Guidelines for the Blood Transfusion Services

11.4: Recommended serological techniques for reagent testing

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11.4: Recommended serological techniques for reagent testing

11.4.1: Potency titrations

11.4.1.1: Introduction

The use of a semi-automatic pipette is recommended; one volume being in the order of 40 µL.

A separate pipette tip should be used for each reagent.

If the reagent is formulated with a medium to enhance its reactivity then the diluent for the determination of the potency titre should be a formulation identical to the reagent but with antibody protein replaced by non-antibody protein, e.g. fetal calf serum or bovine serum albumin. Otherwise, dilutions may be prepared in saline containing a final concentration of 20 g/L bovine serum albumin that has not been deliberately polymerised or otherwise potentiated.

Beginning with the undiluted blood grouping reagent, doubling dilutions (1 in 2, 1 in 4, 1 in 8 etc.) should be prepared. When preparing doubling dilutions, after the addition of the reagent or diluted reagent to an equal volume of the diluent, the tip of the pipette is emptied and blotted before the dilution is mixed and a volume transferred to prepare the subsequent dilution.

The potency titre is the reciprocal of the highest dilution of the reagent that effects a grade 2 reaction using tube and microplate or a grade 1 endpoint in column agglutination technologies.

The dilution caused by the addition of the cell suspension should not be considered in determining the potency titre.

11.4.1.2: Potency test methods for manual and microplate blood grouping reagents

Manual method – direct test

- Add one volume of each dilution of the reagent to a separate tube.
- Add one volume of 2–3% test red cell suspension to each tube.
- Mix thoroughly and incubate for the appropriate temperature and duration.
- Centrifuge and determine the reaction grade.

Manual method – indirect anti-human globulin test

- Add two volumes of each dilution of the reagent to a separate tube.

- Add one volume of 2–3% test red cell suspension in saline, or two volumes of 1.5–2% test red cell suspension in LISS.
- Mix thoroughly and incubate at 37°C for 45 minutes if the red cells are suspended in saline, or for 15 minutes if suspended in LISS.
- Wash the red cells four times.
- Add two volumes of anti-human globulin reagent to the button of test red cells. Mix. Centrifuge and determine the reaction grade.

Microplate method

Equipment

- Rigid polystyrene microplates with 'U'-shaped wells.
- Centrifuge with microplate carriers having a radius of at least 10 cm.
- Microplate shaker.
- Concave microplate reading mirror or automated plate reader.
- Red cells for microplate use, bromelin-treated if required.

Method

- Using a microplate, add one volume (25–50 µL) of each dilution of the reagent to one volume of 2–3% test red cells.
- Mix the contents of the wells using a microplate shaker. Incubate at 19–25°C for 15 minutes.
- Centrifuge the microplate at 100g for 40 seconds. Gently dislodge the red cells from the bottom of the wells using a microplate shaker.
- Determine the reaction grade using a concave mirror or automatic plate reader.

11.4.1.3: Avidity determination

- Mix over an oval area of approximately 20 mm × 40 mm on a glass slide, one volume of the undiluted reagent and one volume of a 30–45% red cell suspension in allogeneic serum or ABO group-compatible plasma.
- Maintain the slide at the recommended temperature for a slide test. If a range of incubation temperatures is given, for those blood grouping reagents where the antibody-antigen reaction is favoured by a colder temperature, the higher temperature should be used; for other blood grouping reagents, the lower temperature should be used.
- Determine the time from mixing at which macroscopic agglutination first appears and record the reaction grade at 1 minute.

11.4.1.4: Test used in performance evaluation and batch release testing of anti-human globulin

Tests for IgM and IgG red cell heterospecific antibodies

- These test for heterospecific antibodies which can cause haemolysis or agglutination of unsensitised red cells in the indirect antiglobulin test.

Method

- Divide 12 test tubes into two sets of six.
- Into each of the first set of tubes, add one volume of washed 2–3% untreated red cells in saline from two group A_1 RhD positive, two group B RhD positive and two group O RhD positive individuals.
- Into each of the second set of tubes add one volume of washed 2–3% enzyme-treated red cells (papain, bromelin or ficin) in saline from the same group A_1 RhD positive, group B RhD positive and group O RhD positive individuals.
- Add two volumes of the anti-human globulin reagent, as intended to be supplied for use, to each test tube. Mix thoroughly. Incubate the reactants for five minutes at 19–25°C.
- Centrifuge the tubes.
- Determine the reaction grade.

Control of enzyme treatment

Weak IgG anti-D known to be reactive with enzyme-treated red cells should effect a positive reaction with each washed, enzyme-treated, red cell sample by the following method:

- To separate tubes, add one volume of the weak IgG anti-D to one volume of each of the washed, 2–3% suspension of enzyme-treated, RhD positive red cell samples. Mix thoroughly. Incubate for five minutes at 37°C. Centrifuge the tubes. Determine the reaction grade.
- The weak anti-D used for this purpose must be absorbed to remove anti-A or anti-B.
- Each of the enzyme-treated RhD positive red cell samples should be agglutinated by the weak IgG anti-D.

Tests for unwanted positive reactions

These test for excess anti-C3d and anti-C3c, which can cause unwanted positive reactions in the indirect antiglobulin test, and for the presence of any undesirable antibodies in the reagent.

Method for preparation of the red cell suspensions from segmented bleed line samples

- Select integral segment lines from two packs of group A1, two packs of group B and two packs of group O blood stored at 2–6°C for at least 10 days.
- Wash each of the red cell samples with saline sufficient to remove serologically reactive traces of plasma.
- Prepare suspensions of each red cell sample as 2–3% in saline and as 1.5–2% in LISS.

Incubation of red cells and fresh group-compatible serum

- Each of the six red cell samples described above is tested as a saline and a LISS suspension with a different, fresh, group-compatible serum.

- For each anti-human globulin reagent to be assessed, prepare two sets of six tubes.
- To the first tube of the first set of six tubes and the first tube of the second set of six tubes, add 1 mL of a fresh, single-donor group-compatible serum. Add 1 mL of a second fresh, single-donor group-compatible serum to the second tube of each set, and so on for the six different, fresh, group-compatible sera.
- To the first tube of the first set of six tubes, add 0.5 mL of a red cell sample as a 2–3% suspension in saline. Add 1 mL of the same red cell sample as a 1.5–2% suspension in LISS to the first tube of the second set of six tubes. Add 0.5 mL of the second red cell sample as a 2–3% suspension in saline to the second tube of the first set of tubes and 1 mL of the same red cell sample as a 1.5–2% suspension in LISS to the second tube of the second set of tubes, and so on for each of the six different, red cell samples.
- Incubate the first set of tubes (saline suspended red cell samples) for 45 minutes at 37°C. Incubate the second set of tubes (LISS suspended red cell samples) for 15 minutes at 37°C.
- Wash the red cell samples with saline sufficient to remove serologically reactive traces of serum. Resuspend the red cells to 2–3% in saline.

Tests with anti-human globulin reagents

- For each anti-human globulin reagent, prepare two sets of six tubes. To each of the first set of six tubes, add in sequence one volume of the 2–3% suspension of washed red cells from the saline test above.
- To each of the second set of six tubes, add in sequence one volume of the washed 2–3% suspension of washed red cells from the LISS tests above.
- Add two volumes of undiluted anti-human globulin, as supplied for use, to each of the 12 tubes. Mix thoroughly.
- Centrifuge the tubes.
- Determine the reaction grade.

anti-IgG potency: polyspecific anti-human globulin and anti-IgG reagents for use in tube or microplate techniques

The anti-IgG reference reagent (see section 11.3.5) should be tested in parallel with the test reagent, each being titrated against red cells sensitised with potent IgG anti-D.

Method

Test cells

- A 2–3% suspension in saline of washed pooled group O R_1r red cells is prepared from four individuals.

anti-D

- anti-D suitable for use in this application should have a potency titre of greater than 512.

- To 4 mL of the potent IgG anti-D add 2 mL of the 2–3% suspension of pooled group O R_1r red cells.

- Mix and incubate at 37°C for 45 minutes.

- Wash the red cell sample with saline sufficient to remove serologically reactive traces of serum. Prepare suspensions of each red cell sample as 2–3% in saline.

Technique

- Prepare 1 mL volumes of twofold serial dilutions of the test anti-human globulin reagent and anti-IgG reference preparation from 1 in 8 to 1 in 4096 (ten tubes).
- Prepare a set of ten tubes for each anti-human globulin reagent to be assessed.
- Place two volumes of each dilution into each of the series of ten tubes.
- Add one volume of the 2–3% suspension of pooled sensitised R_1r red cells to each tube, mix and centrifuge.
- Determine the potency titre.

Controls

The washed, strongly sensitised 2–3% suspension of R_1r red cells gives a negative result when centrifuged and gives negative results using the direct anti-human globulin technique with anti-complement (anti-C3c, anti-C3d, anti-C4c and anti-C4d) reagents and with anti-human globulin diluent in place of the anti-human globulin reagent. (The anti-complement specificities may be present as mixtures in one or more reagents.)

Test for anti-IgG potency by chequerboard titration studies with red cells sensitised with weak IgG antibodies (anti-D, anti-K and anti-Fy^a)

Selection of weak IgG antibody preparations

Antibody preparations should not be diluted to attain the following potency requirements. The use of single-donor antibody preparations is preferred.

The following are selected:

- an IgG anti-D to give an anti-human globulin potency titre of 8–32 using a pool of group O R_1r red cells from four individuals

- an IgG anti-K containing a final concentration of 0.014M EDTA neutralised to pH 7, to give an anti-human globulin potency titre of 8–32 using K+k+ red cells

- an IgG anti-Fy^a containing a final concentration of 0.014M EDTA neutralised to pH 7, to give an anti-human globulin potency titre of 8–32 using Fy(a+b+) red cells.

Test cells

Prepare 10 mL of a 2–3% suspension of washed R1r red cells pooled in equal proportions from four individuals. Similarly, prepare 10 mL of a 2–3% suspension of washed Kk red cells and 10 mL of a 2–3% suspension of washed Fy(a+b+) red cells.

Sensitisation of test cells

anti-D

- Using a set of five containers each of 20 mL to 25 mL volume, prepare 4 mL volumes of serial twofold dilutions of the anti-D from undiluted to 1 in 16.
- Add 2 mL of the 2–3% suspension of pooled R_1r red cells in saline to each container. Mix and incubate at 37°C for 45 minutes.
- Wash the red cells four times with 20 mL volumes of saline at each wash and remove the last supernatant.
- Add 2 mL of saline to the packed washed red cells to prepare the 2–3% suspensions of sensitised red cells.

anti-K

As above, but using the anti-K with the K+k+ red cells.

anti-Fy^a

As above, but using the anti-Fy^a, with the Fy(a+b+) red cells.

Preparation of anti-IgG and/or anti-human globulin dilutions

For each anti-IgG and/or anti-human globulin under test and the anti-IgG reference preparation, prepare 2 mL volumes of twofold serial dilutions from undiluted, that is as supplied for use, to 1 in 16.

Test method for anti-IgG or antiglobulin potency by chequerboard titration

anti-D sensitised red cells

- Prepare five sets of five tubes for each anti-human globulin reagent under test and the anti-IgG reference reagent.
- Place two volumes of the anti-human globulin reagent, undiluted to 1 in 16, in the appropriate tubes for each of the five sets of five tubes.
- Using the 2–3% suspension of red cells sensitised with the undiluted anti-D for the first set of five tubes, the 2–3% suspension of red cells sensitised with the anti-D diluted 1 in 2 for the second set of five tubes, and so on, finishing with the 2–3% suspension of red cells sensitised using the anti-D diluted 1 in 16 for the fifth set of five tubes, add one volume of the washed red cells to each of the sets of anti-human globulin dilutions (see Table 11.4).
- Mix thoroughly. Centrifuge the tubes, appropriately.
- Determine the reaction grade.

Table 11.4 Chequerboard test format

Set	anti-D used to coat red cells	Dilution of anti-human globulin reagent				
		N	2	4	8	16
1	Undiluted					

2	1 in 2					
3	1 in 4					
4	1 in 8					
5	1 in 16					

anti-K sensitised red cells

As above, but using the anti-K sensitised K+k+ cells.

anti-Fy^a sensitised red cells

As above, but using the anti-Fy^a sensitised Fy(a+b+) cells.

Controls

The unwashed 2–3% red cell suspensions sensitised with the undiluted anti-D, anti-K and anti-Fy^a give negative results in a spin-tube test. The washed sensitised cells should not react with the diluent or the anti-complement components of the anti-human globulin reagents.

Test for anti-complement potency; polyspecific anti-human globulin reagents for use in tube tests[7]

Preparation of the complement sensitised red cells

Various very low ionic strength medium techniques are used to prepare the iC3b, C4b, C3d and C4d sensitised red cells that are necessary for the assessment of anti-complement activity.

The C3 and C4 activation states produced on red cells by the various methods are shown in Table 11.5.

As a minimum, red cell samples from two individuals are to be prepared and tested as described below.

anti-C4b potency

Method

- Prepare a set of three tubes for each anti-human globulin reagent under test.
- Prepare doubling dilutions of the anti-human globulin reagent from undiluted to 1 in 4.
- Place two volumes of each anti-human globulin dilution in the appropriate tubes.
- Add one volume of 2–3% EC4b red cells to each tube. Mix thoroughly. Centrifuge the tubes.
- Determine the reaction grade.

Controls

The EC4b cells do not react with anti-C3c, anti-C3d, anti-IgG or saline or the inert anti-human globulin diluent using the direct anti-human globulin technique. They react with anti-C4c and anti-C4d reagents.

anti-C4d potency

Method

- Place two volumes of undiluted anti-human globulin in a tube.
- Add one volume of 2–3% EC4d red cells. Mix thoroughly. Incubate for 5 minutes at 19–25°C.
- Centrifuge the tubes. Determine the reaction grade.

Controls

The EC4d cells do not react with anti-C3c, anti-C3d, anti-C4c, anti-IgG or saline or the inert anti-human globulin diluent using the direct anti-human globulin technique. The undiluted anti-human globulin does not agglutinate unsensitised red cells that have been trypsin-treated, using the direct anti-human globulin technique.

anti-C3d potency

Method

- Prepare a set of seven tubes for each anti-human globulin under test and the anti-C3d reference reagent (see section 11.3.5) which is tested in parallel, at the dilution for the ‘immediate test’ stated in its accompanying instructions for use.
- Place two volumes of each anti-human globulin dilution in each of the tubes (undiluted, that is as intended to be supplied for use, to 1 in 64).
- Add one volume of the 2–3% EC3d/EC4d red cells to each tube. Mix thoroughly and centrifuge the tubes, appropriately.
- Determine the reaction grade.

Controls

The EC3d/EC4d cells do not react with anti-C3c, anti-C4c, anti-IgG, saline or anti-human globulin diluent using the direct anti-human globulin technique. They do react with anti-C3d.

Table 11.5 Complement C3 and C4 activation

Method of preparation	Initial state	State after trypsin treatment
Very low ionic strength medium* 37°C	iC3b/C4b	iC3d/C4d
Very low ionic strength medium* 37°C	C3dg	C3d
Very low ionic strength medium* 37°C with EDTA	C4b	C4d

* These media are not to be confused with low ionic strength solution (LISS).		