

AlfredHealth	Alfred Pathology Services			
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TESTING FOR COLD AGGLUTININS

PRINCIPLE:

Cold HaemAgglutinin Disease (CHAD) is the most common autoimmune haemolytic anaemia (AIHA) associated with cold reacting antibodies. The antibodies involved are **almost always IgM** and may show specificities of:

- Anti-I (present on adult cells).
- Anti-i (present on cord cells).
- Anti-P (present on adult and cord cells). (1,2)

This cold-reactive autoantibody usually binds to the red cells in the peripheral circulation, where there is a lower temperature, causing complement to bind to the red cells. As the red cells circulate back to warmer areas of the body the IgM dissociates and the complement remains. IgM cold reactive autoantibodies that are associated with immune haemolysis often react at >30 degrees celsius and have a high titre of >1000 when tested at 4 degrees. Occasionally, pathologically cold autoantibodies can have a low titre with a high thermal amplitude.

This haemolysis can occur as either acute or chronic haemolysis.

The acute form of haemolysis is often secondary to a pre-existing disease such as:

- Lymphoproliferative disorders (eg. chronic lymphoid leukaemia, lymphoma).
- Infections – Mycoplasma Pneumonia (anti-I) and Infectious Mononucleosis (anti-i).

The chronic form of haemolysis causes mainly mild to moderate haemolysis. It is seen mainly in the elderly and is sometimes associated with:

- Lymphoproliferative diseases.
- Waldenstroms macroglobulinaemia.

IgM antibodies to autoantigens are normally present in the plasma, although at low and nonpathologic levels. Therefore, autoanti-I can be seen in healthy patients as well as those with CHAD. Non-pathologic forms of autoanti-I rarely react to titres above 64 at 4 degrees and are usually non-reactive with I negative (cord) cells at room temperature. Results should be considered in the context of other clinical findings.

Generally accepted diagnostic criteria include the following:³

- Evidence of hemolysis (eg. high reticulocyte count, high LDH, high indirect bilirubin, low haptoglobin).
- Positive direct antiglobulin (Coombs) test for C3d only (or, in a minority, C3d plus weak IgG).
- Cold agglutinin titre of ≥ 64 at 4°C.

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SPECIMEN:

Pink cap EDTA - 6 mL – Specimen is sent to Blood Bank in a thermos with sand warmed to 37 °C. These thermos' are available from the 37°C incubator in the Biochemistry proteins laboratory.

Once collected and entered into the computer the specimen is stored in a 37°C water bath in Blood Bank until testing is about to commence. Specimen is spun immediately before the test is set up.

Note: A non-gel serum may be used though is not first choice due to potential difficulty of obtaining patient red cells from the clot.

REAGENTS:

Group O Surgiscreen III Screening Cells (I antigen)

Group O Immulab Cord cells (i antigen)

Forward group cells (as well as A2 cells in patient is group A)

Auto cells

0.9% Saline

TESTING:

Testing for Cold Agglutinins is a three-step process:

- a) Perform a DAT.
- b) Screening to determine if a Cold Agglutinin is present.
- c) In cases of a **positive** Cold Agglutinin Screen, titrating the Cold Agglutinin.

Note: Before a Cold Agglutinin Screen is done a Haematology registrar or Haematologist should be informed of patient details to determine if the test is required.

Direct Antiglobulin Test:

Procedure:

- 1) Wash the test cells three times with saline and make up the test cells to a 3-5% suspension in 0.9% saline.
- 2) Label 3 tubes with the Patient Identification and label one polyspecific, one anti-IgG and one anti-C3d.
- 3) Add 2 drops of the appropriate reagent to its labelled tube.
- 4) Add one drop of the test cell to each tube.
- 5) Spin in the DiaCent-12 centrifuge for 15 seconds and read and record agglutination.
- 6) Let sit for 5 minutes. After incubation re-spin and record reaction scores.
- 7) The strongest reaction score observed at immediate or 5 minutes is reported.

If a strong reaction is seen with anti-C3d this could be causing spontaneous agglutination in the anti-IgG tube. A saline control could be used to confirm the presence of spontaneous agglutination and a warm-washed DAT is recommended in this instance to determine if the anti-IgG is really reacting.

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Cold Agglutinin Screen:

Procedure:

- 1) Label tubes with the specimen accession number. Then label individual tubes with:
 - a) Cord
 - b) Auto
 - c) Forward group cells (as well as A2 cells in patient is group A)
 - d) Screening Cell 1
 - e) Screening Cell 2
 - f) Screening Cell 3

- 2) Take specimen from 37°C water bath and centrifuge **immediately** for 5 minutes at 3600 rpm.
- 3) Add 4 drops of plasma to all tubes.
- 4) Add one drop of the appropriate 3-5% cell suspension to the appropriately labelled tube.
- 5) Incubate all tubes in a 37°C water bath for 15 minutes.
- 6) After incubation centrifuge all tubes on low speed for 15 seconds.
- 7) Examine all tubes for agglutination and record result scores (Appendix 1: Cold Agglutinin Screen sheet).
- 8) After reading all tubes, incubate the tubes at room temperature for 15 minutes.
- 9) After incubation centrifuge all tubes on low for 15 seconds.
- 10) Examine all tubes for agglutination and record result scores (Appendix 1: Cold Agglutinin Screen sheet).
- 11) After reading all tubes, incubate the tubes at 4 degrees for 15 minutes.
- 12) After incubation centrifuge all tubes on low for 15 seconds.
- 13) Examine all tubes for agglutination and record result scores (Appendix 1: Cold Agglutinin Screen sheet).

Interpretation:

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Table 1. Examples of common reaction patterns seen and their possible causes.⁴

	Occurs in blood groups:	Cord	Auto	A1	A2	B	SC1	SC2	SC3
Anti-I	All groups	+/-	++++	++++	++++	++++	++++	++++	++++
Anti-i	All groups	++++	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Anti-H	A1, A1B, B	++++	+/-	+/-	++	++	++++	++++	++++
Anti-IH	A1, A1B, B	+/-	+/-	-	++++	++	++++	++++	++++
Anti-A1	A2, A2B	-	-	+++	-	-	-	-	-
Anti-Le(a)	All groups	+/-	-	+++	-	+++	-	-	+++

- Cold Agglutinins should increase in strength as the temperature decreases, with maximum agglutination seen at 4 degrees.
- If reaction strength remains weak across the thermal range consider paraprotein effect as the cause of agglutination. Saline replacement could resolve this.
- If a 2+ reaction strength difference is seen between Adult (I) and cord (i) cells, the cold agglutinin can be specified as autoanti-I or -i.
- If variation in reaction /dosing is seen in the reactions of the screening cells, consider investigation of a cold-reacting alloantibodies e.g. anti-M, anti-Le(a), anti-Le(b) or anti-P1.
- No reaction seen in the Auto (with reactions seen in other cells) could indicate a cold allo-antibody and needs to be investigated as such.
- Consider the density of different antigens:
 - I antigen - abundant on all **adult** red cells (branched)
 - i antigen - abundant on **neonate** red cells up to 13-20 months (linear repeats)
 - H antigen - O > A2 > B > A2B > A1 > A1B > Oh (or H-deficient patients)
 - Lewis antigens/M antigen/P1 antigen - vary depending on three cell screen selected

A **positive screen** for cold agglutinins is considered when:

- ★ Reaction strength greater than 2+ is observed in any tube after incubation at room temperature
- ★ Positive reactions are observed in any tube after incubation at 37°C.

If the cold agglutinin screen is positive, consult the senior scientist or haematologist to determine if a titre is required. This test is not urgent and can be done in normal hours.

Examples:

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Reagent Cell	Lot	Expiry	37°C Sal	RT Sal	4°C Sal	Comment
Cord	NA	NA	0	0	1+	i antigen
Auto	NA	NA	0	0	1+	
A1	NA	NA	0.5	2+	4+	Include if patient is blood group A or AB Stronger reaction in A1 cells than A2 May indicate an Anti-A1 (H and I antigen)
A2	NA	NA	0	0	1+	
B	—	—	—	—	—	Include if patient is blood group B or AB (I and B antigen)
Screening cell 1	NA	NA	0	0	1+	Group O cells Dosage may indicate cold-reacting alloantibody. (H and I antigen)
Screening Cell 2	NA	NA	0	0	1+	
Screening Cell 3	NA	NA	0	0	1+	

Patient A: Blood group A POS, cold panel reactions looks like an anti-A1, may have unexpected reaction in reverse group; a subtype should be performed as further investigation.

Reagent Cell	Lot	Expiry	37°C Sal	RT Sal	4°C Sal	Comment
Cord	NA	NA	0.5	0.5	0.5	i antigen
Auto	NA	NA	0.5	0.5	0.5	
A1	NA	NA	0.5	0.5	0.5	Include if patient is blood group A or AB Stronger reaction in A1 cells than A2 May indicate an Anti-A1 (H and I antigen)
A2	NA	NA	0.5	0.5	0.5	
B	—	—	—	—	—	Include if patient is blood group B or AB (I and B antigen)
Screening cell 1	NA	NA	0.5	0.5	0.5	Group O cells Dosage may indicate cold-reacting alloantibody. (H and I antigen)
Screening Cell 2	NA	NA	0.5	0.5	0.5	
Screening Cell 3	NA	NA	0.5	0.5	0.5	

Patient B: Blood Group A POS, reaction strength consistent at all temperatures, most likely paraprotein effect, saline replacement to confirm..

Reagent Cell	Lot	Expiry	37°C Sal	RT Sal	4°C Sal	Comment
Cord	NA	NA	0	0	2+	i antigen
Auto	NA	NA	0	2+	4+	
A1	NA	NA	0	2+	4+	Include if patient is blood group A or AB Stronger reaction in A1 cells than A2 May indicate an Anti-A1 (H and I antigen)
A2	NA	NA	0	2+	4+	
B	—	—	—	—	—	Include if patient is blood group B or AB (I and B antigen)
Screening cell 1	NA	NA	0	2+	4+	Group O cells Dosage may indicate cold-reacting alloantibody. (H and I antigen)
Screening Cell 2	NA	NA	0	2+	4+	
Screening Cell 3	NA	NA	0	2+	4+	

Patient C: Reacts strongly with all adult cells at room temperature. Likely anti-I present. A cold agglutinin titre needs to be performed.

Reagent Cell	Lot	Expiry	37°C Sal	RT Sal	4°C Sal	Comment
Cord	NA	NA	0	0	1+	i antigen
Auto	NA	NA	0	0	1+	
A1	NA	NA	0.5	2+	4+	Include if patient is blood group A or AB Stronger reaction in A1 cells than A2 May indicate an Anti-A1 (H and I antigen)
A2	NA	NA	0	0	1+	
B	—	—	—	—	—	Include if patient is blood group B or AB (I and B antigen)
Screening cell 1	NA	NA	0	0	1+	Group O cells Dosage may indicate cold-reacting alloantibody. (H and I antigen)
Screening Cell 2	NA	NA	0.5	2+	4+	
Screening Cell 3	NA	NA	0.5	2+	4+	

Patient D: Varying reactions seen in the screening cells suggesting cold alloantibody present. A room temperature IS panel should be performed as further investigation.

Cold Agglutinin Titration:

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Procedure:

The specimen is to be tested against 3 cells (autologous, Adult and cord cells). To do this a master dilution must be made with sufficient volume to allow each dilution to be tested against adult, cord and auto cells.

Preparation of Master Dilution:

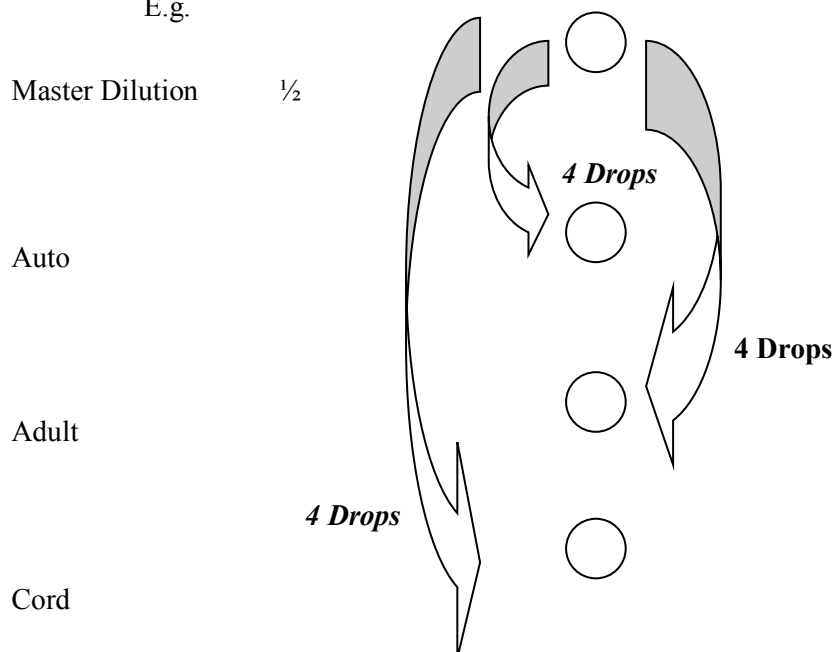
- 1) Label 12 x 75 mm glass tubes for doubling dilution from 1/2 to 1/512 to create the “master” dilution.
- 2) Add 700 µL of saline to all tubes.
- 3) Add 700 µL of the patient serum to be tested to the 1/2 tube and mix well. Remove 700µL from the 1/2 tube and add to the next tube (1/4 dilution) and mix well. Repeat this process until the last dilution of 512.

Note: Do not double dilute past 1/512. If higher dilutions are required make a new dilution up of 1/1000 and use this as the first tube and double dilute 1/2000, 1/4000, etc.

Cold Agglutinin Titration:

- 1) Label three rows of tubes as follows:
 Auto from 1/2 to 1/512.
 Adult from 1/2 to 1/512
 Cord from 1/2 to 1/512.
- 2) Add four drops of each “master” dilution to the appropriately labelled dilution tubes of Auto, Adult and Cord.

E.g.



- 3) Add 1 drop of washed patient cells to each tube labelled “Auto”.

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- 4) Add 1 drop of **one** of the surgiscreen screening cells to each tube labelled "Adult" Select the screening cell that reacted strongest on the screen.
- 5) Add 1 drop of the Immulab Cord cells to each tube labelled "Cord".
- 6) Mix well by gentle shaking.
- 7) **IF NO REACTION SEEN AT 37 DEGREES SKIP TO STEP 11**
- 8) Incubate at 37 degrees for 30 minutes.
- 9) Spin for 15 seconds in an immufuge.
- 10) Read each tube for agglutination starting from the highest dilution. Record all reactions on the Cold Agglutinin Titre result sheet (Appendix 2).
- 11) Incubate at RT for 30 minutes.
- 12) Spin for 15 seconds in an immufuge.
- 13) Read each tube for agglutination starting from the highest dilution. Record all reactions on the Cold Agglutinin Titre result sheet (Appendix 2).
- 14) Place all tubes in a rack and incubate at 4 °C for 30 minutes.
- 15) After 30 minutes remove a few of the tubes (to prevent unread tubes from warming up) spin for 15 seconds in an immufuge. And read for agglutination.
- 16) Read each tube for agglutination starting from the highest dilution. Record all reactions on the Cold Agglutinin Titre result sheet (Appendix 2).
- 17) Repeat steps 11 and 12 until all tubes are read.

Interpretation:

Report the titration result as the reciprocal of the last dilution that was positive with a reaction score of 1+.
i.e. 1/32 = titre 32

Normal range (room temperature):

Auto 2-32
Adult 2-32
Cord 0- 8

Refer results to haematologists for clinical interpretation of results and comment.

References:

- 1) American Association Blood Banks Technical Manual, 17th Edition, 2011 . Method 4.6 Determining the specificity of cold reactive auto agglutinins. P 921 – 923.
- 2) American Association of Blood Banks Technical Manual, 17th Edition, 2011. Method 4.7 Cold agglutinin titre procedure. P 923-925
- 3) The diagnosis and management of primary autoimmune haemolytic anaemia. Hill Et Al. *Br J Haematol.* 2017;176(3) P 395.
- 4) A 13-Question Approach to Resolving Serological Discrepancies in the Transfusion Medicine Laboratory, Yudin Et Al. *Lab Med* Summer 2014 Vol 45 N3, P 193 - 206.

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Appendix 1:

Cold Panel Result Sheet

Patient Name : _____ Patient Ur : _____

Patient Accession : _____ Date : _____ Scientist Initial : _____

Reagent Cell	Lot	Expiry	37°C Sal	RT Sal	4°C Sal	Comment
Cord						i antigen
Auto						
A1						Include if patient is blood group A or AB Stronger reaction in A1 cells than A2 May indicate an Anti-A1 (H and I antigen)
A2						
B						Include if patient is blood group B or AB (I and B antigen)
Screening cell 1						Group O cells Dosage may indicate cold-reacting alloantibody. (H and I antigen)
Screening Cell 2						
Screening Cell 3						

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Appendix 2:

Name: _____ UR: _____ Accession: _____

37 Degrees (if applicable)

	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Cord Cell									
Adult O									
Auto Cell									

Room Temperature

	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Cord Cell									
Adult O									
Auto Cell									

4 Degrees

	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Cord Cell									
Adult O									
Auto Cell									

If patient is a patient undergoing Pulmonary Bypass results must be phoned to theatre pump room in the morning prior to the patient's procedure.

Signature: _____

Note: Titre is the last result that is positive with a reaction score of 1+