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Complement CH50 Assay

Kit Manual

www.HaemoScan.com

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Introduction

Complement haemolytic activity is a functional test of the classical or alternative pathway of the complement system in plasma or serum. The classical pathway method (CH50) is based on lysis of sensibilized sheep erythrocytes in the presence of Ca++ and 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 CH50 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 CH50 (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 classical pathway of small samples (25 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 use only.
- The kit should not be used beyond its expiry date.
- Do not combine reagents from Complement CH50 kits with

different lot numbers.
- The erythrocytes are of ovine 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 tubes
• 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.

Reagent Preparation

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 0,700 and 0,900, when measured in a microplate in a spectrophotometer. If the OD415>0,900, add 2,5 mL of Dilution buffer and repeat the OD415 measurement.

Test samples: Human serum or plasma is used in dilutions of 4, 8, 16, 32, 64 and 128 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 4 to 128 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 CH50 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 CH50, the value of y = 50%, so:

- $CH50 = \exp((50\% - b) / a)$

Upon request an example calculation spreadsheet is available.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 4x	1 8x	1 16x	1 32x	1 64x	1 128x	9 4x	9 8x	9 16x	9 32x	9 64x	9 128x
B	2 4x	2 8x	2 16x	2 32x	2 64x	2 128x	10 4x	10 8x	10 16x	10 32x	10 64x	10 128x
C	3 4x	3 8x	3 16x	3 32x	3 64x	3 128x	11 4x	11 8x	11 16x	11 32x	11 64x	11 128x
D	4 4x	4 8x	4 16x	4 32x	4 64x	4 128x	12 4x	12 8x	12 16x	12 32x	12 64x	12 128x
E	5 4x	5 8x	5 16x	5 32x	5 64x	5 128x	13 4x	13 8x	13 16x	13 32x	13 64x	13 128x
F	6 4x	6 8x	6 16x	6 32x	6 64x	6 128x	14 4x	14 8x	14 16x	14 32x	14 64x	14 128x
G	7 4x	7 8x	7 16x	7 32x	7 64x	7 128x	15 4x	15 8x	15 16x	15 32x	15 64x	15 128x
H	8 4x	8 8x	8 16x	8 32x	8 64x	8 128x	neg cont rol	neg cont rol	neg cont rol	pos cont rol	pos cont rol	pos cont rol

Figure 1. Suggested 96-well template for the CH50 assay.

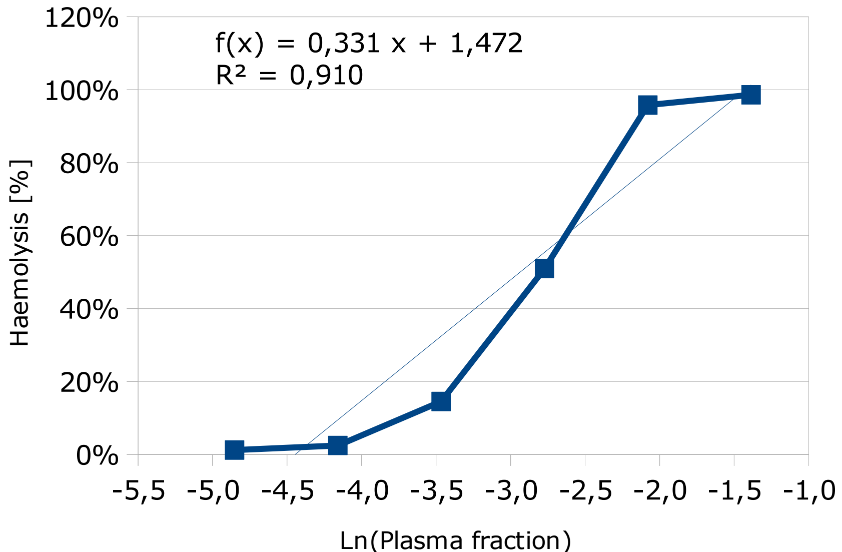

Figure 2. Example graph for the calculation of CH50. In this example the plasma fraction required to obtain 50% haemolysis:

$CH50 = \exp((0,5 - 1,472) / 0,331) = 5,31\%$