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Biomaterial Hemolytic Assay

Kit Insert

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Summary

Hemolytic activity is a requirement to be tested for any blood contacting medical device. The test is based on erythrocyte lysis induced by contact, leachables, toxins, metal ions, surface charge or any other cause of erythrocyte lysis. The current description is based on direct contact of biomaterial and an erythrocyte suspension. The method is based on release of hemoglobin, which can be measured spectrophotometrically. This method is suited to evaluate the haemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2002.

Introduction

Interactions between blood and biomaterials may induce erythrocyte lysis. Particularly during prolonged contact or during contact of blood with large surfaces, this so called process of hemolysis may induce adverse events, due to anemia and the release of iron and other bioactive substances. Optimal conditions to perform a hemolysis assay have been described. Slightly diluted human blood in a blood/material ratio of 1 mL/cm^2 in a rotating chamber is recommended for performing the hemolysis assay.

Principle of the Test

An erythrocyte suspension is incubated for 24 hours with test material during rotation at 37ºC. Before and after incubation samples are collected and centrifuged to obtain supernatant, containing free hemoglobin. The hemoglobin concentration is measured by means of a spectrophotometer. Test samples are compared to reference materials. Positive reactive and less-reactive reference materials are included in the kit. It is recommended to include at least two reference materials in each analysis.

The results of the tested materials in relation to the reference materials may be used to evaluate the hemolytic activity. It must be noted that pass/ fail criteria are based on 2% hemolysis. Thus, also the total hemoglobin concentration of the used erythrocyte suspension must be determined.

The kit is designed to determine hemolytic activity of small biomaterial samples. Larger samples can also be used as long as the ratio between erythrocyte suspension and material size is respected.

Precautions

- Keep the kit or at least the erythrocytes in an as cold as possible freezer. The shelf life of 6 months is based on storage at -80ºC.
- Once the erythrocytes are thawed they should not be refrozen, but be kept at 2-8ºC. Other reagents can be frozen again.
- The kit is intended for research use only.
- The kit should not be used beyond its expiry date.
- Do not combine reagents from Hemolysis Kits 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 (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	3 mL	3 tubes
• Wash Buffer	30 mL	1 bottle
• Dilution Buffer I	20 mL	1 bottle
• Dilution Buffer II	20 mL	1 bottle
• Dilution Buffer III	40 mL	1 bottle
• Lysis fluid	8 mL	1 vial
• Hemoglobin (10 mg/mL)	0.5 mL	1 bottle
• Assay Buffer	10 mL	1 bottle
• Reference 1, Silicon elastomer (SE)	0.5 cm^2	5 pcs
• Reference 2, Buna N	0.5 cm^2	5 pcs
Reference 3, Medical steel (MS)	0.5 cm^2	5 pcs
• RBS cleaning solution (chlorinated trisodium phosphate, sodium metasilicate)	2 mL	1 vial

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, 450, and 380 nm
- Tweezers.
- Micro-centrifuge + vials (1.5 mL).
- Optional, for cleaning of test samples: Sonicator.
- Ultra pure water.
- Ethanol (70%).

Test Procedure

Preparation of Materials for Testing

The Hemolysis test can be used for coated or uncoated biomaterials. It might be important that clean samples be tested. The following procedure is recommended to clean biomaterials without coating:

1. Dilute RBS 50 times in UP water.
2. Sonicate the biomaterial for 15 min in the 2% RBS solution and wash three times with ultra pure water.
3. Sonicate the biomaterial for 5 min in 70% ethanol and wash three times with ultra pure water.
4. Dry the material in the air.

Notes:

- Cleaned materials should always be handled with clean tweezers.
- This cleaning procedure is a recommendation only; each user should determine his or her own optimal procedure. This procedure may affect a (biological) coating.
- The reference materials provided in the kit are clean and ready for use.

Reagent Preparation

Select the required reagents and number of bottles to be used. Keep spare erythrocyte tubes between -80ºC and -20ºC. The volumes given in the procedure are based on biomaterial pieces of 0.5 x 0.5 cm (surface area 2-sided approximately 0.5 cm^2).

Allow all reagents to obtain room temperature.

Prepare reagents as follows:

- **Erythrocyte suspension:** Add 5 mL Wash Buffer to the erythrocyte suspension. Mix gently by end-over-end tumbling of the tube. Centrifuge the tube, for 10 minutes at 1200xg. Remove the supernatant. Repeat this procedure. Then slowly add 5 ml Dilution buffer I mix gently, centrifuge, and perform the same wash procedure once with 5 mL Dilution Buffer II and once with Dilution Buffer III. The pellet is than resuspended in 5 mL Dilution buffer III. (The quality of the final suspension is controlled by centrifuging 0.5 mL in an

eppendorf, which should result in an almost colorless supernatant). Each washed vial is sufficient for 10 biomaterial samples.

- **Test samples:** Samples are preferably placed in a 2 mL syringe. After the erythrocyte suspension has been added, the remaining air is removed from the syringe, which is capped or closed with parafilm. In a standard tube the suspension should obtain air bubbles as little as possible. The negative control is a syringe without any material.
- **Hemoglobin calibrators:** The hemoglobin standard is used undiluted and further stepwise (1:1) diluted with Lysis fluid in separate vials. Use these dilutions as standard curve (Table 1).

Table 1. Preparation of Hemoglobin Calibrators.

		Hemoglobin Concentration (mg/mL)
CAL1	350	10
CAL2	150 Cal 1 + 150 lysis fluid	5
CAL3	150 Cal 2 + 150 lysis fluid	2.5
CAL4	150 Cal 3 + 150 lysis fluid	1.25
CAL5	150 Cal 4 + 150 lysis fluid	0.63
CAL6	150 Cal 5 + 150 lysis fluid	0.31
CAL7	150 Cal 6 + 150 lysis fluid	0.15
BLK	150 lysis fluid	0

Assay Procedure

Notes in Advance:

- The reference materials provided in the kit have been cleaned by the protocol described above and are ready for use.
- The volumes given in this procedure are based on pieces of material of 0.5 x 0.5 cm (2-sided: surface area approximately 0.5 cm^2). Larger materials need more volume. Test materials should be prepared in pieces of approximately the same size. A correction for surface area is made in the final calculations.
- Always avoid intense air contact and bubbles, since this may increase Hemolysis.

1. Place the selected reference and (cleaned) test materials in syringes or tubes using tweezers. Use syringes without material as negative control (CTRL).
2. Add 0.5 mL erythrocyte suspension to each vial with specimen (test material, reference material or negative control). Be sure that the biomaterial is completely immersed in the suspension. A syringe is closed by the plunger, air is removed and a cap or parafilm is used to close the outlet.

3. Add 10 µL of erythrocyte suspension to 990 µL of lysis fluid, to be used as total hemoglobin concentration.
4. Incubate the vials end-over end rotating at 37°C for 24 hours.
5. Transfer the erythrocyte suspension to a small vial (such as eppendorf), take care to avoid bubbles.
6. Centrifuge the vials at high speed (>4000xg) for 1 minute.
7. Add 20 µL of supernatant from sample or standard to a well of a microtiter plate.
8. Add 180 µL Assay Buffer.
9. Mix on a plate shaker.
10. Measure OD at 415/450/380 nm (Harboe method) or OD 415 if the other filters are not available. The calculated OD* from the Harboe method is (2 x 415) – (450 + 380).

Calculations*

1. Plot the calculated OD* against the hemoglobin concentrations of the calibrators. The calibration curve should be a straight line (OD*= A x CAL + B).
2. Correct for the hemoglobin concentration of the negative control.
3. Calculate the hemoglobin concentration of the biomaterials and reference materials in mg/cm^2.
4. Calculate the hemoglobin concentration in $\%/cm^2$ by the total hemoglobin concentration value corrected for 100 x dilution.

Assay Criteria

- The correlation coefficient of the calibration curve should be ≥0.98.
- The result of the negative control should be ≤0.1% of total hemoglobin.

Characteristics

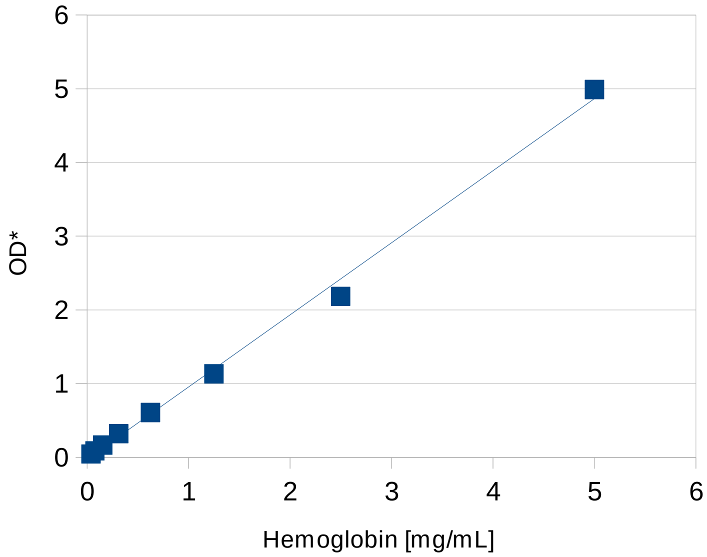

Figure 1. Example of Hemoglobin standard curve.

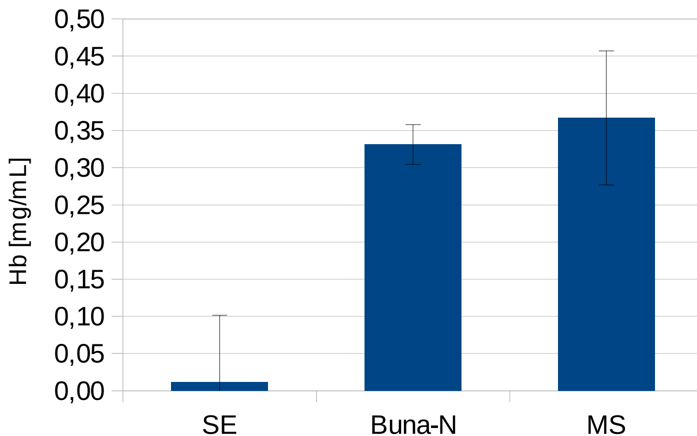

Figure 2. Example of hemolysis of the reference materials, corrected for negative control.