ANTIBODY DETECTION AND IDENTIFICATION

I. Antibody Screen
-Only 0.3 - 2% of people tested will have unexpected antibodies in their serum.

A. Purpose - To detect irregular or unexpected antibodies in the serum/plasma of an individual. The goal, in most cases, is to maximize detection of clinically significant antibodies, which are antibodies that can cause decreased red cell survival, while minimizing the detection of clinically insignificant antibodies.

1. Red cells may be destroyed intravascularly, through the activation of complement.

2. There may be extravascular destruction of red cells by the macrophages of the RES system.

B. Population groups requiring an antibody screen:

1. Prenatal patients- to identify IgG antibodies which can cross the placenta and attach to fetal cells, causing destruction of fetal RBC (Hemolytic Disease of the Fetus and Newborn – HDFN)

2. Pre-transfusion candidates - to evaluate the need for providing antigen negative blood. Clinically significant antibodies may cause a hemolytic transfusion reaction if the patient is transfused with red blood cells carrying the corresponding antigen. The antibody screen is superior to the crossmatch for detecting unexpected antibodies for the following reasons:

   a. Reagent RBCs express most clinically significant antigens

   b. Reagent RBCs have homozygous expression of significant antigens

   c. Strength of antigens decrease on storage; reagent cells in preservative

3. Donor blood

   a. Units containing alloantibodies cause a passive transfer of antibodies and should be made into components having a minimal amount of plasma

   b. Donor could be evaluated for reagent preparation and inclusion in the rare donor file
C. Testing

1. Patient’s serum or plasma (unknown antibody) is tested against reagent red cells (known antigens). Typically 2-3 sets of cells are tested. Method is an application of the indirect antiglobulin test.

2. Characteristics of Screening Cells:
   a. 2-5% suspension of Group O red blood cells
   b. Both Rh Positive and Rh Negative cells in the screen cell set. When using a three cell set, the Rh genotypes are: R₁₁₁₁, R₂₂₂₂, rr
   c. Homozygous expression of antigens in major blood group systems when possible (e.g., Kidd, Duffy, MNSs). Antigens from other systems are also present.
   d. Each set of cells comes with an antigram – an antigen profile or matrix that lists the antigens present on each reagent red cell. The antigram is lot specific!
      
      - + indicates the antigen is present on the RBC.
      - 0 indicates antigen is not present.
   e. May use a 2 cell screen, but will not have the same level of variety/distribution of antigens.
   f. May use a pool of 2 screening cells when screening donors. These may show mixed field agglutination when one cell in pool has the antigen and the other does not.

3. May include an immediate spin phase.
   a. Detects cold reacting IgM antibodies.
   b. This step is often omitted, to limit detection of insignificant antibodies.

4. Must include a 37°C phase.
   a. Allows for sensitization of red cells with antibody.
   b. Often a potentiator (enhancement reagent) is added prior to incubation.
   c. Strong IgG antibodies may be detected as well as strong cold antibodies (ones with wide thermal amplitude).
d. Antibodies that activate complement may demonstrate hemolysis at this phase.

e. Depending on the test method employed, reactions may not be evaluated at this phase.

5. Must include an Anti Human Globulin (AHG) phase.

a. Allows for “bridging” between sensitized red cells (agglutination).

b. Detects clinically significant (IgG) antibodies. May also detect insignificant antibodies that activate complement, if AHG reagent contains anti-complement.

6. If all cells tested yield negative reactions during all phases of testing, the screen is interpreted as negative.

7. A positive reaction with one or more cells during any phase is interpreted as a positive antibody screen.

a. All cells should be tested at all phases of testing, even if a positive reaction has been obtained. The persistence and strength of the reaction may provide clues as to the identity of the antibody.

b. An antibody identification panel should be performed to determine the specificity of the antibody(-ies) detected by the antibody screen.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Antibodies Detected</th>
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<tbody>
<tr>
<td>IS</td>
<td>Lewis, Lu^a, M, N, &amp; P_1&lt;br&gt;Cold auto antibodies (I, H, IH)</td>
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<tr>
<td>37</td>
<td>May see D, E, &amp; K&lt;br&gt;Hold over strong cold antibodies</td>
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<tr>
<td>AHG</td>
<td>Rh, Kell, Kidd, Duffy, Ss, Lu^b, &amp; Xg^a&lt;br&gt;May see cold antibodies, if they activate complement, and if the AHG reagent contains anti-complement.</td>
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D. Methods

1. Tube Testing Procedure
   
a. Patient’s serum (or plasma) is combined with the reagent red blood cells.
   
b. Test is centrifuged and read at room temperature.
   
c. Test is incubated at 37°C.
      1) If no enhancement is used, 45 min to 1 hour.
      2) If enhancement is used, 10-30 minutes.
   
d. After incubation, the test may be centrifuged and read again (depending on enhancement used).
   
e. The test is washed at least 3 times with saline. AHG reagent is added, and the test is centrifuged and read.
   
f. Negative tests are controlled with check cells.

2. Gel Testing Procedure
   
a. Patient’s serum is combined with the reagent red blood cells in a reaction chamber. Below the chamber is a microtubule filled with dextran acrylamide gel.
      1) The reagent RBCs are suspended in LISS to form a 0.8% cell suspension.
      2) The reaction chamber/microtubule is mounted in a card. There are typically 6 reaction chambers per card.
   
b. The card is incubated at 37°C for 15 minutes, up to 1 hour.
   
c. The card is centrifuged for 10 minutes, forcing the red cells into the gel, which contains anti-IgG. Unbound immunoglobulins remain in the reaction chamber (no wash step is necessary).
   
d. If an antigen/antibody reaction has occurred, the agglutinates become trapped in the gel, due to the interaction of anti-IgG and the IgG coated cells. The screen would be considered positive, and an antibody identification panel performed.
   
e. If no antigen/antibody reaction has occurred, the red cells pass through the gel, and form a pellet at the bottom of the microtubule.
3. Solid Phase Testing Procedure
   a. Patient’s serum is added to a set of microtiter wells, which have been coated
      with various red cell antigens.
   b. LISS is added as an enhancement.
   c. The microtiter plate is incubated at 37°C for 15-60 minutes.
   d. Each well in the plate is washed at least 2 times.
   e. IgG coated indicator red blood cells are added to each well.
   f. The microtiter plate is centrifuged for 1-3 minutes.
   g. If an antigen/antibody reaction has occurred, the IgG coated red cells will be
      “trapped” in the matrix, forming a diffuse pattern throughout the well. The
      screen would be considered positive, and an antibody identification panel
      performed.
   h. If no reaction has occurred, the indicator cells will form a dense pellet at the
      bottom of the well.

E. Techniques to eliminate clinically insignificant antibodies:

1. Pre-warming technique:
   a. Prewarm cells and serum separately before mixing together
   b. Incubate at 37°C up to 1 hour without an enhancement
   c. Wash with warm (37°C) saline
   d. Use monospecific AHG (anti-IgG)

2. Autoadsorption - either warm or cold, to remove autoantibodies which may mask the
   presence of clinically significant antibodies.
   a. Cold - removal of cold reacting autoantibody (usually auto anti-I)
      1) Use patient’s cells if patient has not been transfused
      2) Use rabbit cells (REST) if patient has been transfused
      3) Retest absorbed serum for unexpected antibodies that may have been
b. Warm - removal of warm autoantibody

1) Use patient’s cells which have been enzyme treated if patient has not been transfused

2) Use homologous absorption if patient has been recently transfused
   - Helpful to know patient’s phenotype

3) Retest absorbed serum for unexpected antibodies that may have been masked.

3. Saline replacement - after centrifuging serum and cells, serum is replaced with saline, re-centrifuged and re-read. The rouleaux tendency of the red cells caused by abnormal protein content of serum will disperse. (False positive).

F. Limitations:

1. Will not detect ABO incompatibility

2. May not detect antibodies to low incident antigens (not present on reagent cells)

3. May not detect antibodies exhibiting dosage, if antigens not expressed in a homozygous manner.

4. May not detect antibodies in titers below clinically detectable levels. Studies have shown that 42% of antibodies decrease in titer enough that the antibody is no longer detectable 5 years later (unless re-stimulated).

II. Antibody Identification

A. Panel cells:

1. Group O red cells selected to contain the major blood group antigens

2. Used to specifically identify antibodies directed against red cell antigens

3. Usually consist of eight to twenty vial sets

4. Supplied with a lot-specific antigen profile sheet showing the phenotype of each donor

5. Preserved in Alsever’s solution; stable for 2-4 weeks at 4°C
B. Testing: Generally, the method used to detect the antibody in the screen is used in testing the panel cells.

1. May include an auto control (patient cells + patient serum/plasma)
   a. Detect autoantibodies
   b. Detect newly forming alloantibodies in the recently transfused.
   c. If auto control is positive, perform a DAT
   d. If DAT is positive with anti-IgG AHG, perform an elution and identify the antibody coating the cells.

2. Additional techniques to enhance sensitivity of weakly reacting antibodies include:
   a. Increase serum to cell ratio (only if no enhancement is used)
   b. Increase incubation time
   c. Alteration of pH, e.g., lowering pH to 6.5 will enhance some anti-M
   d. Switch enhancement media, e.g., PEG, enzymes

III. Antibody Interpretation

A. Patient’s history:

1. Previous records of unexpected antibodies
2. Transfusion, transplantation and pregnancy history
3. Medication history
4. Diagnosis (e.g., AIHA)
5. Ethnicity (e.g., Black population has higher incidence of anti-Js\textsuperscript{b}, -U, -Le\textsuperscript{a})
1. Exclusion by “cross out” technique
   - Using cells that did not react with the patient’s plasma, eliminate antigen specificities that are present on the cell
     a. Rule out specificity if cell has homozygous antigen expression
     b. The following specificities can be excluded when the antigen is present in a heterozygous manner: K, Kp\(\text{a}\), Js\(\text{a}\), & Lu\(\text{a}\)
     c. When anti-D is present in the patient’s plasma, it may be difficult to find a cell that is D - and C + c - (r’r’) in order to exclude C. In this situation only, it is acceptable to exclude C using a cell with heterozygous expression (D- C+ c+…r’r). This is true for the E antigen also.

2. Inclusion pattern
   - compare the pattern of reactivity obtained with the test serum with that of the remaining antigen specificities. Consider the following:
     a. phases where reactions occurred
     b. reaction strength
     c. dosage
     d. reactivity of auto control

3. “Three positive; three negative” rule
   - Testing the patient’s serum with at least three antigen-positive and three antigen negative cells will result in a probability \(P\) value of .05. (A \(P\) value is a statistical measure of the probability that a certain set of events will happen by random chance.) The interpretation of the antibody identification will be correct 95% of the time.

4. Antigen type the patient to confirm the ability to produce the antibody.
   a. **Remember Landsteiner’s Law!** The patient’s cells should lack the antigen in order for the immune system to see the antigen as foreign and produce an antibody to it.
   
   b. Use a pre-transfusion sample, or reticulocytes.

   c. Test a cell that lacks the antigen and a cell that possesses the antigen and its allele
(heterozygous) as controls for the anti-sera.

d. A positive DAT or recent transfusion may invalidate the typing results.

C. Variation in reaction strength:

1. Dosage: reactions stronger with cells having homozygous antigen expression (e.g., MN, Lu, Rh, Kidd, Duffy)

2. Variability: expression of antigens differ from person to person, (e.g., P, I, Lu)

3. Multiple antibodies: a stronger reaction when more than one antigen is present on the same cell. However, dosage effect may also occur on same cell.

D. Phases of reactions: Consider which antibodies are detected at various phases and temperatures.

IV. Additional Investigative Tools

A. Selected cell panels

1. Additional panel cells that may be tested in order to eliminate additional antibody possibilities.

2. In patients with known antibody, selected cells may be tested to prove no additional antibodies have formed.

B. Cord cells: aid in identifying auto anti-I

C. A₁, A₂, B cells: for determining presence of unexpected anti-A, -B, -A,B, or -H

D. Antigen type the patient’s cells to see what antibodies could be produced

E. Reference center - rare donor cells

F. Enzyme enhancement
   - used when increased sensitivity is needed for detection or separation of antibody mixtures

1. Sources:
a. Ficin - figs (most commonly used)

b. Bromelin – pineapple

c. Papain – papaya

d. Trypsin - hog stomach

2. Techniques – used to remove antibody from cells sensitized in vivo or promote in vitro sensitization of cells for identification

a. One step procedure - enzymes added directly to serum / red cells in place of routine enhancement reagent.

b. Two step procedure - red cells pre-treated with enzymes before addition of test serum

3. Mechanism of action

a. Cleave glycoproteins (sialic acid) residues from the cell reducing red cell’s negative charge

b. Destroys or depresses red cell antigens: Fy\textsuperscript{a}, Fy\textsuperscript{b}, M, N, S, and Xg\textsuperscript{a}

c. Exposes or enhances expression of Kidd, Lewis, Rh, Ii, P\textsubscript{1} and ABO antigens.

G. Neutralization:

1. Soluble substances (antigens) may bind with antibody, making the antibody unavailable to react with antigens on the reagent red cells. With the antibody neutralized, other antibodies may be detected, if present.

2. Different fluids contain antigens as soluble substance:

   a. Saliva - Lewis
   b. Hydatid cyst fluid - P\textsubscript{1}
   c. Urine - Sd\textsuperscript{a}
   d. Serum - Chido and Rodgers
   e. Human milk – I

H. Summary of the logical sequence of antibody detection and identification.
1. Antibody screen  
2. Panel  
3. Select Cells - if necessary  
4. “Three pos; three neg” rule  
5. Antigen type patient - if possible

V. Clinical Characteristics of Alloantibody vs. Autoantibody

A. Warm (IgG) alloantibodies - clinically significant  
   1. Negative auto control - unless patient has positive DAT  
   2. Most common antibodies identified at AHG phase (may be at 37°C)  
      a. Rh - largest number of alloantibodies are made against Rh antigens  
      b. Kell - account for 2/3 of non-Rh antibodies  
   3. If screen / panel cells are negative but antiglobulin crossmatch (AGXM) is incompatible consider:  
      a. Antibody to low frequency antigen  
      b. Positive DAT on donor unit  
      c. Bacterial contamination of unit  
   4. If all cells are positive consider:  
      a. Multiple antibodies  
      b. Antibody to high incident antigen, e.g., HTLA

B. Warm autoantibodies  
   1. Positive auto control  
   2. Autoantibodies may show specificity to Rh system - most common is auto anti-e  
   3. If all panel cells are positive consider:
a. Broad Rh specificity

b. A cold antibody binding complement also present (poly specific AHG)

c. The need to remove auto antibody to determine the presence of an existing alloantibody
-38% of patients with a warm auto antibody will also have alloantibodies.

C. Cold (IgM) alloantibodies:

1. Negative auto control

2. Most common antibodies in I.S. phase of panel which may activate complement and continue to be reactive through the AHG phase are:
   Anti-Le, -P₁, -M and N

D. Cold autoantibodies

1. Autoanti-I - assumes pathologic significance in cold agglutinin disease or mixed autoimmune hemolytic anemia and Mycoplasma pneumonia.

   a. All panel cells positive

   b. Cord cells negative or weakly positive

   c. Auto control strongly positive

2. Anti-i - relatively weak cold agglutinin; also seen as transient potent antibody in infectious mononucleosis

3. Autoanti-IH

   a. All panel cells positive

   b. Cord cells negative or weakly positive

   c. Auto control negative or weakly positive

4. Autoanti-H - seen in A₁ and A₁B individuals (not the alloanti-H of an Oₜ individual which reacts over a wide thermal range with all red cells except those of other Oₜ individuals)

   a. All panel cells positive
b. Cord cells strongly positive  
c. Auto control negative or weakly positive  
   - H substance strongest on group: O > A2 > B > A2B > A1 > A1B

5. Anti-P- potent IgG hemolysin with wide thermal range (biphasic hemolysin). Seen in PCH

6. Cold agglutination of undetermined specificity
   a. All panel cells positive
   b. Cord cells and auto control strongly positive

VI. **Additional Considerations:**

A. Albumin agglutination:
   1. Rare phenomenon caused by antibody to sodium caprylate, a stabilizer in many commercial albumin preparations
   2. Most reactive at I.S. and 37ºC; a few at AHG phase
   3. Auto control positive; DAT negative

B. Preservative antibodies - antibody produced to preservative solution

C. Panagglutination - an antibody capable of agglutinating all red blood cells including patient’s own cells

D. Polyagglutination - a state in which an individual’s red cells are agglutinated by all sera regardless of blood type

E. Rouleaux - coin-like stacking of red blood cells in the presence of plasma expanders or abnormal plasma proteins