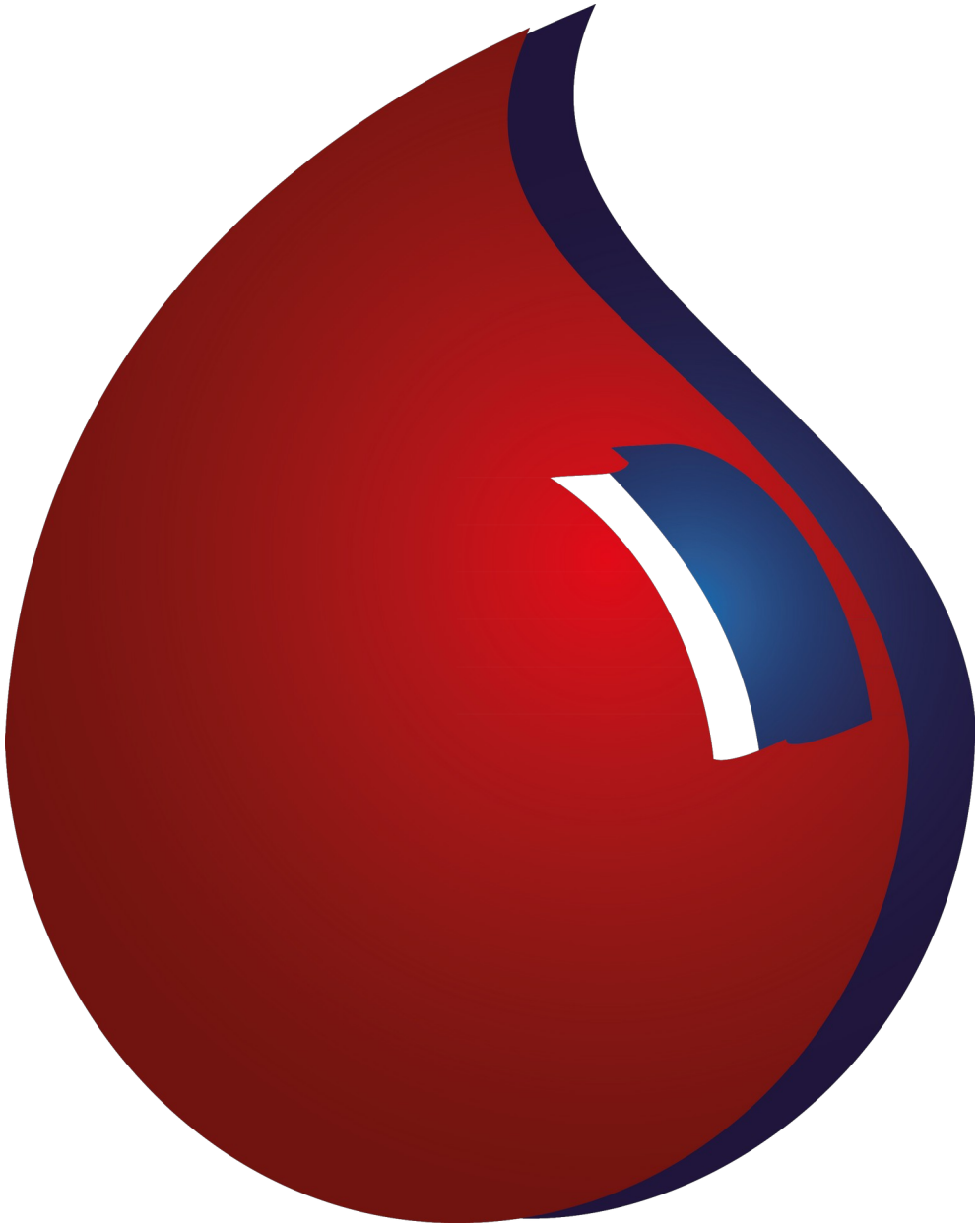

HaemoScan

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Hemolysis Assay for Solutes

Manual

www.HaemoScan.com

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Introduction

Determining haemolytic activity is a requirement when during the development process of new solutes/pharmaceuticals. This assay is based on erythrocyte lysis induced by contact, leachables, toxins, metal ions, surface charge or by any other cause. In vivo haemolysis may induce (severe) adverse events, due to anemia and the release of iron or other bioactive substances.

The current description is based on direct contact of a solute and an erythrocyte suspension. The method is based on release of haemoglobin, which can be measured by means of a spectrophotometer.

Principle of the Test

An erythrocyte suspension is incubated with the solute at 37ºC for 1 hour. After incubation the samples are centrifuged to obtain supernatant. The free haemoglobin concentration in the supernatant is measured by means of a spectrophotometer at a wavelength of 415 nm. Alternatively, a wavelength of 540 nm can be used.

The test kit can be used to screen solutes/pharmaceuticals at their highest intended concentration. Subsequently, when certain compounds are found to induce haemolysis, the test kit can be used to find the EC50 by preparing a dose-response curve.

Precautions

- The kit is intended for research use only.
- The kit should not be used beyond its expiry date.
- Do not combine reagents from Haemolysis assays with different lot numbers.
- The erythrocytes are of human origin and have been tested and confirmed negative for HbsAg, HCV, HIV I/II, HTLVI/II and Treponema. However, blood should always be treated as a potential biohazard during use and for disposal.
- 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	1,5 mL	1 tube
• Assay Buffer (0.9% NaCl)	100 mL	1 bottle
• Lysis fluid (Positive control)	3 mL	1 bottle
• Reference sample (Triton X-100, 0,625%)	0,5 mL	1 tube

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 + microplates (round bottom and flat bottom) + microplate stickers.

Test Procedure

Note: The protocol below assumes the preparation of erythrocyte suspension for analysis of the maximum amount of samples (2x96 wells). Alternatively, smaller batches of samples can be analyzed, requiring a smaller volume of erythrocyte suspension. Calculate the required volume accordingly. Store the unused erythrocytes as concentrate.

Reagent Preparation

Erythrocyte suspension:

1. Add 1 mL erythrocyte concentrate to 5 mL Assay 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 20 mL Assay buffer, this is sufficient for 2 microplates.
4. Mix 50 µL of the obtained erythrocyte suspension with 150 µL Lysis fluid, this should give an OD415 between 0,500 and 0,600, when measured in a flat bottom microplate in a spectrophotometer. If the OD415>0,600, add 1 mL of Assay buffer and repeat the OD415 measurement. If the OD415<0,500, add more erythrocyte concentrate and repeat the OD415 measurement.

Note: Instead of the Assay buffer, the user can use the buffer in which the sample is suspended or dissolved.

Test samples: For the 'Screening' procedure, samples are suspended or dissolved in Assay buffer, or another buffer system of the users choosing, at the highest concentration of the intended application. For the 'EC50' procedure, a serial dilution is prepared of the samples. The dilution factor is up to the user.
The negative control is (Assay) buffer, the positive control is Lysis fluid.
Reference sample: Dilute the reference sample 10 times in Assay buffer, resulting in a concentration of 0,0625% Triton X-100. Subsequently, prepare a six point serial dilution with a dilution factor of 4.

Assay Procedure

1. Pipette 100 µL of test sample, reference sample and controls in a round bottom microplate.
2. Pipette 100 µL of the erythrocyte suspension to each well.
3. Cover the plate with a microplate sticker.
4. Incubate for 1h in an incubator at 37 °C.
5. Centrifuge the plate at 400xg for 10 minutes to pellet the unlysed cells.
6. Transfer 100 µL of supernatant to a well of a flat bottom microplate.
7. 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 EC50 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 EC50, the value of y = 50%, so:
   - $EC50 = \exp((50\% - b) / a)$

Upon request an example calculation spreadsheet is available.

Note: As the data is expected to follow a sigmoidal curve, a 4PL model for fitting the data would be better suited. However, this is less convenient in most spreadsheet programs.

	1	2	3	4	5	6	7	8	9	10	11	12
A	$1_{10^{-4}}$	$1_{10^{-5}}$	$1_{10^{-6}}$	$1_{10^{-7}}$	$1_{10^{-8}}$	$1_{10^{-9}}$	$9_{10^{-4}}$	$9_{10^{-5}}$	$9_{10^{-6}}$	$9_{10^{-7}}$	$9_{10^{-8}}$	$9_{10^{-9}}$
B	$2_{10^{-4}}$	$2_{10^{-5}}$	$2_{10^{-6}}$	$2_{10^{-7}}$	$2_{10^{-8}}$	$2_{10^{-9}}$	$10_{10^{-4}}$	$10_{10^{-5}}$	$10_{10^{-6}}$	$10_{10^{-7}}$	$10_{10^{-8}}$	$10_{10^{-9}}$
C	$3_{10^{-4}}$	$3_{10^{-5}}$	$3_{10^{-6}}$	$3_{10^{-7}}$	$3_{10^{-8}}$	$3_{10^{-9}}$	$11_{10^{-4}}$	$11_{10^{-5}}$	$11_{10^{-6}}$	$11_{10^{-7}}$	$11_{10^{-8}}$	$11_{10^{-9}}$
D	$4_{10^{-4}}$	$4_{10^{-5}}$	$4_{10^{-6}}$	$4_{10^{-7}}$	$4_{10^{-8}}$	$4_{10^{-9}}$	$12_{10^{-4}}$	$12_{10^{-5}}$	$12_{10^{-6}}$	$12_{10^{-7}}$	$12_{10^{-8}}$	$12_{10^{-9}}$
E	$5_{10^{-4}}$	$5_{10^{-5}}$	$5_{10^{-6}}$	$5_{10^{-7}}$	$5_{10^{-8}}$	$5_{10^{-9}}$	$13_{10^{-4}}$	$13_{10^{-5}}$	$13_{10^{-6}}$	$13_{10^{-7}}$	$13_{10^{-8}}$	$13_{10^{-9}}$
F	$6_{10^{-4}}$	$6_{10^{-5}}$	$6_{10^{-6}}$	$6_{10^{-7}}$	$6_{10^{-8}}$	$6_{10^{-9}}$	$14_{10^{-4}}$	$14_{10^{-5}}$	$14_{10^{-6}}$	$14_{10^{-7}}$	$14_{10^{-8}}$	$14_{10^{-9}}$
G	$7_{10^{-4}}$	$7_{10^{-5}}$	$7_{10^{-6}}$	$7_{10^{-7}}$	$7_{10^{-8}}$	$7_{10^{-9}}$	$REF_{10^{-4}}$	$REF_{10^{-5}}$	$REF_{10^{-6}}$	$REF_{10^{-7}}$	$REF_{10^{-8}}$	$REF_{10^{-9}}$
H	$8_{10^{-4}}$	$8_{10^{-5}}$	$8_{10^{-6}}$	$8_{10^{-7}}$	$8_{10^{-8}}$	$8_{10^{-9}}$	NC	NC	NC	PC	PC	PC

Figure 1. Example 96-well template for EC50 dose-response curves.

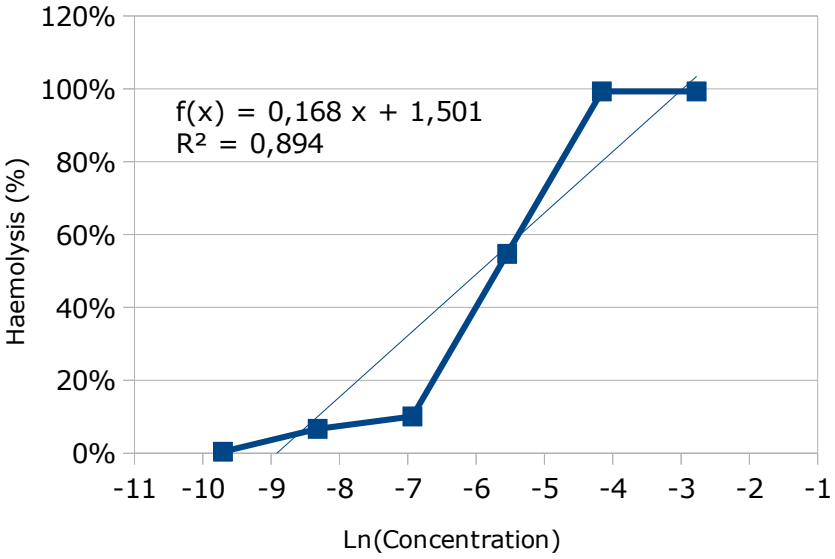

Figure 2. Example graph for the calculation of EC50. In this example the concentration required to obtain 50% haemolysis:

EC50 = exp((0,5 – 1,501) / 0,168) = 0,0026 %