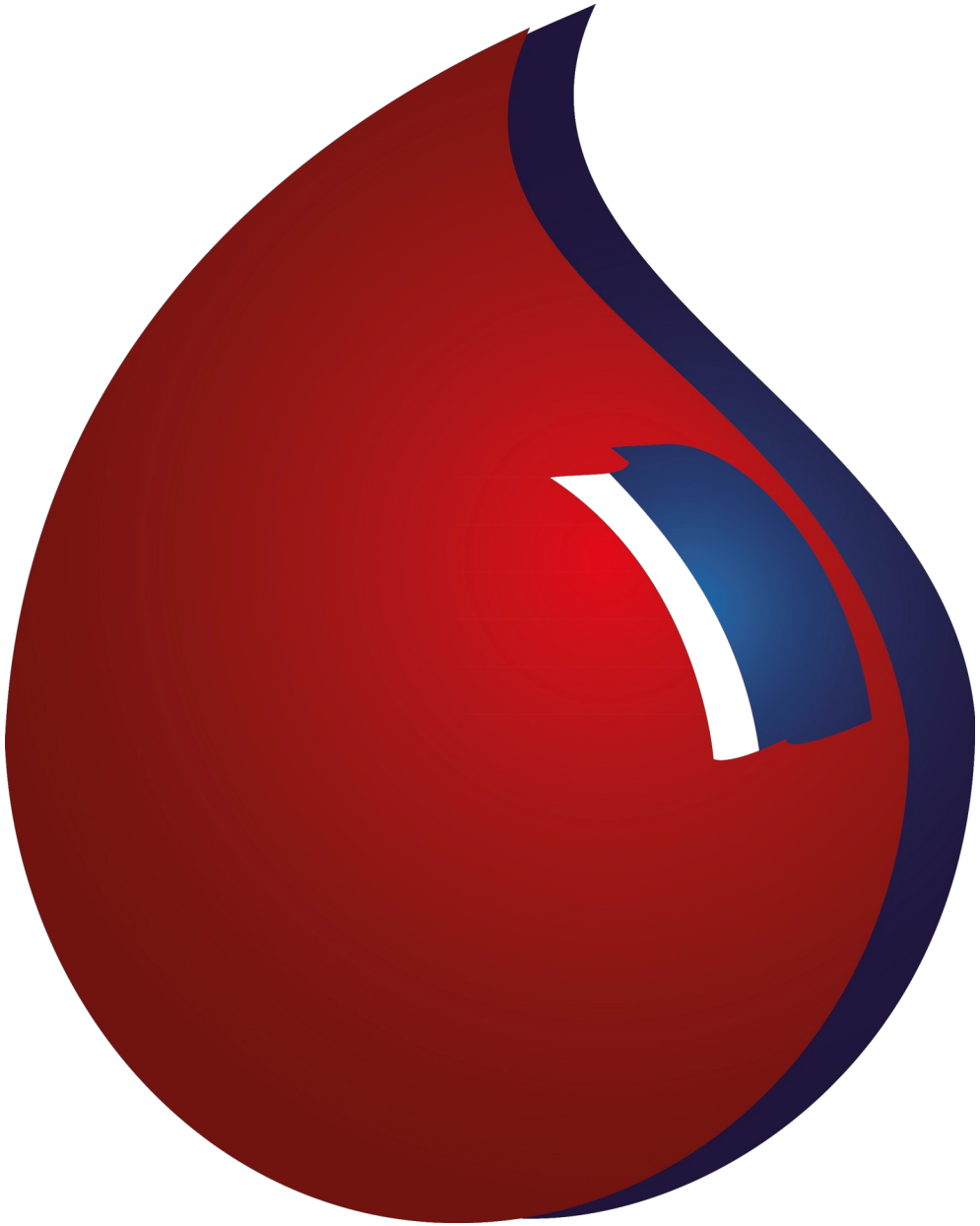

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Complement AP50 Assay

Kit Manual

Version: April 2020

Introduction

Complement haemolytic activity is a functional test of the classical or alternative pathway of the complement system in plasma or serum. The alternative pathway method (AP50) is based on lysis of rabbit erythrocytes in the presence of Mg++. It is helpful as screening test when complement depletion or deficiency is suspected. The test is sensitive to the reduction, absence and/or inactivity of any component of the pathway.

This method is also suited to assess the effects of pharmaceuticals on inhibition or consumption of complement components. Additionally, haemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2017 after blood, plasma, or serum contact with biomaterials can be evaluated. Activation of the complement system by biomaterials or pharmaceuticals can result in consumption of complement proteins, which reduces the AP50 level.

Principle of the Test

An erythrocyte suspension is incubated for 30 minutes with serial diluted serum or plasma at 37 °C. The activation of the complement system will result in haemolysis. After incubation the samples are centrifuged to obtain supernatant. The free haemoglobin concentration in the supernatant, which is directly proportional to complement activity, is measured by means of a spectrophotometer at a wavelength of 415 nm. Plotting the dilution factor of plasma against the degree of haemolysis allows the calculation of the AP50 (i.e. the dilution of plasma to obtain 50% lysis of erythrocytes).

The positive reference is total lysis induced by lysis fluid and the negative reference is obtained after incubation with dilution buffer.

The kit is designed to determine complement activity via the alternative pathway of small samples (75 µL) and can be performed in a 96 well (round bottom) microplate. The assay can also be performed in microcentrifuge tubes.

Precautions

- The kit is intended for research purposes only.
- The kit should not be used beyond its expiry date.
- Do not combine reagents from Complement AP50 kits with

different lot numbers.
- The erythrocytes are of rabbit origin and these animals have been tested and approved for consumption.
- Chemicals and reagents have to be treated as hazardous waste according to biohazard safety guidelines or regulations.
- Wear disposable gloves when handling specimens and reagents.
- Use disposable pipette tips throughout the procedure to avoid contamination of reagents.

Contents of the Kit

• Erythrocyte concentrate	1,2 mL	1 tube
• Dilution Buffer	100 mL	1 bottle
• Lysis fluid	3,5 mL	1 bottle
• Stop Solution	100 mL	1 bottle

- Reference 1, freeze dried plasma with low complement activity
- Reference 2, freeze dried plasma with normal complement activity

Additional Materials and Equipment

The following materials and equipment are required but are not provided with the kit:

- Adjustable pipettes with disposable tips.
- Incubator at 37°C.
- Spectrophotometer capable of measuring at 415 nm.
- Micro-centrifuge + microcentrifuge tubes (1,5 mL) or centrifuge + microplates.

Test Procedure

Note: The protocol below assumes the preparation of erythrocyte suspension for analysis of the maximum amount of samples (105). Alternatively, smaller batches of samples can be analysed, requiring a smaller volume of erythrocyte suspension. Calculate the required volumes accordingly.

<u>Reagent Preparation</u>

Erythrocyte suspension:

1. Add 1 mL erythrocyte concentrate to 5 mL Dilution Buffer and mix gently by end-over-end tumbling of the tube.
2. Centrifuge the tube for 5 minutes at 400xg and aspirate or decant the supernatant. Repeat this procedure if the supernatant was not colourless.

3. Resuspend the pellet in 35 mL Dilution buffer, this is sufficient for 7 microplates or 105 samples when the suggested microplate layout is used (Fig 1.)
4. Mix 25 µL of the obtained erythrocyte suspension with 75 µL demineralized water, this should give an OD415 between 0,400 and 0,500, when measured in a microplate in a spectrophotometer. If the OD415>0,500, add 2,5 mL of Dilution buffer and repeat the OD415 measurement.

Test samples: Human serum or plasma is used in dilutions of 2, 3, 4,5, 6,8, 10,1 and 15,2 times. A serial dilution is prepared in a round bottom microplate, resulting in 50 µL per well. The positive control is lysis fluid instead of plasma, the negative control is dilution buffer instead of plasma.
Reference plasma: Both reference plasma samples are reconstituted with 250 µL distilled water and are also used in dilutions ranging from 2 to 15,2 times.

Assay Procedure

1. Pipette 50 µL of test sample, reference sample and controls in a round bottom microplate (see Fig.1 for a plate layout example).
2. Pipette 50 µL of the erythrocyte suspension to each well.
3. Cover the plate with a microplate sticker.
4. Incubate for 30 minutes in an incubator at 37 °C.
5. Pipette 100 µL stop solution to all wells.
6. Centrifuge the plate (or microcentrifuge tubes) at 400xg for 10 minutes to pellet the unlysed cells.
7. Transfer 100 µL of supernatant to a well of a flat bottom microplate.
8. Measure OD 415 nm.

Calculations

1. Correct, for all test and reference samples, for the OD415 of the negative control.
2. Calculate the amount of haemolysis for each sample and dilution.
3. Plot the natural logarithm (ln) of the dilution as the independent variable (x) and the percent haemolysis as the dependent variable (y). See Figure 2.
4. Calculate the AP50 from the slope (a) and intercept (b) of the best-fit line as follows:
   - $y = a \cdot \ln(x) + b$
5. Solve for x:
   - $x = \exp((y - b) / a)$
6. For AP50, the value of y = 50%, so:

- AP50 = exp((50% - b) / a)

Upon request an example calculation spreadsheet is available.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 2x	1 3x	1 4,5x	1 6,8x	1 10,1x	1 15,2x	9 2x	9 3x	9 4,5x	9 6,8x	9 10,1x	9 15,2x
B	2 2x	2 3x	2 4,5x	2 6,8x	2 10,1x	2 15,2x	10 2x	10 3x	10 4,5x	10 6,8x	10 10,1x	10 15,2x
C	3 2x	3 3x	3 4,5x	3 6,8x	3 10,1x	3 15,2x	11 2x	11 3x	11 4,5x	11 6,8x	11 10,1x	11 15,2x
D	4 2x	4 3x	4 4,5x	4 6,8x	4 10,1x	4 15,2x	12 2x	12 3x	12 4,5x	12 6,8x	12 10,1x	12 15,2x
E	5 2x	5 3x	5 4,5x	5 6,8x	5 10,1x	5 15,2x	13 2x	13 3x	13 4,5x	13 6,8x	13 10,1x	13 15,2x
F	6 2x	6 3x	6 4,5x	6 6,8x	6 10,1x	6 15,2x	14 2x	14 3x	14 4,5x	14 6,8x	14 10,1x	14 15,2x
G	7 2x	7 3x	7 4,5x	7 6,8x	7 10,1x	7 15,2x	15 2x	15 3x	15 4,5x	15 6,8x	15 10,1x	15 15,2x
H	8 2x	8 3x	8 4,5x	8 6,8x	8 10,1x	8 15,2x	neg control	neg control	neg control	pos control	pos control	pos control

Figure 1. Suggested 96-well template for the AP50 assay.

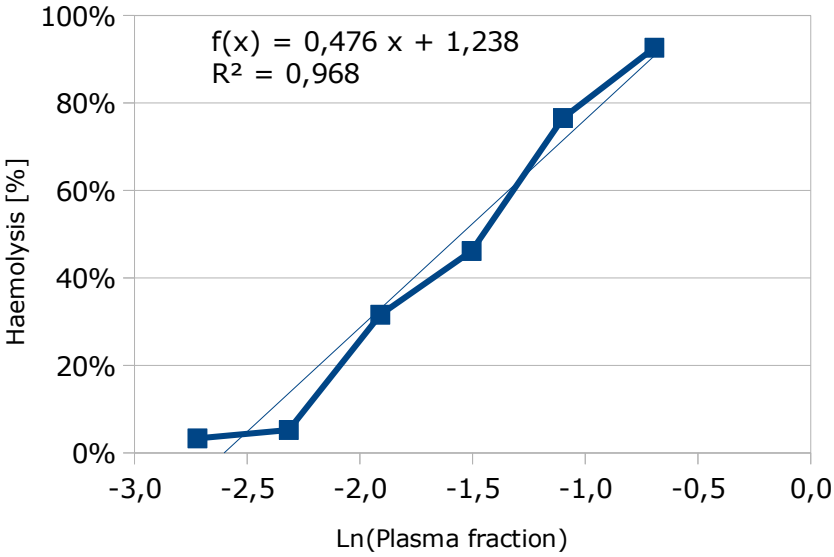

Figure 2. Example graph for the calculation of AP50. In this example the plasma fraction required to obtain 50% haemolysis:

AP50 = exp((0,5 – 1,238) / 0,476) = 21,21%