



A common European approach to the regulatory testing of nanomaterials

# NANoREG

# Guidance Document

Work in progress

Version 2.2

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# 1 Introduction

## 1.1 Purpose of the guidance document and implementation

This document gives the NANoREG partners a specific description on the nanomaterials to be tested and the dispersion protocols to be used as well as the minimum characterization and reporting requirements to be applied for the *in vivo*, *in vitro* and eco-toxicological studies in NANoREG.

The guidance given is divided into mandatory, recommended, and optional procedures and characterization, acknowledging the current state of the art and available equipment in different laboratories. However, we urge all participants to excel in their studies to reach maximum impact and likelihood of scientific merit.

It is important to note that only tests on the approved NANoREG Nanomaterials, using no less than the mandatory dispersion protocols and the specified characterization and reporting requirements are eligible for funding in NANoREG. Possible deviations must be argued and approved by the relevant Work Package leader (WPL) and the management committee (MC).

## 1.2 Why common procedures and a minimum level of harmonisation?

The NANoREG project is aimed at developing building blocks for the regulatory testing and assessing of Nanomaterials. To this end, the project will be “testing the tests” to, on one hand determine the most appropriate ways to assess the environmental, health and safety effects of nanomaterials, and on the other hand to generate information for read across and Safe by Design (SbD).

To achieve read-across, it is necessary to:

- use the same test materials
- harmonize the test method(s)
- report specific measurements using the specific characterization methods
- report the data in a specific format for final logging

By these four points we will all gain insight into the reliability of the specific test methods by comparing results, to be able to use the results of one task as input for another and last but not least, to generate a robust dataset on which the NANoREG project can build the conclusions and policy recommendations.

# 2 NANoREG Core Nanomaterials

## 2.1 Eligible Manufactured Nanomaterials

A list of 19 manufactured Nanomaterials (MNM), denoted “the NANoREG Core Nanomaterials” have been selected for the NANoREG project so far (Table 1). The use of these 19 Core MNM is mandatory as they serve as the backbone for the test of the tests.

It is acknowledged that use of other materials may still be necessary to address specific regulatory questions or methodological developments in NANoREG. Currently, a set of 42 MNM and additional tailor-made MNM are available for these purposes. Again these materials are mandatory to ensure comparability across the project, but their use has to be justified and approved by the WPL, under which the work will be conducted, and finally by the MC.

In principle, only the experiments with the NANoREG Core Nanomaterials or on the list of

alternative materials (approved by the WPL and the MC), will be regarded as eligible costs in the framework of NANoREG.

All the NANoREG Core MNM are "industry relevant" and most are readily available from the JRC or Fraunhofer nanomaterial sample repository. Some MNM will be distributed from elsewhere. For most of the materials, especially the NM series, a large amount of data is already available (e.g. from the OECD testing project, NANOGENOTOX, PROSPECt and other projects).

**Table 1: The 19 NANoREG Core Nanomaterials.**

Type of MNM	MNM Identification codes used by NANoREG
<b>Titanium Dioxide</b>	NM101, NM102, NM103
<b>Silicon Dioxide</b>	NM200, NM203
<b>Zinc Oxide</b>	NM110, NM111
<b>Cerium Dioxide</b>	NM212
<b>Barium Sulphate</b>	NM220
<b>Silver</b>	NM300K, NM302
<b>Nanotubes (single and multi-walled)</b>	NM400, NM401, NM410
<b>Nanofibrillar cellulose</b>	NFC Fine, NFC Medium-coarse, UPM Biofibrils AS, UPM Biofibrils NS, UPM Bleached Birch Pulp
<b>Final material closing knowledge gaps</b>	Under evaluation

## 2.2 NANoREG Web ordering

All NANoREG materials can be ordered through the NANoREG Nanomaterials Information and Web-Order (NIWO) system. It can be reached directly from <http://www.nanoreg-materials.eu/> or via the partner area of the [NANoREG homepage](#). The NIWO system also offers up-to-date information in Technical Data Sheets (TDS) on the key physico-chemical data on each of the MNMs. Partners have to register and must be accepted as a NANoREG user to use this system. Please see and follow the instructions for registration and use carefully (<http://www.nanoreg-materials.eu/About>).

## 2.3 Handling of sample vials in the use phase

The JRC and Fraunhofer distribute the samples of nanomaterials to users in (small) sample vials or 'flasks' that are sealed under inert gas atmosphere (e.g. Argon). The stability of the samples over time can only be reasonably guaranteed by these distributors as long as the seal of the container is undamaged (i.e. the vial or flask still unopened). Other distributors using atmospheric gas need to qualify their materials periodically.

The contents of JRC and Fraunhofer vials (small NM quantities of a few tens of milligrams to about one gram) are supposed to be used within a few hours and only once after first opening,

i.e. once the seal has been broken. This principle would also be optimal for materials supplied by other distributors.

In cases the distributors ship much larger quantities (tens or hundreds of grams in a single flask), the user is entirely responsible for taking utmost care in the way the NM is stored after first opening of the flask. In particular, due considerations must be taken at all times during laboratory operations of the risks of contamination of the NM with, for instance, dust from ambient air, humidity or other chemical substances, while the flask lid is open or during transfer operations of the NM into other containers.

When sample transfer or re-opening of sample vials is needed, it is recommended to conduct sub-sampling in an Argon bath as depicted in the ENPRA dispersion protocol.

### 3 Selected SOPs and characterization requirements

#### 3.1 Eligible dispersion protocols

The NANoREG Management Committee has agreed on four dispersion protocols to be used in NANoREG (Table 2). The selection was based on a long list of protocols and their assessments based on pre-selected criteria for documentation, and final recommendations from a workshop held in November 2013 in Copenhagen.

The use of these dispersion protocols in the specified application areas is mandatory for all the NANoREG partners in their NANoREG experiments. The protocols can be downloaded from CIRCABC (*C-Supporting Documents/SOPs and Benchmark Data*).

**Table 2: Overview of dispersion protocols**

Type of test	Protocol
<b>Calibration of sonicators for in vitro and in vivo studies</b>	Calorimetic method combined with adjustment using the NM200 benchmark material NANOGENOTOX batch medium
<b>In vitro studies</b>	NANOGENOTOX
<b>In vivo studies</b>	NANOGENOTOX or ENPRA
<b>Calibration of sonicators for ecotox studies</b>	Calorimetic method combined with adjustment using the NM200 benchmark material in water
<b>Eco-toxicity studies</b>	A NANoREG water and a NOM*-water protocol for CNT

\* Natural Organic Matter

## 3.2 Minimum characterization requirements for dispersions and exposure media

### 3.2.1 Test item preparation and exposure characterization protocols

To ensure high-quality assessment of the in-use performance of the dispersion protocols in *in vivo*, *in vitro* and eco-toxicological tests, and finally to catch the potential outliers, the MC has decided on the means to produce a limited set of mandatory characterization data and how they should be reported.

The minimum characterization requirements are:

- Analysis of the hydrodynamic size(-distribution) of NM in the batch dispersion.
- Analysis of the initial hydrodynamic size(-distribution) of NM in the exposure medium.
- Analysis of the final hydrodynamic size(-distribution) in the exposure medium.

Additionally it is recommended to perform analysis of the fate (agglomeration, sedimentation, test conditions, chemical reactivity, and dissolution) of the NM in the exposure medium. A full list of characteristics to be reported and recommended is given in Table 3.

The MC selection of these minimum characterization requirements was based on the recommendations of the SOP workshop held 28 November 2013 at NRCWE in Copenhagen, Denmark, and fully reflects the minimum requirements typically requested for publication in scientific publications.

To help the users of the dispersion protocols to identify whether they have produced batch dispersions with expected size-distributions, a set of benchmark data will be made available from WP2 as a preliminary D2.6 and D2.7 report (see below).

Task 2.4 in WP2 has identified the DLS as one of the most robust and accessible instruments for characterization of hydrodynamic sizes of particles in liquid dispersions. DLS is also one of the instruments with the widest ranges in concentration and hydrodynamic sizes. However, higher size-resolution and accuracy, especially for multimodal size-distributions, may be achieved using other techniques such as analytical (ultra)centrifuges. The requested and possible alternative method to be used for the minimum characterization is listed in Table 4.

Answers to issues raised during the 3rd NANoREG consortium meeting and subsequent hearing time (ended June 19, 2014) have been listed in a separate Q&A document that is available on CIRCABC (C-supporting documents/Guidance document).

## 4 Sharing of benchmark and partners test data

As the NANoREG NM will be tested and characterized in a great number of different assays/tests (different materials, different *in vitro* and eco-toxicological exposure media, different cells and environmental species etc.), it is not possible to establish validation of the dispersion protocols for all MNM. Similarly, it is impossible to establish benchmark values for hydrodynamic size-distributions in all the test media for all MNM within the framework of the NANoREG project.

To meet the project goals and to perform a live „test of the test“, WP2 has offered to produce DLS benchmark data on the batch dispersion protocols for the NANoREG project as part of D2.6 and D2.7. Additional studies will be made on specific test media as part of D2.8. This work will be shared with WP3, WP4 and WP5. In return WP3, WP4 and WP5 are requested to send their DLS data on the batch dispersions to the WP2 leader ([kaj@nrcwe.dk](mailto:kaj@nrcwe.dk) and [mgm@nrcwe.dk](mailto:mgm@nrcwe.dk)) to participate in a NANoREG analysis on the performance of the applied dispersion protocols to be submitted for publication at a later stage in the project (see Chapter 5).

Table 3: Characterization requirements in toxicological tests using dispersions

<b>Element in the workflow</b>	<b>Recommendation (R) and Mandatory requirement (M); Optional (O)</b>
<b>Nanomaterial check</b>	Despite already characterized, it is recommended to perform a simple rough verification of the test material (e.g., TEM and SEM for qualitative verification of size and composition by EDS/EDX; XRD for crystalline phase, DLS at optimal zeta-potential or surfactant where benchmark exist, Raman scattering for phase (and defects) in CNT etc. (R)
<b>Batch dispersion<sup>*,£</sup></b>	Ten repeated measurements of hydrodynamic size (DLS) are made without pause in combination with verification or measurement with TEM, SEM or AFM which-ever is most suitable. In vitro (M) and eco-tox (M).
<b>Initial exposure medium<sup>£</sup></b>	Ten consecutive measurements of hydrodynamic size (DLS) are made (if technically possible) without pause on the same sample in combination with verification or measurement with TEM, SEM or AFM which-ever is most suitable. In vitro (M) and eco-tox (M)
<b>Final exposure medium<sup>£</sup></b>	Ten consecutive measurements of hydrodynamic size (DLS) are made (if technically possible) without pause on the same sample in combination with verification or measurement with TEM, SEM or AFM which-ever is most suitable. In vitro (M) and eco-tox (M).
<b>Stability of dispersion during assay<sup>*,£</sup></b>	It is recommended to follow size distribution and sedimentation of NMs in the exposure medium during the test. (R) Sedimentation rates can be assessed from calculations, analytical centrifugation or tests using the DLS. By DLS one may analyze both agglomeration and sedimentation. From experience, a time-resolution of 20 minutes after the first hour is a suitable time-resolution (e.g., 20 min.). Within the first hour a time-resolution of 10 minutes is normally suitable.
<b>Contextual conditions and reactivity in the during testing<sup>£</sup></b>	Measure several of the following parameters (pH, T, conductivity, redox potential and the CO <sub>2</sub> /O <sub>2</sub> concentrations) during testing. In vitro (R) and eco-toxicity (M).
<b>Dissolution in batch dispersion and test media<sup>£</sup></b>	Dissolution can be assessed by taking hydrous samples from the test or parallel tests conducted at the same conditions as the exposure conditions. The residual test material must be removed from the medium immediate after taking the sample. (R)

\* Benchmark data on core test materials are produced by WP2 and made available on CIRCABC (*C-Supporting Documents/SOPs/Benchmark data*);

£ Protocol is available at CIRCABC (*C-Supporting Documents/SOPs*)

€ Some data will be produced by Task 2.4. Please contact the WP2 leader if interested.

The WP2 benchmark data will be made using the techniques listed in Table 4. Partners can use the benchmark data to assess whether they have a suitable and representative dispersion for their experiments. The benchmark data will be uploaded and data completed as soon as possible and updated as data progress.

Table 4: Types of measurements to be made for characterization of the different dispersion

Situation/Assay	DLS	Analytical centrifuge	NTA	UV-vis	TEM/SEM/AFM (one method as suitable)
<b>NM size distributions in batch dispersion</b>	M <sup>&amp;</sup>	O	X	NA	M <sup>&amp;</sup>
<b>NM size distributions in vitro exposure media</b>	M <sup>&amp;,£</sup>	O	X	NA	M <sup>&amp;,£</sup>
<b>Size distribution in aquatic eco-toxicological test systems</b>	M	O	X	NA	M <sup>&amp;,£</sup>
<b>NM Concentration in aquatic eco-toxicological test systems</b>	NA	NA	NA	M <sup>&amp;,€</sup>	NA

NA: Not applicable

M: Mandatory (Protocols distributed and available at the *C-Supporting Documents/SOPs*)

O: Optional

X: Applicability for expected size-distributions and in concentration ranges has not been confirmed

<sup>&</sup> SOPs are available at CIRCABC (*C-Supporting Documents/SOPs*)

<sup>£</sup> At least relevant low, intermediate and high exposure concentrations should be covered.

<sup>€</sup> The characterization should be accompanied with suitable chemical analysis

#### 4.1 Statistical considerations

At this stage it is not possible to define statistical requirements regarding precision, accuracy, reproducibility etc. on all manufactured nanomaterials. Data on these aspects will become available during the "testing of tests". However, the aim of the sonicator "calibration" and benchmark data provided by WP2, is to harmonize the critical starting point for all the tests and enable immediate assessment of the quality of the test dispersions made.

Of course this does not overrule the necessity to take the statistical reliability into account when designing and applying test methods and to make statistical information regarding the test results from each laboratory (see next section).

## 5 Data sharing and data logging

For the purpose of the NANoREG project it is of paramount importance that an exchange of information between partners is possible. For this reason paragraph 8.3 of the Consortium Agreement states that:

*All Parties agree to an open and free sharing of data (Foreground) generated by the NANoREG project, and will transfer all data to the project database (NANoREG data platform) as detailed in the DOW.*

Data sharing and central logging is required for two key purposes.

First, to understand the applicability of the dispersion protocols, a deeper analysis of the in-use variability between the different laboratories will be made based on the batch dispersion data

from all the tests made by the WP3, WP4, and WP5 partners. Therefore, partners conducting toxicological tests are requested to submit their probe-sonicator calibration data as well as their DLS files with the batch dispersion data to the leader of Task 2.4 ([kaj@nrcwe.dk](mailto:kaj@nrcwe.dk) and [mgm@nrcwe.dk](mailto:mgm@nrcwe.dk)). A scientific paper documenting the in-use experience (and statistics) with the dispersion protocols is intended in collaboration with all partners submitting their data to WP2.

Second, to enable mapping of results (WP1) and to establish a better understanding of the relationships between physicochemical properties and the toxicological test results for grouping and safety by design (WP5 and WP6), reporting to a centralised data sharing platform must also be made as part of the project.

For this general data logging, ISA-TAB-Nano spreadsheet templates will be prepared and made available from CIRCABC (*expected before November 2014*). For this reporting, not only the exposure and test results, but also the relevant metadata and statistical information will be logged. The reporting requests are reduced as much as possible, respecting the workload of the NANoREG partners.

The data will be stored in CIRCABC (folder will be announced later) and be available for all partners as far as partners have not explicitly restricted the use of the data.

## 6 Status of SOPs and benchmark data

### 6.1 Status of dispersion protocols

The NANOGENOTOX dispersion protocol: "[The NANOGENOTOX dispersion protocol for NANoREG\\_10-07-2014.pdf](#)" (New modification for NANoREG (July 14, 2014) is made available on CIRCABC; C-Supporting Documents/SOPs)

The ENPRA dispersion protocol: "[The ENPRA dispersion protocol for NANoREG\\_10-07-2014.pdf](#)" (New modification for NANoREG (July 14, 2014) is made available on CIRCABC; C-Supporting Documents/SOPs)

The NANoREG-Ecotox dispersion protocol version 6. "[20150429 NANoREG-Ecotox Dispersion SOP version 6](#)" *This protocol replaces the PROSPECT dispersion protocol for ecotoxicity testing!* The document is available on CIRCABC; C-Supporting Documents/SOPs

### 6.2 Status on probe calibration protocols

Probe calibration protocol for the ENPRA and NANOGENOTOX protocols: "[SOP for calibration of Probe-sonicators for in vitro and in vivo testing\\_10-07-2014.pdf](#)" and Excel template: "[Template for Probe-Sonicator Comparison\\_10-07-2014.xlsx](#)" (Available on CIRCABC ; C-Supporting Documents/SOPs.

Probe calibration protocol for producing the stock dispersions for ecotoxicological testing is pending (draft available from Andy Booth, SINTEF; Final version expected in September, 2014)

### 6.3 Status on sampling SOPs

SOP for preparation of TEM-grids: "[SOP for TEM sample preparation CODA-CERVA.pdf](#)" is available on CIRCABC; C-Supporting Documents/SOPs.

SOP for preparation of SEM samples. The need and SOP has not been identified. Will be made on request.

SOP for preparation of AFM samples. The need and SOP has not been identified. Will be made on request.

#### 6.4 Status on characterization SOPs

SOP for determination of hydrodynamic size-distribution and dispersion stabilities: "SOP for DLS measurements using MALVERN NANO ZS\_2014-13-07.pdf" (Available on CIRCABC ; C-Supporting Documents/SOPs)

Template for TEM characterization of NM dispersions collected on TEM grids is pending. (Expected in September, 2014)

SOP for determination of NM concentrations in exposure media by UV-vis is pending (draft available from Andy Booth, SINTEF) (Expected in September, 2014)

#### 6.5 Status on benchmark values

Benchmark values for the NANOGENOTOX dispersion protocol: "DLS Benchmark Data for the NANOGENOTOX dispersion protocol\_2014-11-07.pdf"; (Draft available on CIRCABC; C-Supporting Documents/SOPs/Benchmark data; . Expected date for completion: Ultimo August, 2014).

Benchmark values for the ENPRA dispersion protocol: "DLS Benchmark Data for the ENPRA dispersion protocol\_2014-11-07.pdf"; (Draft available on CIRCABC; C-Supporting Documents/SOPs). Expected date for completion: Ultimo September, 2014).

Benchmark values for the water and NOM-water dispersion protocols: (Data not available; Expected date for completion October, 2014).

## 7 Corrections and Amendments

### 7.1 Changes made from Version 1.0 to Version 2.0; July 10, 2014.

Table 2. The ecotoxicity team has changed their protocol to be based on water (at least MilliQ-filtered de-ionized water) and the same probe-sonication treatment as applied in the NANOGENOTOX and ENPRA protocols. However, the ecotoxicology testing team follows a slightly different probe-sonicator calibration protocol as compared to the one established for the ENPRA and NANOGENOTOX dispersion protocols. This has corrected and further clarified in Table 2. (Changes made by Andy Booth and Keld Alstrup Jensen, NRCWE).

Table 3. Text in Nanomaterial check. The parenthesis has been corrected so the last sentence reads: "Raman scattering for phase (and defects) in CNT etc instead of Raman scattering for phase (and defects in CNT etc). (Changes made by Keld Alstrup Jensen, NRCWE).

Table 4. Mandatory DLS measurement of exposure media has been corrected to be mandatory for relevant low, intermediate and high concentrations. (Changes made by Keld Alstrup Jensen; NRCWE).

Footnote in Table 4: <sup>&</sup> SOPs are available at CIRCABC (*C-Supporting Documents/SOPs*) (Changes made by Keld Alstrup Jensen; NRCWE).

A chapter 6 Status of SOPs and benchmark data has been added to facilitate a better overview of the SOPs, templates, and benchmark values and their status.

A chapter 7 Corrections and Amendments has been added to make it easier for the reader to follow important changes in the Technical Guidance Document as it is still a living document.

# Questions and Answers

Response to the questions and remarks made  
by partners on the NANOREG Guidance  
document

Version: 1.0 (Q&As 01-12; related to Guidance Document version 1.0)

Date: 2014-07

Version: 1.1 (Q&As 13-29; related to Guidance Document version 2.0)

Date: 2015-03

Version: 2.2 no changes

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## 1 High throughput tasks affected?

*High throughput tasks will be seriously affected by current dispersion protocol recommendations. This implies buying a new, more powerful sonicator for some task 5.6 partners, Further discussions are to take place between WP2 and WP5 to achieve a compromise.*

Answer:

It is difficult to answer this question without specific information on the test design. The NANOGENOTOX and ENPRA protocols define preparation of 6 ml batch dispersion with a concentration of 2.56 mg/ml. At a typical maximum dose of 320 µg/ml one preparation is enough for 24 serial dose-tests with 6 doses from 1 to 320 µg (see table below).

In vitro dose (mg/ml)	Dilution factor	# ml of 1 ml total	
0.01	256	0.00390625	
0.02	128	0.0078125	
0.04	64	0.015625	
0.08	32	0.03125	
0.16	16	0.0625	
0.32	8	0.125	n portions in 6 ml
		0.24609375	24.38

The NANOGENOTOX dispersion protocol does not specify a specific probe, but it recommends use of a specific probe to ease comparison. The publically available NANOGENOTOX deliverable report number 3, describes a procedure for probe-sonicator harmonization by measurement of the consumed energy. As presented at the third NANoREG consortium meeting, a calorimetric probe sonicator calibration procedure has been established for use in NANoREG. By using this procedure, suitable settings and durations of sonication can be established for several different probe sonicators. Please read and follow the technical guidance document carefully.

## 2 Benchmark data: which test media; who will provide them?

*WP2 should state clearly in which test media they will perform additional studies (D2.8), including antibiotics and serum (with respective concentrations) regarding this issue, a table with all potential WP5 media was provided to WP2. Besides NM dose range should also be stated. Moreover, lab experiments are performed at 37C, and this should also be accounted for in WP2.*

*Moreover there may not be benchmark data on DLS measurements at the beginning and end of experiments in all test media from WP2, so we need to exchange these data amongst all WP5 partners? Who will provide benchmark data then?*

Answer:

As explained at the NANoREG consortium meeting 03, this part is WP2 research and it will not be possible to cover all different combinations outlined by WP5 and also covering WP4. WP2 will conduct experiments to hopefully identify the future procedure for exposure fate analysis. The work will be performed on a select suite of frequently used media compositions and naturally experiments will be conducted at realistic conditions. It was explained at the consortium meeting that benchmark data would be established for the batch dispersions and that the size-distributions and fate in exposure media would be part of the experiment in the experiment. Data will be reported to WP1 (ISA TAB) from where assessment of the data produced by WP3, WP4 and WP5 will be performed.

## 3 TEM/SEM/AFM: when?

*TEM/SEM/AFM measurements for each experimental condition are not feasible, clear indications on when to perform these needs to be included in the guidance document (this has already been put forwards to Keld).*

Answer:

Samples are prepared for subsequent confirmation and explorative analysis. The analysis is mandatory on the batch dispersion to enable subsequent morphological characterization of the initial exposure state and verification of DLS results. Imaging of the exposure media is also essential to help understand the exposure characteristics and fate of the nano material tested. These samples are to be made on low, intermediate and high exposure concentrations to investigate potential differences in exposure characteristics and changes over time. Further specification cannot be given as the exposure concentrations are highly variable.

#### 4 How many DLS measurements?

*How many DLS measurements do we have to do for each time point and NM concentration? Do we have to redo it each time we perform a new experiment?*

Answer:

As explained in the technical guidance document, you are requested to make ten consecutive analyses of a sample to represent the beginning and the end of an experiment. This is taking one sample, place it in the DLS, measure it 10 repeated times without pause at the beginning of the test and then again at a time-point corresponding to the end of the test. The analysis is done on each test material. The analysis can be done in different ways:

Place a cuvette with a suitable and representative volume in the DLS, perform 10 measurements, let it sit with the DLS equipment turned on until end of the test and perform the analysis once more.

Place a cuvette with a suitable and representative volume in the DLS, perform 10 measurements. Take out the vial very carefully and place it carefully in the cell-incubator until next intermediate measurement or at the end of the test and perform the analysis once more.

Place a cuvette with a suitable and representative volume in the DLS, perform 10 measurements, fix the analytical conditions and continue measurement for every 20 minutes and finalize the study by 10 repeated measurements on the same samples.

Protocols for this will be made available on the CIRCABC.

#### 5 Requirements NFC

*NFC has special requirements, it should not be sonicated and the use of DLS is still not clear. Further conversations are to be carried out between relevant WP5 partners and WP2.*

Answer:

Even-though the DLS signal is not easily understood, measurement may still be possible. Further detail and potential deviation from protocol for NFC will be announced as soon as possible.

#### 6 Hydrodynamic behaviour

*Additional information on the hydrodynamic behavior as function of time is requested. It is suggested to do continuous measurements at 20 min time intervals. How long must these measurements be (24h)? Do we have to evaluate the stability of dispersion for each experimental concentration and replicate?*

Answer:

These measurements should be done to achieve better understanding of the agglomeration and sedimentation behavior of the test materials in the specific test media. If possible and identified as a key information for the specific study, it is recommended to repeat the experiment to get more substantial data for assessment of the exposure conditions. It is now specified that data should be generated for low, intermediate and high concentrations to cover the potential differences in behavior with exposure concentrations.

## 7 EM equipment not always available

*Concerning TEM measurements, the real problem for some partners is the availability to perform EM measurements in their institutes. The recommendation to do that for each experiment in each task leads to serious modifications in the initial study plan to comply with such new issues.*

Answer:

The work is not so time-consuming as it sounds. It is recommended to seek collaboration within NANoREG or find a reliable contract institute with strong track-record. Characterization templates will be made available to harmonize the data generated.

## 8 DLS measurements non-adherent cells

*Regarding non-adherent cells, it is not clear how DLS measurements will be carried out at the beginning and end of experiments.*

Answer:

This is true and a challenge. How is this exposure condition normally assessed today. Characterization of true exposure conditions may be possible only by TEM. It is suggested to run a parallel cell-free test, which can indicate the behavior without cells. A key issue here is whether the nano materials stay dispersed and available in the medium.

## 9 NM settling

*Regarding test on NM settling we agree that one test is enough. We also believe that each new batch dispersion as well as each start/end exposure medium could be documented by DLS and not by TEM in order to minimize costs.*

Answer:

It is recommended to make the samples and keep them for back-up analysis if outliers suddenly appear.

## 10 What if dispersion goes wrong?

*Partners are concerned regarding indications to quickly stop an experiment if the dispersion goes wrong because when a toxicological experiment is planned many conditions are to be taken into account such as cell growth, time of exposure, differentiation of ex-vivo isolated mouse cells etc. The suggested approach will have an excess load of work and some delay in work plan. A possibility could be to consider as good deviation of ca. 20% (instead of the proposed 10%) from the benchmark data of Zeta average diameter.*

Answer:

The stop of experiment should be if one observed that the batch dispersion is out of range. This is to prevent making tests on a bad sample and thereby prevent waste of money producing doubtful results. The final acceptable boundaries for deviation on Zeta average cannot be distributed before after summer as the data does not yet exist. However, after following the calorimetric probe-calibration SOP we distribute, it should generally be possible to be within 10% in Zeta-average size. More detailed specification will follow as soon as possible.

## 11 Sample preparation for TEM

*Regarding sample preparation for TEM, it is advantageous to make the TEM grid hydrophilic (e.g., by glow discharge) to prevent artificial agglomeration on the TEM-grid during drying.*

Answer:

Yes, this is correct and included in the SOP for TEM sample preparation.

## 12 Marine tests media

Regarding marine tests media, samples will be heavily dominated by NaCl crystals and no visible nano materials. It would be more feasible to only recommend TEM analysis if medium compositions permits, instead of mandatory.

Answer:

It is very likely that the amount of NaCl precipitates can be reduced as compared to this experience following the recommended preparation procedure. Charge the grid and make the sample by drop on grid method. We prefer to keep it mandatory to make such analysis. Instead one should optimize the sample preparation step. One could use centrifugation to concentrate dispersed particles and make bottom samples to analyse settled nano materials. This part is under the responsibility of the specific test laboratories.

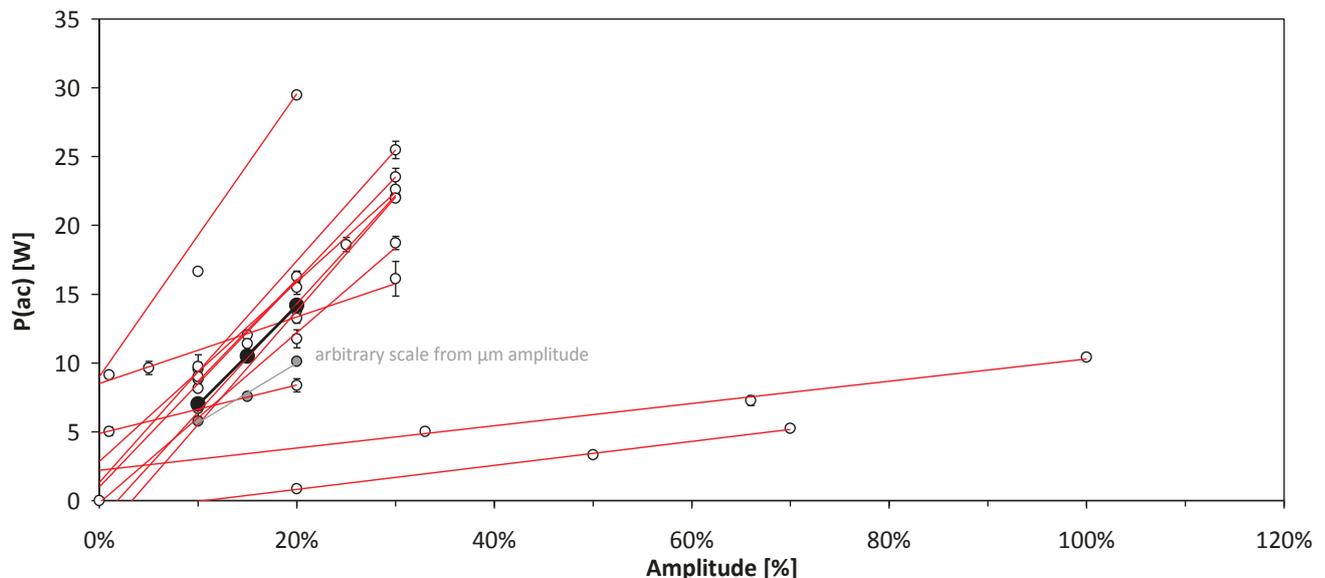
## 13 Probe sonicator calibration

What are the experiences with probe sonicator calibration?

Answer:

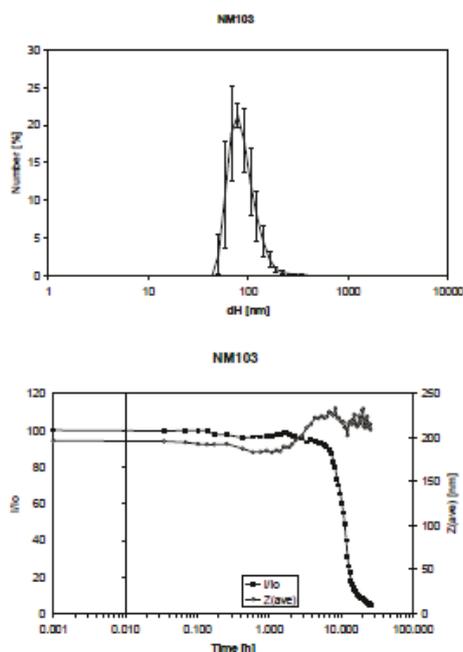
- Eighteen partners have submitted their probe-calibration data
- A few had problems, but issues were easily solved
- Lessons learned from the calibration exercise:
  - Probe-sonicators have different performances! Even sonicators of the same brand have different performance.
  - Calorimetric calibration alone appears insufficient for calibration of the de-agglomeration efficiencies as several partners had to adjust the sonication times to meet the  $240 \pm 30$  nm size-range criterion and  $PDI < 0.46$  (adjust is especially needed if different probe-diameters are used).
  - It appears impossible to replace the probe with a Cup-horn sonicator if similar de-aggregation of NM200.

Calorimetric calibration data are given below



## 14 Benchmark data

- One complete data set exists
- Data from 2-3 partners expected ultimo November
- New update of benchmark data primo December
- OBS
- NM103
- NM104



## DLS Benchmark data on NM dispersions prepared using the NANOGENOTOX dispersion protocol

Work in progress

If you have questions of problems, please e-mail [kaj@nrcwe.dk](mailto:kaj@nrcwe.dk), [yke@nrcwe.dk](mailto:yke@nrcwe.dk) and [CC.mgm@nrcwe.dk](mailto:CC.mgm@nrcwe.dk)

Key words: NANOGENOTOX dispersion protocol, DLS, hydrodynamic diameter, average zeta-size, PDI, size-distribution, benchmark data

Version 0.1

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Keld Alstrup Jensen, National Research Centre for the Working Environment, DENMARK (WP2)

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## 15 Frequency calibration sonicator

We have performed sonicator SOP and achieved the Pac 7.35 With the replicates done at the same day and the DLS data (z-average and PDI) are within the range of benchmark using 16 minutes sonication time. Do we have to repeat this calibration in another day as well? Does it matter how long the water to be used stands on the bench for example can it stand overnight and then be used for sonication calibration? We suspect that it may influence.

Answer

Calibration is demanded only once. The performance of the sonicator should not change. Water for testing must be equilibrated to temperature at sonicator. I see no problem with over-night storage (of-course covered up with Alu-foil or parafilm)

## 16 Weighting less than 15.36 mg NMs

Do we have to weigh 15.36 mg for sonication or can we weigh another amounts in this case less than 15.36 mg in order to make it more suitable for dilutions we are going to use.

Answer:

Yes; it is important to use the standard amount of 15.36 mg. If you change concentrations, you change the dispersability. Without knowing your dose-range, there should not be a problem in diluting to very low concentrations.

## 17 Preparing several aliquots at one time

*Can we weigh and make several aliquots (sub-vials) at once and then use that vial for sonication. This is because it makes it more practical to weigh many vials at one time keep them closed and safe and then use them when needed.*

Answer

Yes, but storage should be short-term and it would be best to keep the materials in desiccator and or under Ar atmosphere as indicated in the dispersion protocols.

## 18 Difference in data of DLS measurements

*How do we act if we get some difference in the data of DLS measurement within our experiments which may be due for example to a change of the serum batch? Can some recommendations be provided?*

Answer

This is not an issue. For the cell assays you simply document what is going on. There is no golden solution for this.

## 19 One time use of Vials

*SOPs for keeping the vials after opening with argon flow would be great.*

Answer

One possible set-up is illustrated in ENPRA and NANOGENOTOX dispersion protocols. We recommend one-time use to reduce variability.

## 20 NP solutions in water stable for longer period?

*Some information indicate that the NP solutions in water are stable for 2 weeks and that it is possible to vortex this solution within those 2 weeks to get a similar solution as initial. Is it true and the case for all NPs for NanoReg? What is the storage for this solution required during those 2 weeks?*

Answer

No, don't do this. The protocol clearly says use within 30 min to 1 hour. It is true that some samples may be stable for long, but long storage will cause problems (reactivity, dissolution; shown in session 5.1).

## 21 Low concentration of NPs for DLS measurements

*What about the low concentration of NPs for DLS measurement if the media interfere with the measurement as shown in WP 5? Do we measure only for the highest concentration?*

Answer

I have not seen your data. Normally not a problem, but if you go very very low in concentration, I suggest to take the lowest possible concentration, where data can be obtained.

## 22 Check dispersion for each experiment

*Do we need to check our dispersion in water for each experiment we conduct as the main point for WP5 will be the behavior in media?*

Answer

Yes, this is the idea. It is quality control to catch possible poor dispersions and offer you a chance to abort a bad experiment

## 23 TEM measurements

*According the previous points, what about the TEM measurement?*

Answer:

Take samples of a set of good experiments for the analysis. (This Q&A needs to be elaborated in the next version).

## 24 SOP for SEM

*Will the SOP for SEM be available on the CIRCABC website?*

Answer

We only request a qualitative description of the nature of the dispersed particles/fibers. We could make a small list of characteristics to report, if needed. We, however, rely most on the neutral DLS measurement.

## 25 SOP for Zeta-potential

*Will the SOP for Zeta-potential be available on CIRCABC, if any? How many times Z-potential should be measured?*

Answer

We did not ask for zeta-potential only the zeta-average size.

## 26 Fluorescence nano particles

*Another issue is that IIT Does not guarantee stability of their fluorescence np following nanogenotox protocol, for this they provide their own protocol, which we understand we should all follow.*

Answer:

All experiments must be done following the dispersion protocol(s). If needed you may perform additional studies without medie. Is it regulatory test or scientific question? Has it been tested whether the fluorescence dissappear?

## 27 (DLS-) characterisations and High-throughput screening

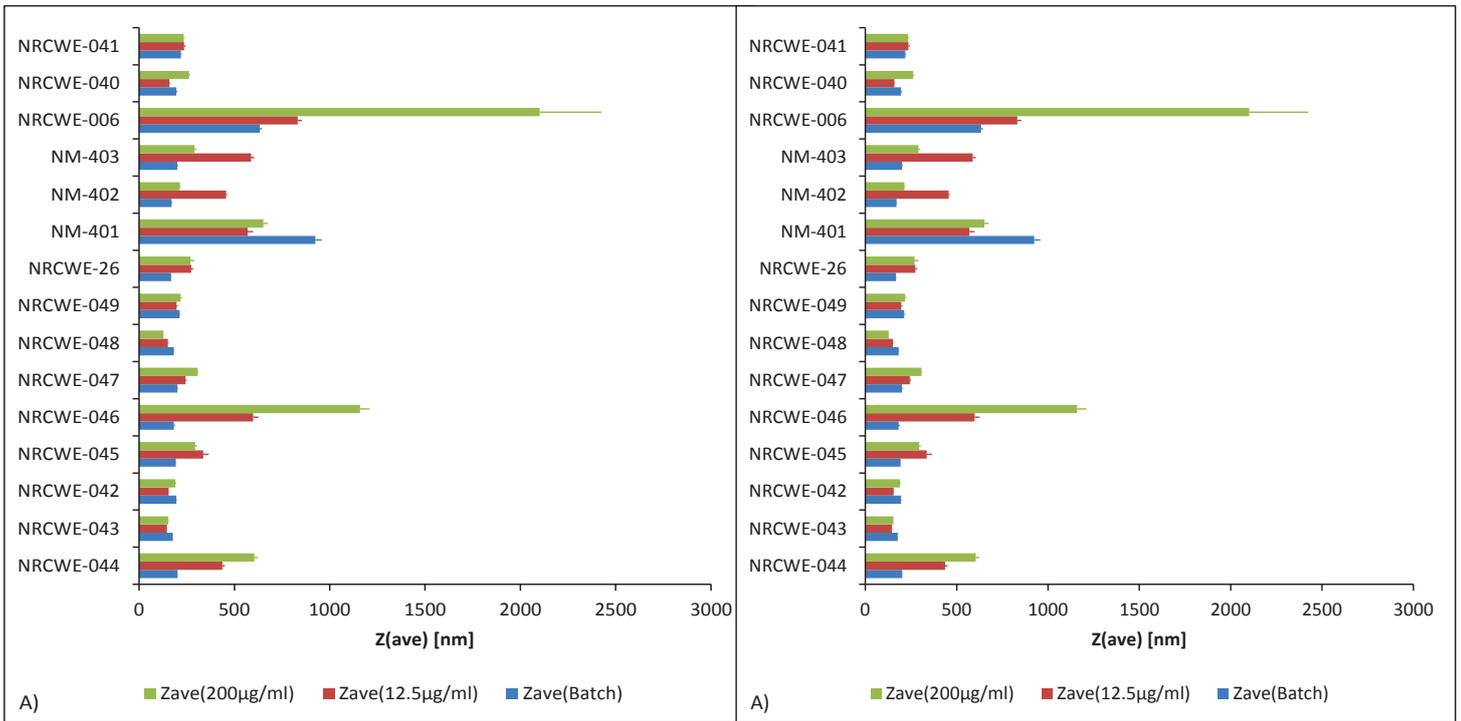
*Incorporating the (DLS-)characterisations called for by the guidance document will introduce a bottleneck into high-throughput screening (i.e. the biological screening is HT but not the DLS...).*

- <https://docs.google.com/document/d/1HCPTaqaGLfIXAGjLrLiGuyekMvpqZ9Polxf-YupCfuk/edit>. Error on page - answer needs to be elaborated in next version.
- We've collaborated on suggestions as to what would help reducing this bottleneck and wonder if these amendments could be acceptable
  - <https://docs.google.com/document/d/1VgtntsVkyIDcmPV1Hw7HFDfLQY4ux7oMLIxRpdilspw/edit> . Error on page - answer needs to be elaborated in next version.

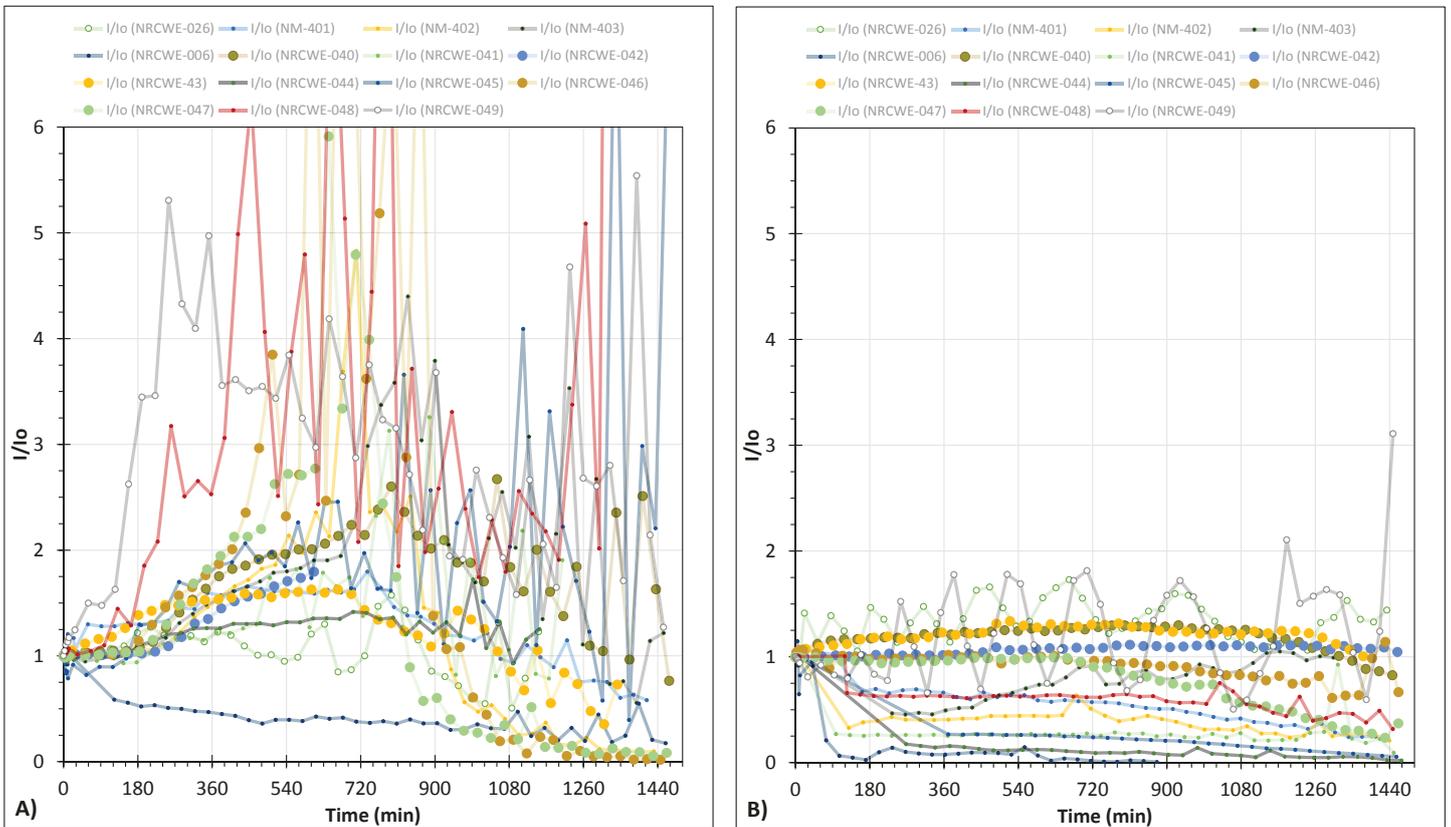
## 28 Clarification of what is requested

- Each batch dispersion is measured to control for dispersion quality. These data will be reported to the NANoREG database.  
This enables you to abort an experiment with a bad dispersion. The dispersion sometimes fail!
- The stability of the "particles" in cell media are best documented by a set of parallel studies for each of the exact cell media YOU are using.  
This is done in a DLS cuvette as explained in the DLS SOP. I.e. these tests are performed in cell-free media.
- EM samples are made of representative samples of the batch dispersion (diluted as indicated in the TEM sample preparation protocol) and representative samples of the in vitro exposures media  
This analysis is to visually understand what is measured by the DLS and gives you better understanding of your exposure characteristics

## 29 Size-information in batch and exposure medium



## 30 Sedimentation behaviour in exposure medium



## Document history of the NANoREG Guidance Document

<i>Version</i>	<i>Date</i>	<i>Distribution</i>	<i>Remarks</i>
1	05.05.2014	All Partners of the Consortium	First version of the Guidance Document
2	13.07.2014	All Partners of the Consortium	Chapter 7 describes the changes and amendments from Version 1.0 to Version 2.0
2.1	16.04.2015	All Partners of the Consortium	<ul style="list-style-type: none"><li>• Questions and Answers V1.0 updated as a result of Knowledge Cafe during 4th Consortium Meeting November 2014 - resulted in V1.1, approved by MC 013</li><li>• Guidance Document V2.0 and updated version of Questions and Answers combined into 1 document together with Document history</li></ul>
2.2	04-06-2015	Uploaded to CIRCABC	<ul style="list-style-type: none"><li>• Par 6.1: Introduction of NANoREG-Ecotox dispersion protocol v6 replaces Prospect dispersion protocol.</li><li>• Version numbers of Guidance Document, Q&amp;As and Document History synchronised</li></ul>

