A common European approach to the regulatory testing of nanomaterials

# The NANOGENOTOX dispersion protocol for NANoREG

If in trouble, please send feed-back to

kaj@nrcwe.dk, yke@nrcwe.dk and CC mgm@nrcwe.dk

Grant Agreement n° 2009 21 01

Version 1.0

Date: 11 July, 2014

Keld Alstrup Jensen, National Research Centre for the Working Environment, Copenhagen, Denmark (NANoREG WP2)

# Table of Content

# 1 Introduction

This SOP was developed for preparation of general batch dispersions for *in vitro* and *in vivo* toxicity testing in NANOGENOTOX. The method aims to produce a highly dispersed state of any MN by ethanol (EtOH) prewetting to handle hydrophobic materials followed by dispersion in sterile-filtered 0.05% w/v BSA-water at a fixed concentration of 2.56 mg/ml using probe sonication. The protocol may not always produce the smallest particle size in the dispersion as possible, but it is a generically applicable procedure that ensures reasonable dispersion of a wide range of test materials.

The protocol is developed with the aim to use the dispersion immediately after its production. The current protocol can ensure stable dispersion characteristics for 0.5 to 1 hour. Re-dispersing due to onset of agglomeration and/or sedimentation is possible by vortexing the dispersion for 10 seconds.

Testing of powder NM after longer storage times in liquids should be avoided in general due to risk of partial alteration of the original MN or its coatings and other hydrochemical reactions.

# 2 Materials

A 2.56 mg/ml stock dispersion is prepared by prewetting powder in 0.5 vol% ethanol (≥ 96% purity) followed by dispersion in 0.05 wt% BSA-water during 16 minutes of probe sonication. For harmonization of dispersion energy and stabilities it is recommended to produce a 6-8 ml dispersion in the 20 ml tall glass scintillation vials. It takes 15.36 mg to produce a 6 ml 2.56 mg/ml stock dispersion (see below for further details).'

## 2.1 List of Ingredients and supplies

Pure and sterile-filtered water
Bovine Serum Albumin (sterile)
Ethanol (96 vol%)
1 flask for batch dissolution of BSA
1 flask for sterile-filtered 1% w/v stock BSA-water solution
1 flask for 0.05% w/v BSA-dispersion medium
Sterile filter (0.2 μm)
Vials: 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.) for weighing out MN and sonication
Steel and glass spatula
Pipette and pipette tips
Weighing boat/weighing paper
Electrostatic neutralizer
Weigh
Control or reference weights
Probe sonicator
Ice (Ice-water)

## 2.2 Water quality

It is recommended to use the purest available water. The protocol has been tested using both Nanopure Diamond UV and Millipore MilliQ-filtered water (Figures 1 and 2). No differences were observed in the quality of the batch dispersions. However, if chemical analyses of especially Fe and Zn are to be conducted, it may be recommended to use Nanopure or

controlled water because water from some Millipore systems has been found to be contaminated with these elements at promille levels even after mounted after de-ionization units.

Quality control of the water may be done before use. Especially in case that analysis and experiments may be influenced by trace-elements at low concentrations.

For general sampling and validation, we suggest collection of water in acid cleaned chemically stable bottles suitable for elemental analysis. Control the water quality (e.g., particles by DLS, elemental concentration by Atomic Absorption Spectrometry (AAS) or Inductive Coupled Mass Spectrometry (ICP-MS), CFU and endotoxin by growth in petri dishes or specific analysis) before use. If the water sample passes the quality test, the water is evaluated as pure and can be used for the experiment.

### *2.2.1 Nanopure Diamond UV water:*

The Thermo Nanopure Diamond® UV-water system (Thermo Scientific) water purification system is made by Thermo Scientific and designed with 4 stages of de-ionization combined with UV light-treatment and final particle filtration Filter: 0.2 μm filter (γ-irradiated Barnstead D3750 Hollow fibre filter). The water quality is listed as:

Resistivity: ≤ 18.2 MΩ-cm at 25ºC
Pyrogens: < 0.001 EU/ml
Total Organic Carbon: < 3.0 ppb
Other: nuclease-free (RNase andDNase).

*Figure 1. Example of Thermo Nanopure Diamond® UV-water system (Thermo Scientific).*

### *2.2.2 MilliQ-water:*

Most Millipore water purification systems in toxicology laboratories are designed to filter water that has already been de-ionized. It contains an internal filtration unit and a final 0.22 μm MilliPark Gammagold (Millipore) filter for particle filtration. The quality of the Millipore water is listed as:

Resistivity: 18.2 MΩ-cm at 25ºC
Pyrogens: <0.02 EU/ml
TOC: 5-10 ppb
Silicates: <0.1 ppb
Heavy metals ≤ 0.1 ppb
Microorganisms: ≤1 cfu/ml

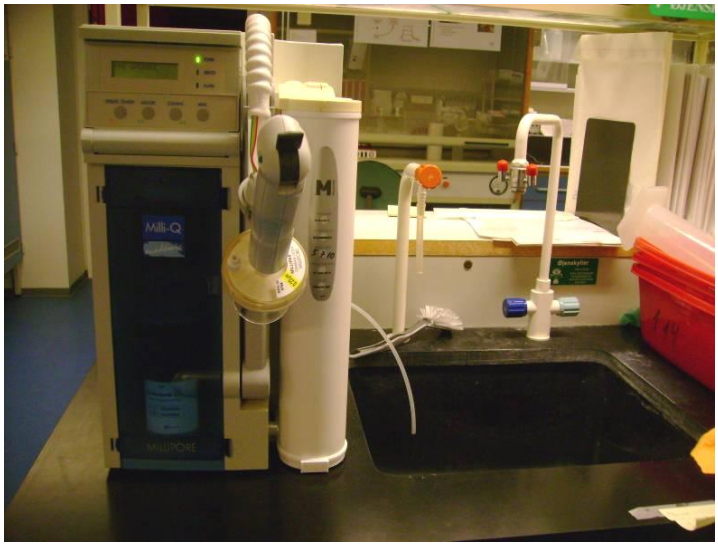


*Figure 2. Example of a Millipore water filtration system (Millipore).*

### 2.3 Type of serum albumin

Different types of serum albumin may be selected to fit the specific toxicological *in vitro* and *in vivo* tests systems (e,g, calf, mouse and rat). It is important that the selected albumin has passed tests for purity and sterility. This protocol was developed using Bovine Serum Albumin (BSA) with the following specifications:

Bovine Serum Albumin (Fraction V), Sigma (catalogue number: A-9418)
Cell culture tested
Analysis: 16 wt% Nitrogen and 1.9 wt% $H_2O$.
Number of amino acid residues: 607
Molecular weight: 66,399 Da
Isoelectric point in water at 25 °C: pH = 4.7
Physical dimensions: 140 x 40 x 40 Å (prolate ellipsoid where a = b < c)

# 3 Production of dispersion medium

### 3.1 Production of sterile-filtered BSA water

The production of the 0.05% w/v BSA-water (the dispersion medium) is done in two steps: 1) Preparing a sterile-filtered 1% w/v BSA stock solution and 2) Dilution to reach a 0.05% w/v BSA dispersion medium.

### 3.1.1 Procedure for making a 1% w/v BSA water stock solution

1) Add from pipette 50 ml water Nanopure (or MilliQ) to a 100 ml mixing flask (e.g, re-useable acid-washed blue-cap flasks or similar).
2) Weigh out 1 g BSA (powder) in a weighing boat and pour it into the flask with 50 ml water, rinse the weighing boat into the mixing bottle with Nanopure (or MilliQ) water to retrieve as much BSA as possible into the mixing flask.
3) Fill Nanopure water (or MilliQ) into the mixing flask up until 100 ml to reach a 1 % w/v BSA-water solution.
4) Gently stir or swirl the BSA-solution for a few minutes (be careful to avoid foam by not using agitated stirring) and leave the mixing flask in the refrigerator over-night for complete dissolution of the BSA.
5) Sterile filter the 1% w/v BSA-water solution into a new flask through a 0.2 μm sterile disposable filterware with collection flasks after complete dissolution of BSA in the mixing flask. We found that this sterile filtration causes about 28% loss of BSA and hence, the true BSA concentration in the final so-called 0.05% w/v BSA solution is in fact of 0.036% w/v as determined by a Pierce BCA protein Assay Kit for microplate reading. We have kept the 0.05% notation in accord with the direct dilution procedure below.

*Figure 3. Flask with raw 1% w/v BSA solution and a 1% w/v sterile-filtered BSA-solution in a centrifuge vial. After sterile filtration the 1% w/v BSA stock solution can be kept in a refrigerator for at least 2 weeks.*

### 3.1.2 Procedure for making the "0.05% w/v BSA-water dispersion medium"

The 0.05% w/v BSA solution to be used for test item preparation is achieved by simple dilution of the sterile-filtered 1% w/v BSA batch solution. *Remember that the 0.05% w/v BSA solution in reality contains ca. 0.036% BSA w/v due to loss in the sterile-filtration.*

Example: 2 ml 1 % w/v BSA is diluted with 38 ml Nanopure water (or MilliQ) (Dilution factor = 20x) to reach a batch solution of "0.05% w/v."

# 4 Handling of the test materials

Weighing should be done in a ventilated weighing box, a glove box or a fume hood designated for sensitive weighing with an accuracy of at least 0.1 mg or better.

For preparing a 2.56 mg/ml stock dispersion in a 6 ml EtOH-BSA-water, each vial must contain 15.36 mg nanomaterial. For harmonization of the dispersion energy it is recommended to stay as close as possible to a total volume of 6 ml EtOH + BSA-water.

Calculation of the correct volume is done simply according to equation 1:
1: V = m /c

m = mass of nanomaterial (mg)
c = concentration (normally 2.56 mg/ml)
V = volume of dispersion medium (ml)

## 4.1 Materials and supplies

Microweigh with accuracy of 0.1 mg or better
Reference or control weights with masses within the scale of the weighing project
Wet and dry wipes for cleaning
Weighing boat
Steel and glass spatula's
Vials: 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.)[a]
Vials with nanomaterials
Tray for storage of vials
Argon source.[b]

[a] Can be re-used after acid washing or regular washing followed rinsing in ethanol. Add new pp-lock+Alu-foil lids.
[b] Maybe needed for handling NM-series materials distributed from JRC. See below.

## 4.2 Preparation of weighing area

1) Turn on the weighing box, glove box, fume-hood 15-30 minutes before use.
2) Ensure wearing appropriate work dress (two- or three layer gloves[*], lab-coat, laboratory shoes) and that personal respiratory protection equipment is easily accessible in case of accident.
3) Ensure all material to be used for weighing and storage is present (nanomaterials, bottles/vials for weighing material in, cleaning tissue (both wet and dry wipes) before commencing the work.
4) Calibrate the weigh with traceable reference weight and log the data. Check that accuracy is within acceptance.

[*] *It is recommended to use two- or three layers of gloves for dermal protection. 1) Inner glove in textile 2) and or inner glove using long powder free nitril or latex rubber glove, 3) powder free nitril glove.*

## 4.3 Weighing out the nanomaterial powders

1) Open a clean empty vial for preparation of the stock dispersion and place it on the weigh.
2) Tara the weigh

3) Carefully open the vial without shaking it (NM-materials are packed in argon atmosphere and a special strategy may be required for weighing and potential re-use for NM-samples – see below).
4) Remove the electrostatic charge on the vial using a neutralizer (e.g., ionization blower) and carefully weigh out the required mass with a spatula in steel or glass.
5) Close the lid on both vials
6) After completion of weighing materials, clean the weigh and work area for potential spills using wet and dry wipes.
7) Waste is packed in a suitable waste bag and discarded according to local or institutional directions.
8) Ventilate the work-area (ventilated weighing station, fume hood, glove box etc.) for 15 minutes after weighing and cleaning has been completed.

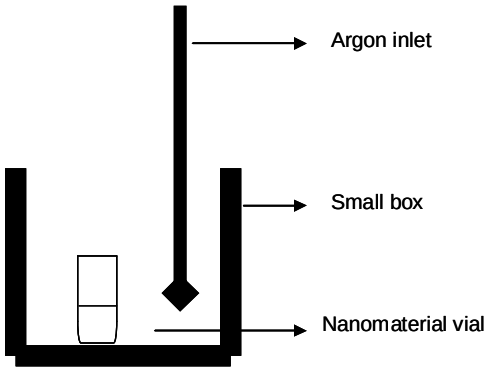


*Figure 4. Illustration of a simple setup for sample retrieval and re-sealing NM material vials under argon.*

### 4.4 Handling liquid dispersions (example for NM-300K)

The vial with NM300K contains 0.5 g silver to 5 ml de-ionised water (75%) with stabilizing agent 7% ammonium nitrate and 8% emulsifiers (4% each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene(20) Sorbitan mono-Laurat (Tween 20). The silver concentration is roughly 10% w/w (100mg/ml), which means that each vial contains 500 mg of silver (See data sheets for exact concentrations). The material is very viscous, and weighing is therefore recommended for dilution.

For dilution use at least 25 ml dispersion media. - E.g. 1 ml NM-300K to 38.0625 ml dispersion media will give a concentration of 2.56 mg/ml.

**WORK FAST**, once the material vial is open! Keep the vial upright during handling to minimize escape of the argon.

1) Weigh the vial for the stock suspension with cap
2) Remove cap from stock suspension vial
3) Remove cap from material vial
4) Pipette calculated volume from material vial to mixing vial. (At least material enough for 25 ml dispersion)
5) Close the material vial. If you choose to use the silver again, seal it under argon.
6) Close the mixing vial
7) Weigh the mixing vial and calculate mass difference

8) Add calculated amount of dispersion medium (2% serum water v/v) and stir it gently by hand.
9) Pipette 6 ml from mixing vial to stock suspension vial.
10) Close the stock suspension vial.
11) Proceed to probe-sonication (section 6).

# 5 Dispersing the test materials

6 ml is required to disperse 15.36 mg powder at the target concentration of 2.56 mg/ml. 0.5 vol% EtOH (96% or higher) is used for pre-wetting: This corresponds to 6 ml x 0.5/100 = 0.03 ml (30 µl) EtOH. Then 99.5 vol% sterile-filtered BSA-water (0.05% w/v) is used as dispersion medium corresponding to 6 – 0.03 = 5.97 ml BSA-water.

Pre-wetting is introduced to enable dispersion of hydrophobic materials in water-based systems. Here EtOH pre-wetting is used for all materials to harmonize the treatment for all powder materials.

### *5.1.1 Pre-wetting procedure (volumes for 15.36 mg powder)*

1) Carefully open the glass scintillation vial with pre-weighed powder (ideally 15.36 mg).
2) Tilt the scintillation vial ca. 45° and add 30 µl EtOH drop-by-drop onto the particles in the vial by pipette.
3) Screw on the lid and gently mix the EtOH and powder by simoultaneous gently tapping the vial on the table-top while rotating the tilted 45° vial from side to side between your fingers for approximately one minute.
4) Add 970 µl 0.05 % BSA water by pipette while slowly rotating and swirling the 45° tilted scintillation glass. Be careful to avoid foaming of BSA. The last ml BSA-water or so is added along the top of the inner wall of the vial to collect the powder in the fluid at the vial bottom.
5) Add the remaining 5 ml 0.05 % BSA water by pipette along the sidewalls of the scintillation vial to wash down any particles that may be stuck to the sidewalls.
6) Place the vial on ice for at least 5 minutes while the sonicator and ice-water is prepared.

### *5.1.2 Probe Sonication*

Remember for the sonication vial, it is requested to use the specific 10 ml Schott Duran glass beakers D=2.6 cm listed under materials to enable comparability.

**Important:** The **suggested sonifier** is a 400 W, 20 kHz Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). The horn can be seen at www.sonifier.com/pdf/DISRUPTO.PDF. The sonicator can be seen at http://www.sonifier.com/s450_digital.asp.). **Other sonifiers** may be used. **All sonifiers must calibrated** using the ***NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing***.

The samples are continuously cooled in an ice-water bath to minimize heat development during sonication (Fig. 5). For the ice-water bath, add pre-cooled MilliQ to the insulated box (e.g., styrofoam) with ice in order to ensure a more direct cooling of the sample.

1) Fill a 250 ml glass beaker with ice and place it upside-down in a insulation box (e.g., styrofoam)
2) Add ca. 85-90 vol% ice into the insulation box
3) Add ca. 10-15 vol% cold (e.g.,. refrigerated) water into the insulation box
4) Carefully place the glass scintillation vial with powder on top of the upside-down glass-beaker and pack the ice-water around the vial to keep the dispersion cooled. One may fix the vial using a clip or burette holder to ensure that the vial is not moving during sonication.
5) Insert the sonication probe as close as possible one third into the dispersion. Never immerse it less than the upper quarter and never lower than half-way into the dispersion (find the correct height using a vial with BSA-water alone).
6) Start sonication and run it for 16 min at the settings identified of your specific probe-sonifier using the *NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing*, while controlling that the sonication probe does not touch the walls of the scintillation vial.
7) Remove the scintillation vial and add the lid.
8) Clean the sonication probe by sonication for 5 minutes (similar sonication settings) with the probe fully immersed in a 50:50 water-EtOH (>96%) mixture followed by rinsing in EtOH using a dispenser and a collection bottle underneath. The probe is allowed to air-dry in the fume-hood. Other in-house cleaning methods may also apply.

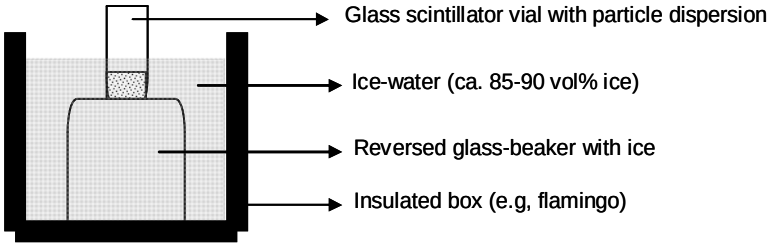


*Figure 5. Illustration showing a principle set-up for placement of sonication vial in ice-water bath. Different configurations are possible. NB Flamingo is styrofoam!*

**Important:** The sonicator horn must not touch the sides and bottom of the glass when ON. Make sure that *the horn is immersed as close as possible to the upper one third in the medium; not above the upper quarter and not below the upper half of the medium*. Do not start the sonicator before the probe has penetrated the liquid. Use approximately 6 ml dispersion medium and never less than 4 ml.

# 6 Quality control

After sonication, the dispersions must be checked ***using dynamic light-scattering (DLS) or alternative techniques*** following the requests in the NANoREG Technical Guidance Document. For the first assessment, it is suggested to use the standard viscosity of water. It should be noted, however, that the viscosities of some dispersion may differ significantly from that of water (and even for some materials also from time to time). Viscosity measurements

are usually time-consuming and will have to be made in parallel or by subsequent correction of the DLS data.

Samples for electron microscopy (TEM or SEM) or Atomic Force Microscopy must be prepared at the same time as DLS measurements are done. Check for protocols on CIRCA as specified in the NANoREG Technical Guidance Document.

# 7 Stability of the stock suspension

The stock dispersions are meta-stable due to the relatively high particle concentrations. Suspensions normally remain stable for 30 to 60 minutes depending on the NM. To ensure homogeneity, one should always gently shake or Vortex the stock dispersion at low to intermediate speed for 10 sec before use. It has been shown that this, normally re-establishes the original dispersion characteristics. It is assumed that this observation can be extrapolated to other NM.

Alternatively ultrasound bath treatment may be used, but the result may vary with type of equipment.

# 8 Application of the stock dispersion

The stock dispersion may now be used for *in vitro* and *in vivo* exposure studies. Please follow the NANoREG Technical Guidance Document regarding the documentation and qualification requirements for the stock dispersion medium and exposure media.

## 8.1 In vitro studies

Procedure: The 2.56 mg/ml stock suspension should be diluted at least 10 times with full normal cell media. Highest tested concentration will thus be 256 µg/ml. It is recommended that media controls are included in the studies to determine if the dilution with the dispersion medium has any effect on your cells/assay. So far no reports have been given on that.

## 8.2 In vivo studies

Procedure: The stock suspension will be used as is (Highest concentration; 128 µg/50 µL) or diluted to lower concentrations (1-64 µg/mL).

*It should be noted that the dispersion characteristics of these diluted dispersions have not been documented yet. Dilution may be made using the dispersion medium (sterile-filtered 0.05% BSA-water) or phosphate-buffered saline solutions. It is anticipated, however, that better dispersion and stabilities is generally expected for stock dispersion diluted in pure de-ionized sterile-filtered water.*

# 9 References

Jacobsen NR, Pojano G, Wallin H and Jensen KA (2010). Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA report. March 2010. National Research Centre for the Working Environment. 8 pp.

# Changes to protocol

No changes from vs. 1.0 has been made

## Important notes

Improved wetting and particle recovery from sidewalls can be made by adding the 0.05% w/v BSA solution in two steps. A first step with 970 µl for prewetting and a second step with 5 ml to wash down walls of the vials. Also store the vial ca. 5 min on ice while preparing the probe sonicater.

It has been found that the sonication efficiency and dispersion stability may vary with the vial type. It requested for harmonization that batch dispersions are made in the exact 20 ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.).

It is imperative that the sonication probe is immersed as close as possible to one third into the medium in the Scint-Burk sonication vial, but not above. At this position, one will avoid splashing and still retrieve as high efficiency of the sonication probe as possible. Lower positions may increase deterioration of the probe tip and lower the de-agglomeration efficiency.

The type of probe-sonicator is not fixed. The setting for a specific sonicator and tip dimension is identified following the "SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing". However, it is strongly recommended to use a 13 mm diameter probe-tip. Larger tip diameter will increase the risk of touching the vial side walls and breaking the vial during use. A smaller tip is prone to decrease the de-agglomeration efficiency.

The probe tip should be inspected regularly for deterioration. If the tip starts to deteriorate, it must be replaced to avoid unnecessary contamination by probe debris. Careful cleaning and maintenance of the probe is imperative to ensure a good and clean test material.

This generic protocol may not always give the best dispersion possible regarding achievement of minimal size and stability, but it produces acceptable dispersions which satisfy the needs for cross-comparison in toxicity testing in being reasonably dispersed, made in the same way, with the same amount of dispersants etc. It should also be noted that MNs are sometimes strongly aggregated or agglomerated and dispersion to sub-100nm particles of MN may not always be possible nor relevant.