This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 862419. This publication reflects only the author's views and the European Union is not liable for any use that may be made of the information contained therein.

# SAbyNA

H2020-NMBP-15-2020

# SOP: Haemolysis assay

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*Version 2*

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|---|---|
| Lead beneficiary | RIVM |
| Contributing beneficiaries | IOM |

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# 1. Introduction

This standard operating procedure (SOP) describes the haemolysis assay as it was performed in the SAbyNA project. The purpose of the assay is to assess membranolytic potential of nanomaterials (NMs).

# 2. Materials

## 2.1 Reagents

- Fresh whole sheep blood in Alsever's solution (Thermo Scientific, 12977755) stored at 4°C for a maximum of one week.
- PBS
- Triton-X

## 2.2 Equipment

- Vortex
- Bath sonicator
- 15 and 50 mL tubes
- 2 mL Eppendorf tubes
- Flat bottomed 96-well plates
- Centrifuge for 50 mL tubes and 96-well plates
- Ice
- Plate shaker
- Absorbance plate reader

# 3. Procedure

1. Prepare the assay: Collect ice, label Eppendorf tubes with numbers 1-9 for each NM tested, let NM samples come to room temperature.
2. Prepare 0.1% Triton-X by combining 5 mL PBS with 5 µL Triton-X in a 15 mL tube.
3. Prepare NM stock solution:
    a. For powders, weigh NMs and add PBS to obtain 15 mg/mL.
    b. For particles already in suspension, dilute to 15 mg/mL in PBS.
    c. Vortex thoroughly and sonicate for 10 min using bath sonication.

**Note**: The use of glass vials for NM weighing and bath sonication is standard practice at RIVM, but Eppendorf tubes may be used as well.

4. Prepare NM dilution range (Table 1):
    a. 10 mg/mL is obtained by combining 333 µL of the 15 mg/mL stock with 167 µL PBS (Epp 2)
    b. Dilute the 15 mg/mL stock in a 1:1 serial dilution (combine 500 µL of PBS with 500 µL of the previous Eppendorf tube) to obtain concentrations for Epps 3-9.

**Table 1. NM concentration range tested.**

| Eppendorf tube (or glass vial) | NM concentration (mg/mL) |
|---|---|
| 1 | 15 |
| 2 | 10 |
| 3 | 7.5 |
| 4 | 3.75 |
| 5 | 1.88 |
| 6 | 0.94 |
| 7 | 0.47 |
| 8 | 0.23 |
| 9 | 0.12 |

5. Wash the Sheep's blood:
    a. Transfer 1 mL blood to a 15 mL tube. A total of 1.5 mL is needed per intended 96-well plate
    b. Add 1 mL cold PBS to each tube.
    c. Centrifuge tubes at 250 x g for 5 min.
    d. Remove supernatant and repeat this washing step.
6. Resuspend each pellet in 3.6 mL cold PBS and combine the obtained red blood cells (RBCs) into 15 mL tubes. Keep on ice until needed.

**Note**: The red blood cells should not be combined in vessels larger than 15 mL, as the weight of the red blood cells on top of each other may cause the ones at the bottom to rupture.

1. Add 150 µL of samples (NM suspension and 0.1% Triton-X) to the wells of a 96-well plate in triplicates. Make sure to vortex well. PBS is used as negative control. See Figure 1 for an example of a plate layout.

**Note**: If performing multiple plates in parallel, fill two plates at a time to avoid clashing timing schedules later on in the protocol. It is important that each plate has its own Triton-X (positive) and PBS (negative) control conditions.

2. Add 75 µL RBCs suspension to the wells containing the NM suspensions using a multichannel pipette.
3. Incubate for 10 min at room temperature whilst agitating at 300 rpm.
4. Centrifuge the plate(s) at 250 x g for 15 min.
5. Transfer 100 µL of the supernatant to a new 96-well plate using a multichannel pipet.
6. Read absorbance at wavelength 540 nm and 650 nm to correct for particle interference.
7. Express data as % haemolysis, where the Triton-X treated RBCs are set at 100% haemolysis.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | NM1 | | | | | | | | | Triton | |
| C | | | | | | | | | | | | |
| D | | 15 | 10 | 7.5 | 3.75 | 1.875 | 0.9375 | 0.46875 | 0.234375 | 0.117188 | | |
| E | | NM2 | | | | | | | | | PBS | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

**Figure 1. Example of plate layout used in the SAbyNA project.**