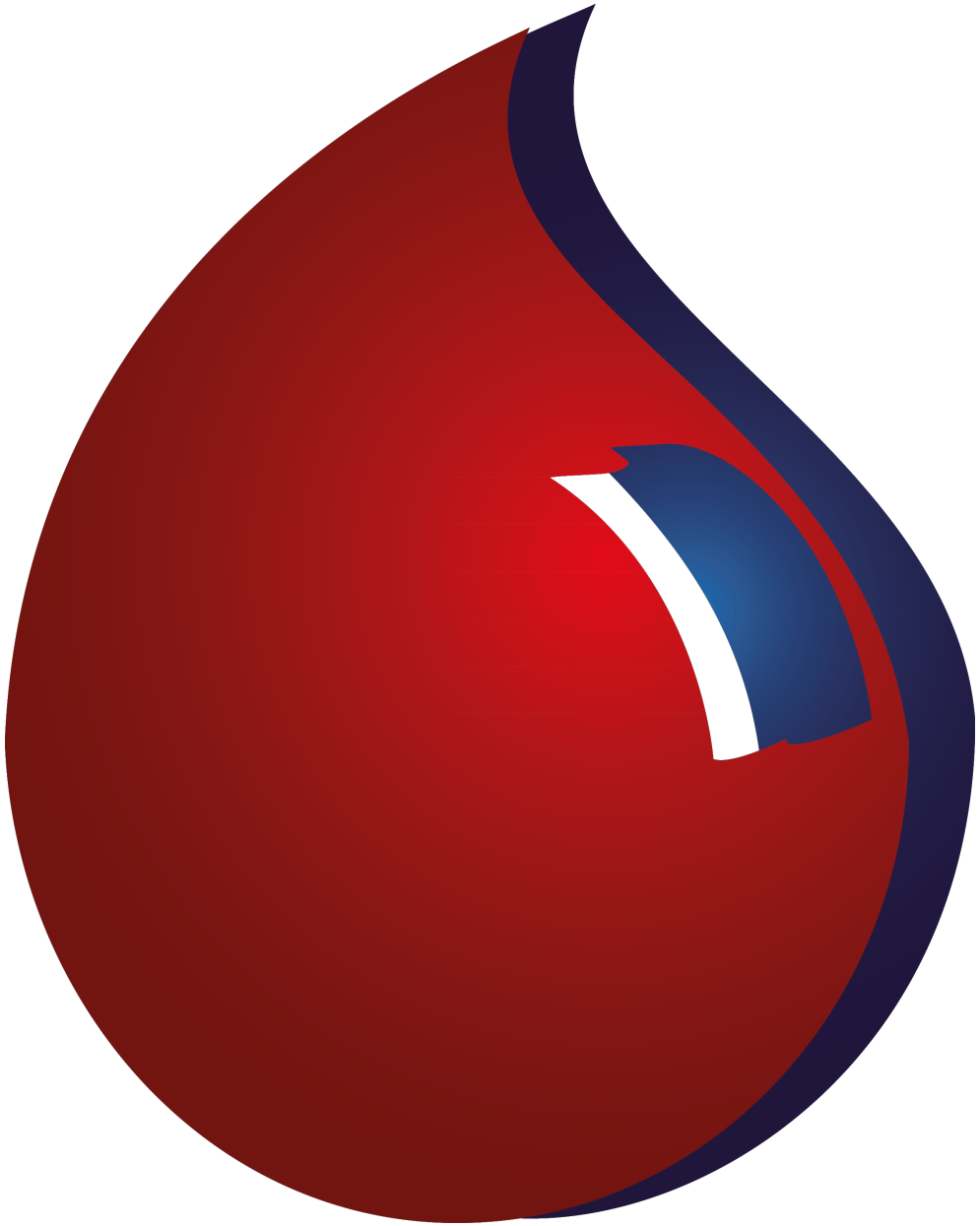

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Complement AP50 Assay

Kit Insert

Version: February 2016

Summary

Complement haemolytic activity is a functional test of the classical and alternative pathway of complement in plasma or serum. The alternative pathway method (AP50) is based on haemolysis of rabbit erythrocytes in the presence of Mg++. This method is suited to evaluate the haemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2002 and to assess the effects of pharmaceuticals on inhibition or consumption of complement proteins.

Introduction

Interactions between blood and a biomaterial may activate the complement system. Particularly during prolonged contact or during contact of blood with large surfaces, this may induce adverse events. This is due to generation of an inflammatory reaction and loss of host defense mechanism. Similarly, pharmaceuticals or their carriers may affect the complement system. This results in consumption of complement proteins, which reduces the AP50 level (i.e. the dilution of plasma/serum to obtain 50% lysis of erythrocytes).

Principle of the Test

An erythrocyte suspension is incubated for 30 minutes with serial diluted serum or plasma at 37ºC. It is known that human complement is able to activate the alternative pathway. After incubation samples are centrifuged to obtain supernatant, containing free hemoglobin. The hemoglobin concentration is measured by means of a spectrophotometer.

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

The kit is designed to determine haemolytic activity of small samples (50 µL or less) and can be performed in a 96 well microtiter plate.

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 (latex) gloves when handling specimens and reagents.
- Never pipette by mouth and avoid contact of skin and mucous membranes
- Use disposable pipette tips throughout the procedure to avoid contamination of reagents.

Contents of the Kit

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

- Reference 1, low complement plasma
- Reference 2, normal complement plasma

Additional Materials and Equipment

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

- (Calibrated) adjustable pipettes with disposable tips.
- Incubator at 37°C.
- Spectrophotometer capable of measuring at 415 nm.
- Micro-centrifuge + vials (1.5 mL) or centrifuge for microtiter plates.

Test Procedure

Reagent Preparation

- **Erythrocyte suspension:** Add slowly, while mixing, the erythrocyte suspension to 5 mL Dilution Buffer. Mix gently by end-over-end tumbling of the tube. Centrifuge the tube for 10 minutes at 400xg. Remove the supernatant. Repeat this procedure if the OD415 of the supernatant is >0,500. Resuspend the pellet in 40 mL Dilution buffer, this is sufficient for 7 microtiter plates. Dilute 25 µL of erythrocyte suspension in 75 µL lysis fluid, this should give an OD415 between 0,400 and 0,500, when measured in a microtiter plate in a spectrophotometer. Prewarm the suspension at 37°C just before use.

- **Test samples:** Human plasma is used in dilutions of 2, 3, 4,5, 6,8, 10,1, 15,2 times, made by serial dilution in a round bottom plate of 2 parts in 1 part dilution buffer, 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 test sample or reference dilutions and controls in a round bottom plate (see Fig. 1 for format example).
2. Add 50 µL of the erythrocyte suspension to each well.
3. Cover the plate with a microplate sheet.
4. Incubate for 30 minutes in an oven at 37°C.
5. Pipette 100 µL stop solution to all wells.
6. Centrifuge the plate at 400xg for 10 minutes.
7. Transfer 100 µL of supernatant to a well of a flat bottom microtiter plate.
8. Measure OD 415 nm.

Calculations

1. Correct, for all test and reference materials, for the OD415 of the negative control.
2. Calculate the amount of haemolysis for each sample and dilution.
3. Plot for each sample the haemolysis (%) on the x-axis and sample concentration (%) on the y-axis.
4. Fit a linear curve (y=ax+b) through the data points.
5. Calculate AP50 = a * 0,5 +b.

Upon request an example calculation spreadsheet is available.

Characteristics

	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	12 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.