

**Cold Agglutination Titer**  
detecting Cold Reacting Antibodies

**Objectives:**

1. Perform a serial dilution to determine the amount of cold reacting antibody present in a patient specimen with the results obtained falling within  $\pm 1$  tube of instructor's value.
2. Properly dispense the correct amounts of diluent and red blood cells and transfer the necessary amount of serum from tube to tube, using precision and care.
3. Calculate the dilution of each tube in the serial dilution once all reagents and patient sample have been added.
4. Recognize the clumping of red blood cells as agglutination and properly interpret and record each tube as being positive or negative for agglutination according to the criteria in the procedure.
5. Recognize the endpoint for the test and correctly interpret and record the titer.
6. If appropriate, evaluate reagent package inserts / instructional materials to determine the substance being analyzed, the principle of the procedure, the expected value, significance of abnormal results, limitations of the procedure, and troubleshooting procedures to follow if / when control results are unacceptable.
7. Appropriately record and report results as instructed.
8. List 2 limitations of the procedure and describe how the results will be affected.
9. Utilize lecture notes, textbook and laboratory information to answer study questions.

**Introduction:**

Serology testing for cold agglutinins are commonly requested in suspected cases of primary atypical pneumonia, where this rapid screening test has proven useful. Cold agglutinin antibodies are found in the serum of approximately 55% of the patients with primary atypical pneumonia, a respiratory disease caused by *Mycoplasma pneumoniae*. These antibodies cause agglutination of adult red blood cells at 4°C, but not at normal body temperature (37°C). Cold agglutinin antibody levels are often detectable by the end of the first or second week of the disease, increasing to their maximum by the second to fourth week and decreased or absent by the sixth week. In *M. pneumoniae*, a positive correlation exists between the level of cold agglutinin antibodies and the severity of the disease, the extent of pulmonary involvement and duration of illness. Extremely high titers are sometimes found in cases of hemolytic anemia. A fourfold or greater rise in titer from paired sera (where one sample is taken early in the disease and another sample is drawn several days or a week later) is significant of acute disease.

Cold agglutinins may also be produced by other diseases including liver disorders, chronic sepsis, acquired hemolytic anemia, leishmaniasis and black water fever. Most of these diseases have symptoms that easily distinguish them from those of primary atypical pneumonia.

Most cold agglutinins have a specificity for the "I" antigen found on most all adult human red cells. The specimen must be kept warm until the serum containing the antibody can be separated from the patient's red cells which contain the "I" antigen. Proper handling requires the blood be collected in tubes kept warm from the moment of collection until the physical separation of the serum from the cells. If the specimen is allowed to cool, the antibody may attach to the "I" antigens causing a falsely decrease titer. Should this happen to the sample, it must be placed in a 37°C incubator for 30 minutes before removing the serum for testing. The incubation at 37°C will cause the cold agglutinin to dissociate from the patient's cells.

**Principle:**

When serial dilutions of serum containing a cold agglutinin antibody with anti-I specificity are mixed with 1% group O adult red cells and refrigerated, a positive reaction of agglutination will occur in those tubes containing sufficient antibody. The end point is determined as the last tube demonstrating the agglutination, and the reciprocal of the dilution is reported as the titer.

**Materials:**

- |    |                                      |    |                               |
|----|--------------------------------------|----|-------------------------------|
| 1. | Twelve (12) 12 x 75 Test tubes       | 5. | Serological pipets            |
| 2. | Test tube rack                       | 6. | Refrigerator                  |
| 3. | 0.85% Saline                         | 7. | 37°C waterbath                |
| 4. | 1% Group O red blood cell suspension | 8. | Sharpie or water proof marker |

**Procedure:**

1. Number and label twelve 12x75 mm tubes with patient's initials. Place patient's full name on tube #1, and "cell control" on tube #12.
2. Place 0.3 mL of saline in each tube.
3. Use a clean serological pipet to add 0.3 mL patient's serum to the first tube. Mix thoroughly by raising and lowering the serum-saline solution three times in the pipet, taking care to avoid creating bubbles.
4. Using the same pipet, transfer 0.3 mL from tube #1 to tube #2. Again raise and lower to solution into the pipet three times to mix.
5. Continue to use the same pipet to repeat the procedure of transferring 0.3 mL from tube #2 to tube #3, then from 3 to #4, etc. through tube #11. After adding and mixing tube # 11, discard 0.3 mL. NO serum goes into tube # 12.
6. Use a clean serological pipet to add 0.3 mL of a 1% group O human red blood cell suspension to each tube.
5. Mix well by shaking the rack. Evaluate the fluid level in the tubes. If the titer process is performed correctly, the level will be the same in all tubes. Show the tubes to your instructor.
6. Incubate at 4°C (refrigerator temperature) for 30 minutes
7. After 30 minutes, remove the rack from the refrigerator and immediately centrifuge all tubes for 30 seconds.
8. Starting with tube #1, shake the tube gently and read for macroscopic agglutination. Record the highest dilution in which agglutination is detected. Precede with reading the tubes quickly and with little handling of the tubes as possible, as the reaction is reversible.

9. Tube # 12 is the negative control and its result must be NEGATIVE. A positive result in this tube invalidates the test result.
10. Incubate all positive tubes in a 37°C waterbath for 15 minutes. Remove, spin and immediately read for agglutination. They should be negative at this point.

### Interpretation:

Read tests immediately on removal from the cold, spun and read in numerical order. A positive test will result in a cell button on the bottom of the tube that is difficult to dislodge by gentle shaking. Large or small clumps will be seen while gently shaking. As soon as a tube has been determined as being "positive", precede to reading the next tube. The last tube showing agglutination is the *endpoint* of the test. The *titer* is reported out as the reciprocal of the last dilution showing a positive result.

### Limitations of the Procedure:

1. Dispensing incorrect quantities of diluent or red blood cell solution or transferring more or less than the required amount of diluted serum will adversely affect the outcome of this test, resulting in a falsely increased or decreased titer.
2. The technique for shaking the tubes to detect agglutination is critical. Harsh shaking may cause weak or fragile agglutinates to break apart, resulting in a false negative result in the tube and a false decrease in the reported titer.
3. The reaction between a true cold agglutinin and the red blood cells is reversible. To prove the presence of a true cold agglutinin, all tubes showing agglutination at 4°C must be negative after incubation at 37°C for 15 - 30 minutes. If agglutination remains, the antibody involved is not a true cold agglutinin.
4. Tube #12 is the cell control and must demonstrate a negative reaction. A positive result in tube #12 indicates spontaneous agglutination of the red cells. The test procedure must be repeated using a different cell suspension.
5. Test should be performed regularly, because an increase in titer throughout the duration of the illness is of greater significance than a positive result on a single specimen.
6. A fourfold or greater rise in the titer of cold agglutinins is suggestive of a recent *M. pneumoniae* infection.

<u>Tube #</u>	<u>Dilution</u>	<u>Titer</u>
1	1 / 2	4
2	1 / 4	8
3	1 / 8	16
4	1 / 16	32
5	1/32	64
6	1/64	128
7	1/128	256
8	1/256	512
9	1/512	1024
10	1/1024	2048
11	1/2048	4096

Name \_\_\_\_\_ Date \_\_\_\_\_

**Cold Agglutinin Titer**  
Recording/Interpreting Results

1. In the chart below record the reactions on the "Observed Result" row for your visual observation of each tube after centrifugation and shaking has been performed. Record "**A**" for agglutination and "**NA**" for no agglutination. *Only results written in ink will be accepted*, pencils are not allowed for recording results in the clinical laboratory.
2. After you have set up your tubes for the dilution procedure show them to your instructor **BEFORE** placing them in the refrigerator for incubation.
3. The last tube showing agglutination (**A**) is the endpoint.
4. The titer is determined as the reciprocal of the last tube showing agglutination.
5. The Cell Control is reported as "positive" or "negative".

<b>Patient's Name</b> _____							<b>Patient ID #</b> _____					
<b>Tube Number</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>Observed Result</b>												
<b>Endpoint</b> _____												
<b>Titer of endpoint</b> _____												
<b>Cell Control</b> _____												

**Cold Agglutinin Titer**  
Study Questions

Name \_\_\_\_\_ Date \_\_\_\_\_

Questions are worth one point, unless otherwise indicated.

1. Are the results valid? YES NO
2. What are cold agglutinins?
3. In what disease process are cold agglutinins most commonly encountered, and what organism causes this disease? (2 points)
4. List two other diseases in which cold agglutinins may be produced.
5. Describe the proper collection and storage procedure for a cold agglutinin test. (2 points)
6. Explain the purpose of the 37°C incubation step including the expected outcome for a cold agglutinin. (2 points)
7. Explain the purpose and expected outcome of tube #12. (2 points)
8. Why would a patient with a positive cold agglutinin test be retested?
9. The tech mistakenly refrigerates a blood specimen for a cold agglutinin test. (2 points)
  - a. If the procedure is performed on this specimen, what effect would the improper storage have on the tests results?
  - b. Other than recollect and properly handle another sample, what could the tech correct the problem to obtain valid results?