This document is not intended to be a comprehensive method and is published as a brief protocol for antibody titration only.

**Alloantibodies**

Unexpected anti-erythrocytic alloantibodies are human blood group antibodies that are produced in response to exposure to foreign red cell antigens. They may also be called "alloimmune antibodies" or "unexpected antibodies". These alloantibodies may be formed for a range of reasons, but are most commonly stimulated by immune system exposure to antigens found on foreign red cells following blood transfusion or from foetal red cells introduced into the maternal circulation during pregnancy.

Some of these alloantibodies may be harmless or medically unimportant, but some may cause Haemolytic Transfusion Reactions (HTR) or Haemolytic Disease of the Foetus and Newborn (HDFN).

HDFN is a clinical syndrome where the survival of the foetal red cells is shortened by the action of specific alloantibodies that are maternally derived, affecting the foetus by placental transfer. The main class of antibody of interest when diagnosing HDFN is IgG as it will cross the placental barrier and may be more concentrated in the foetal circulation than in the maternal circulation. IgM antibodies that may be present in maternal circulation will not cross the placental barrier.

**Purpose of Antibody Titration**

- Determine if an antibody is increasing in strength in the maternal circulation.
- Increasing levels of antibody indicate an active maternal immune response.
- Assist in determining if it is appropriate to monitor a foetus for potential HDFN.

**Precautions when Performing an Antibody Titration**

- Antibody titration is NOT performed to predict the severity of HDFN. In particular, a single titre of a maternal antibody does not indicate the level of foetal harm and should not be used by clinicians to determine patient management strategies or clinical intervention.
- Methods that detect IgM antibodies should NOT be used for antibody titration studies. Where possible, appropriately stored, previously tested samples should be run in parallel with current specimens for comparison of results.
- Titration studies should be performed as suggested below. Other methods using enzyme-treated red cells, LISS, LISS additives, other enhancement methods or Column Agglutination Technology (CAT) methods should NOT be used for titration purposes.

**Titrating Antibodies other than anti-D**

The body of evidence for the utility of antibody titration is mainly focused on anti-D and some other Rh class antibodies. Titration studies of other antibodies should be undertaken only after discussion with the obstetrician as to the significance of the results and how the data obtained will affect patient management. There is little data available concerning critical titres for non-RhD antibodies encountered in pregnancy.

**Reagents Required**

1. Patient serum diluent - 5% Bovine Albumin (BA) in buffered saline.
2. Red cell suspension diluent – either CSL Celpresol, Alsevers Solution or buffered saline.
3. A pool of R2R2 red blood cells washed and formulated to a 3% suspension in CSL Celpresol, Alsevers Solution or buffered saline.

**Reagent Notes**

- A dilution buffer containing BA is used to maintain a minimum level of protein in the test system, even though the patient serum is being serially diluted. Some alloantibodies will fail to react when protein levels are reduced by dilution in saline and therefore give falsely reduced antibody titre results.
- The BA used to make 5% BA in buffered saline should be of serological grade from a reputable manufacturer of immunohaematology reagents. Non-serological grade BA may contain caprylate as a preservative and give non specific reactions due to anti-caprylate antibodies that are sometimes found in patient serum.
- A pool of R2R2 cells is used as these cells are generally commonly available and the antigen expression varies less than other common RhD positive phenotypes like R1R1. A pool should be made of at least 2 but preferably 3 to 5 individual cells, if available. This gives better reproducibility and allows improved detection of rising antibody titres during pregnancy.
Antibody Titration Method

This method was developed by the Australian NICE (National Immunohaematology Continuing Education) consensus forum. Whilst the ‘NICE Method’ does not define the indicator cell phenotype, CSL believe the use of a pool of R\textsubscript{R} cells provides a significant improvement in the reproducibility of this test.

1. Prepare master dilutions of the patient serum or plasma from neat to 1 in 1024 (1:1024). This will require 10 tubes. Use a minimum volume of 250μl and a diluent of 5% protein in buffered saline (pH 7.0 - 7.2).

2. Prepare a 3% washed cell suspension in CSL Celpresol™, Alsevers Solution or buffered saline, (pH 7.0 - 7.2).

3. These cells should be a pool of equal volumes of R\textsubscript{R} cells for anti-D titres or cells homozygous for the antigen being tested.

4. Transfer 200μl of serum/plasma dilution into a tube.

5. Add 50μl of the cell suspension to each tube.

6. Mix and incubate at 37°C for 30 minutes.

7. Wash 3 times in buffered saline and add 2 drops of AHG, mix, spin at low speed (500RCF) for 20 seconds.

8. Read and score reactions.

9. The end point titre is reported as the reciprocal of the highest dilution showing an agglutination score of 1 or greater on the 0 to 4 scale. For example, if the tube containing a 1:32 dilution is the last tube showing a 1 score or greater the titre is reported as 32 rather than 1:32.

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**Master Dilutions**

<table>
<thead>
<tr>
<th>Sample (Neat)</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
<th>1:128</th>
<th>1:256</th>
<th>1:512</th>
<th>1:1024</th>
</tr>
</thead>
</table>

**Expected Scores**

| 4   | 4   | 4   | 4   | 3   | 2   | 2   | 1    | 0    | 0     |

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**Reaction Reading and Scoring**

Antibody titre reactions should be read macroscopically using a consistent “tip and roll” technique.

- Use a light box to provide a well-lit background to facilitate reaction grading.
- Optical magnification is not recommended.
- Scoring of antibody titres is important to obtain accurate results.
- Tubes showing reactions weaker than a score of 1 are ignored.
- It is recommended that the below score definitions are used and pay attention to the text rather than the graphic to improve grading accuracy and reproducibility.
Other Related Immunohaematology Testing

If a maternal group and antibody screen has not been previously performed or if blood transfusion or RhD Immunoglobulin is required, a pre- or post-delivery sample should be tested to determine if the maternal circulation contains clinically relevant alloantibodies that may harm the foetus.

A cord blood sample should be taken from babies of RhD Negative women, women with known antibodies or in cases where there is insufficient documentation of maternal blood group or antibody status. The cord sample should be tested for blood group and Direct Antiglobulin Test (DAT). Elution studies may be useful. If the DAT is positive, haemoglobin and bilirubin estimation should be performed.

Cord Blood Sample is RhD Positive

When the cord blood sample of the baby of an RhD Negative woman is RhD Positive, RhD Immunoglobulin administration is indicated.

Cord Blood Sample is RhD Negative

When the cord blood is RhD Negative, it is recommended that testing for the presence of the weak RhD antigen by the Indirect Antiglobulin Test (IAT) be performed (Du test). If positive, RhD Immunoglobulin is indicated.

If DAT is positive, it may also indicate foetomaternal ABO incompatibility. Difficulty with RhD typing of DAT positive samples may occur due to false positive reactions. The use of a monoclonal Anti-D reagent may overcome this problem.

Antibody elution from the neonatal red cells can be performed to confirm the identity of the antibody coating the cord red cells. Common methods used for elution include Lui Freeze Thaw and the Acid Glycine Elution methods depending on the suspected specificity of the causative antibody.

RhD Immunoglobulin

RhD Immunoglobulin is a therapeutic product to prevent RhD Negative women for making anti-D antibodies in response to foetal maternal hemorrhage from an RhD Positive foetus.

This product is an infusion of so called “passive” IgG anti-D. As it is IgG, the infused anti-D antibodies can cross the placenta and enter the foetal circulation. They may coat RhD Positive foetal cells and give a positive DAT. However, these DAT positive red cells survive normally and there has been no report of foetal or neonatal anaemia or HDFN due to therapeutic anti-D.

Antibody Titrations Using Alternative Methods

The goal of antibody titration is to detect increasing levels of IgG class antibodies in maternal samples. The testing method is designed to be specific for IgG antibodies and reproducible. The gold standard method is a carefully performed tube technique. The test uses controlled volumes, controlled indicator cells and uses the IAT phase to detect only IgG antibodies.

Routine users of Column Agglutination Technology (CAT) such as DiaMed™ ID-MTS™, OCD BioVue™, Grifols™ and other brands may use these methods and give precise results, however the following issues should be considered:

Case Study

Consider the case where a maternal sample contains a clinically relevant antibody but it is due from a recent exposure to foreign red cell and only IgM is present. It is important to detect this antibody in an antibody screen and this antibody may be of clinical relevance if a transfusion is required. The IgM antibodies will not cross the placenta and harm the foetus.

The tube antibody titre technique will generally not detect these IgM antibodies. An antibody titre using one of the CAT systems will detect these antibodies and give the user no indication of the immunoglobulin class. This situation will give significantly different tube and CAT titre results, which may cause misinterpretation and lead to incorrect patient management.

2. Reproducibility

The intention of antibody titre studies is to detect increasing levels of maternal antibodies. Reproducibility is required, not sensitivity.

When samples containing IgG alloantibodies are tested, there are often significant differences between titre results when using tube and CAT methods. If sequential samples are tested using different techniques than changes in titre may be due to the technique rather than real increasing antibody levels.

Case Study

A sample may be collected during the first trimester of a pregnancy and have an anti-D titre of 4 using the recommended tube method.

If a further sample is collected during the third trimester and tested using a CAT system is may have an anti-D titre of 64.

A clinician may not be aware of the technical difference of the test methods and interpret these results as an indication of significantly increasing levels of maternal IgG antibodies. This may lead to a range of unwarranted and costly actions ranging from unnecessary foetal monitoring, performance of hazardous procedures (such as amniocentesis) or unwarranted pregnancy termination.
References