Notes:

1. Sulphydryl reagents used at low concentration may weaken antigens of Kell system. For investigation of antibodies in Kell system, it may be necessary to use alkylation with iodoacetic acid, followed by dialysis.
2. Gelling of serum or plasma sample may be observed during treatment with DTT. This can occur if the DTT has been prepared incorrectly, and has a concentration above 0.01 M. Gelling may also occur if serum and DTT are incubated too long. An aliquot of the sample undergoing treatment can be tested after 30 minutes of incubation, if the activity thought to be due to IgM has disappeared, there is no need to incubate further. Gelled samples cannot be tested for antibody activity because overtreatment with DTT causes denaturation of all serum proteins.

Elution Techniques

● Citric Acid Elution Method

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. Elution solution: citric acid (monohydrate), 1.3 g, KH_2PO_4 0.65 g saline to 100ml, store at 4°C.
2. Neutralizing solution: Na_2PO_4 , 13.0 g; distilled water to 100 ml; store at 4°C.
3. Supernatant saline from final wash of the red blood cells to be tested.

Procedure:

1. Chill all reagents to 4°C before use.
2. Place 1 ml of packed red blood cells in a 13 x 100 mm test tube.
3. Add 1 ml of eluting solution and note the time.
4. Stopper the tube and mix by inversion for 90 seconds.
5. Remove the stopper and promptly centrifuge the tube at 900-1000g for 45 seconds.
6. Transfer supernatant fluid to a clean test tube and add 5-6 drops of neutralizing solution; save red blood cells for use in adsorption studies if needed.
7. Check pH; adjust it, if necessary, to pH 7.0 by adding more neutralizing solution.
8. Centrifuge at 900-1000 g for 2-3 minutes to remove precipitate that forms after neutralization. Harvest the supernatant eluate and test it in parallel with supernatant saline from final wash.

Notes:

1. Once the red blood cells have been rendered DAT-negative, they may be tested for the presence of blood group antigens, except those of the Kell blood group system. Expression of antigens in the Kell system is markedly weakened after citric acid treatment.
2. Citric acid modified red blood cells may also be treated with protease and used in autologous adsorption studies.

● Cold Acid Elution

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. Glycine-HCl (0.1M, pH 3.0), prepared by dissolving 3.75 g of glycine and 2.922 g of sodium chloride in 500 ml of distilled water. Adjust pH to 3.0 with 12N HCl. Store at 4°C.
2. Phosphate buffer (0.8 M, pH 8.2), prepared by dissolving 109.6 g of Na_2HPO_4 and 3.8 g of KH_2PO_4 in approximately 600 ml of distilled water. Adjust pH, if necessary, with either 1N NaOH or 1N HCl. Dilute to a final volume of 1 litre with distilled water. Store at 4°C.
3. Normal saline, at 4°C.
4. Supernatant saline from final wash of red blood cells to be tested.

Procedure:

1. Place the red blood cells in 13 x 100 mm test tube and chill them in an ice bath for 5 minutes before adding glycine-HCl.
2. Add 1 ml of chilled saline and 2 ml of chilled glycine-HCl to 1 ml of washed red blood cells.
3. Mix and incubate the tube in an ice bath for 1 minute.
4. Quickly centrifuge the tube at 900-1000 g for 2-3 minutes.
5. Transfer the supernatant eluate into a clean test tube, and add 0.1 ml of pH 8.2 phosphate buffer for each 1 ml of eluate.
6. Mix and centrifuge at 900-1000 g for 2-3 minutes.
7. Transfer the supernatant eluate into a clean test tube, and add test in parallel with the supernatant saline from the final wash.

Notes:

1. Keep glycine in ice bath during use, to maintain correct pH.
2. Phosphate buffer will crystallize during storage at 4°C. Redissolve it at 37°C before use.
3. Addition of phosphate buffer restores neutrality to the acidic eluate. Unneutralized acidity may cause haemolysis of the reagent red blood cells used in testing the eluate. The addition of 22% bovine albumin (one part to four parts of eluate) may reduce such haemolysis.

● Glycine-HCl/EDTA Elution

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. Disodium EDTA (10% w/v): Na_2EDTA , 2 H_2O , 10 g distilled water to 100 ml.
2. Glycine-HCl (0.1 M at pH 1.5): Glycine 3.754 g; NaCl 2.922 g; distilled water to 500 ml; adjust to pH 1.5 with 12 N HCl; store at 4°C.
3. TRIS base: Hydroxymethyl aminomethane, 12.1g; distilled water to 100 ml.
4. Supernatant saline from final wash of the red blood cells to be tested.

Procedure:

1. Mix 4 ml of glycine-HCl and 1 ml of EDTA in 16 x 100 mm test tube.
2. Immediately add 1 ml of washed red blood cells and mix well.
3. Incubate at room temperature for 1-2 minutes.
4. Centrifuge the tube at 900-1000 g for 2-3 minutes.
5. Transfer the supernatant eluate into a clean test tube and adjust to pH 7.5 with 1 M TRIS base.
6. Mix and centrifuge at 900-1000 g for 2-3 minutes.

7. Transfer the supernatant eluate into a clean test tube, and test it in parallel with the supernatant saline from the final wash.

Notes:

1. Once the red blood cells have been rendered DAT-negative, they may be tested for the presence of blood group antigens, except those in the Kell system. Treatment with glycine-HCl/EDTA denatures Kell system antigens.
2. Red blood cells modified with glycine-HCl/EDTA may be treated with protease and used in autologous adsorption studies.

● Heat Elution

Specimen:

Packed DAT positive red blood cells washed six times with saline

Reagents:

1. 6% bovine albumin, prepared by diluting 22% or 30% bovine albumin with saline.
2. Supernatant saline from final wash of the red blood cells to be tested.

Procedure:

1. Mix equal volumes of washed packed cells and 6% bovine albumin in 13 x 100 mm test tube.
2. Place the tube at 56°C for 10 minutes. Agitate the tube periodically during this time.
3. Centrifuge the tube at 900-1000 g for 2-3 minutes, preferably in a heated centrifuge.
4. Immediately transfer the supernatant eluate into a clean test tube, and test in parallel with supernatant saline from final wash.

● The Donath-Landsteiner Test

Specimen:

Serum separated from freshly collected blood sample maintained at 37°C.

Reagents:

1. Freshly collected normal serum, to use as a source of complement
2. 50% suspension of washed group O red blood cells that express the P antigen.

Procedure:

1. Label three sets of three 10 x 75 mm test tubes as follows: A1-A2-A3; B1-B2-B3; C1-C2-C3.
2. To tubes 1 and 2 of each set, add 10 volumes of the patient's serum.
3. To tubes 2 and 3 of each set, add 10 volumes of fresh normal serum.
4. To all tubes, add one volume of 50% suspension of washed P-positive red blood cells and mix well.
5. Place the three 'A' tubes in a bath of melting ice for 30 minutes, and then at 37°C for 1 hour.
6. Place the three 'B' tubes in a bath of melting ice, and keep them in melting ice for 9 minutes.
7. Place the three 'C' tubes at 37°C, and keep them at 37°C for 90 minutes.
8. Centrifuge all tubes, and examine the supernatant fluid for haemolysis.

Interpretation:

The Donath Landsteiner test is considered positive when the patients serum, with or without added complement, causes haemolysis in the tubes that were incubated first in melting ice and then at 37°C (i.e. tubes A1 and A2), and there is no haemolysis in any of the tubes maintained throughout at 37°C or in melting ice. The A3, B3 and C3 tubes serve as a control for complement activity and should not manifest haemolysis.

Notes:

1. The biphasic nature of the haemolysin associated with PCH requires that serum be incubated with cells at cold temperature and then at 37°C.
2. Active complement is essential for demonstration of the antibody. Because patients with PCH may have low levels of serum complement, fresh normal serum should be included in the reaction medium as a source of complement.
3. To avoid loss of antibody by cold autoadsorption before testing, the patient's blood should be allowed to clot at 37°C, and the serum separated from the clot at this temperature.

Chequer Board titration for quality control of Anti-IgG potency in polyspecific AHG reagent and evaluation of complement potency with complement coated cells

Reagents and materials required for chequer board titration:

1. Anti-D IgG reagent with albumin titer 256-512.
2. Polyspecific AHG reagent
3. Freshly collected O Rh₀ D positive cells
4. Normal saline
5. 12 x 100 mm and 12 x 75 mm test tubes
6. Pipettes 1 ml and 5 ml
7. Table centrifuge
8. Timer
9. Water bath or laboratory incubator

Reagent Preparation Procedure:

Preparation of 2% cell suspension

1. Collect 2 ml of freshly drawn venous blood in a clean 12 x 100 mm test tube with suitable anticoagulant.
2. Centrifuge at 3000 rpm for 2-3 minutes to form a cell button.

3. Discard the supernatant.
4. Resuspend the cell button in 5 ml of normal saline.
5. Centrifuge at 3000 rpm for 2-3 minutes.
6. Repeat the washing of cells (Step 4 and 5) twice more so that the cells are washed three times.
7. After the final centrifugation, remove the supernatant without disturbing the cell button.
8. Take 0.5 ml of packed cells and resuspend them in 25 ml of normal saline to get a 2% cell suspension.

Dilutions of Anti-D (IgG) reagent

1. Take a set of ten, 12 x 100 mm test tubes and number them from 1 to 10.
2. Add 2 ml of normal saline to each of the tubes from tube number 2 to 10.
3. Add 2 ml each of Anti-D (IgG) reagent to tube number 1 and 2.
4. Mix the content of tube number 2 and transfer 2 ml of diluted reagent to tube number 3 with the help of pipette.
5. Continue this serial dilution till tube number 10.
6. Discard 2 ml of diluted reagent from tube number 10.

Cell Sensitization:

1. To each of the above dilutions of Anti-D (IgG) add 2 ml of well mixed freshly prepared 2% cell suspension.
2. Mix well all the tubes and cover them with aluminium foil.
3. Incubate the tubes at 37°C for 30 minutes, with periodic mixing.
4. Centrifuge the tubes at 3000 rpm for 2-3 minutes.
5. Remove the supernatant and resuspend the cell button in 5 ml of normal saline.
6. Centrifuge at 3000 rpm for 2-3 minutes.
7. Repeat the washing (Step 4 and 5) atleast four times.
8. Resuspend the cell button from each tube in 2 ml of normal saline to get a 2% suspension of sensitized cells.

Note:

Thorough washing of sensitized cells (after incubation) is very important as even slight traces of free Anti-D IgG can lead to false negative results.

Dilutions of Anti Human Globulin Reagent:

1. Take a set of six, 12 x 100 mm test tubes and number them from 1 to 6.
2. Add 2.5 ml of normal saline to each of the tubes from tube number 2 to 6.
3. Add 2.5 ml of Polyspecific AHG reagent to tube number 1 and 2.
4. Mix the content of tube number 2 and transfer 2.5 ml of the diluted reagent to tube number 3.
5. Continue this serial dilution till tube number 6.
6. Discard 2.5 ml of diluted reagent from tube number 6.

Preparation of Complement coated cells:

Reagents and material required:

1. LISS solution
2. Buffered saline
3. O group red blood cells 50% suspension
4. Inert O group serum
5. Test tubes 12 x 100 mm
6. Table centrifuge
7. Water bath or laboratory incubator

Preparation of 50% cell suspension of O group red blood cells

1. Collect 1 ml of freshly drawn venous blood in a clean 12 x 100 mm test tube containing suitable anticoagulant.
2. Centrifuge the tube at 3000 rpm for 2-3 minutes to form cell button.
3. Discard the supernatant.
4. Resuspend the cell button in 5 ml of buffered saline.
5. Centrifuge at 3000 rpm for 2-3 minutes.
6. Repeat the washing of cells (Step 4 and 5) twice more so that the cells are washed three times.
7. After the final centrifugation, remove the supernatant without disturbing the cell button.
8. Add 1 ml of buffered saline to the packed red blood cells to get a 50% O group red cell suspension.

Collection of inert O group serum

1. Collect 2 ml of freshly drawn venous blood in a clean 12 x 100 mm test tube.
2. Immediately centrifuge at 3000 rpm for 2-3 minutes.
3. Collect 0.5 ml of serum in a clean test tube.

Sensitization of O group red blood cells

1. Place 8.5 ml of LISS into a 20-25 ml container.
2. Add 0.5 ml of fresh inert O group serum to it.
3. Mix well and add 1 ml of 50% O group red cell suspension.
4. Mix thoroughly and incubate at 37°C for 30 minutes with occasional further mixing.
5. Centrifuge at 3000 rpm for 2-3 minutes to form a cell button.

6. Discard the supernatant and resuspend the cell button in 20 ml buffered saline.
7. Centrifuge at 3000 rpm for 2-3 minutes.
8. Repeat the washing of cells (Step 6 and 7) thrice more so that cells are washed four times.
9. After the final centrifugation, remove the supernatant without disturbing the cell button.
10. Add 14.5 ml of buffered saline to packed red blood cells to obtain 2-3% suspension of complement coated cells.

Chequer board titration:

1. Take a set of sixty, 12 x 75 mm test tubes, number and arrange them as shown below in the table:

Dilution of AHG		3% Rho (D) + ve sensitized cells							
N	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
N									
1:2									
1:4									
1:8									
1:16									
1:32									

2. Add 0.2 ml each of N (neat) AHG in the respective tubes.
3. Similarly add dilutions of AHG in their respective tubes (horizontal rows).
4. Referring to the above mentioned table add 0.2 ml each of 2% suspension of sensitized cells with Anti-D (IgG) dilutions in their respective tubes (vertical rows).
5. Mix well all the tubes.
6. Centrifuge the tubes at 3000 rpm for 20 seconds.
7. Gently dislodge the cell button and observe for agglutination.
8. Chart the results in the above given table.

Complement potency titration:

DILUTION OF AHG	2% CELL SUSPENSION OF COMPLIMENT COATED CELLS
N	
1:2	
1:4	
1:8	
1:16	
1:32	

1. Add 0.2 ml neat AHG reagent.
2. Similarly add dilutions of AHG in respective tubes.
3. Referring to the above mentioned table add 0.2 ml each of 2% suspension of complement coated cells to all the tubes containing AHG reagent with dilutions.
4. Mix well all the tubes.
5. Centrifuge the tubes at 3000 rpm for 20 seconds.
6. Gently dislodge the cell button and observe for agglutination.

Preparation of Coombs Control cells

Reagents and Materials required:

1. Anti-D (IgG)
2. Freshly collected O Rh₀ (D) positive cells
3. Normal saline
4. 12 x 100 mm test tubes
5. Pipettes 1ml and 5 ml
6. Table Centrifuge, timer.
7. Water bath or laboratory incubator.

Procedure:

Since commercially available Anti-D (IgG) reagents have albumin titer of 256-512, diluting Anti-D (IgG) reagent 1:40 to 1:50 in normal saline is enough to achieve sensitization with O Rh₀(D) positive cells.

1. Take equal volume of 1:40 to 1:50 diluted Anti-D (IgG) in a 12 x 75 mm test tube and 2% cell suspension of O Rh₀(D) positive cells.
2. Mix well and incubate at 37°C for 15 minutes. Periodic mixing during 15 minutes interval ensures thorough sensitization.
3. Remove the supernatant and resuspend the cell button in 5 ml of normal saline.
4. Centrifuge at 3000 rpm for 2-3 minutes.
5. Repeat the washing (Step 3 and 4) atleast four times.
6. Resuspend the cell button in normal saline to obtain a 2% suspension of Coombs control cells.

REACTIONS OF COMMON ERYTHROCYTE ANTIBODIES*									
	Antibody For	Saline Medium	Albumin Medium	AHG Test	Enzyme Test	In Vitro Hemolysis	Optimal C.°		
							4	24	37
Usually IgM	H, I	M	S	F	S	F	M	S	F
	i	M	S	F	S	S	M	S	F
	A, B, A, B	M	F	F	M	S	M	S	F
	Lu ^a	M	S	F	F	N	M	S	M
	Lu ^b	S	S	M	F	N	R	S	M
	M, N	M	S	S	F	N	M	M	F
	P ₁	M	S	S	S	F	M	S	F
	PP ₁ P ^c	M	M	M	M	M	S	M	M
	Le ^a , Le ^b	M	S	S	M	S	M	S	F
	Usually IgG	S, s	S	S	M	S	N	F	S
K, k, Js ^a , Js ^b		F	S	M	F	N	F	S	M
C, D, E, c, e, S			S	M	M	N	F	S	M
Fy ^a , Fy ^b		F	F	M	F	N	N	F	M
Jk ^a , Jk ^b		F	S	M	M	F	N	S	M

*M = Most (>20%), S = Some (5-20%), F = Few (1-5%), R = Rare (<1%), N = Not reported.

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