DIRECT ANTIGLOBULIN TEST
Tube Method

I. Principle:

Polyspecific (broad spectrum) anti-human globulin (AHG) reagents contain antibodies to IgG and various complement components which react with red blood cells (RBCs) sensitized in vivo with these globulins causing agglutination of the RBCs. Monospecific anti-human globulin reagents contain antibodies to IgG only or complement components only.

II. Purpose:

The direct antiglobulin test (DAT) is used to demonstrate the sensitization of red blood cells in vivo with IgG antibodies and/or complement components (C₃b, C₃d, C₄). This test is useful in the investigation of hemolytic transfusion reactions, hemolytic disease of the fetus and newborn, autoimmune hemolytic anemia, and red blood cell sensitization due to drugs.

III. Specimen

The specimen of choice is red blood cells (RBCs) collected in EDTA. Other acceptable anticoagulants include heparin, ACD, and CPD. Specimens collected without anticoagulant should be avoided if testing will not be accomplished immediately, as the complement in serum could bind to the RBCs in vitro if the specimen is stored at room temperature or 4°C, yielding false positive results. Specimens collected in tubes with neutral gel separators are unacceptable, as the gel has been associated with false positive results.

IV. Equipment and Reagents:

- Polyspecific anti-human globulin reagent (AHG)
- Anti-IgG AHG
- Anti-C₃d AHG
- 6% Bovine Serum Albumin
- IgG-coated and C₃–coated RBCs (Coombs’ Control Cells or Check Cells)
- 12 x 75 test tubes
- 0.9% saline
- Dispo pipettes
- Serofuge
V. **Controls:**

1. RBCs yielding a positive DAT result should be tested against 6% Bovine Serum Albumin (BSA). A negative result with the BSA confirms the positive result with the anti-human globulin reagent. A positive result with the BSA indicates the RBCs are aggregating or agglutinating for some other reason, which should be investigated. The results of the DAT are invalid.

2. All tests that are negative at the AHG phase should be tested against Coombs’ Control Cells to verify proper technique has been followed. The Coombs’ Control Cells should yield a positive result. A negative result with Coombs’ Control Cells invalidates the DAT results.

VI. **Procedure:**

1. Prepare a 2-5% suspension of red blood cells to be tested in a labeled tube.

2. Label four test tubes: Poly, Control, anti-IgG, and anti-C\textsubscript{3}. Label each tube with specimen identifying information (patient’s name or initials or donor identification number [DIN])

3. Place 1 drop of the RBC suspension into each tube.

4. Wash the RBCs: Fill each tube 2/3 to ¾ full with 0.9% saline. Centrifuge tubes on high for 60 seconds. Tip the tubes upside down to decant the saline. Use the residual drop of saline to dislodge the RBCs from the bottom of each tube. Fill the tubes 2/3 to ¾ full with saline again. Repeat the wash process 2 to 3 additional times. After the final wash, with the tubes still inverted, blot the tubes dry with an absorbent wipe to ensure that all residual saline has been removed. This prevents the anti-human globulin reagent from being diluted.

5. Set the tubes labeled “anti-IgG” and “anti-C\textsubscript{3}” aside, for use later in additional testing.

6. Add 2 drops of the polyspecific AHG reagent to the tube labeled “Poly”. Add 2 drops of 6% BSA to the tube labeled “control”.

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Agglutination mirror

Microscope
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7. Gently shake the tubes in order to mix the contents, and centrifuge 15-20 seconds on high (3500 rpm). The RBCs will form a button or pellet at the bottom of each tube.

8. Gently resuspend the RBC button and examine for agglutination. Tubes that appear to be negative macroscopically must be examined microscopically. Immediately grade the reactions and record results on the appropriate worksheet.
   a. If the “Poly” tube is negative, incubate both the “Poly” and the “control” tubes at room temperature (RT) for 10 minutes, and then centrifuge again (15-20 seconds on high). Read macro-and microscopically for agglutination. (This allows the reaction with complement time to develop)
   b. If the “Poly” tube is positive and the “control” tube is negative, the DAT should be repeated using monospecific reagents (anti-IgG and anti-C3d) to determine which globulin is coating the RBCs. Proceed to step 10.
   c. If the “control” tube is positive either at immediate spin or following RT incubation, the DAT results are invalid, and cannot be interpreted.

9. If the “Poly” tube is negative following room temperature incubation, add 1 drop of Coombs’ Control Cells to the tube. Mix and centrifuge for 15-20 seconds on high. The Coombs’ Control Cells should yield a positive reaction. If no agglutination is macroscopically observed, the test is invalid and must be repeated. Immediately grade the strength of the reactions and record on the appropriate worksheet.

If the test is positive with polyspecific AHG:

10. Add 2 drops of anti-IgG AHG reagent to the tube labeled “anti-IgG”. Add 2 drops of anti-C3 AHG reagent to the tube labeled “anti-C3”. Gently shake each tube to mix the contents and centrifuge for 15-20 seconds on high (3500 rpm).

11. Gently resuspend the RBC button and examine macro- and microscopically for agglutination. Reactions that appear to be negative macroscopically must be examined microscopically. Immediately grade the reactions and record results on the appropriate worksheet.
   a. If the “anti-C3” tube is negative, incubate both the “anti-C3” and the “control” tubes at RT for 10 minutes, and then centrifuge again (15-20 seconds on high). Read for agglutination macroscopically and
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microscopically. If the test remains negative following incubation, add Coombs’ Control Cells, as in step 9. (Must use C3-coated cells)

b. The “anti-IgG” tube does not require RT incubation. If the test is negative, immediately add Coombs’ Control Cells, as in step 9.
c. If the “anti-IgG” tube is positive, the antibody coating the RBCs may be stripped off the RBC (eluted) and tested against RBCs with known antigen content in order to determine the specificity of the antibody.

VII. Reporting Results

<table>
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<tr>
<th>Reagent:</th>
<th>Poly IS</th>
<th>Poly RT</th>
<th>BSA</th>
<th>&gt;IgG</th>
<th>&gt;C3</th>
<th>Coombs’ Control</th>
<th>Interpretation</th>
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<td></td>
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<td>+</td>
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</tr>
</tbody>
</table>

VIII. Additional Information:

1. Failure to thoroughly wash the RBCs to be tested is a major cause of false-negative antiglobulin tests, as remaining unbound immunoglobulins may neutralize the AHG reagent.

2. False negative reactions may also occur if the washing process is not performed as quickly as possible, leading to the dissociation of bound antibody from the RBCs.

3. All tests must be read immediately since reactions due to IgG will weaken upon standing.

4. A proper RBC suspension is necessary to ensure proper reactivity. Too heavy or too weak of a suspension may result in inaccurate test results.
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5. Any test using RBCs that is read at the antiglobulin phase of testing will be invalid if the RBCs being tested have a positive DAT. These include:

   a. Weak D Test
   b. Antigen Typing
   c. Auto Control
   d. Antiglobulin crossmatch (donor RBCs with a positive DAT)

IX. REFERENCES: