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Complement CH50 Assay

Kit Manual

www.HaemoScan.com

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Introduction

Complement haemolytic assays are functional tests of the classical or alternative pathway of the complement system in serum or plasma. The classical pathway assay (CH50) is based on the lysis of sheep erythrocytes that have been sensitised with an anti-sheep erythrocyte antibody, in the presence of Ca^{2+} and Mg^{2+}. It is a useful screening test when complement depletion or deficiency is suspected, as it is sensitive to the reduction, absence and/or inactivity of any component of the classical and terminal pathways.

This method is also suitable for assessing the effects of pharmaceuticals on the inhibition or consumption of complement components. In addition, it can be used to evaluate the haemocompatibility of biomaterials and medical devices in accordance with ISO 10993-4, by testing blood, plasma or serum after contact with the material. Activation of the complement system by biomaterials or pharmaceuticals can result in consumption of complement proteins, which reduces the CH50.

Principle of the Test

An erythrocyte suspension is incubated with serially diluted serum or plasma for 30 minutes at 37 °C. Activation of the complement system results in lysis of the erythrocytes (haemolysis). After incubation, the samples are centrifuged and the concentration of free haemoglobin in the supernatant, which is proportional to the degree of lysis, is measured spectrophotometrically at 415 nm. Plotting the degree of haemolysis against the logarithm of the plasma fraction allows calculation of the CH50, i.e. the reciprocal of the plasma dilution that results in 50% lysis of the erythrocytes.

The positive control (100% lysis) is obtained with Lysis fluid and the negative control (0% lysis) with Dilution buffer.

The kit is designed to determine the classical pathway activity of small samples (25 µL) 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. It is not intended for diagnostic or therapeutic procedures.
- Store the kit at 4–6 °C. Do not freeze the Erythrocyte concentrate.

- Do not use the kit beyond its expiry date.
- Do not combine reagents from Complement CH50 kits with different lot numbers.
- The erythrocytes are of ovine origin, obtained from animals that have been inspected and approved for human consumption, and have been sensitised with an antibody of rabbit origin.
- The reference plasmas are of human origin. The donor blood was tested and found negative for anti-HIV-1/2, HBsAg, anti-HCV, syphilis, HIV-1 RNA, HBV DNA and HCV DNA. As no test method can completely exclude the presence of infectious agents, handle all components of biological origin, and all serum and plasma samples, as potentially infectious material (biosafety level 2). Some components contain protein of bovine origin.
- The Lysis fluid contains Triton X-100, which may cause endocrine disruption in the environment. Avoid release to the environment and collect spillage.
- Dispose of the kit components, samples and contaminated materials as biological (potentially infectious) waste, e.g. by autoclaving or incineration, in accordance with local and national regulations. For further information, please refer to the Safety Data Sheet (SDS), available at www.haemoscan.com.
- Wear disposable nitrile gloves, a laboratory coat and safety glasses. Do not pipette by mouth.
- 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 human plasma with low complement activity
- Reference 2, freeze-dried human 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

- Microplate reader capable of measuring at 415 nm
- Centrifuge with microplate rotor, or microcentrifuge with 1.5 mL microcentrifuge tubes
- 96-well round-bottom and flat-bottom microplates and microplate sealing film
- Demineralised water

Test Procedure

Note: The protocol below describes the preparation of erythrocyte suspension for the maximum number of samples (105). Smaller batches of samples can be analysed with a correspondingly smaller volume of erythrocyte suspension; calculate the required volumes accordingly.

Reagent Preparation

Erythrocyte suspension:

1. Add 1 mL of Erythrocyte concentrate to 5 mL of Dilution buffer and mix gently by end-over-end inversion of the tube.
2. Centrifuge the tube for 5 minutes at 400 × g and aspirate or decant the supernatant. Repeat this washing step if the supernatant is not colourless.
3. Resuspend the pellet in 35 mL of Dilution buffer. This is sufficient for 7 microplates or 105 samples when the suggested plate layout is used (Figure 1).
4. Mix 25 µL of the erythrocyte suspension with 75 µL of demineralised water. Measured in a microplate, this mixture should give an OD_{415} between 0.700 and 0.900. If the OD_{415} is above 0.900, add 2.5 mL of Dilution buffer and repeat the OD_{415} measurement.

Test samples: Human serum or plasma is tested at dilutions of 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 in Dilution buffer. Prepare the serial dilution in a round-bottom microplate, resulting in 50 µL per well. For the positive control, use Lysis fluid instead of sample; for the negative control, use Dilution buffer.

Reference plasma: Reconstitute each reference plasma with 250 µL of distilled water and test at the same dilutions (1:4 to 1:128).

Assay Procedure

1. Pipette 50 µL of each diluted test sample, reference sample and control into a round-bottom microplate (see Figure 1 for an example plate layout).
2. Add 50 µL of erythrocyte suspension to each well.
3. Cover the plate with microplate sealing film.

4. Incubate for 30 minutes at 37 °C.
5. Add 100 µL of Stop solution to each well.
6. Centrifuge the plate (or microcentrifuge tubes) for 10 minutes at 400 × g to pellet the intact erythrocytes.
7. Transfer 100 µL of each supernatant to a well of a flat-bottom microplate.
8. Measure the OD at 415 nm.

Calculations

1. Subtract the mean OD_{415} of the negative control from the OD_{415} of all test samples, reference samples and the positive control.
2. Calculate the percentage haemolysis for each sample and dilution as OD_{415} (sample) / OD_{415} (positive control) × 100%.
3. Plot the percentage haemolysis as the dependent variable (y) against the natural logarithm (ln) of the plasma fraction as the independent variable (x), where the plasma fraction is the reciprocal of the dilution (e.g. x = 0.25 for a 1:4 dilution). See Figure 2.
4. Determine the slope (a) and intercept (b) of the best-fit line:
   - $y = a \cdot \ln(x) + b$
5. Solve for x:
   - $x = \exp((y - b) / a)$
6. For 50% haemolysis (y = 50%), calculate the plasma fraction x_{50}; the CH50 is the reciprocal of this fraction:
   - $x_{50} = \exp((50\% - b) / a)$, and $CH50 = 1 / x_{50}$

Note: The relationship between haemolysis and ln(plasma fraction) is sigmoidal and only approximately linear in its central part. If the curve shows clear plateaux, restrict the linear fit to the dilutions that give approximately 10–90% haemolysis, or use a four-parameter logistic (4PL) fit.
An example calculation spreadsheet is available on request.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 4×	1 8×	1 16×	1 32×	1 64×	1 128×	9 4×	9 8×	9 16×	9 32×	9 64×	9 128×
B	2 4×	2 8×	2 16×	2 32×	2 64×	2 128×	10 4×	10 8×	10 16×	10 32×	10 64×	10 128×
C	3 4×	3 8×	3 16×	3 32×	3 64×	3 128×	11 4×	11 8×	11 16×	11 32×	11 64×	11 128×
D	4 4×	4 8×	4 16×	4 32×	4 64×	4 128×	12 4×	12 8×	12 16×	12 32×	12 64×	12 128×
E	5 4×	5 8×	5 16×	5 32×	5 64×	5 128×	13 4×	13 8×	13 16×	13 32×	13 64×	13 128×
F	6 4×	6 8×	6 16×	6 32×	6 64×	6 128×	14 4×	14 8×	14 16×	14 32×	14 64×	14 128×
G	7 4×	7 8×	7 16×	7 32×	7 64×	7 128×	15 4×	15 8×	15 16×	15 32×	15 64×	15 128×
H	8 4×	8 8×	8 16×	8 32×	8 64×	8 128×	NC	NC	NC	PC	PC	PC

Figure 1. Suggested 96-well plate layout for the CH50 assay. 1–15: samples (including the reference plasmas) with their dilution factors; NC: negative control; PC: positive control.

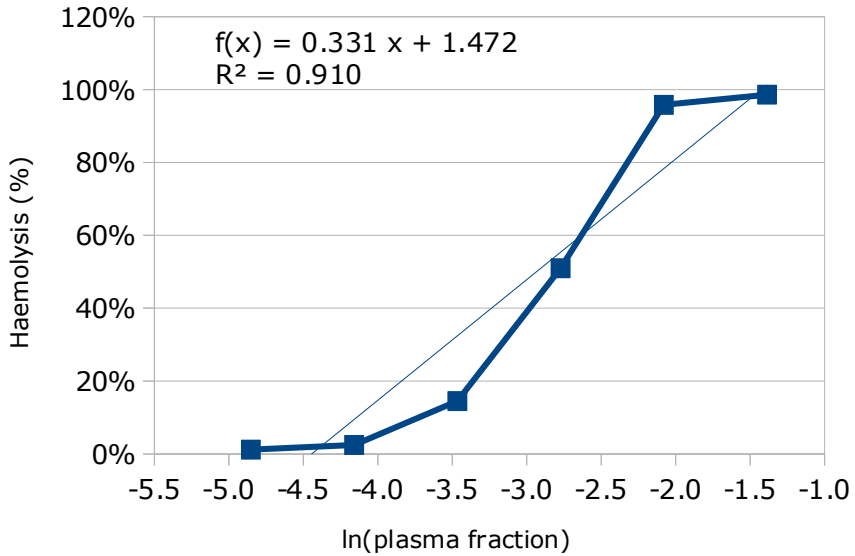

Figure 2. Example graph for the calculation of the CH50. In this example, the plasma fraction that gives 50% haemolysis is:

x_{50} = exp((0.50 − 1.472) / 0.331) = 0.0530 (5.30%), which corresponds to CH50 = 1 / 0.0530 = 18.9.