

JRC TECHNICAL REPORTS

The NanoDefine Methods Manual

*Part 3: Standard Operating
Procedures (SOPs)*



Agnieszka Mech, Hubert Rauscher, Kirsten Rasmussen, Frank Babick, Vasile-Dan Hodoroaba, Antoine Ghanem, Wendel Wohlleben, Hans Marvin, Raphael Brüngel, Christoph M. Friedrich, Katrin Löschner, Douglas Gilliland

2020



EUR 29876 EN

This publication is a Technical report by the Joint Research Centre (JRC), the European Commission's science and knowledge service. It aims to provide evidence-based scientific support to the European policymaking process. The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

Contact information

Name: Hubert Rauscher
Address: via E. Fermi. 2749, TP125
I-21027 Ispra (VA), Italy
Email: JRC-NANOTECHNOLOGY@ec.europa.eu

EU Science Hub

<https://ec.europa.eu/jrc>

JRC117501

EUR 29876 EN

PDF ISBN 978-92-76-11955-5 ISSN 1831-9424 doi:10.2760/02910

Print ISBN 978-92-76-11954-8 ISSN 1018-5593 doi:10.2760/778

Luxembourg: Publications Office of the European Union, 2020

© European Union, 2020



The reuse policy of the European Commission is implemented by the Commission Decision 2011/833/EU of 12 December 2011 on the reuse of Commission documents (OJ L 330, 14.12.2011, p. 39). Except otherwise noted, the reuse of this document is authorised under the Creative Commons Attribution 4.0 International (CC BY 4.0) licence (<https://creativecommons.org/licenses/by/4.0/>). This means that reuse is allowed provided appropriate credit is given and any changes are indicated. For any use or reproduction of photos or other material that is not owned by the EU, permission must be sought directly from the copyright holders.

All content © European Union, 2020

How to cite this report: Mech et al., *The NanoDefine Methods Manual. Part 3: Standard Operating Procedures (SOPs)*, EUR 29876 EN, Publications Office of the European Union, Luxembourg, 2020, ISBN 978-92-76-11955-5, doi:10.2760/02910, JRC117501

Authors

Agnieszka Mech, Hubert Rauscher, Kirsten Rasmussen, Douglas Gilliland

European Commission Joint Research Centre (JRC), Ispra, Italy

Frank Babick

Technische Universität Dresden, Institute of Process Engineering and Environmental Technology, Dresden, Germany

Vasile-Dan Hodoroaba

Federal Institute for Materials Research and Testing (BAM), 12205 Berlin, Germany

Antoine Ghanem

Solvay – Research & Innovation Centre Brussels, 1120 Brussels, Belgium

Wendel Wohlleben

BASF SE, Material Physics Research, 67056 Ludwigshafen, Germany

Hans Marvin

RIKILT - Wageningen UR, 6708 Wageningen, The Netherlands

Katrin Löschner

Technical University of Denmark, National Food Institute, 2800 Kgs. Lyngby, Denmark

Raphael Brüngel, Christoph M. Friedrich

University of Applied Sciences and Arts Dortmund (FH Dortmund), Department of Computer Science, 44227 Dortmund, Germany

Contributors

Claire Gaillard, Francesca Pianella, François Rossi, Thomas Linsinger, Robert Koeber, Gert Roebben

European Commission Joint Research Centre (JRC), Ispra, Italy and Geel, Belgium

Christian Ullmann, Michael Stintz

Technische Universität Dresden, Institute of Process Engineering and Environmental Technology, 01062 Dresden, Germany

Johannes Mielke, Franziska Emmerling, Andreas Thünemann, Ralf Bienert, Patrick Knappe

Federal Institute for Materials Research and Testing (BAM), 12205 Berlin, Germany

Stefan Weigel

RIKILT - Wageningen UR, 6708 Wageningen, The Netherlands

Stephan Wagner, Frank von der Kammer

University of Vienna, Department of Environmental Geosciences, 1010 Vienna, Austria

Manuel Correia, Erik Huusfeldt Larsen

Technical University of Denmark, National Food Institute, 2800 Kgs. Lyngby, Denmark

Kai Dirscherl

Danish National Metrology Institute (DFM), Kogle Alle 5, DK-2970 Hørsholm, Denmark

Johannes Rückert, Thorsten Wagner

University of Applied Sciences and Arts Dortmund (FH Dortmund), Department of Computer Science, 44227 Dortmund, Germany

Jutta Tentschert

Federal Institute for Risk Assessment, Product Safety, 10589 Berlin, Germany

Ralf Kaegi, Toni Uusimäki

Eawag - Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

Eveline Verleysen, Jan Mast

Sciensano (previously CODA-CERVA), 1050 Brussels, Belgium

Alvise Bianchin

MBN Nanomaterialia s.p.a, 31050 Vascon di Carbonera, Italy

George Favre, Charles Motzkus

LNE Laboratoire National de Métrologie et d'Essais, 75724 Paris, France

Legal Note

This document contains general recommendations supporting the user in the decision whether a material is a nanomaterial according to the EC Recommendation on the Definition of Nanomaterial (Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU). OJ L 275, pp. 38-40). However, users are reminded that the texts of the appropriate EC legal acts are the only authentic legal reference and that the information in this document does not constitute legal advice. Usage of the information remains under the sole responsibility of the user. The NanoDefine Consortium Partners do not accept any liability with regard to the contents of this document.

Disclaimer

Certain commercial equipment, instrument brand names and materials are identified in this document as examples or to specify adequately an experimental procedure. In no case does such identification imply recommendation or endorsement by the NanoDefine Consortium, nor does it imply that the material or equipment is necessarily the best available for the purpose and for the material under consideration.



NanoDefine

**Development of an integrated approach
based on validated and standardised
methods to support the implementation of
the EC recommendation for a definition of
nanomaterial**

The NanoDefine Methods Manual

Part 1: The NanoDefiner Framework and Tools

The research leading to these results has received funding from the
European Community's Seventh Framework Programme (FP7/2007-2013)
under Grant Agreement n° 604347

Website: <http://www.nanodefine.eu/>
Project co-ordinator: Wageningen Food Safety Research (WFSR), NL

Table of Contents

Executive summary	4
1 Introduction to the Standard Operating Procedures	5
1.1 About the Standard Operating Procedures	5
1.2 Priority materials	6
1.3 General considerations for dispersion	7
1.3.1 Introduction	7
1.3.2 Sonication type	7
1.3.3 Sonication power and energy requirements	8
1.3.4 Towards harmonisation of sonication	9
1.3.5 Probe sonicator issues	9
1.4 Specific considerations of dispersion in NanoDefine	11
1.4.1 Requirements of the dispersions	11
1.4.2 Evaluation of the dispersion quality	13
1.5 References	14
Dispersion SOPs: Production of an aqueous based dispersion of the NanoDefine priority materials	
2 SOP for production of an aqueous based dispersion of IRMM-380 (Pigment Yellow 83, Fine grade)	16
3 SOP for production of an aqueous based dispersion of IRMM-381 (BaSO ₄ (fine grade))	25
4 SOP for production of an aqueous based dispersion of IRMM-382 (Multi-wall Carbon nanotubes)	32
5 SOP for production of an aqueous based dispersion of IRMM-383 (Nano steel)	38
6 SOP for production of an aqueous based dispersion of IRMM-384 (CaCO ₃)	47
7 SOP for production of an aqueous based dispersion of IRMM-385 (Kaolin)	55
8 SOP for production of an aqueous based dispersion IRMM-386 (Opaque Pigment Yellow 83)	65
9 SOP for production of an aqueous based dispersion of IRMM-387 (BaSO ₄ (ultrafine grade))	74
10 SOP for production of an aqueous based dispersion of IRMM-388 (Coated TiO ₂)	82
11 SOP for production of an aqueous based dispersion of IRMM-389 (basic methacrylate copolymer)	90
12 SOP for production of an aqueous based dispersion of BAM-11 Zeolite	97
13 Generic SOP for calorimetric calibration of an ultrasonic probe sonicator	105
14 Properties of recommended materials	109
Generic SOPs for methods	
15 Generic SOP for DLS method for sample preparation and measurement of substances in suspension	111
16 SOP for Cuvette-AC method for sample preparation and measurement of BaSO ₄ and comparable types of powders in suspension	121
17 SOP for analysis of Fe ₂ O ₃ in Polyethylene Matrix with Electron Microscopy methods	131
18 SOP for extraction of TiO ₂ from Sunscreen for analysis with Electron Microscopy	133
19 Size characterisation of suspended particles by AUC-RI with speed ramp option	136
20 Particle size distribution measurement of BaSO ₄ using Line-Start Disc Centrifuge with Optical Detection	146

21 Measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software.....	156
--	-----

Generic SOPs for nanomaterials in products

22 SOP for analysis of TiO ₂ particles from sunscreen by AF4-MALS-ICP-MS.....	173
23 Sample preparation and spICP-MS analysis of TiO ₂ particles in sunscreen products.....	179
24 Sample preparation and spICP-MS analysis of TiO ₂ nanoparticles in suspensions.....	187
25 Sample preparation and spICP-MS analysis of Al ₂ O ₃ nanoparticles in toothpaste.....	195
26 General Conclusions.....	203
26.1 Dispersion protocols.....	203
26.2 Possible contamination by probe sonicator.....	205
26.3 Material- and technique-dependent observations.....	205
26.4 Sample preparation for NM in products.....	206

About the NanoDefine Methods Manual

The present series of reports, the **NanoDefine Methods Manual**, has been developed within the NanoDefine project 'Development of an integrated approach based on validated and standardized methods to support the implementation of the EC recommendation for a definition of nanomaterial'¹ funded by the European Union's 7th Framework Programme, under grant agreement 604347.

In 2011 the European Commission (EC) published the recommendation (2011/696/EU) for a definition of the term 'nanomaterial'¹, the EC NM Definition, as a reference to determine whether an unknown material can be considered as a 'nanomaterial' for regulatory purposes. One challenge is the development of methods that reliably identify, characterize and quantify nanomaterials (NM) both as substances and in various products and matrices.

The overall goal of NanoDefine was to support the implementation of the EC NM Definition. It can also support the implementation of any NM definition based on particle size. The project has developed an integrated approach, which allows identifying any material as a nano or non-nano material according to the EC NM Definition. NanoDefine explicitly supported the governance challenges associated with the implementation of legislation concerning nanomaterials by:

- addressing the issues on availability of suitable measuring techniques, reference materials, validated methods, acceptable to all stakeholders (authorities, policy makers, commercial firms),
- developing an integrated and interdisciplinary approach and a close international co-operation and networking with academia, commercial firms and standardization bodies.

Thus, the **NanoDefine Methods Manual** provides guidance on practical implementation of the EC NM Definition throughout the nanomaterial characterization process, and on the characterization techniques employed as well as their application range and limits. It assists the user in choosing the most appropriate measurement method(s) to identify any substance or mixture for a specific purpose, according to the EC NM Definition of a nanomaterial. The NanoDefine project also explored how to assess a material against the criteria of the definition through proxy solutions, i.e. by applying measurement techniques that indirectly determine the D_{50} . Those findings were developed through empirically based scientific work and are included in Part 1 of this Manual. As they go beyond the text of the EC NM Definition, they may be used as practical approach to indicate whether a material is a nanomaterial or not, but keeping in mind that they should not be taken as recommendation for the implementation of the EC NM Definition in a regulatory context.

The NanoDefine Methods Manual consists of the following three parts:

- Part 1: The NanoDefiner Framework and Tools
- Part 2: Evaluation of Methods
- Part 3: Standard Operating Procedures (SOPs)

Part 1 covers the NanoDefiner framework, general information on measurement methods and performance criteria and tools developed by NanoDefine such as a materials categorisation system, a decision support flow scheme and an e-tool.

Part 2 discusses the outcome of the evaluation of the nanomaterials characterisation methods for measuring size.

Part 3 presents the 23 Standard Operating Procedures developed within the NanoDefine project. The current document is part 3.

Abbreviations and acronyms used in the Manual

AC	Analytical Centrifugation
AF4	Asymmetrical Flow Field-Flow-Fractionation
AFM	Atomic Force Microscopy
ALS	Angular Light Scattering
Aq.	Aqueous
AR	Aspect Ratio
AUC	Analytical Ultra Centrifugation
BET	Brunauer-Emmett-Teller
BSA	Bovine Serum Albumin
CM	Characterisation Method
CEN	European Committee for Standardization
CFFF	Centrifugal Field-Flow-Fractionation
CLS	Centrifugal Liquid Sedimentation
CPC	Condensation Particle Counter
DEMA	Differential Electrical Mobility Analysis (also spray-DEMA)
DMA	Differential Mobility Analyser
DLS	Dynamic Light Scattering
DSFS	Decision Support Flow Scheme
DUM	Dynamic Ultramicroscopy
EC	European Commission
EC NM Definition	EC Recommendation on the Definition of a Nanomaterial
EDX / EDS	Energy Dispersive X-ray spectrometry
EELS	Electron Energy Loss Spectroscopy
EFTEM	Energy-Filtered Transmission Electron Microscopy
EHS	Environment, Health and Safety
EM	Electron Microscopy
ESD	Equivalent Spherical Diameter
ESI-SMPS	Engineering System International SMPS
ESZ	Electrical Sensing Zone
FFF	Field-Flow-Fractionation
FTIR	Fourier-transform Infrared Spectroscopy
HSE	Health, Safety and Environment
ICP-MS	Inductively Coupled Plasma - Mass Spectrometry
ICP-OES	Inductively Coupled Plasma - Optical Emission Spectrometry
KB	Knowledge Base
LD	Laser Diffraction
LoD	Limit of Detection
LS	Light Scattering

MALS	Multi-Angle Light Scattering
MALLS	Multi angle laser light scattering
MCS	Material Categorisation Scheme
MT	Measurement Technique
MWCNT	Multi-walled Carbon Nanotube
m/z	Mass-to-Charge Ratio
NaDS	Sodium Dodecyl Sulphate
NM	Nanomaterial
NTA	Nanoparticle Tracking Analysis
NP	Nanoparticle
PSD	Particle Size Distribution
PTA	Particle Tracking Analysis
QELS	Quasi Elastic Light Scattering
RI	Refractive Index
SAXS	Small-Angle X-ray Scattering
SDS	Safety Data Sheet
SEM	Scanning Electron Microscopy
SEM-EDX	SEM-Energy Dispersive X-ray Analysis
SedFFF	Sedimentation Field-Flow-Fractionation
SFM	Scanning Force Microscopy
SLS	Static Light Scattering
SMPS	Scanning Mobility Particle Sizer
SOP	Standard Operating Procedure
spICP-MS	Single Particle ICP-MS
TEM	Transmission Electron Microscopy
TRPS	Tuneable Resistive Pulse Sensing
UF	Ultrafine
USB	Ultrasonic Bath Sonicator
USP	Ultrasonic Probe Sonicator
USSp	Ultrasonic Spectroscopy
UV	Ultra Violet
UV-vis	Ultra Violet - Visible
VS	Vial Sonicator
VSSA	Volume-Specific Specific Surface Area

Executive summary

In the NanoDefine project ('NanoDefine') approach a characterisation method includes both sample preparation and analysis with a defined measurement technique. To ensure repeatability, the sample preparation should be done according to Standard Operation Procedures (SOPs). The aim of this report, part 3 of the NanoDefine Manual, is to present the twenty-three SOPs developed within NanoDefine to facilitate and harmonise the particle size distribution measurements. These SOPs have been developed for different purposes, leading to different the types of SOPs that are gathered in this document.

SOPs were developed for the purposes listed below:

- dispersing powders in a liquid phase and ensuring maximum de-agglomeration and disintegration of aggregates and stability (i.e. minimum re-agglomeration)
- ensuring comparability of dispersion procedures
- conducting measurements of particle size distribution (and other properties of the particles) with specific measurement techniques
- extracting particles (or a specific particulate species) from a complex dispersion
- entire methods covering the steps from sample preparation to particles size analysis

Eleven of the SOPs are presented as detailed, material specific dispersion protocols designed to produce liquid (aqueous) dispersions of the NanoDefine priority materials, which are: IRMM-380 (Pigment yellow 83, transparent grade), IRMM-381 (BaSO₄, fine grade), IRMM-382 (MWCNT), IRMM-383 (Nano steel), IRMM-384 (CaCO₃, fine grade), IRMM-385 (Kaolin), IRMM-386 (Pigment yellow 83, opaque grade), IRMM-387 (BaSO₄, ultrafine grade), IRMM-388 (Coated TiO₂, pigment grade), IRMM-389 (Basic methacrylate copolymer particles, BMC) and BAM-11 (Zeolite powder). The SOPs for these materials are based on the high intensity energy input methods of probe and vial sonication. To ensure maximum harmonisation across the project, all materials have an associated dispersion protocol describing probe sonication, as this type of sonicator was available to all partners, while for selected materials (IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 (CaCO₃), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO₂)) also a protocol for vial-sonication was developed. The sonication conditions used were chosen to ensure that the two sonicator systems were supplying a similar volume-specific energy input.

One SOP was developed because sonication, although effective for dispersing the test material, introduces a significant variable in the dispersing process as a wide variety of different sonication instruments exists with different nominal power and probe size. To reduce the variability that this may introduce a SOP 'Generic SOP for calorimetric calibration of an ultrasonic probe sonication' was developed. The use of this SOP ensures (better) harmonisation of the power output when using significantly different sonicator types or probe types/sizes for dispersion compared to those used in the development of in the optimised protocols.

The remainder of the SOPs concerns procedures for combinations of size measurement techniques and materials, and materials in products.

Additionally, the report notes that the use of sonication probes, although the most commonly available, should be done with caution as probe material may be released due to wear of the probe, and above all the onset of this may start after only a few hours of use, and that this wear may not be easy to detect.

1 Introduction to the Standard Operating Procedures

1.1 About the Standard Operating Procedures

The NMs in powder form can be analysed via two routes: as dry powders (which is not further discussed in this report) or in liquid dispersion. The Standard Operating Procedures (SOPs) presented here were developed for dispersing powders for analysis as dispersions. Part 1 of the NanoDefine Methods Manual describes how these dispersions, once available, are analysed with regard to particle size. The SOPs were developed with the purpose of minimising the variability of the dispersion procedure on the measured size, and this is described in section 1.3.

NanoDefine developed eleven SOPs for generating an aqueous dispersion the priority materials based on probe sonication for all the priority materials, see chapters 2 to 12. For four of the materials (IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 (CaCO₃), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO₂)), these SOPs included also vial sonication.

Additionally the following twelve SOPs were developed:

- 1) A generic SOP for calorimetric calibration, see chapter 13.
- 2) The DLS method, which was developed for four NanoDefine priority materials (IRMM-381 (BaSO₄, fine grade), IRMM-384 (CaCO₃, fine grade), IRMM-385 (Kaolin), IRMM-388 (Coated TiO₂, pigment grade) but which can also be applied to comparable types of materials, considering that adaptations might be needed, see chapter 15.
- 3) The Cuvette-AC method which was developed for two of the NanoDefine priority materials (IRMM-381 (BaSO₄, fine grade), IRMM-387 (BaSO₄, ultrafine grade) but which can also be applied to comparable types of materials, considering that adaptations might be needed, see chapter 16.
- 4) A method for the analysis of Fe₂O₃ in polyethylene matrix with electron microscopy, which illustrates protocols for preparation of products for microscopy methods and covers sample preparation and fully automatic particle size distribution (PSD) analysis of Fe₂O₃ nanoparticles in high density polyethylene, see chapter 17.
- 5) A method for the analysis of TiO₂ in sunscreen with electron microscopy, see chapter 18.
- 6) A method for size characterisation of suspended particles by AUC-RI with speed ramp option.
- 7) Particle size distribution measurement of BaSO₄ using Line-Start Disc Centrifuge with Optical Detection.
- 8) Measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software.
- 9) Analysis of TiO₂ particles from sunscreen by AF4-MALS-ICP-MS.
- 10) Sample preparation and splCP-MS analysis of TiO₂ nanoparticles in sunscreen products.
- 11) Sample preparation and splCP-MS analysis of TiO₂ nanoparticles in suspensions.
- 12) Sample preparation and splCP-MS analysis of Al₂O₃ nanoparticles in toothpaste.

All SOPs are presented in the document as stand-alone, self-explanatory documents which can be easily extracted from the report; thus repetitions of some text and images occur. All NanoDefine

technical reports, including the ones for the SOPs, can be found on the project website at <http://www.nanodefine.eu/index.php/nanodefine-publications/nanodefine-technical-reports>^a.

1.2 Priority materials

The materials which have been chosen as priority substances in NanoDefine (Table 1) provide examples of the major classes of nanomaterials: metals, metal oxides, metal salts, polymers, carbonaceous materials (MWCNT) and ceramics. Such a wide range of materials provided a challenge for the development of dispersion protocols as, firstly, each material potentially may require a different type of stabiliser and secondly, the binding strength between materials may be widely different making careful optimization of sonication time and power critical to maximize disaggregation without inducing undesirable fusion of particulates, see e.g. Babick et al. (2016)².

Table 1: NanoDefine priority materials

Material	Description
IRMM-380 Pigment yellow 83 (transparent grade)	Organic diarylide dye with hydrophobic character. Sub-100 nm particulates
IRMM-381 BaSO ₄ (fine grade)	Inorganic hydrophilic metal salt with low water solubility
IRMM-382 MWCNT	Highly tangled fibrous carbonaceous materials which are strongly hydrophobic in nature
IRMM-383 Nano steel	Highly anisotropic (platelets) particulates with negatively charged surface at neutral pH
IRMM-384 CaCO ₃ (fine grade)	Inorganic hydrophilic metal salt with low but non-negligible water solubility
IRMM-385 Kaolin	Highly anisotropic ceramic/mineral (platelets) particulates with negatively charged metal-oxide surface at neutral pH
IRMM-386 Pigment yellow 83 (opaque grade)	Organic diarylide dye with hydrophobic character. Mainly non-nano (>100 nm) particulates
IRMM-387 BaSO ₄ (ultrafine grade)	Inorganic salt/mineral with low water solubility
IRMM-388 Coated TiO ₂ (pigment grade)	Inorganic metal oxide with thin hydrophilic coating, code Kronos 2360
IRMM-389 Basic methacrylate copolymer particles (BMC)	Hydrophilic organic particles, insoluble in water and highly soluble in most organic liquids
BAM-11 Zeolite powder	Nanoporous ceramic/mineral particulates of irregular shape with negatively charged metal-oxide surface at neutral pH

^a NanoDefine technical reports are available at the site <http://www.nanodefine.eu/index.php/nanodefine-publications/nanodefine-technical-reports>

1.3 General considerations for dispersion

1.3.1 Introduction

The issue of dispersion is particularly important in the evaluation of nanoparticle size as many nanomaterials are normally found in the form of dried powders which need to be brought into stable dispersions in liquid before they can be measured by many of the most common particle size measuring methods such as dynamic light scattering (DLS), laser scattering (LS), centrifugal liquid sedimentation (CLS) and analytical ultracentrifuge (AUC). The dispersion procedure is a pivotal step in the process of measuring the particle size distribution and the dispersion procedures must be effective, efficient, reproducible, and the final dispersion should have a particle size distribution that is as close as possible to the true size distribution of constituent particles.

The first step in developing a dispersion procedure is the choice of media, pre-dispersion and wetting. Primary considerations concerning the media are possible limitations imposed by the instrumental method to be used later and the need to ensure that the particles are inert towards the media, i.e. do not dissolve or swell.

The second step is choosing the method to use for the mechanical de-agglomeration of the particles. Here, the primary concern is the input energy (both in terms of local stress intensity and energy density) and whether it is sufficient to disaggregate without causing damage to the particles or their coating, if relevant. For the reasons previously discussed, the usual choice for most materials will be ultrasonic using either probe or vial sonicators. The lower power of bath sonication will, in practical terms, probably be insufficient for most materials but may be the only choice when evaluating certain 'soft' nanomaterials such liposomes or drug and food supplement carriers.

The final step in the process, stabilisation, is by far the most complex area to advice on due to the very large number of possible variables and the following issues need to be considered. The first consideration is always compatibility of the stabilisation method with the measurement method. The second consideration will be the effectiveness of the stabilisation and it includes consideration of the time scale for which stability must be guaranteed. Simplicity should also be considered and in the cases that adequate performance can be achieved with commonly available simple stabilisers these should be used in preference to high performance proprietary products.

1.3.2 Sonication type

The scientific literature, e.g. Hartmann et al. (2015)³, regarding dispersion shows a common theme that, in the vast majority of cases effective dispersion of dry nano-powders into a liquid, requires high energy sonication; low energy methods such as bath sonication are not effective. Thus, in NanoDefine development of protocols has concentrated on the use of probe sonication as the primary method with additional data being provided for the use of vial-sonicator where the developing laboratory had this instrumentation available.

For general use in the dispersion of nanopowders it strongly recommended when using a probe sonicator to carefully consider the maximum input power level. Successful sonication can be obtained with a nominal maximum input power level of 100-200 W, which does not affect the size distribution of the constituent particles³. For the relatively small volumes (1-10 mL) considered in the protocols a lower value of 50 W may be acceptable subject to verification. Ultrasonic probe devices having a nominal power above 200 W may still be used, as this may not affect the local

stress intensities by imploding cavitation bubbles nor the relationship between particle size and energy density. The important issue is the calorimetric calibration for a combination of given instruments, probes, beakers, sample volumes, immersion depth of the probe and temperature.

Sonication may be done in constant mode or in pulsed mode. The principal advantage of constant mode sonication is that it is possible with most models of probe sonicators, while a more limited number of instruments may have the option of also using a pulsed mode. Where pulsed mode is available there may be a number of advantages. Firstly, the use of pulsed mode allows the use of higher acoustic intensity but with reduced temperature increase as heat may transfer to the environment during the off-cycles. This is of particular relevance in vial sonication, which relies on heat transfer through the metal of the probe, as active cooling of the sample (by e.g. immersion in an ice bath) is not normally possible. This issue may be important where temperature sensitive samples are treated. Secondly, the off-cycle in pulse-mode allows the dissolution of bubbles, which can create acoustic shielding, and thus it may overall improve efficiency of the process.

1.3.3 Sonication power and energy requirements

It appears likely that once a certain minimum intensity of sonication (amplitude) is achieved then further increases in the power settings have a limited influence on the final minimum particle size which can be achieved but influences more the time required to achieve the minimum particle size. In the work of Guillemin et al. (2012)⁴ on the de-agglomeration of ZnO nanopowders there is a theoretical and experimental examination of the effect of sonication power which shows that the breakage frequency during sonication increases with the square root of the thermal power confirming the limited advantages of adding additional power. In other studies, e.g. Taurozzi et al. (2011)⁵ it has been noted that the use of higher power levels may actually be detrimental to efficient re-dispersion as the excess energy may result in fusion of de-agglomerated particles with the irreversible formation of aggregates.

Another factor which may influence the sonication efficiency is the temperature, as it affects properties of the liquid, thus cavitation, critical bubble size and viscous dissipation of micro-jets as well as properties of the particulate phase, e.g. strength of interparticle bonds. In many of the studies for nanotoxicology cooling in an ice-bath is recommended but in practice the use of such low temperatures may be important mainly to avoid thermal degradation the protein molecule (albumin) which is added as a surfactant. In the work of Raman and Abbas (2008)⁶ studies of the sonication of Al₂O₃ show that the *energy transfer* from the probe sonicator to the liquid peaked at low temperature decreasing by 10-15 % when the temperature was increased from 10 °C to 50 °C. The actual efficiency of *particle breakage* showed a maximum around 25 °C with only a moderate decrease of 10-15 % occurring as the temperature increased to 50 °C. More significantly it was found that reducing the liquid temperature from 25 °C to 10 °C produced a decrease of around 80 % in the efficiency of particle breakage compared to the maximum value achieved. From this it would seem that the use of cooling during sonication should be done with caution as excessive reduction in temperature may be detrimental to the overall efficiency of the process.

From these considerations it is evident that the determination of the sonication conditions must firstly verify that the minimum intensity value is achieved and thereafter verify from that value what is an appropriate power level to reach the minimum size in a experimentally reasonable time without significant formation of fresh agglomerates due re-fusion of particles by the ultrasonic energy. For the work in NanoDefine it has been considered realistic that the sonication time should not exceed 1 hour and ideally should be less than 30 minutes.

1.3.4 Towards harmonisation of sonication

The final and possibly most important point is that there is a clear need to develop methods that standardise the sonication in a manner which can reduce the variability introduced by the wide variety of different types of sonicators used in different laboratories. Some sonication description reports consider the use of electrical power input as a means to define the sonication power but, in general, this is likely to be unsuccessful due to the potential for variations in the efficiency of converting electrical energy into ultrasonic energy applied to the probe, and, more importantly, the variation in transferring that energy from the probe into the sample solution.

This problem has led to the proposal by a number of groups that the sonication process should be in some way harmonised by measuring the energy effectively transferred to the sample. The possibility of using calorimetric methods to evaluate the relative power of different sonicators has proved to be a simple and attractive strategy as a first step towards this harmonisation. These methods are based on the assumption that the acoustic energy absorbed by a liquid sample of known mass is converted into thermal energy. By monitoring the temporal increase in temperature during sonication it is possible to obtain a relative measure of the power output of a sonicator. It is possible that the efficiency of energy transfer will be influenced by many factors, such as liquid viscosity, volume, shape, and container materials. However, by standardising on a relatively large volume, but still smaller than 100 mL, in a similarly shaped vessel the influence from such variables may be sufficiently minimised to make the method suitable for inter-comparison of different models of sonicators.

These methods, often based on variations of the basic methods described by Taurozzi et al.⁷, show a promising route to determine the power output of different sonicators and more importantly provide a means to define sonication conditions based on measurable independent physical parameters rather than a specific setting on an instrument.

The situation of the vial sonicator system is in some ways simpler as, to the authors' knowledge, currently only one manufacturer exists and thus there is no need to harmonise between such instruments. The problem remains how to compare vial sonicator performance with that of probe sonicators. While it was not possible to make a direct comparison through a standard method (chapter 13) it was possible to measure the rate of temperature increase when operating with a single full (2 mL) vial so that an approximate measure of power output could also be determined.

The remaining sonication system, cup-horn, has not been considered in NanoDefine as this instrumentation was not available amongst the method developers.

1.3.5 Probe sonicator issues

While there is no doubt that high intensity sonication is a necessary step in producing dispersions of nanomaterials starting from dry powders there is another potential issue which should be considered when deciding exactly which method is the best to be applied. As discussed previously, high intensity sonication can be achieved by 3 main methods which are (a) standard probe sonication, (b) vial sonication and (c) cup-horn sonication. Of these three methods the first is by far the most common due to the greater flexibility of use (variable probes size and processing volume), ease of liquid cooling and general availability. The major disadvantage of this method is that the sonicator probe is placed in direct contact with the liquid containing the sample creating a risk that the sample would be contaminated should there be either ionic or mechanical release of material

from the probe^b during operation. The onset of this effect cannot be predicted and may in many cases be difficult to detect other than by regular verification of probe integrity. It was noted that this problem can be serious, as can be seen in the examples shown below which were observed after 30 minutes of sonication of IRMM-380 (6 mm diameter probe) and IRMM-386 (3 mm diameter probe) samples (see Figure 1).

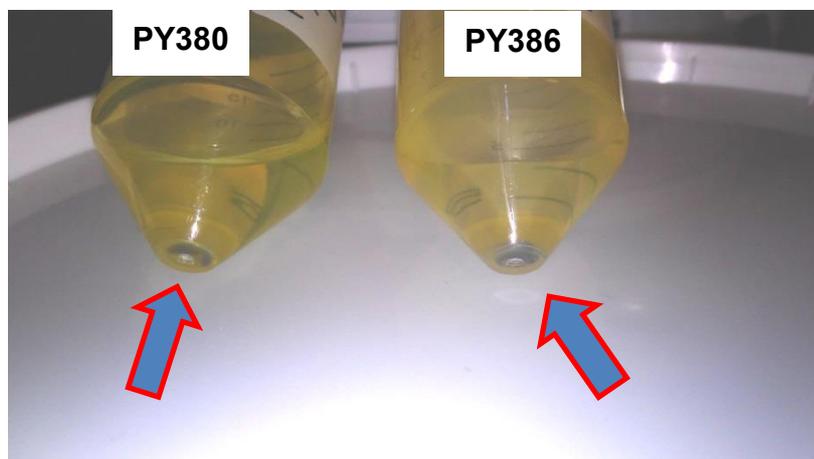


Figure 1: Photograph of pigment yellow 83 samples after probe sonication showing sediment of residue

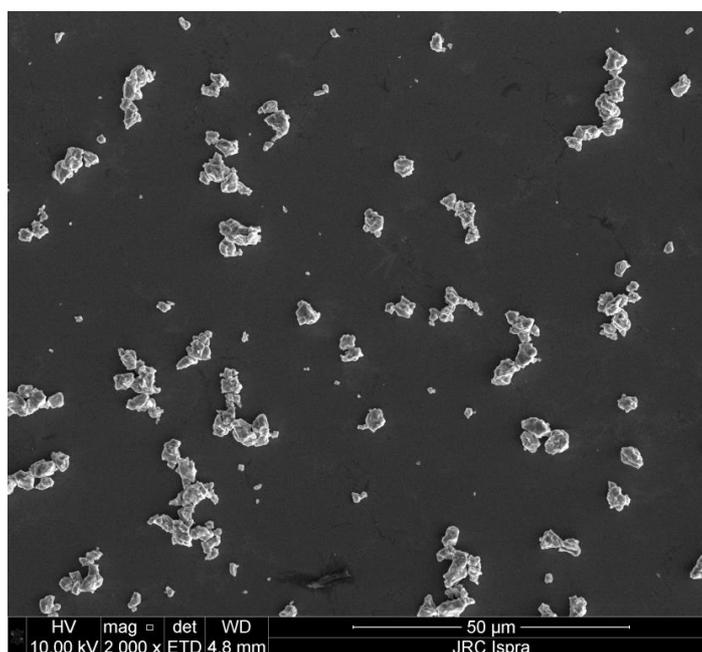


Figure 2: SEM Image of residue produced by probe sonicator

In this particular case the probe heads had been operated over 4-6 weeks and used only for the sonication of IRMM-380 and 386 (pigment yellow 83, transparent and opaque grades respectively), IRMM-384 (CaCO_3 (fine grade)) and IRMM-388 (Coated TiO_2). The deterioration was noted on two different probes with different diameters (3 mm and 6 mm). One of the probes was operated in pure water for a further 50 minutes period and the residue collected for analysis by SEM. An

^b Probes are often made from titanium based materials

example of the residues can be seen in the SEM image shown in Figure 2. In additional studies of these samples, elemental analysis of these fragments by SEM-EDX confirmed them to be composed of mainly titanium as would be expected of debris from the type of probe used. In this example it should be noted that the use of the probes with the hard and abrasive TiO₂ (IRMM-388) may have contributed to a more rapid than normal degradation of the tip surface; with other, softer priority materials this may not be observed.

Given the experience of probe degradation it is advisable that, when appropriate equipment is available and sample volumes and temperature sensitivity permit, non-contact methods of sonication should be adopted to avoid any risk of this problem. In cases where direct contact probe sonication is to be used then it may be preferable to use a probe sonicator which has an exchangeable tip so that this may be easily inspected and if necessary replaced whenever necessary. In this way regular substitution may be undertaken with a lower cost than in the case of substituting mono-block probes.

1.4 Specific considerations of dispersion in NanoDefine

1.4.1 Requirements of the dispersions

Besides aiming at dispersions with a maximum number of unbound single particles and a minimum of agglomerates/aggregates, the requirements of the analytical techniques (Table 2) were taken into consideration for the development of the protocols. In particular, several methods, such as AF4 and CLS, made it desirable, although not essential, that the dispersion medium would be water-based. Required particle mass concentrations were in the range of ≤ 1 to 10 mgmL⁻¹ and sample volumes in the range of 100 μ L to 2 mL. With regard to the stability of the dispersion and the need for chemical stabilisers, it was concluded that for most instrumental methods a temporal stability of 30 minutes was sufficient and as such placed less stringent requirements on the performance and therefore the choice of surfactants. Thus, while it is possible that the most effective stabilisation could be realised by using highly optimised proprietary surfactants or surfactant mixtures, the limited temporal stability required means that in most cases relatively simple surfactants could be used. Furthermore, since the protocols needed to be transferred to numerous laboratories it was desirable that they were as simple, safe and widely applicable as possible thus also favouring the choice of relatively simple and commonly available molecular surfactants rather than more complex or less commonly available proprietary commercial surfactants, polymers or surfactant mixtures.

In the scientific literature, many methods can be found for dispersing specific types of particles in specific liquids but there is little information on more generically applicable protocols. In fact, the development of true dispersion protocols in the recent literature has been driven almost exclusively by the needs of the nanotoxicology testing community and their need to produce short-term stability dispersions which, apart from the nanoparticles, contain only compounds which are compatible (non-toxic) with biological systems. Thus, it is clear that the range of possible stabilising agents available to researchers is severely restricted as the stabilising agent must not contribute to any observed toxicity to cells and in most cases the stabilising agent must also work within the pH conditions, which may not be suited to some materials. Given these limitations, it is quite possible that the quality of dispersion achieved with these protocols is sub-optimal for many materials.

Table 2: Minimum requirements for various measurement techniques identified in NanoDefine

Instrument method	Measurement volume (approx.)	Stability time required (minimum)	Preferred media	Final concentrations required
CLS	100 μ L	<30 min	Aq.	<1 mgmL ⁻¹
AUC	100 μ L	<30 min	Aq./Org	<10 mgmL ⁻¹
Cuvette Centrifuge	1 mL	<30 min	Aq./Org	<10 mgmL ⁻¹
DLS/LS/MALS	1 mL	<30 min	Aq./Org	<10 mgmL ⁻¹
AF4	100 μ L	<30 min	Aq./Org*	<10 mgmL ⁻¹
TEM	<100 μ L	<30 min by drop	Aq./Org	\leq 10 mgmL ⁻¹
spICP-MS	1-2 mL (to dilute)	<30 min prior to dilution	Aq.	\leq 1 mgmL ⁻¹
PTA	1- 2 mL	<30 min prior to dilution	Aq./Org*	\leq 1 mgmL ⁻¹
ESI-SMPS	1-2 mL	<30 min	Aq./Org*	\leq 1 mgmL ⁻¹
SAXS	2 mL	30 min	Aq./Org	\leq 10 mgmL ⁻¹

***Only selected organic media may be acceptable**

Another issue to consider is the final application of the dispersion, which in NanoDefine is to produce a sample which is appropriate to specific particle size measuring techniques. In the case of nanotoxicity testing the use of a protein stabiliser, such the commonly used BSA, is easily justified as cell culture media is very often rich in albumin or blood serum and consequently the testing of particles pre-coated with protein is scientifically valid. However when considering the requirements for dispersion for measurement purposes, then the fact that the generic toxicity testing protocols are actually coating the particle with a fairly large protein (BSA) should be taken into account as this coating may have an influence on the measurement result obtained. For example, dispersion stabilisation with a protein may be a quite acceptable and effective method for preparing inorganic materials for EM based analysis, where the low relative contrast of the organic materials means it is not a strong source of interference in the accurate measurement of particle size. In contrast, the use of protein-coated samples with DLS or CLS/AC methods is likely to produce results which would be influenced by the presence of protein but in a way which would be difficult to quantify accurately. In the case of DLS, the presence of the protein as a coating on the surface of the particles would result in an apparent increase in the hydrodynamic diameter of the particle. Since the protein has a size of a few nanometres in its natural folded state it would be reasonable to assume that this would not have a great effect on large particles; however, when coating small particles of a few nanometres, the potential error is very large. In addition, any remaining free protein, having a size of a few nanometres in monomer form but being able to form larger dimer and trimers, may be a source of confusion for techniques with non-specific particle detectors. If the same situation is considered for centrifugal sedimentation based methods such as CLS and AC the problem becomes more complicated as the coating not only changes the apparent hydrodynamic diameter of the particle but also changes its mean density, which is critical to the size determination. In addition, depending on the quantity of protein used, there is a possible influence

on dispersion medium viscosity and optical properties which may introduce additional uncertainties to some measurement methods such as DLS, AC, PTA etc. Finally, the ability of albumin or similar proteins to effectively coat many types of particles means that it also can coat the inside surface of the measurement instrument and consequently render the surface prone to non-specific bonding of particles. This phenomenon can lead to problems of instrument operation, loss of material, cross contamination, memory effects and in certain circumstance may produce aggregates.

1.4.2 Evaluation of the dispersion quality

The aim of the dispersion protocols developed in NanoDefine was to disperse the materials in such a way that the resulting dispersions of (nano)particles are stable and contain only or mainly constituent particles. In the development of the dispersion protocols, optimisation of the procedures has been done by identifying likely combinations of sonication and stabilisers. These have then been developed into working protocols by systematic variation and optimisation of parameters based on the resultant mean particles size using dynamic light scattering (DLS) or centrifugal liquid sedimentation (CLS). This approach is suitable for the development of protocols but it is based on ensemble / fractionation techniques, which cannot distinguish between single particles and agglomerates/aggregates and is thus poorly suited to evaluate the true extent to which the dry powder has been dispersed into constituent particles. To evaluate the final state of the dispersed material in a more definitive and unambiguous manner, the dispersion was evaluated by qualitative and quantitative TEM analysis. Table 3 gives an overview of the qualitative evaluation of dispersions achieved using the NanoDefine optimised protocols and evaluated by TEM.

Table 3 Qualitative evaluation, based on TEM, of dispersions achieved using optimised protocols

Material Code	Description	Qualitative assessment of the effectiveness of dispersion protocol based on TEM analysis
IRMM-380	Pigment Yellow 83 (transparent)	Results in a combination of single constituent particles, and aggregates/agglomerates.
IRMM-381	BaSO ₄ (fine)	The protocol for IRMM-381 – BaSO ₄ (fine grade) allows dispersion of the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles).
IRMM-382	MWCNT	Both dispersion protocols result in a combination of single constituent particles, and smaller and larger agglomerates.
IRMM-383	Nano steel	Allows dispersion of the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 particles). Due to the layered structure of the material, it remains debatable if some of the apparent intensity fluctuations in the TEM images of the particles are caused by different grains making up one platelet, or by even smaller constituent particles attached to the dispersed platelets. Visual inspection of the liquid dispersion after sonication suggested that the material is not completely dispersed in the medium.
IRMM-384	CaCO ₃	Allows dispersion of the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles).

IRMM-385	Kaolin	Allows dispersing the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles)
IRMM-386	Pigment Yellow 83 (opaque)	Allows dispersion of the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles)
IRMM-387	BaSO ₄ (Ultrafine)	The 'Protocol for IRMM-387 – BaSO ₄ (ultrafine grade) ' allows dispersing the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles).
IRMM-388	Coated TiO ₂	Allows dispersing the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-20 constituent particles)
IRMM-389	Basic Methacrylate Copolymer, BMA	The dispersion protocol of BMA by TUD allows dispersing the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles).
BAM-11	Zeolite	The protocol for BAM-11 – zeolite results in a combination of single constituent particles, and aggregates/agglomerates.

1.5 References

¹ Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU). Official Journal of the European Union, L 275, pp.38-40

² Babick F., Mielke J., Wohlleben W., Weigel S., Hodoroaba V.-D., How reliably can a material be classified as a nanomaterial? Available particle-sizing techniques at work. [J Nanopart Res.](#) 2016; 18: 158. doi: [10.1007/s11051-016-3461-7](https://doi.org/10.1007/s11051-016-3461-7)

³ Hartmann N. B., Alstrup Jensen K., Baun A., Rasmussen K., Rauscher H., Tantra R., Cupi D., Gilliland D., Pianella F., Riego Sintes J.M., 'Techniques and Protocols for Dispersing Nanoparticle Powders in Aqueous Media – is there a Rationale for Harmonization?', J. Toxicology and Environmental Health, Part B: Critical Reviews. Pp. 1-28, 2015.

⁴ Guillemin J.-P., Schaer E., Marchal P., Lemaître C., Nonnet H., Ledieu A., A mass conservative approach to model the ultrasonic deagglomeration of ZnO nanoparticle suspension in water. Powder Technology, 2012, Vol. 219, Pages 59-64

⁵ Taurozzi J. S., Hackley V.A., Wiesner M.R., Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment – issues and recommendations. Nanotoxicology. 2011 Dec;5(4):711-29. DOI: 10.3109/17435390.2010.528846

⁶ Raman V., Abbas A., Experimental investigations on ultrasound mediated particle breakage. Ultrasonics Sonochemistry. Vol. 15, Issue 1, 2008, Pp. 55-64. <https://doi.org/10.1016/j.ultsonch.2006.11.009>

⁷ Taurozzi J. S., Hackley V.A., Wiesner M.R., Preparation of nanoparticle dispersions from powdered material using ultrasonic disruption. National Institute of Standards and Technology. 2012. Available at: <http://nvlpubs.nist.gov/nistpubs/specialpublications/nist.sp.1200-2.pdf>

Dispersion SOPs: Production of an aqueous based dispersion of the NanoDefine priority materials

2 SOP for production of an aqueous based dispersion of IRMM-380 (Pigment Yellow 83, Fine grade)

2.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-380 starting from dry powder form.

2.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based, surfactant stabilised colloidal suspension of IRMM-380, fine grade Pigment Yellow 83. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

2.3 Abbreviations

CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
NM	Nanomaterial
MALS	Multi-angle Light Scattering
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No. 25638-17-9)
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS.
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
SOP	Standard Operating Procedure
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

2.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2 mL or 6 mL) of a surfactant stabilised aqueous suspension (0.1 mgmL⁻¹ of IRMM-380)

of IRMM-380, Pigment Yellow 83 (Transparent grade). The procedure foresees starting from a dry powder sample of the IRMM-380 materials and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a low concentration of the commercial surfactant NEKAL BX. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL, while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

2.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 μ L, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

2.4.1.1 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

2.4.2 Material supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

2.4.3 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity $18.2 \text{ M}\Omega\text{cm}^{-1}$, $0.2 \mu\text{m}$ in-line filtration)
- Pigment Yellow 83 transparent grade distributed by IRMM with project ID no. IRMM-380
- High purity methanol (analytical grade)
- Ice-water mixture for cooling the sample during sonication.
- Surfactant: 30 wt% aqueous solution of NEKAL-BX (Sodium Butyl naphthalene sulphonate (CAS No. 25638-17-9)

2.4.4 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

2.4.4.1 Determination of suitable sonicator power settings

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 WmL^{-1} for 2 mL sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was Hielsche UPS200S instrument whose power output characteristics were determined as described in chapter 13 and the results shown in Figure 1.

In this procedure for dispersion of IRMM-380 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

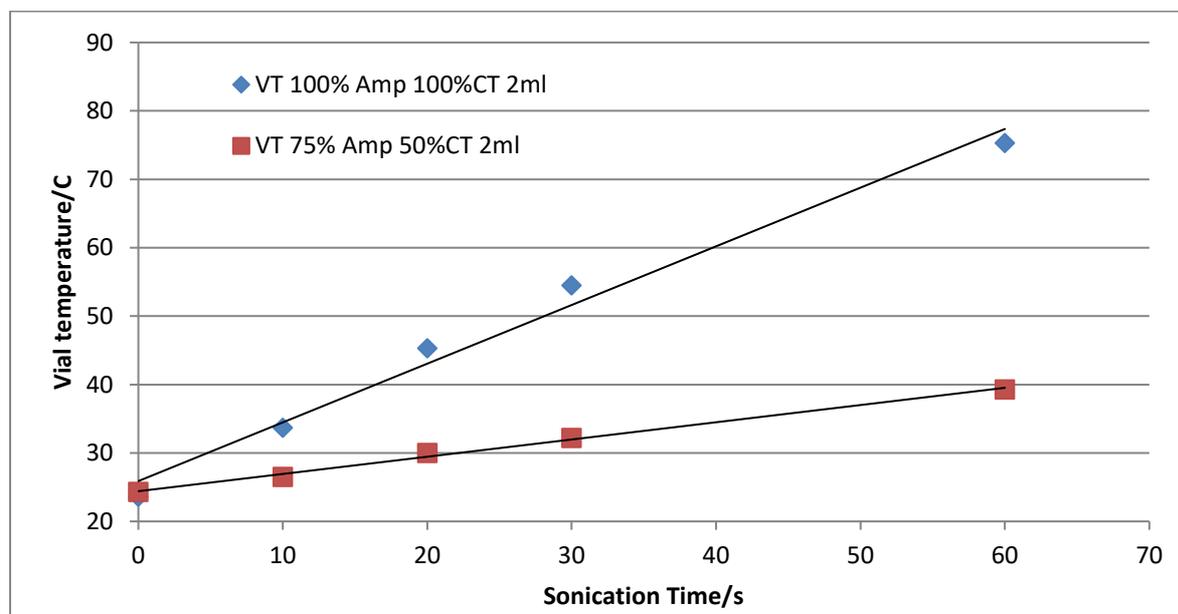


Figure 1: Temperature increase of 2 mL water in vial sonicator at (a) 100 % Amplitude and 100 % cycle-time and (b) 75 % Amplitude and 50 % cycle time. Specific power absorbed is (a) 3.8 WmL^{-1} and (b) 1.1 WmL^{-1} respectively

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what are the correct amplitude and cycle time settings required to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to approximate these values before proceeding with the dispersion procedure.

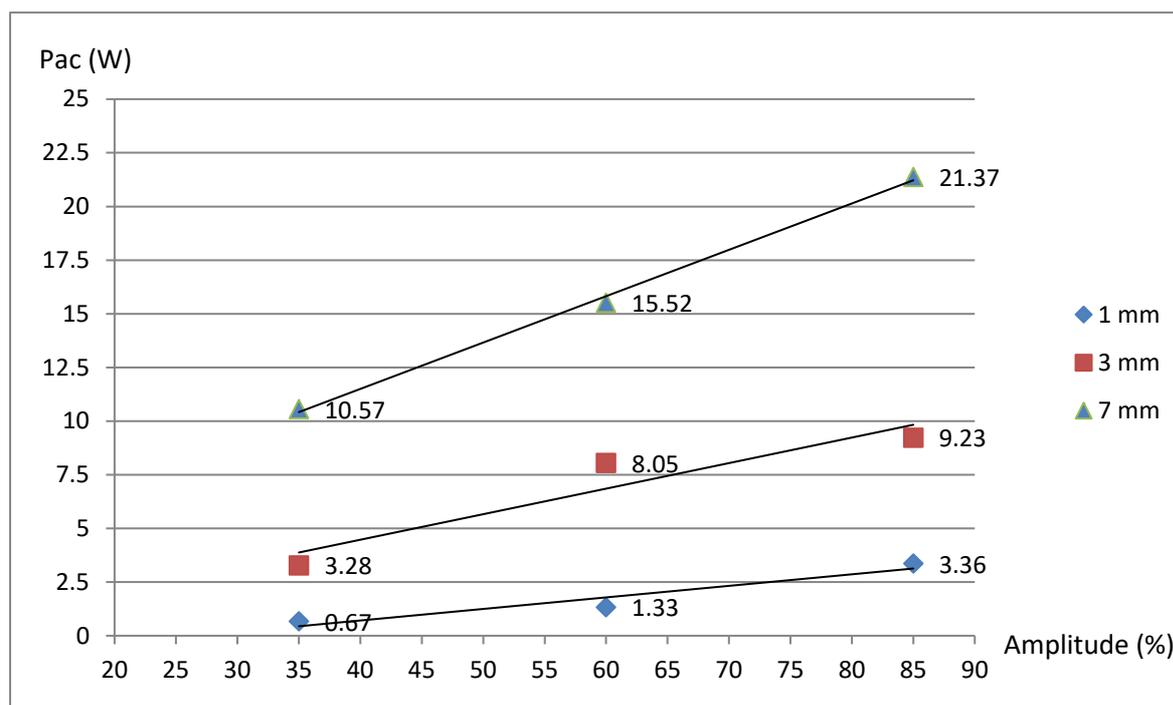


Figure 2: Acoustic power absorbed when using different probe diameters and amplitude settings

2.4.4.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 2.4.4.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

2.4.4.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh an empty 22 mL glass vial and add approximately 15 μL of Nekal BX solution (30 wt%) using a pipette. Reweigh the vial and calculate by difference the amount of NEKAL BX before adding sufficient pure methanol to give a mass concentration of 0.5 mgmL^{-1} : This solution will hereafter be referred to as solution A.

Weigh approximately 10 mg of IRMM-380 into a 22 mL glass vial and add sufficient pure methanol to give a concentration of 1 mgmL^{-1} . It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (2'): Add solution A to solution B in a ratio of $10 \mu\text{LmL}^{-1}$.

Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (2'): This will hereafter be referred to as solution B.

Prepare a heated water bath under a chemical safety hood and heat to 40-50 °C. Suspend the lower half of vial in the water bath until the MeOH evaporates leaving a layer of surfactant coated particles on the bottom of the vial. Add sufficient MilliQ water to get 10 mgmL⁻¹ solids in water and seal the vial with a suitable lid. Re-disperse the solids into the water by immersing the bottom half of the vial in a USB and sonicating for 2 minutes or until the solids appear uniformly distributed in the water. This will hereafter be referred to as solution C

Take an empty 22 mL vial and add 5.94 ml of pure water followed by 60 µL of solution C to give a final concentration of 0.1mg/mL IRMM-380 in water. This will hereafter be referred to as solution D

Sonication using probe sonicator: Take the 22 mL glass vial containing solution D and mount the probe sonicator head inside the vial as shown in Figure 3. The probe head should be immersed in the solution to a depth of approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. In this procedure the sonicator used was a Hielscher UPS200S and this was operated in pulsed mode with an amplitude of 75 % and a cycle time of 50 % which, as described in the Section 2.4.4.1, produces a mean adsorbed power of 7.8 W (50 % cycle time) corresponding to 1.3 WmL⁻¹ when normalised to the specified volume of 6 mL. A sonication time of 20 minutes was determined to be the optimum treatment time for this material under the described conditions.



Figure 3: Photograph showing recommended positioning of probe sonicator in sample

When attempting to use a probe sonicator which is different from the model used in the development of this method users must firstly determine the power output characteristics of their

own instrument using the method described in chapter 13. From this data, instrument settings should be determined which can approximate the power output values detailed above and in section 2.4.4.1.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Alternative method using vial sonicator: Place 2 mL of solution D in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 4).The vial should sonicated for 15 min at 75 % amplitude with the cycle time being set at 50 %. As cooling cannot be applied in the case of vial sonicator the use of a 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

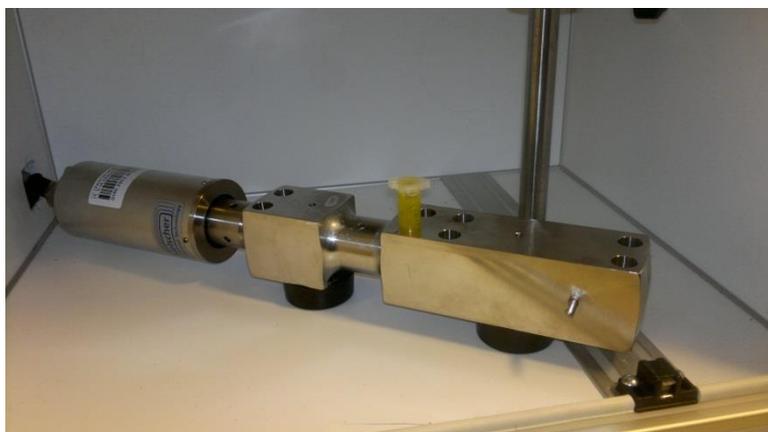


Figure 4: Positioning of sample in vial-sonicator

2.4.5 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 2.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in section 2.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease in mean size.

2.4.6 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepare in previous steps have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of

30 minutes following completion of the primary dispersion procedure outlined in the previous section 2.4.4. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of brief vortexing followed by a single additional step of bath sonication. In the case of IRMM-380, material aging of up to 6 days may be fully reversed if the sample vial is vortexed for 2 minutes and the treated for 15 minutes in a laboratory scale bath sonicator at room temperature.

2.4.7 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

2.5 Validation Status

This method has not yet been subjected to validation

2.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

2.7 Information on expected particle size distribution

Table 1 Values of mean particle size as determined by CLS and DLS

Dispersion approaches	Methanol+nekalBX method + 15' VS
Mean particle size (IRMM-380) by CLS (weight-size distribution)	50 nm
Mean particle size (IRMM-380) by DLS (Z-average)	47 nm

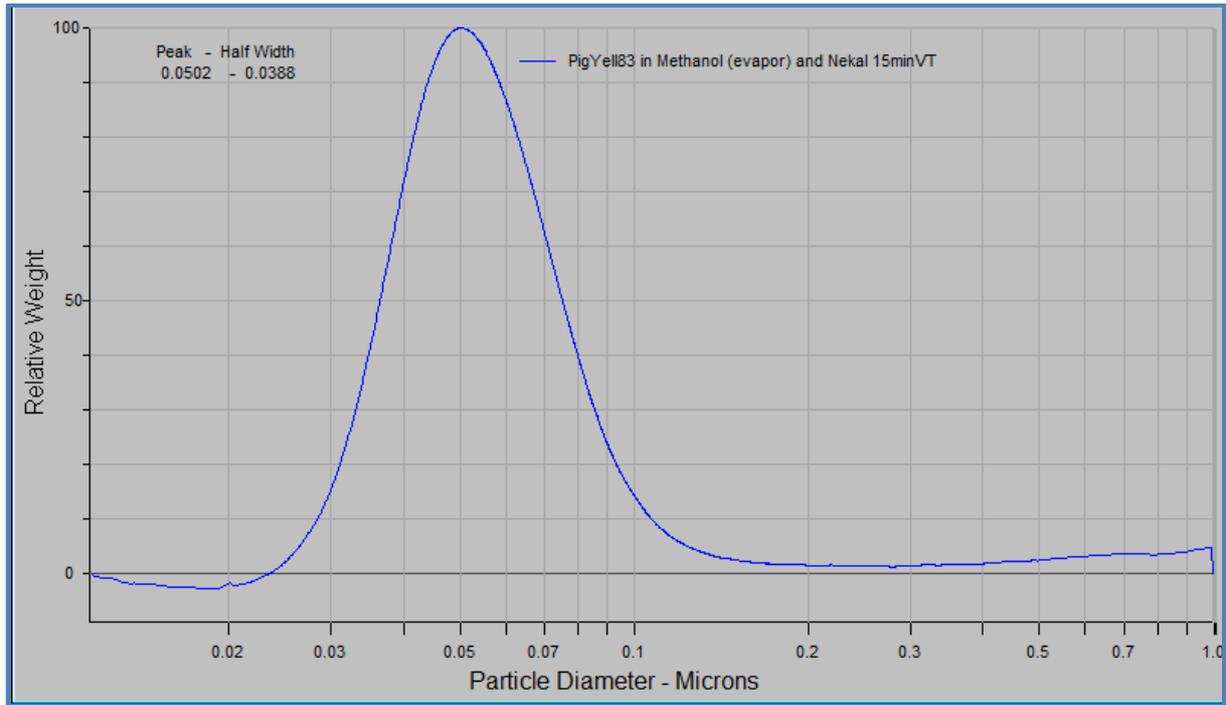


Figure 4: Particle size distribution as determined by CLS

3 SOP for production of an aqueous based dispersion of IRMM-381 (BaSO₄ (fine grade))

3.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-381 starting from a dry powder form.

3.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant-stabilised, water-based colloidal suspension of IRMM-381, fine grade BaSO₄. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS, does not significantly change (according to DLS measurements) over a time period of at least 60 minutes from completion of the dispersion procedure.

3.3 Abbreviations

DLS	Dynamic Light Scattering
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS)
PSD	Particle Size Distribution
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

3.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of an aqueous suspension (2.6 mgmL⁻¹) of IRMM-381, BaSO₄ (fine grade). The procedure foresees starting from a dry powder sample of IRMM-381 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing the commercial stabilising agent sodium hexametaphosphate (SHMP). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using a probe sonicator and allows to produce a batch volume of 6 mL.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particle size values while longer treatment times will either degrade the quality (re-formation of larger aggregates/agglomerates) or will not provide a significant further reduction in the mean size. The amount of SHMP used for dispersion can have an effect on the dispersion quality and obtained particle size distribution. This procedure has been developed to produce the lowest mean particle size distribution for dispersion in 2 mgmL⁻¹ SHMP. If required, the concentration of SHMP can be lowered down to 0.2 mg⁻¹. The particle size distributions obtained upon dispersion in 2 and 0.2 mgmL⁻¹ SHMP have been evaluated by DLS and were found to be comparable.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 60 minutes (aged, after re-dispersion by vortexing) with the results showing no major variation in the means size distribution. Sedimentation may be observed if the dispersion stands during some minutes after preparation. In this case re-dispersion is possible by vortexing.

3.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W
- Ultrasonic bath sonicator
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

3.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

3.4.3 Material Supplies

- 20 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

3.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm⁻¹, 0.2 μm in-line filtration)
- BaSO₄ (fine grade) distributed by IRMM with project ID no. IRMM-381
- Ice-water mixture for cooling the sample during sonication
- Sodium hexametaphosphate powder (CAS No. 68915-31-1, purity ≥ 96 %, e.g. 305553 Aldrich)

3.4.5 Materials and methods

This section describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

3.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was Microson XL 2000 (Qsonica, LLC (Newtown, USA) with nominal maximum power of 100 W. The sonicator was fitted with a probe head with diameters of 6.4 mm (length of 117 mm and maximum peak-to-peak amplitude of 60 μm).

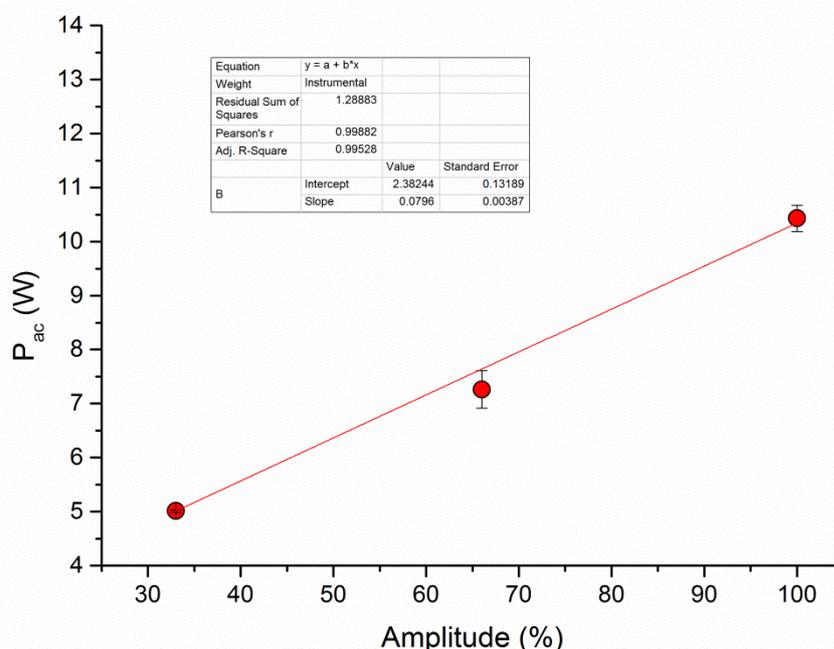


Figure 1: Calculated delivered output power P_{ac} of the probe sonicator at different amplitude settings. This calibration curve was used to determine the output setting value which corresponds to $P_{ac} = 10.3$ W (in this example: amplitude of 100 %)

The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in in Figure 1. In this procedure for the dispersion of IRMM-381 the sonicator was operated at a set amplitude value of 100 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 10.3 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce an output of 10.3 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

3.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 3.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

3.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Prepare stabilising agent solution (2 mgmL⁻¹ SHMP) by dissolving the appropriate amount of SHMP powder into MilliQ water. Shake vigorously to ensure that all powder is solubilized. Subsequently, filter the prepared SHMP solution using a 0.2 µm filter to ensure that no large particulates are present.

Weigh approximately 15.6 mg of IRMM-381 into a 20 mL glass vial. It is recommended that an ionizer be used to neutralize electrostatic charge during weighing of fine powders. Add the respective volume of SHMP solution to give an IRMM-381 concentration of 2.6 mgmL⁻¹ (6 mL for exactly 15.6 mg of IRMM-381) adjusting the volume to compensate for small deviations in the final weighed mass). Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Take the 20 mL glass vial containing the 2.6 mgmL⁻¹ IRMM-381 suspension and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is full immersed in the cooling water. The sample should then be sonicated at a constant power 10.3 W for 20

minutes. The correct power setting should be determined from calibration curve which was previously determined by the method described in chapter 13. The resulting dispersion should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.



Figure 2: Ultrasonic probe sonication setup for dispersion of NM powders

3.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 3.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 3.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

3.4.7 Recovery of dispersions after aging beyond verified period of stability.

The temporal stability of the dispersions prepared in previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 1 h following completion of the primary dispersion procedure outlined in section 3.4.5.3. Sedimentation may be observed if the dispersion stands during some minutes after preparation. In this case re-dispersion is possible by vortexing.

3.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

3.5 Validation status

This method has not yet been subjected to validation

3.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

3.7 Information on expected particle size distribution

Table 1 Summary of the DLS results obtained for IRMM-381 – BaSO₄ (fine grade) suspensions in MilliQ water (N=1) and 2 mgmL⁻¹ hexametaphosphate (N=2) prepared at different probe sonication times

Sonication Time	0 min	2.5 min	5 min	10 min	15 min	20 min	25 min	30 min
Intensity-weighted mean diameter: by DLS(Z _{ave} , cumulants method)	657.6 ± 17.1	493.8 ± 11.3	466.2 ± 1.0	415.6 ± 1.7	399.0 ± 1.6	377.4 ± 1.3	363.9 ± 2.0	365.9 ± 13.2

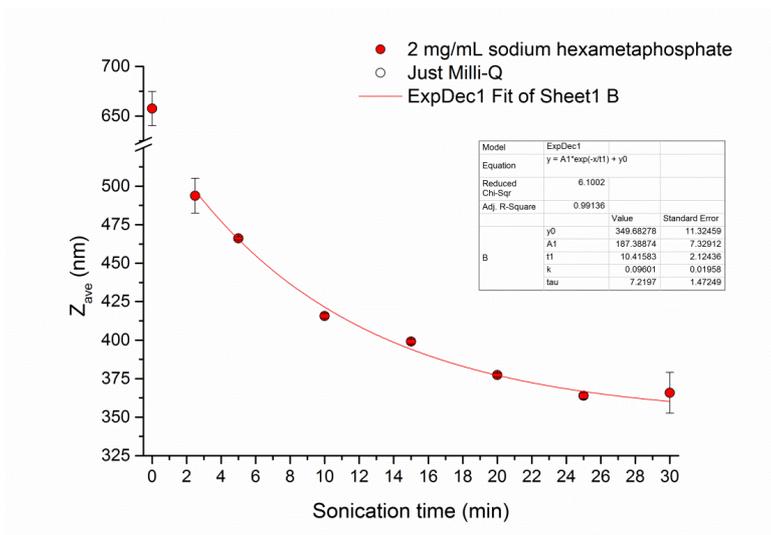


Figure 3: Z_{ave} values obtained by DLS for IRMM-381 – BaSO₄ (fine grade) suspensions in MilliQ water (N=1) and 2 mgmL⁻¹ hexametaphosphate (N=2) prepared at different probe sonication times

Table 2 Mean diameter corresponding to the major peak of the intensity-weighted size distribution obtained by the NNLS method (DLS) for dispersed IRMM-381– BaSO₄ (fine grade)

Sonication Time	0 min	2.5 min	5 min	10 min	15 min	20 min	25 min	30 min
Peak mean (nm)	661.8 ± 10.4	506.0 ± 7.3	489.0 ± 7.5	442.0 ± 4.5	429.1 ± 6.7	408.2 ± 5.1	392.2 ± 2.3	387.2 ± 3.0

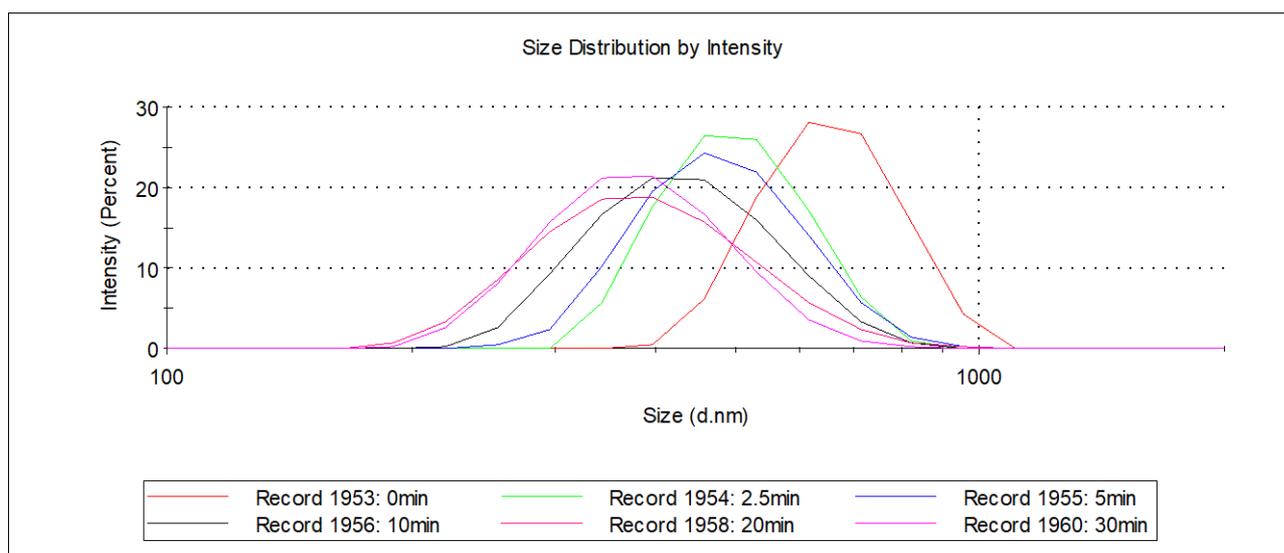


Figure 4: Intensity-weighted size distribution obtained by DLS for IRMM-381 – BaSO₄ (ultrafine grade) suspensions in 2 mgmL⁻¹ hexametaphosphate (N=2) prepared at different probe sonication times

4 SOP for production of an aqueous based dispersion of IRMM-382 (Multi-wall Carbon nanotubes)

4.1 Aim

The aim of the procedure is to describe a laboratory scale methods to produce a colloidally stable water-based dispersion of the multi-walled carbon nanotubes starting from IRMM-382 in dry powder form.

4.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant-stabilised, water-based colloidal suspension of IRMM-382, Multi-wall Carbon nanotubes. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose absorbance, as determined by UV-Vis measurements, does not significantly change over a time period of at least 30 minutes from completion of the dispersion procedure.

4.3 Abbreviations

MWCNT	Multi-wall Carbon nanotubes
NM	Nano/Material
SOP	Standard Operating Procedure
Tannic acid	Natural extract of plant material (CAS No. 1401-55-4)
TEM	Transmission Electron Microscopy
Triton X-100	Commercial surfactant (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, CAS No. 9002-93-1)
USP	Ultrasonic probe sonicator
UV-Vis	Ultraviolet-Visible absorption spectroscopy

4.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of a surfactant stabilised aqueous suspension (1 mgmL⁻¹ or 0.2 mgmL⁻¹ of IRMM-382) of IRMM-382, Multi-wall Carbon nanotubes. The procedure foresees starting from a dry powder sample of IRMM-382 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a low concentration of the stabilisers/surfactants Tannic Acid or Triton X-100. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The sonication time and power values detailed in the procedure have been determined to produce the most stable dispersion where minimum precipitation of MWCNT is observed in an experimentally relevant time (<1 hour). The use of a higher power may produce froth (depending on the dispersant) and the carbon nanotubes may attach to the generated bubbles, hence leading to a less effective dispersion of the MWCNT, while longer treatment times may potentially either degrade the quality by breaking MWCNT.

To evaluate the stability of the dispersions, UV-Vis absorbance has been measured immediately after sonication (pristine) and again after a rest periods of up 21 days (aged) with the results showing no major variation in the UV-Vis absorbance confirming the stability of the dispersion.

4.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W. Alternatively a 200 W vial sonicator may be used
- Adjustable volume pipettes of 100 μ L, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication

4.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Ultraviolet-visible spectrometer (UV-Vis)
- Transmission Electron Microscope (TEM)

4.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of dispersant
- Disposable plastic weighing boats or similar for weighing of Tannic acid and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

4.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 M Ω cm⁻¹, 0.2 μ m in-line filtration)
- Multi-wall Carbon nanotubes distributed by IRMM with project ID no. IRMM-382.
- Ice-water mixture for cooling the sample during sonication
- 1 wt% aqueous solution of Triton X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol, CAS No. 9002-93-1)
- A 300 mgL⁻¹ aqueous solution of Tannic Acid (CAS No. 1401-55-4)

4.4.5 Materials and methods

This section describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures.

4.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was Hielscher UPS200S with nominal maximum power of 200 W. This sonicator could be fitted with probe heads with diameters of 1mm, 3 mm or 7 mm diameter. The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in Figure 1. In this procedure for the dispersion of IRMM-382 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of either 35 % or 50 %. From the calibration curve it was determined that under these operating conditions the instrument was producing a measured power output of 10.6 W or 13.7 W respectively.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce the output power specified in the materials and method section of this SOP. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

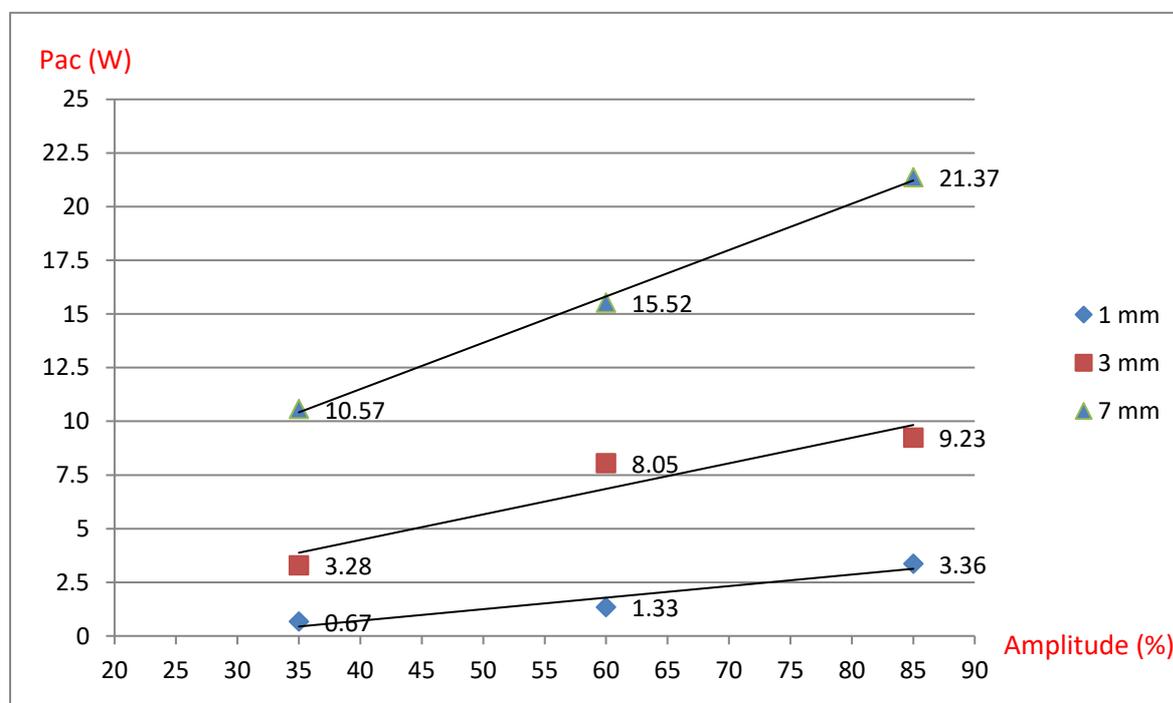


Figure 1: Acoustic power absorption characteristics of model UPS200S Heilscher ultrasonic processor

4.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 4.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

4.4.5.3 Detailed dispersion procedure for IRMM-382-MWCNT

This SOP describes two methods of dispersing the priority material IRMM-382 (MWCNT) using different stabilising agents. The first method, based on the use of Triton X100 surfactant, is able to produce dispersions with a higher mass of nanomaterials (1 mgmL^{-1}) but requires a relatively high concentration of surfactant (10 mgmL^{-1}). The second method using tannic acid can disperse a lower quantity of solids (0.2 mgmL^{-1}) but uses a much lower relative mass of stabiliser (0.3 mgmL^{-1}).

Pre-dispersion of IRMM-382-MWCNT in 1 wt% aqueous Triton X100: Weigh accurately 6 mg of IRMM-382 into a 22 mL glass vial and add sufficient of Triton X-100 (1 % wt) aqueous solution using a pipette to give a concentration of 1 mgmL^{-1} . Treat solution for 10 minutes in an ultrasonic bath to ensure wetting of the nanomaterial

Pre-dispersion of IRMM-382-MWCNT in (300 mgL^{-1}) aqueous tannic acid: Weigh accurately 2 mg of IRMM-382 into a 22 mL glass vial and add sufficient aqueous Tannic acid (300 mgL^{-1}) solution using a pipette to give a concentration of 0.2 mgmL^{-1} . Treat the solution for 10 minutes in an ultrasonic bath to ensure wetting of the nanomaterial.

Sonication using probe sonicator: The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning steps noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Take the 22 mL glass vial containing 6 mL of either solution A or B and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is full immersed in the cooling water.

Ultrasonic dispersion of IRMM-382-MWCNT in Triton X100: A 6 mL sample of solution A should be sonicated for 60 minutes with power setting adjusted to give constant power of 10.6 W (35 % amplitude on Heilscher UPS200S sonicator)

Ultrasonic dispersion of IRMM-382-MWCNT in aqueous tannic acid: A 6 mL sample of solution B should be sonicated for 30 minutes with power setting adjusted to give constant power of 13.7 W (50 % amplitude on Heilscher UPS200S sonicator)

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determines a similar calibration curve for their own instrument. The correct power settings for probe sonicators should be determined from calibration

curves measured by the method described in chapter 13. The resulting colloidal solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.



Figure 2: Photograph showing recommended positioning of probe sonicator in sample

4.4.6 Optional verification of dispersion quality

The physical form of the MWCNT particles means that light scattering and sedimentation methods are not reliable for assessing the size of the conventional. The only suitable method is by EM and where the operator has access to TEM instrumentation it is strongly recommended that the dispersion quality and stability be evaluated by TEM.

4.4.7 Recovery of dispersions after aging beyond verified period of stability

In the work of Rastogi et al. (Journal of Colloid and Interface Science 328 (2008) 421–428) it was reported that UV-Visible absorption spectroscopy was a suitable method for evaluating the dispersion quality of MWCNT and a similar approach was adopted to check the temporal stability of IRMM-382 dispersed by these procedures. To do this the UV-Visible spectra were periodically acquired from dispersed samples and the absorption values at 500 nm determined. Measurement of the UV-Visible absorption of the dispersed solutions of MWCNT (both Triton X100 and tannic acid stabilised) shows a near constant level of absorption (500 nm) over a period of 21 days confirming that little or no tendency to agglomeration and sedimentation occurs with the materials dispersed following this procedure. It is therefore not considered necessary to evaluate any procedure for recovering aged samples.

4.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

4.5 Validation status

This method has not yet been subjected to validation.

4.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

4.7 Information on expected particle size distribution

No examples of size distributions can be reported here as dispersions of MWCNT cannot be reliably measured using DLS or CLS methods, and TEM analysis was only able to provide qualitative information.

5 SOP for production of an aqueous based dispersion of IRMM-383 (Nano steel)

5.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a water-based dispersion of IRMM-383 (Nano steel) starting from dry powder form. The presented protocol allows dispersing the material in a highly dispersed state.

5.2 Scope

This scope of the Standard Operating Procedure (SOP) is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based suspension of IRMM-383, Nano steel. The method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution.

The physico-chemical properties of this material do not allow obtaining a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

This instability is not compatible with several of the particle size measurement methods adopted in the NanoDefine project. The protocol remains useful for methods such as AFM and EM, where the sample is transferred to a solid carrier by sedimentation. Such sedimentation results in a preferential orientation of the platelets composing this material, which biases the measurements of conventional 2D EM-based methods. Because the Z-dimension is not measurable, the minimal external dimension referred to in the EC NM definition, cannot be estimated by EM.

5.3 Abbreviations

AFM	Atomic Force Microscopy
EM	Electron Microscopy
NM	Nanomaterial
PSD	Particle Size Distribution
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
USP	Ultrasonic probe sonicator
VM	Vortex mixer

5.4 Description

The following SOP describes a method for the preparation of small volumes (10 mL) of an aqueous dispersion of IRMM-383 at a concentration of 2.56 mgmL⁻¹. The procedure foresees starting from a dry powder sample of IRMM-383 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a

shorter time may produce measurably larger mean particles size, while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

The stability of the dispersions after sonication is evaluated visually immediately after sonication (pristine). The physico-chemical properties of this material do not allow obtaining a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

The dispersion efficiency is evaluated based on the particle size distribution determined by TEM.

5.5 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 20 kHz and equipped with a probe with a tip diameter of approximately 13 mm (e.g. Vibracell 75041 ultrasonifier, Fisher Bioblock Scientific, Aalst, Belgium)
- Adjustable volume pipettes of 100 μL , with disposable tips.
- Pipettes of 10 mL
- Box for ice-cooling of samples during sonication.
- Vortex mixer

5.5.1 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument

5.5.2 Material Supplies

- 20 mL glass vial (e.g. 10560503-X500, Wheaton Science Products, Millville, New Jersey, distributed by Fisher Scientific) with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of EM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs
- Parafilm M
- Flask rings (e.g. Heathrow scientific lead rings)
- Vial holder

5.5.3 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity $18.2 \text{ M}\Omega\text{cm}^{-1}$, 0.2 μm in-line filtration)
- Nano steel distributed by IRMM with project ID no. IRMM-383
- Ice-water mixture for cooling the sample during sonication

5.5.4 Materials and methods

This section describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures.

For laboratories equipped with a TEM, additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

5.5.4.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was a Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium). This sonicator is fitted with a probe head with a diameter of 13 mm diameter. The power characteristics of this sonicator probe have been experimentally determined. The resulting calibration curves can be seen in Figure 1. The output energy in function of the sonication time and the output power in function of the selected amplitude are shown in Figure 1A and Figure 1B, respectively.

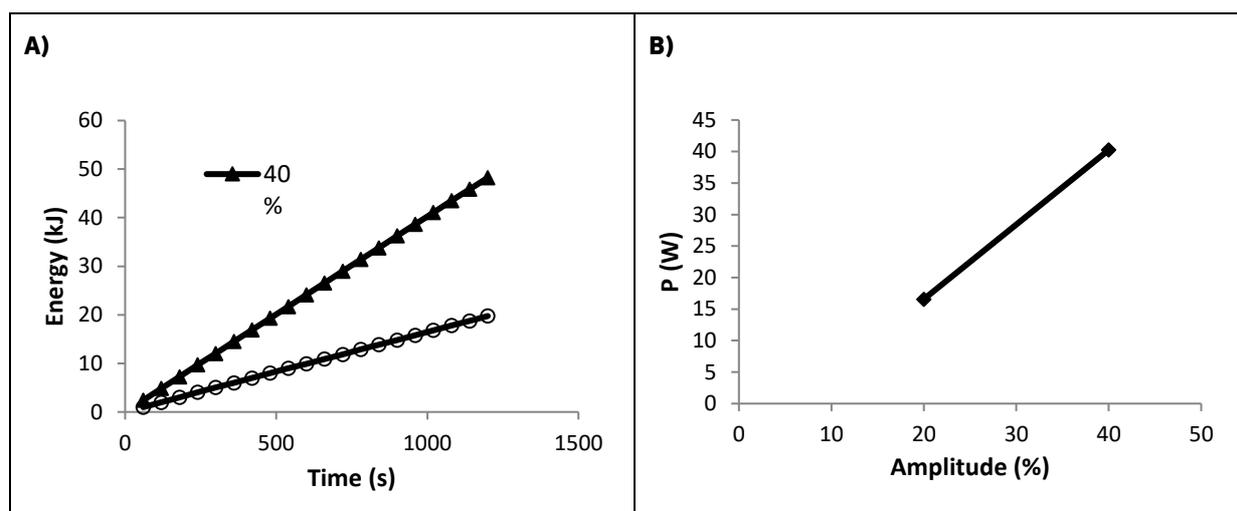


Figure 1: Calibration curves for the probe sonicator Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium) fitted with a probe head with a diameter of 13 mm, showing (A) the output energy in function of the sonication time and (B) the output power in function of the selected amplitude

In this procedure for the dispersion of IRMM-383, the 13 mm probe head (CV33) is positioned in the bottom half of the dispersion and the sonicator is operated at a set amplitude value of 40%. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 40 W. Sonication is stopped after 10 minutes, when an added specific energy of 25 ± 2 kJ is read out from the sonicator apparatus.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determines a similar calibration curve for their own instrument. Once the calibration procedure has been completed, an examination of the resulting

amplitude–power curve must be done in order to determine the correct amplitude setting required to produce an output of 40 W. The amplitude setting of the sonicator should be adjusted to this value before proceeding with the dispersion procedure.

5.5.4.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 5.5.4.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

5.5.4.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the appropriate cleaning steps noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

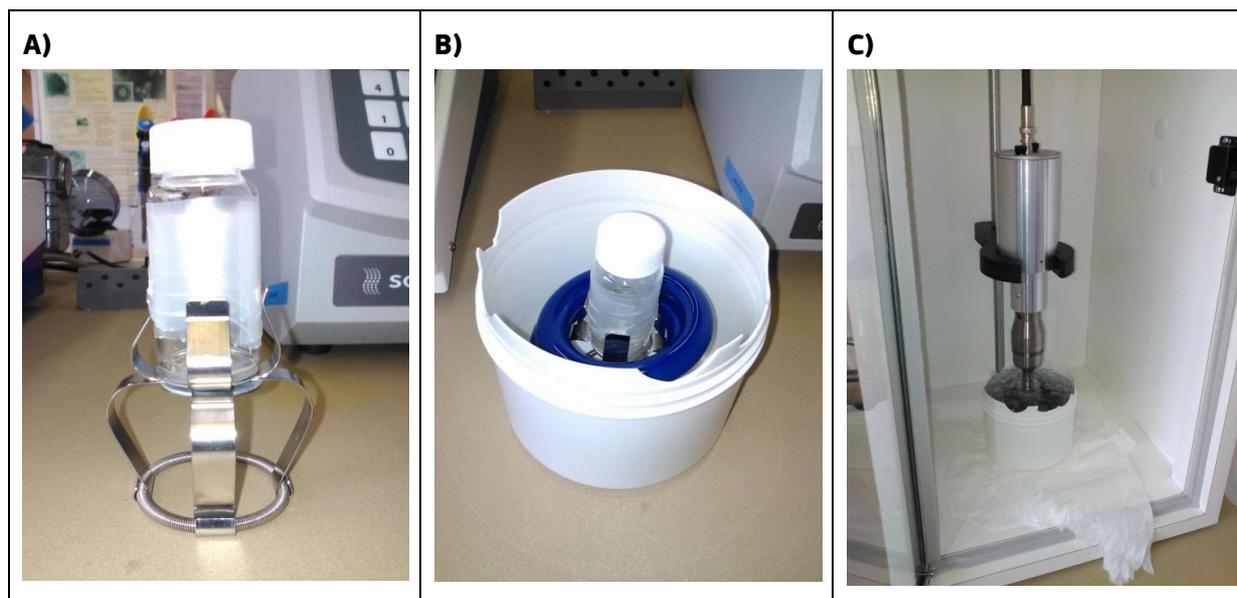


Figure 2: Setup for Sonication using the probe sonicator

Weigh approximately 25.6 mg of IRMM-383 into a 20 mL glass vial and add 10 mL of pure water to give a concentration of 2.56 mgmL⁻¹. It is recommended to use an Ionizer to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Wrap parafilm M around the 20 mL glass vial to avoid movement during sonication, and place the vial in the vial holder (Figure 2A). Place the vial holder in the box for ice-cooling using the flask rings (Figure 2B). Add a mixture of crushed ice and water in the box to cool the dispersion during sonication. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. Mount the probe sonicator head inside the vial

(Figure 2C). The probe head should be immersed in the dispersion to a depth of at least 1 cm. The sample should then be sonicated at a constant power of 40 W for 10 minutes. The correct power setting should be determined from the calibration curve which was previously determined.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (20 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Figure 2 (A) shows the 20 mL glass vial containing the dispersion is placed in the vial holder. Parafilm M is wrapped around the vial to avoid sliding during sonication. (B) The vial and vial holder are placed in the box for ice-cooling using the flask rings. (C) A mixture of crushed ice and water is added in the box to cool the dispersion during sonication. The vial is fully immersed in the cooling water. The probe sonicator head is immersed in the dispersion to a depth of at least 1 cm.

5.5.5 Optional verification of dispersion quality

Where the operator has access to a TEM instrument, it is strongly recommended that the dispersion be evaluated by TEM and the results compared with that in section 5.8.

To become suitable for TEM analysis, the dispersion has to be diluted 10 times after sonication to obtain a concentration of 0.256 mgmL^{-1} . TEM specimens can be prepared following the drop-on-grid method¹. This method includes pre-treating pioloform and carbon coated, 400 mesh copper grids (Agar Scientific, Essex, England) with 1 % Alcian blue (Fluka, Buchs, Switzerland) to increase hydrophilicity and rinsing 5 times with distilled water. The grid is then placed on 15 μL of dispersion during 10 minutes, and is rinsed 2 times afterwards with distilled water.

The described laboratory scale method produces a water-based dispersion of IRMM-383 starting from dry powder. The presented protocol disperses the material in a highly dispersed state. The material's physico-chemical properties do not allow obtaining a stable dispersion. The protocol remains useful for methods such as AFM and EM where specimens can be prepared by transferring a fraction of the sample to a solid carrier by sedimentation. TEM evaluation of the dispersion quality shows the smallest dispersible particles (referred to a single constituent particles) and some small agglomerates thereof (consisting of 2-10 particles) (Figures 3 and 4). Note that conventional TEM is not able to measure the smallest dimension (platelet thickness) of the particles due to preferential orientation of particles on the grid. The size distribution (Figure 4) should be interpreted as the distribution of the Feret min values of the particles' 2D projections on the EM grid.

If the expected mean aggregate/agglomerate size is significantly larger (>15 %) than that shown in section 5.8 of this SOP, the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

5.5.6 Recovery of dispersions after aging beyond verified period of stability

The material's physico-chemical properties do not allow producing a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

5.5.7 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

5.6 Validation status

This method has not yet been subjected to validation

5.7 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

5.8 Information on expected particle size distribution

The qualitative TEM analysis describes the physico-chemical characteristics of the particles, such as the aggregation/agglomeration state, and the size and shape of the free single particulates, aggregates and agglomerates. Table 1 summarizes the qualitative TEM analysis of IRMM-383.

Quantitative TEM analysis is performed using methods described by Verleysen et al. and De Temmerman et al.²⁻⁴. Figure 3 shows representative TEM images of IRMM-383. The corresponding size distribution is shown in Figure 4 and is determined by a semi-automated approach using imageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). This approach can be briefly summarized as follows:

- To suppress background noise, a mean filter is applied before analysis. The use of other filters was not necessary for the examined material.
- A threshold for the detection of the particles based on mass-thickness contrast in the image is chosen manually.
- Particles are only detected in a pre-defined Region of Interest (ROI), which allows excluding border particles.
- For every micrograph, the 'Fill holes' option is switched on.

Table 1 Summary of the qualitative TEM analysis of IRMM-383 Nano steel

Examined property	Description
Distribution of particles on the grid?	evenly distributed
Concentration of particles on the grid?	OK
Aggregation/agglomeration state?	single constituent particles and agglomerates
Sub-fraction	a sub-fraction of smaller particles is present in the sample (size 10-50 nm). It remains uncertain whether these small particles are nano steel or a contaminant.
Manually measured size of the constituent particles?	the size ranges from 60 nm to 1.5 μm
Manually measured size of the aggregates/agglomerates?	the size ranges from 100 nm to 2.5 μm
2D shape of the PP?	irregular polygonal
2D shape of the aggregates/agglomerates?	complex
Surface structure of the constituent particles?	rough
Surface structure of the aggregates/agglomerates?	rough
Diffraction contrast?	diffraction contrast, which indicates that the material is crystalline, can be observed in the constituent particles
Efficiency of the dispersion protocol?	allows dispersion of the material up to the level of single constituent particle and some small agglomerates (consisting of 2-10 constituent particles). Visual inspection suggested however that the material is not completely dispersed in the medium. The nano steel flakes start to sink down before and immediately after sonication.

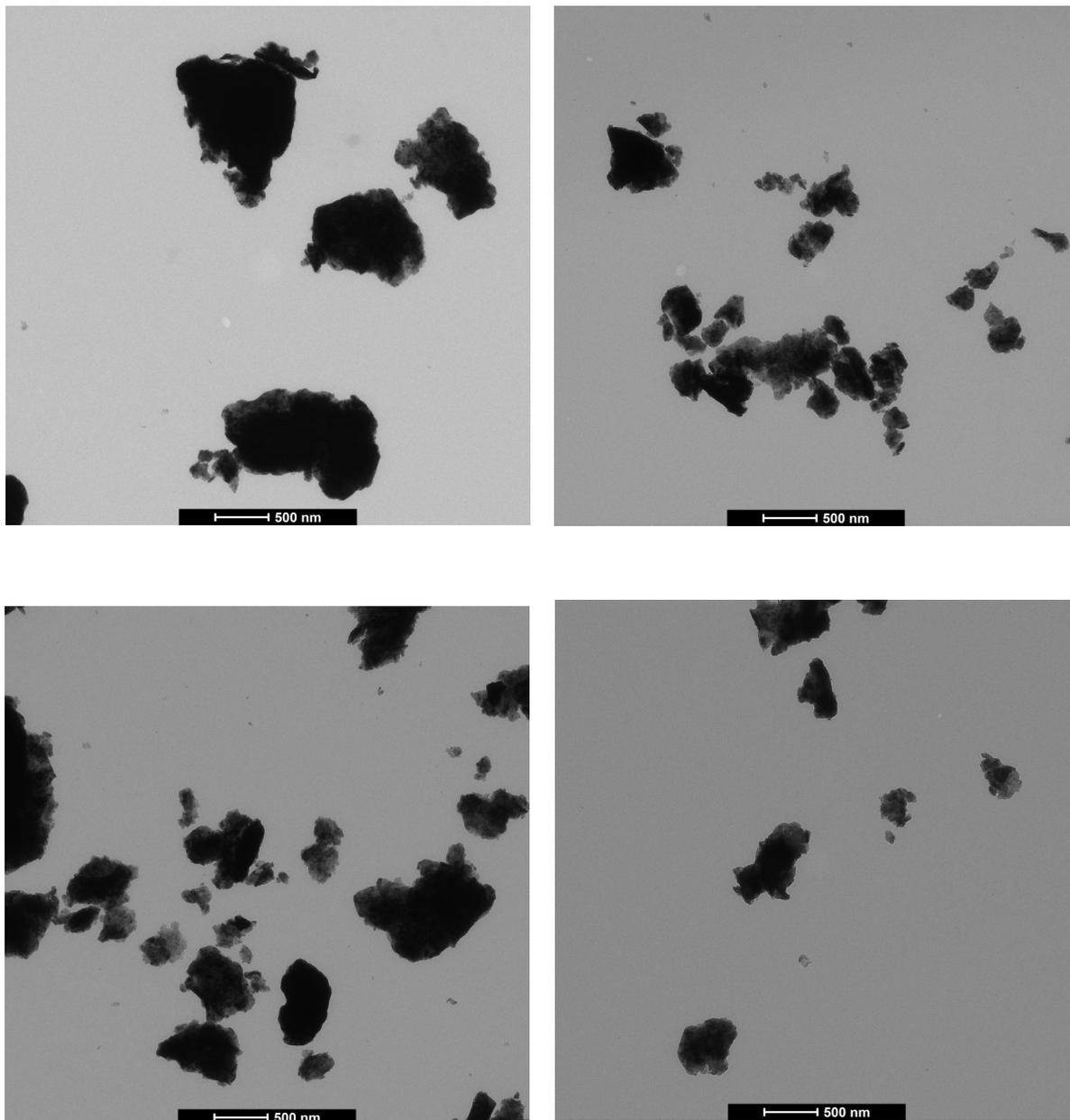


Figure 3: Representative TEM images of IRMM-383 Nano steel particles dispersed using the presented SOP

Descriptive statistical analysis of the Feret min of the particles is obtained using a home-made script in the python programming language. The raw data is represented as a histogram ('Number based distribution') (Figure 4). It should be noted that TEM is probably not able to measure the smallest dimension (platelet thickness) of all particles due to preferential orientation of platelet-like particles on the grid.

A sub-fraction of smaller particles is present in the sample (size 10-50 nm). It remains uncertain whether these small particles are nano steel or a contaminant.

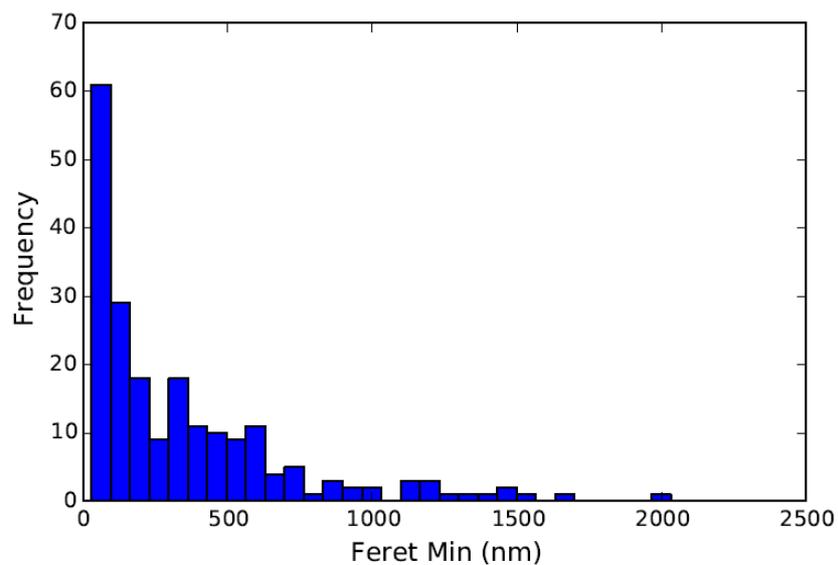


Figure 4: Representative size distribution (Feret min) of the single particulates of IRMM-383 Nano steel obtained by quantitative TEM. Note that the Feret min parameter is measured in the X-Y plane and is not suitable to estimate of the minimal external dimensions of platelet-like particles situated in the Z-plane

6 SOP for production of an aqueous based dispersion of IRMM-384 (CaCO₃)

6.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-384 starting from dry powder form.

6.2 Scope

The scope of this SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-384, calcium carbonate CaCO₃. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

6.3 Abbreviations

Calgon	Sodium hexametaphosphate (CAS No. 10124-56-8).
CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
MALS	Multi-angle Light Scattering
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No. 25638-17-9)
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS.
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

6.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2 mL or 6 mL) of a surfactant stabilised aqueous suspension (50 mgmL⁻¹ of IRMM-384) of IRMM-384, Calcium carbonate (CaCO₃). The procedure foresees starting from a dry powder sample of IRMM-384 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing the commercial surfactant Sodium hexametaphosphate (Calgon). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjusting the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

6.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

6.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

6.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM and any chemicals in powder form
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

6.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity $18.2 \text{ M}\Omega\text{cm}^{-1}$, $0.2 \mu\text{m}$ in-line filtration)
- Calcium carbonate (CaCO_3) distributed by IRMM with project ID no. IRMM-384
- Ice-water mixture for cooling the sample during sonication
- Surfactant: aqueous solution of Sodium hexametaphosphate (Calgon)

6.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

6.4.5.1 *Determination of suitable sonicator power settings*

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 WmL^{-1} for 2 mL sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was Hielscher UPS200S instrument whose power output characteristics were determined as described in chapter 13 and the results are shown in Figure 1.

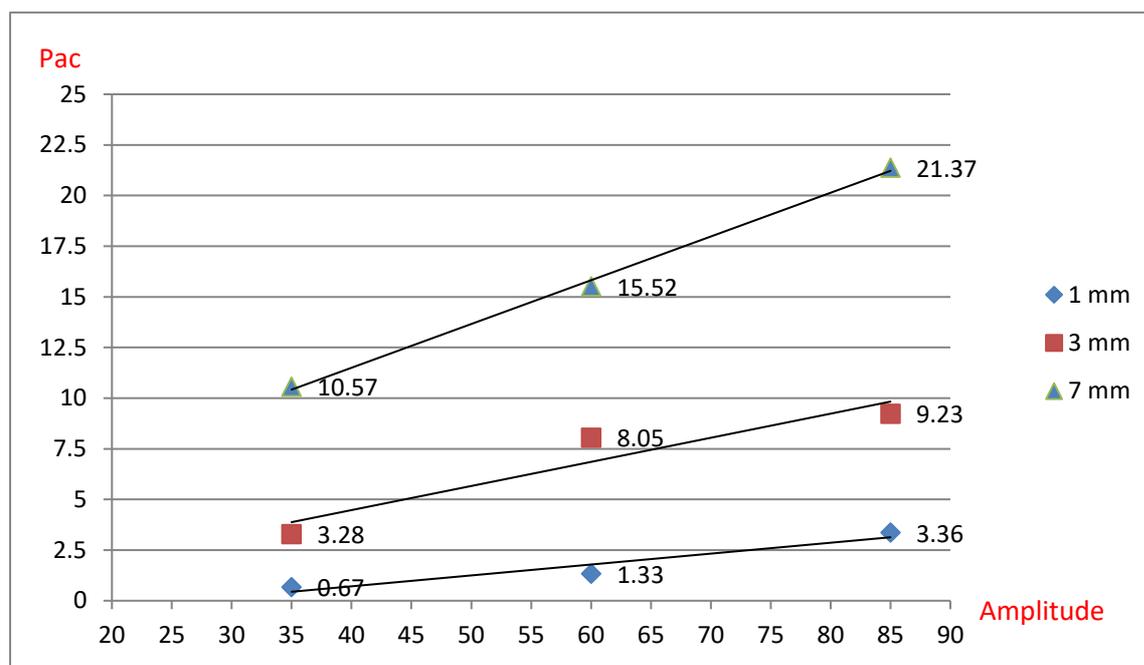


Figure 1: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time

In this procedure for dispersion of IRMM-384 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what are the correct amplitude and cycle time settings to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to match these values before proceeding with the dispersion procedure.

6.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 6.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

6.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

To produce 10 mL of dispersant weigh an empty 22 mL glass vial and add approximately 20 mg of Sodium hexametaphosphate (SHMP) using a spatula. Reweigh the vial and calculate by difference amount of SHMP before adding sufficient ultrahigh purity water to give a concentration of 2mgmL^{-1} ; Vortex (2'). This solution will hereafter be referred to as solution A.

Weigh approximately 300 mg of IRMM-384 into a 22 mL glass vial and add sufficient solution A to give a concentration of 50mgmL^{-1} . It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (10'). This will hereafter be referred to as solution B.

Sonication using probe sonicator: Take the 22 mL glass vial containing 6 mL of solution B and mount the probe sonicator head inside the vial as shown in Figure 2.



Figure 2: Photograph illustrating the mounting of probe sonicator in sample

The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. The sample should then be sonicated at a amplitude of 75 % and 50 % cycle time for 15 minutes (Hielscher UPS200S). At this amplitude setting the peak power output is 18 W which corresponds to measured mean power of 7.9 W when using a 50 % cycle time. For use with other types of sonicator correct settings should be determined from calibration curves determined by the method described in chapter 13. The resulting solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low amplitude (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

Sonication using vial sonicator: Place 2 mL of solution C in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 3). The vial should be sonicated for 10 min at 75 % amplitude with the cycle time being set at 50 %. Cooling cannot be applied in the case of vial sonicator and the use of a 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

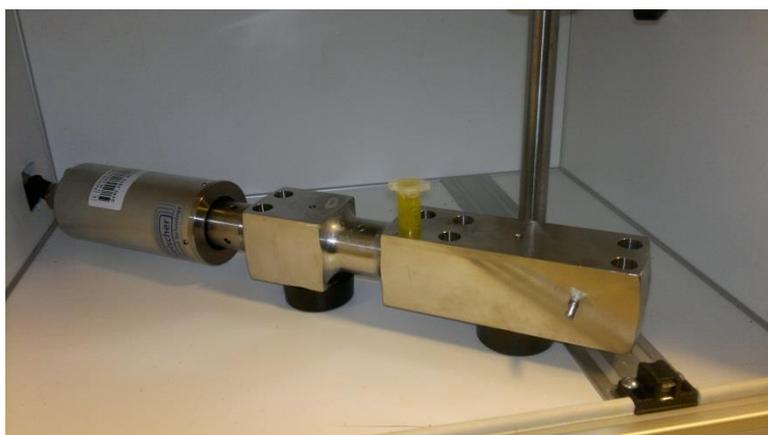


Figure 3: Positioning of sample in vial-sonicator

6.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 6.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in section 6.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

6.4.7 Recovery of dispersions after aging beyond verified period of stability.

The temporal stability of the dispersions prepared in previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in section 6.4.5. In the case of materials which have been allowed to age for longer than 30 minutes it is possible that some degree of re-agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-384, material aging of up to 6 days may be

fully reversed if the sample vial is vortexed for 2 minutes and then treated for 10 minutes in a laboratory scale bath sonicator at room temperature.

6.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

6.5 Validation status

This method has not yet been subjected to validation

6.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

6.7 Information on expected particle size distribution

A typical mass based particle size distribution of the (vial sonicator) re-dispersed CaCO₃ is shown in Figure 4, while Table 1 lists typical values of mean particles size obtained using the probe and vial sonicator methods.

Table 1: Mean particle size values of dispersed CaCO₃ as determined by CLS

	Sonication Method			
Mean Particle Size in nm of IRMM-384 by CLS (weight-size distribution)	USP 7 mm 15'	VS 10' (*)		
	348	392	359	361

(*) results of three different analyses

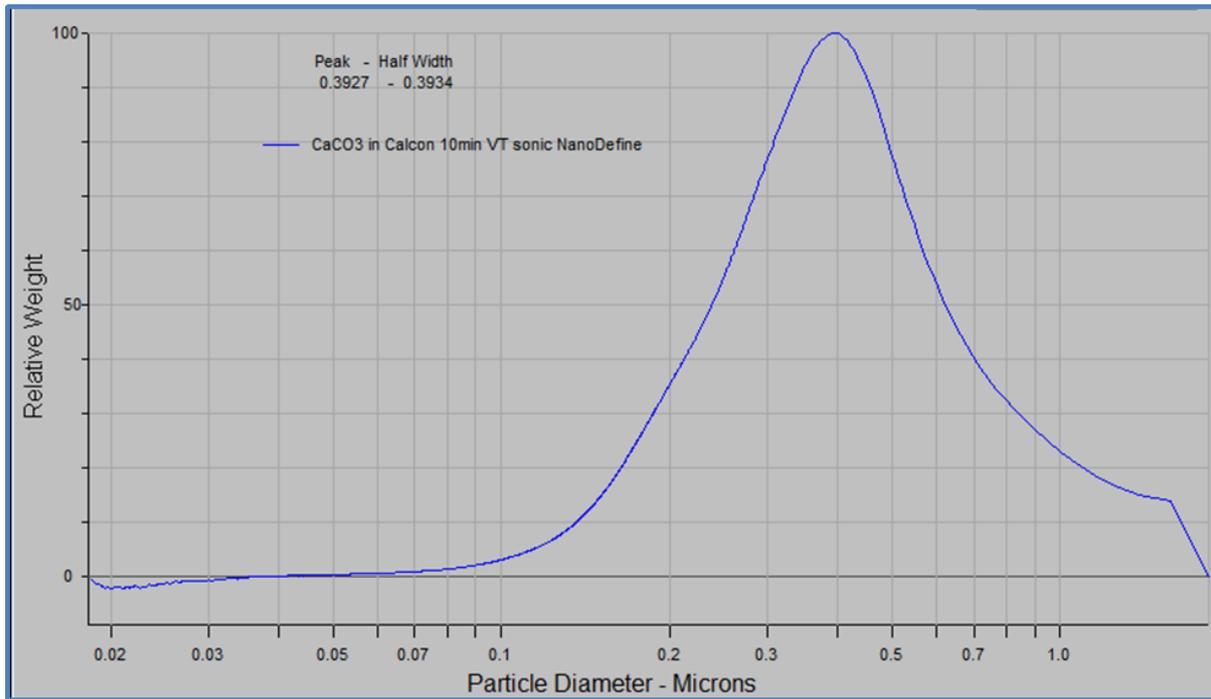


Figure 4: Typical particle size distribution of dispersed CaCO₃ determined by CLS

7 SOP for production of an aqueous based dispersion of IRMM-385 (Kaolin)

7.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a stable water-based dispersion of IRMM-385 starting from dry powder form.

7.2 Scope

This scope of the Standard Operating Procedure (SOP) is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based dispersion of Kaolin (NanoDefine designation IRMM-385). The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution does not significantly change over a time period of at least 30 minutes from completion of the dispersion procedure.

7.3 Abbreviations

EM	Electron Microscopy
NM	Nanomaterial
PSD	Particle Size Distribution
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
USP	Ultrasonic probe sonicator
VM	Vortex mixer

7.4 Description

The following SOP describes a method for the preparation of small volumes (10 mL) of an aqueous dispersion of IRMM-385, Kaolin, designated at a concentration of 2.56 mgmL⁻¹. The procedure foresees starting from a dry powder sample of IRMM-385 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used, and using this data, adjust the power settings of the sonicator to produce an output value which is specified in the procedure.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

The stability of the dispersions after sonication is evaluated visually immediately after sonication (pristine), and again after a rest period of 10, 20 and 30 minutes (aged). The particles stay in

dispersion and precipitation is not observed. Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation.

The dispersion efficiency is evaluated based on the particle size distribution determined by TEM.

7.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 20 kHz and equipped with a probe with a tip diameter of approximately 13 mm (e.g. Vibracell 75041 ultrasonifier, Fisher Bioblock Scientific, Aalst, Belgium)
- Adjustable volume pipettes of 100 μ L with disposable tip
- Pipettes of 10 mL
- Box for ice-cooling of samples during sonication
- Vortex mixer

7.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

7.4.3 Material Supplies

- 20 mL glass vial (e.g. 10560503-X500, Wheaton Science Products, Millville, New Jersey, distributed by Fisher Scientific) with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs
- Parafilm M
- Flask rings (e.g. Heathrow scientific lead rings)
- Vial holder

7.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 $M\Omega\text{cm}^{-1}$, 0.2 μm in-line filtration)
- Kaolin distributed by IRMM with project ID no. IRMM-385
- Ice-water mixture for cooling the sample during sonication

7.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures.

For laboratories equipped with a TEM additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

7.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was a Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium). This sonicator is fitted with a probe head with a diameter of 13 mm diameter. The power characteristics of this sonicator probe have been experimentally determined. The resulting calibration curves can be seen in Figure 1. The output energy in function of the sonication time and the output power in function of the selected amplitude are shown in Figure 1A and Figure 1B, respectively.

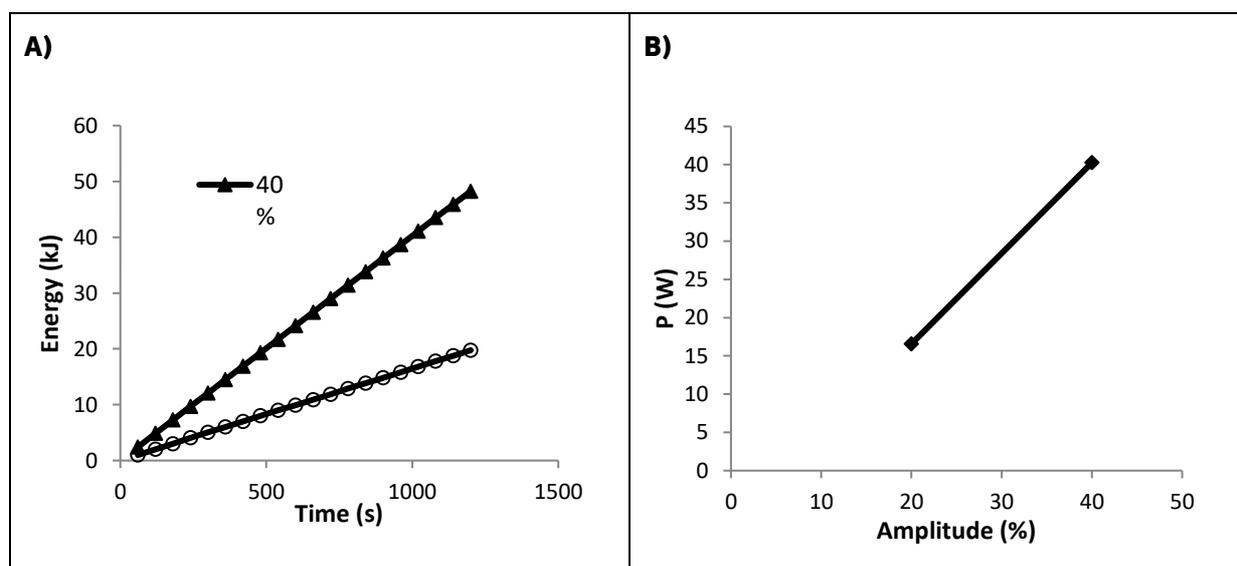


Figure 1: Calibration curves for the probe sonicator Vibracell 75041 ultrasonifier (750 W, 20 kHz, Fisher Bioblock Scientific, Aalst, Belgium) fitted with a probe head with a diameter of 13 mm, showing (A) the output energy in function of the sonication time and (B) the output power in function of the selected amplitude

In this procedure for the dispersion of IRMM-385, the 13 mm probe head (CV33) is positioned in the bottom half of the dispersion and the sonicator is operated at a set amplitude value of 40%. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 40 W. Sonication is stopped after 10 minutes, when an added specific energy of 25 ± 2 kJ is read out from the sonicator apparatus.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determines a similar calibration curve for their own instrument. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine the correct amplitude setting required to produce an output of 40 W. The amplitude setting of the sonicator should be adjusted to this value before proceeding with the dispersion procedure.

7.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 7.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

7.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh approximately 25.6mg of IRMM-385 into a 20 mL glass vial and add 10 mL of pure water to give a concentration of 2.56 mgmL⁻¹. It is recommended that an Ionizer is used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Wrap parafilm M around the 20 mL glass vial containing the dispersion to avoid movement during sonication, and place the vial in the vial holder (Figure 2A). Place the vial holder in the box for ice-cooling using the flask rings (Figure 2B). Add a mixture of crushed ice and water in the box to cool the dispersion during sonication. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. Mount the probe sonicator head inside the vial (Figure 2C). The probe head should be immersed in the dispersion to a depth of at least 1 cm. The sample should then be sonicated at a constant power of 40 W for 10 minutes. The correct power setting should be determined from the calibration curve. The resulting solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (20 %) for 5 minutes in high purity ethanol and then 5 minutes in water before being dried with a flow of clean compressed nitrogen or air.

As can be seen in Figure 2A the 20 mL glass vial containing the dispersion is placed in the vial holder. Parafilm M is wrapped around the vial to avoid sliding during sonication. The vial and vial holder are placed in the box for ice-cooling using the flask rings (Figure 2B). A mixture of crushed ice and water is added in the box to cool the dispersion during sonication. The vial is fully immersed in the cooling water (Figure 2C). The probe sonicator head is immersed in the dispersion to a depth of at least 1 cm.

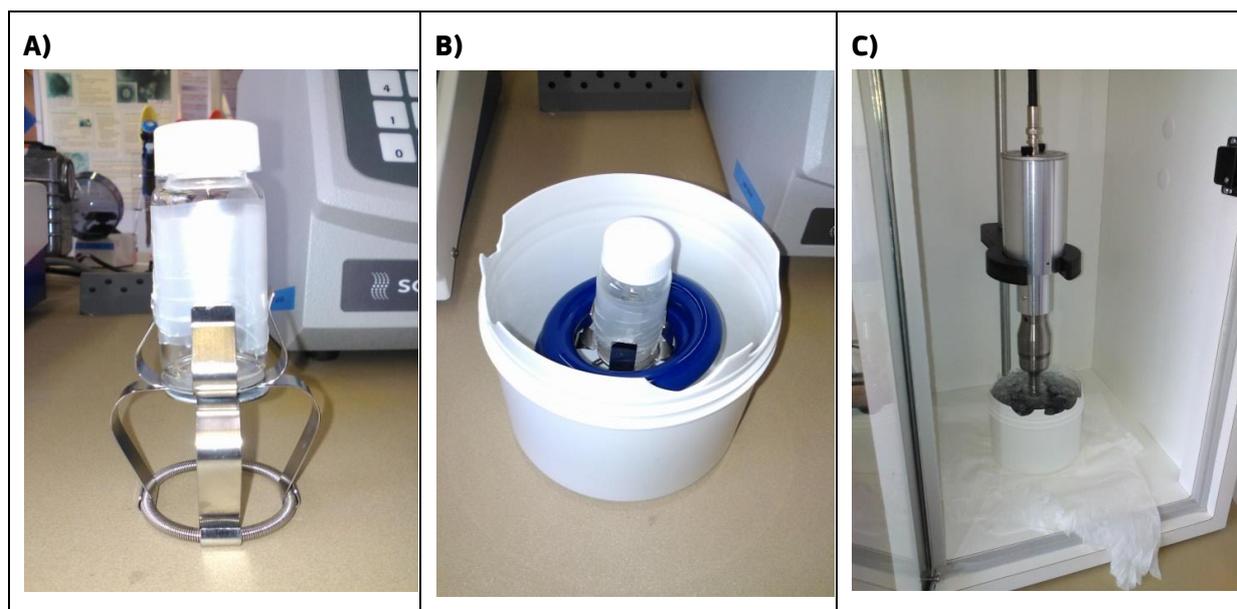


Figure 2: Setup for sonication using the probe sonicator

7.4.6 Optional verification of dispersion quality

Where the operator has access to a TEM instrument, it is strongly recommended that the dispersion be evaluated by TEM and the results compared with that in section 7.7.

To become suitable for TEM analysis, the dispersion has to be diluted 10 times after sonication to obtain a concentration of 0.256 mgmL^{-1} . TEM specimens can be prepared following the grid-on-drop method¹. This method includes pre-treating pioloform and carbon coated, 400 mesh copper grids (Agar Scientific, Essex, England) with 1 % Alcian blue (Fluka, Buchs, Switzerland) to increase hydrophilicity and rinsing 5 times with distilled water. The grid is then placed on 15 μL of dispersion during 10 minutes, and is rinsed 2 times afterwards with distilled water.

TEM evaluation of the dispersion quality shows the smallest dispersible particles (referred to as single constituent particles) and some small agglomerates thereof (consisting of 2-10 particles) (Figure 3 and Figure 4). If the expected mean aggregate/agglomerate size is significantly larger (>15 %) than that shown in section 7.7 of this SOP, the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

7.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepared in previous steps has been verified visually, and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in section 7.4.5. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur.

7.5 Validation status

This method has not yet been subjected to validation

7.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

7.7 Information on expected particle size distribution

The qualitative TEM analysis describes the physico-chemical characteristics of the particles, such as the aggregation/agglomeration state, and the size and shape of the free single particulates, aggregates and agglomerates. Table 1 summarizes the qualitative TEM analysis of IRMM-385.

Quantitative TEM analysis is performed using methods described by Verleysen et al. and De Temmerman et al.¹⁻³ shows representative TEM images of IRMM-385. The corresponding size distribution is shown in Figure 4 and is determined by a semi-automated approach using imageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). This approach can be briefly summarized as follows:

- To suppress background noise, a mean filter is applied before analysis. The use of other filters was not necessary for the examined material.
- A threshold for the detection of the particles based on mass-thickness contrast in the image is chosen manually.
- Particles are only detected in a pre-defined Region of Interest (ROI), which allows excluding border particles.
- For every micrograph, the 'Fill holes' option is switched on.

Table 1: Summary of the qualitative TEM analysis of IRMM-385 Kaolin

Examined property	Description
Distribution of particles on the grid?	evenly distributed
Concentration of particles on the grid?	OK
Aggregation/agglomeration state?	single constituent particles, aggregates and agglomerates
Sub-fraction	no sub-fraction of smaller particles/contaminants is present in the sample
Manually measured size of the constituent particles?	the size ranges from 25 nm to 750 nm
Manually measured size of the aggregates/agglomerates?	the size ranges from 100 nm to 7.5 μ m
2D shape of the PP?	irregular polygonal
2D shape of the aggregates/agglomerates?	fractal-like or more complex
Surface structure of the constituent particles?	rough
Surface structure of the aggregates/agglomerates?	rough
Diffraction contrast?	diffraction contrast, which indicates that the material is crystalline, can be observed in the constituent particles
Efficiency of the dispersion protocol?	allows dispersing the material up to the level of single constituent particle and some small aggregates/agglomerates (consisting of 2-10 constituent particles)

Descriptive statistical analysis of the Feret min of the particles is obtained using a home-made script in the python programming language. The raw data is represented as a histogram ('Number based distribution') (Figure 4A). A log-normal curve is fitted iteratively the scatter plot (Figure 4B). It should be noted that TEM is probably not able to measure the smallest dimension (platelet thickness) of all particles due to preferential orientation of platelet-like particles on the grid.

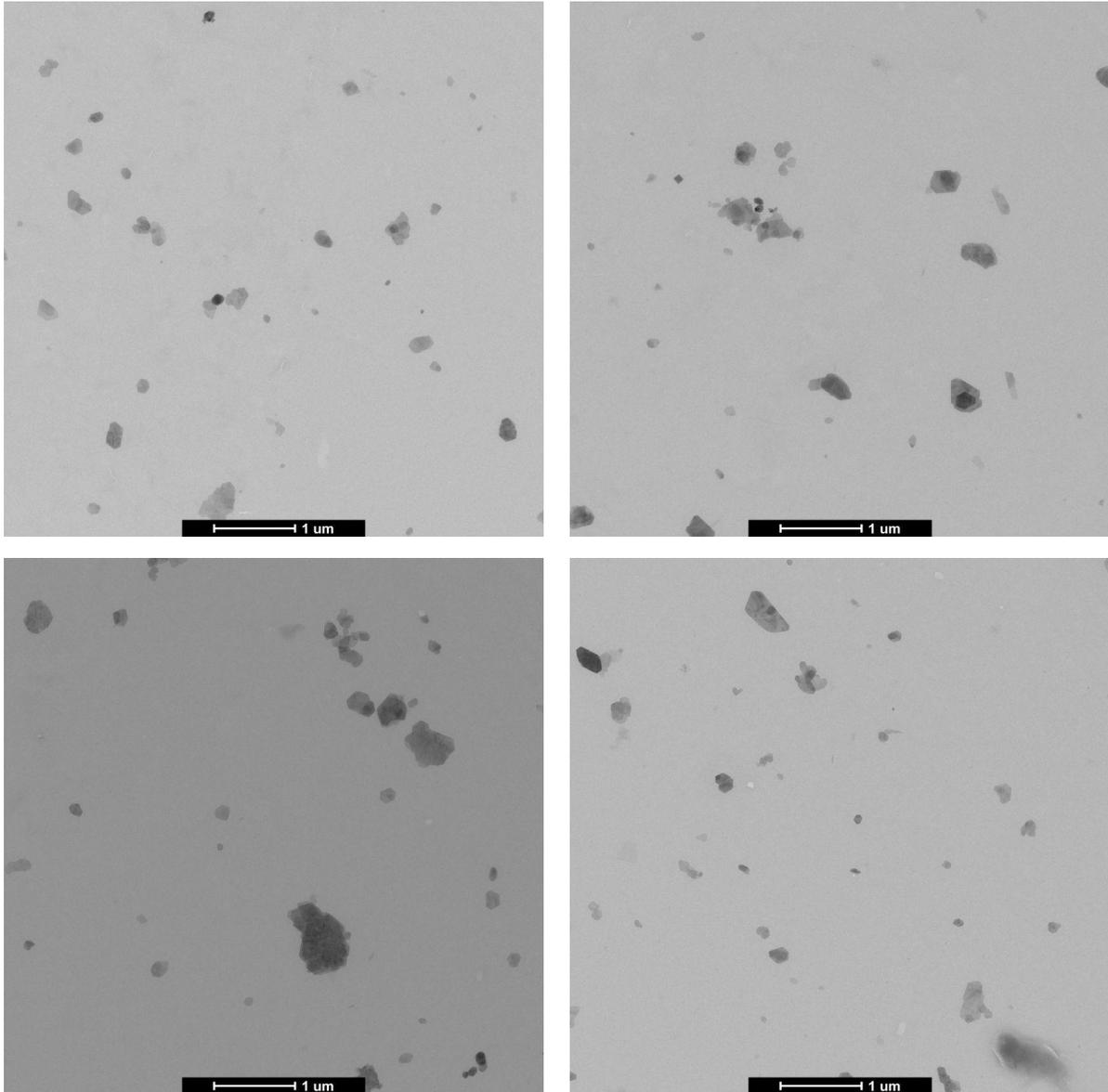
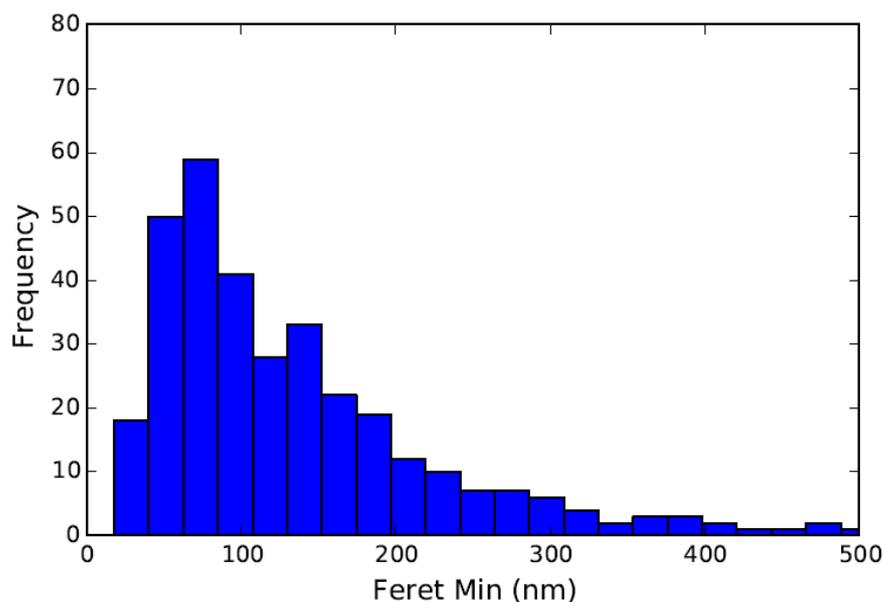
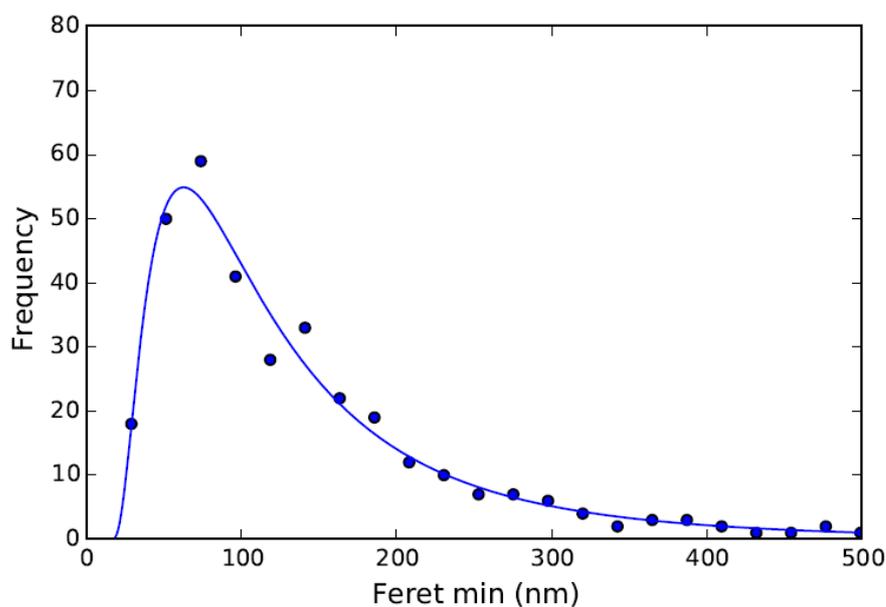


Figure 3: Representative TEM images of the dispersed particles of IRMM-385 Kaolin



A



B

Figure 4: Size distribution (Feret min) of the single particulates of IRMM-385 Kaolin obtained by quantitative TEM analysis represented by a histogram in the upper panel and in the lower panel by a scatter plot with a fitted log-normal function

7.8 References

¹ E. Verleysen, E. Van Doren, N. Waegeneers, P.-J. De Temmerman, M. Abi Daoud Francisco, J. Mast, TEM and SP-ICP-MS Analysis of the Release of Silver Nanoparticles from Decoration of Pastry. *Journal of agricultural and food chemistry*, 63(13), 3570-3578 (2015).

² E. Verleysen, P.-J. De Temmerman, E. Van Doren, M. Abi Daoud Francisco, J. Mast, Quantitative characterization of aggregated and agglomerated titanium dioxide nanomaterials by transmission electron microscopy. *Powder Technology* 258, 180-188 (2014).

³ P.-J. De Temmerman, E. Van Doren, E. Verleysen, Y. Van der Stede, M. Francisco, J. Mast, Quantitative characterization of agglomerates and aggregates of pyrogenic and precipitated amorphous silica nanomaterials by transmission electron microscopy, *Journal of Nanobiotechnology*, 10 (2012).

8 SOP for production of an aqueous based dispersion IRMM-386 (Opaque Pigment Yellow 83)

8.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-386 starting from dry powder form.

8.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-386 opaque Pigment Yellow 83. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

8.3 Abbreviations

CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
MALS	Multi-angle Light Scattering
MeOH	Methanol
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No. 25638-17-9)
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

8.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2 mL or 6 mL) of a surfactant stabilised aqueous suspension (0.1 mg mL^{-1} of IRMM-386) of IRMM-386, Pigment Yellow 83 (Opaque grade). The procedure foresees starting from a dry powder sample of IRMM-386 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a low concentration of the commercial surfactant NEKAL BX. The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and amplitude values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

8.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 3mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 μL , 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

8.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

8.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

8.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity $18.2 \text{ M}\Omega\text{cm}^{-1}$, $0.2 \mu\text{m}$ in-line filtration)
- Pigment Yellow 83 opaque grade distributed by IRMM with project ID no. IRMM-386
- High purity methanol (analytical grade)
- Ice-water mixture for cooling the sample during sonication.
- Surfactant: 30 wt% aqueous solution of NEKAL-BX (Sodium Butyl naphthalene sulphonate (CAS No. 25638-17-9)

8.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

8.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 WmL^{-1} for 2 mL sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was

Hielscher UPS200S instrument whose power output characteristics were determined as described in chapter 13 and the results are shown in Figure 1.

In this procedure for dispersion of IRMM-386 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude and cycle time settings are required to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to approximate these values before proceeding with the dispersion procedure.

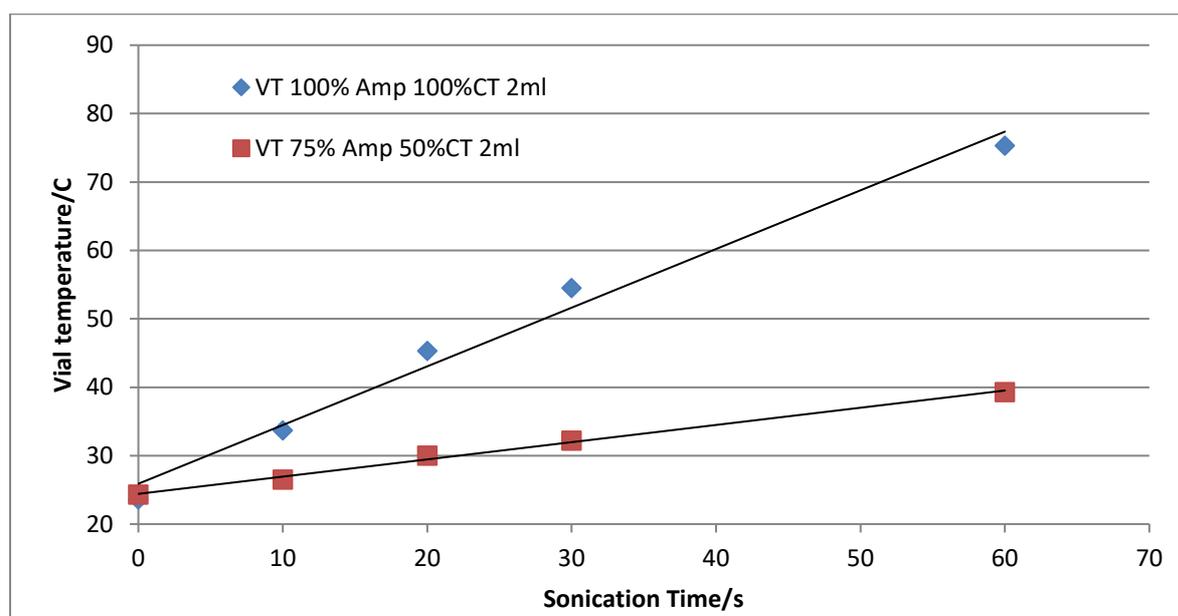


Figure 1: Temperature increase of 2 mL water in vial sonicator at (a) 100 % Amplitude and 100 % cycle-time and (b) 75 % amplitude and 50 % cycle time. Specific power absorbed is (a) 3.8 WmL^{-1} and (b) 1.1 WmL^{-1} respectively

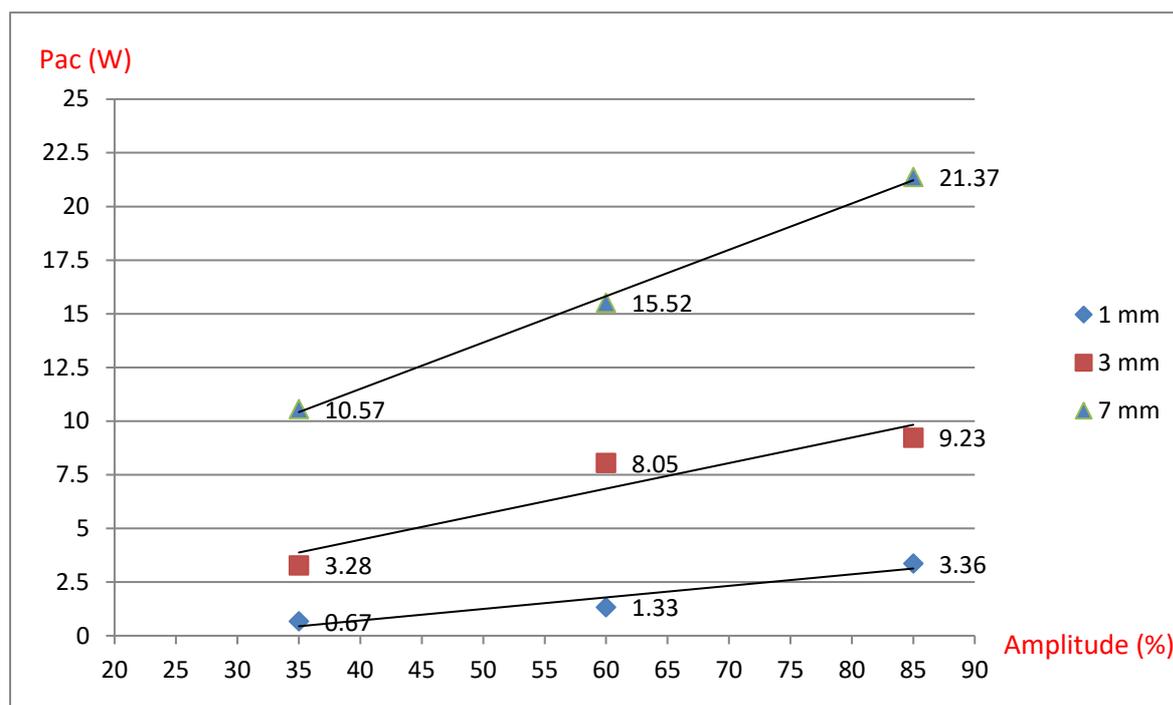


Figure 2: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time Acoustic power absorbed when using different probe diameters and amplitude settings

8.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 7). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

8.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh an empty 22 mL glass vial and add approximately 15 μL of Nekal BX solution (30 wt%) using a pipette. Reweigh the vial and calculate by difference the amount of NEKAL BX before adding sufficient pure methanol to give a concentration of 0.5 mgmL^{-1} . This solution will hereafter be referred to as solution A.

Weigh approximately 10 mg of IRMM-386 into a 22 mL glass vial and add sufficient pure methanol to give a concentration of 1 mgmL^{-1} . It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing

(2') and then sonicating in USB (2'): Add solution A to solution B in a ratio of 10 μmL^{-1} . Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (2'): This will hereafter be referred to as solution B.

Prepare a heated water bath under a chemical safety hood and heat to 40-50 °C. Suspend the lower half of vial in the water bath until the MeOH evaporates leaving a layer of surfactant coated particles on the bottom of the vial. Add sufficient MilliQ water to get 10 mgmL^{-1} solids in water and seal the vial with a suitable lid. Re-disperse the solids into the water by immersing the bottom half of the vial in a USB and sonicating for 2 minutes or until the solids appear uniformly distributed in the water. This will hereafter be referred to as solution C.

Take an empty 22 mL vial and add 5.94 mL of pure water followed by 60 μL of solution C to give a final concentration of 0.1 mgmL^{-1} IRMM-386 in water. This will hereafter be referred to as solution D.

Sonication using probe sonicator: Take the 22 mL glass vial containing solution D and mount the probe sonicator head inside the vial as shown in Figure 3. The probe head should be immersed in the solution to a depth of approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is fully immersed in the cooling water. In this procedure the sonicator used was a Hielscher UPS200S and this was operated in pulsed mode with an amplitude of 75 % and a cycle time of 50 % which corresponds to a peak power of 18 W, mean adsorbed power of 7.8 W (50 % cycle time) and 1.3 WmL^{-1} when normalised to the specified volume of 6 mL. A sonication time of 20 minutes was determined to be the optimum treatment time for this material under the described conditions.



Figure 3: Photograph showing recommended positioning of probe sonicator in sample

When attempting to use a probe sonicator which is different from the model used in the development of this method users must firstly determine the power output characteristic of their

own instrument using the method described in chapter 13. From this data, instrument settings should be determined which can approximate the power output values detailed above.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

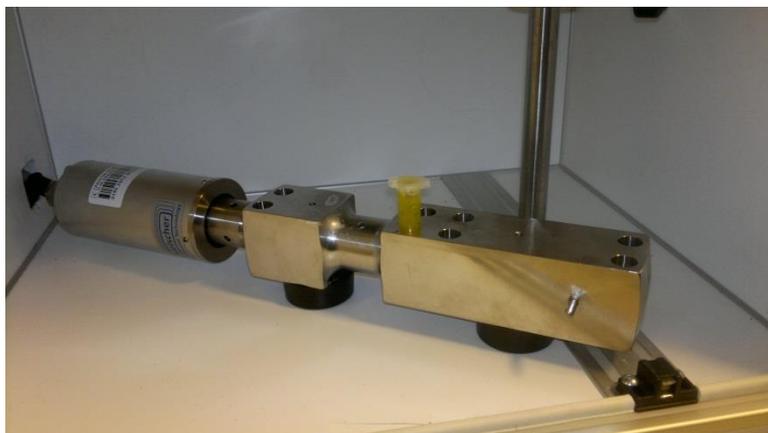


Figure 4: Positioning of sample in vial-sonicator

Sonication using vial sonicator: Place 2 mL of solution D in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 4). The vial should be sonicated for 15 min at 75 % amplitude with the cycle time being set at 50 %. As cooling cannot be applied in the case of vial sonicator the use of a 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

8.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 8.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in chapter 13 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

8.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepared in previous steps have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in the previous

section 8.4.5. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-386, material aging of up to 6 days may be fully reversed if the sample vial is vortexed for 2 minutes and the treated for 10 minutes in a laboratory scale bath sonicator at room temperature. (See Figure 5).

8.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

8.5 Validation status

This method has not yet been subjected to validation

8.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

8.7 Information on expected particle size distribution

Figure 5 shows a typical mass based particle size distribution of the (vial sonicator) as-dispersed IRMM-386 Pigment Yellow 83 (opaque grade) together with particle size distribution of the same material after 24 hours ageing and re-dispersion by 10 minutes USB. Table 1 lists typical values of mean particle size obtained using the probe and vial sonicator methods.

Table 1: Mean particle size of dispersed IRMM-386 by CLS (weight-size distribution)

	Sonication Method	
Mean Particle Size in nm of IRMM-386 by CLS (weight-size distribution)	USP 7 mm 15'	VS 15'
	326	240

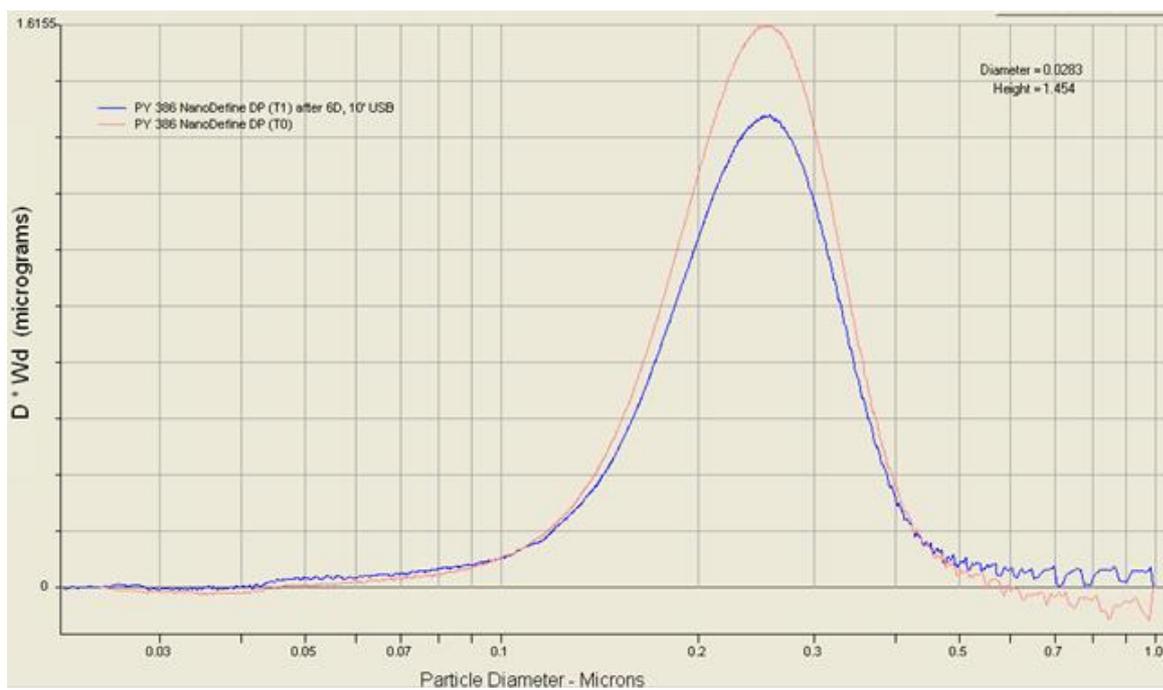


Figure 5: CLS determined particle size distribution analysis of IRMM-386 Pigment Yellow 83 (opaque grade) as-dispersed (T0) and after 24 h ageing followed by 10 min bath sonication

9 SOP for production of an aqueous based dispersion of IRMM-387 (BaSO₄ (ultrafine grade))

9.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloidally stable water-based dispersion of IRMM-387 starting from dry powder form.

9.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-387, ultrafine grade BaSO₄. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS, does not significantly change (according to DLS measurements) over a time period of at least 60 minutes from completion of the dispersion procedure.

9.3 Abbreviations

DLS	Dynamic Light Scattering
MALS	Multi-angle Light Scattering
NM	Nanomaterial
PDI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS)
PSD	Particle Size Distribution
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

9.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of an aqueous suspension (2.6 mgmL⁻¹) of IRMM-387, BaSO₄ (ultrafine grade). The procedure foresees starting from a dry powder sample of IRMM-387 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing the commercial stabilising agent sodium hexametaphosphate (SHMP). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using a probe sonicator and is suited to the production of a batch volume of 6 mL.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particle size values while longer treatment times will either degrade the quality (re-formation of larger aggregates/agglomerates) or will not provide a significant further reduction in the mean size. Similarly, the amount of SHMP used for dispersion has a considerable influence on the quality of the dispersion and obtained particle size distribution. This procedure has been developed to produce the lowest mean particle size distribution for dispersion in 2 mgmL⁻¹ SHMP. The use of lower concentrations of SHMP will lead to IRMM-387 dispersions with larger mean particle size values.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 60 minutes (aged) with the results showing no major variation in the means size distribution.

9.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7mm. The sonicator should have nominal power output of at least 100 W
- Adjustable volume pipettes of 100 µL, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

9.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

9.4.3 Material Supplies

- 20 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

9.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm⁻¹, 0.2 µm in-line filtration)
- BaSO₄ (ultrafine grade) distributed by IRMM with project ID no. IRMM-387
- Ice-water mixture for cooling the sample during sonication
- Sodium hexametaphosphate powder (CAS No. 68915-31-1, purity ≥ 96 %, e.g. 305553 Aldrich)

9.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

9.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was Microson XL 2000 (Qsonica, LLC (Newtown, USA) with nominal maximum power of 100 W.

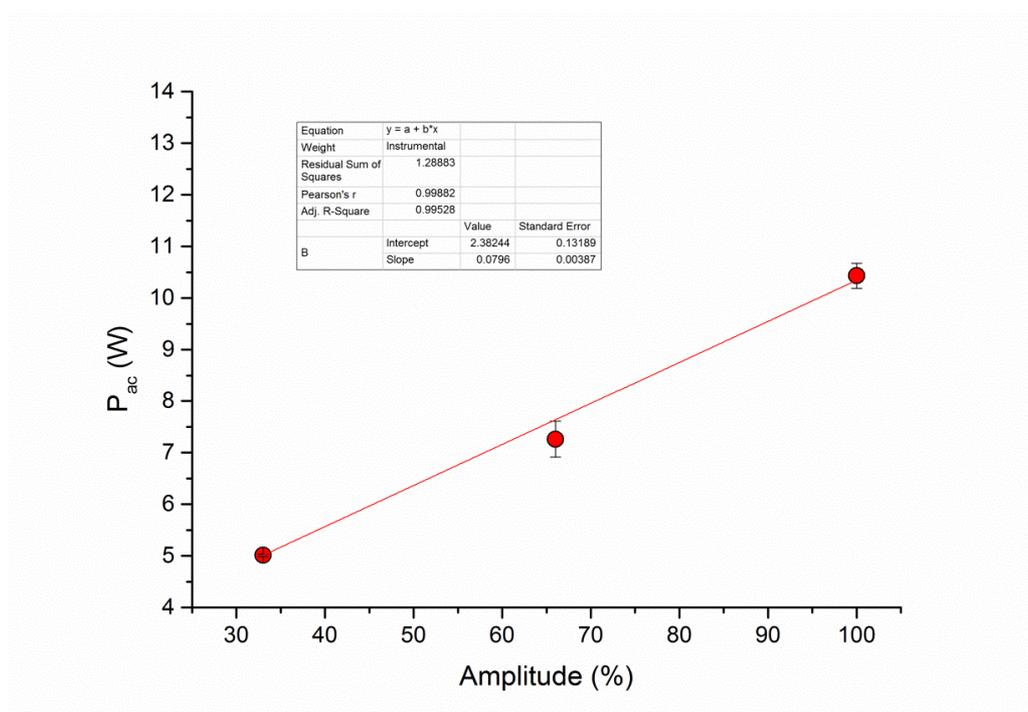


Figure 1: Calculated delivered output power P_{ac} of the probe sonicator at different amplitude settings. This calibration curve was used to determine the output setting value which corresponds to $P_{ac} = 7.6$ W (in this example: amplitude of 66 %)

The sonicator was fitted with a probe head with diameters of 6.4 mm (length of 117 mm and maximum peak-to-peak amplitude of 60 μm). The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curve can be seen in Figure 1. In this procedure for the dispersion of IRMM-387 the sonicator was operated at a set amplitude value of 66 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 7.6 W. Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-

power curve must be done in order to determine what is correct amplitude setting required to produce an output of 7.6 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

9.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 9.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

9.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Prepare stabilising agent solution (2 mgmL⁻¹ SHMP) by dissolving the appropriate amount of SHMP powder into MilliQ water. Shake vigorously to ensure that all powder is solubilized. Subsequently, filter the prepared SHMP solution using a 0.2 µm filter to ensure that no large particulates are present.

Weigh approximately 15.6 mg of IRMM-387 into a 20 mL glass vial. It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Add the respective volume of SHMP solution to give an IRMM-387 concentration of 2.6 mgmL⁻¹ (6 mL for exactly 15.6 mg of IRMM-387, adjust volume to compensate for small deviations in the final weighed mass). Homogenize the mixture by vortexing (2').

Sonication using probe sonicator: Take the 20 mL glass vial containing the 2.6 mgmL⁻¹ IRMM-387 suspension and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial in the vial is full immersed in the cooling water. The sample should then be sonicated at a constant power 7.6 W for 5 minutes. The correct power setting should be determine from calibration curve which was previously determined by the method described in chapter 13. The resulting dispersion should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.



Figure 2: Ultrasonic probe sonication setup for dispersion of NM powders

9.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 9.8.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 9.8 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

9.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of IRMM-387–BaSO₄ dispersions prepared by this method have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 1 h following completion of the primary dispersion procedure outlined in section 9.4.5. At this time no further information is available of the re-dispersability of the solution after ageing for longer time periods.

9.5 Reporting of results

The main objective of the procedure does not foresee any specific metrological step. Consequently it has not been deemed necessary to detail any step relating to the reporting of results.

9.6 Validation status

This method has not yet been subjected to validation

9.7 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

9.8 Information on expected particle size distribution

The DLS results obtained for IRMM-387 dispersions prepared according to procedure described in section 9.4 are summarized in Table 1, Figure 3, Figure 4, Table 2 and Figure 5. The data shown includes the whole set of results obtained at different sonication times with fixed amplitude settings (22.5 kHz probe sonicator, 66 % max. amplitude, 6.4 mm probe) and is reported herein so that the end-user of this SOP can evaluate his own results. The increase in sonication time leads to a progressive decrease in mean particle size (Zave and Peak1 mean values, Table 1, Figure 3, Table 2 and Figure 5). As described in the procedure in section 9.4.5, the optimized time of sonication was found to be 5 min. This sonication time was the minimum time required in order to obtain a stable IRMM-387 dispersion (no sedimentation). The use of treatment times longer than 5 min may result in even lower mean particle size values, but is not advised since the PDI was observed to increase with the extent of treatment (suggesting that the suspension becomes more polydisperse).

Table 1: Summary of the DLS results obtained for IRMM-387 – BaSO₄ (ultrafine grade) suspensions in MilliQ water and 2 mgmL⁻¹ hexametaphosphate (N=5) prepared at different probe sonication times

Sonication Time	0 min	1 min	2 min	3 min	5 min	8 min	16 min
Intensity-weighted mean diameter: by DLS (Z _{ave} , cumulants method)	127.8 ± 0.2	127.5 ± 0.7	125.9 ± 0.9	125.0 ± 1.0	124.3 ± 1.2	122.7 ± 0.6	118.8 ± 1.0
Polydispersity index by DLS (PDI, cumulants method)	0.135 ± 0.006	0.133 ± 0.002	0.126 ± 0.012	0.132 ± 0.008	0.136 ± 0.006	0.151 ± 0.006	0.161 ± 0.003

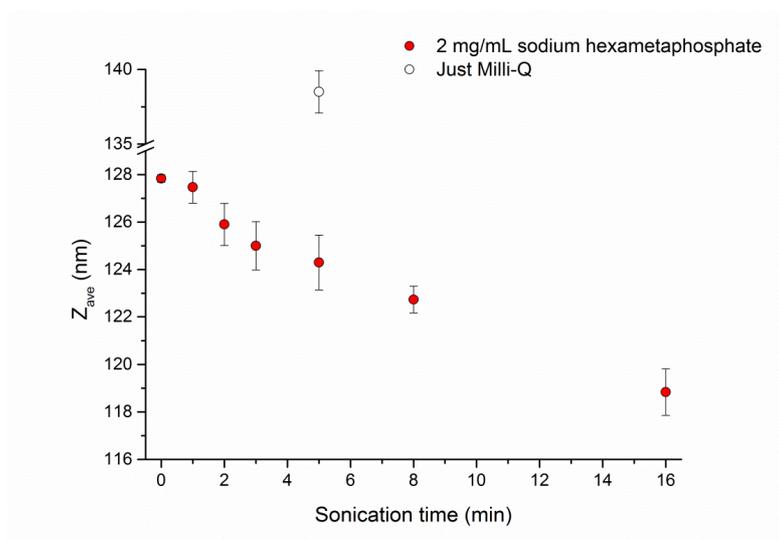


Figure 3: Z_{ave} values obtained by DLS for IRMM-387 – BaSO₄ (ultrafine grade) suspensions in MilliQ water (N=2) and 2mgmL⁻¹ hexametaphosphate (N=3) prepared at different probe sonication times

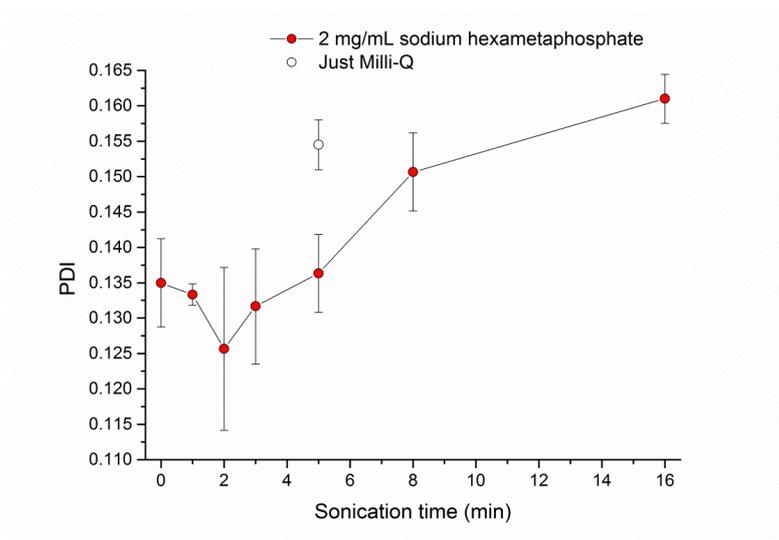


Figure 4: PDI values obtained by DLS for IRMM-387 – BaSO₄ (ultrafine grade) suspensions in MilliQ water (N=2) and 2mgmL⁻¹ hexametaphosphate (N=3) prepared at different probe sonication times

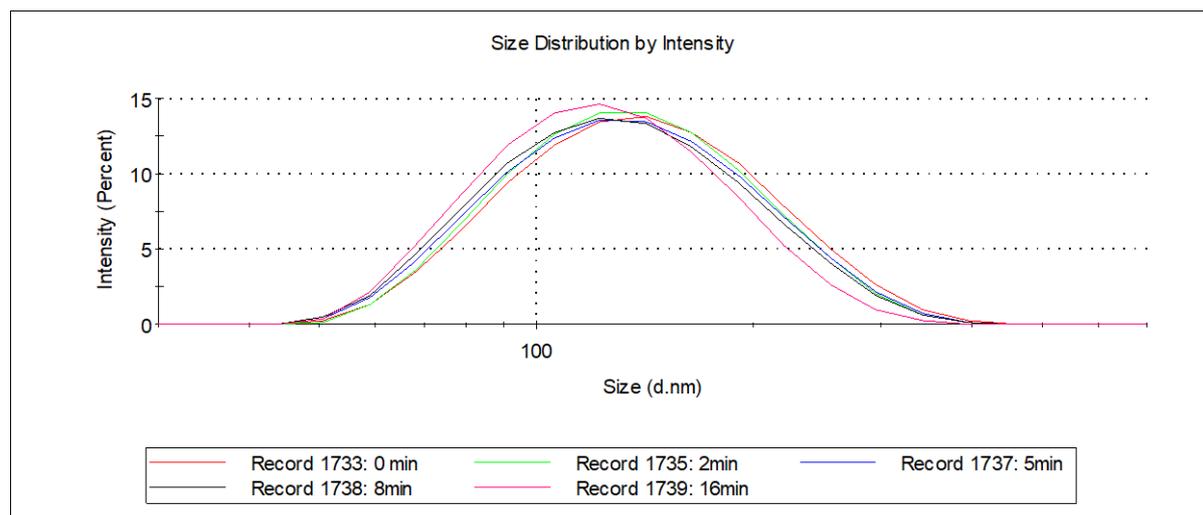


Figure 5: Intensity-weighted size distribution obtained by DLS for IRMM-387 – BaSO₄ (ultrafine grade) suspensions in 2 mgmL⁻¹ hexametaphosphate (N=3) prepared at different probe sonication times

Table: 2 Mean diameter corresponding to the major peak of the intensity-weighted size distribution obtained by the NLS method (DLS) for dispersed IRMM-387– BaSO₄ (ultrafine grade)

Sonication Time	0 min	1 min	2 min	3 min	5 min	8 min	16 min
Peak mean	148.6 ± 1.7	146.8 ± 2.0	144.3 ± 2.6	143.5 ± 2.1	143.5 ± 2.4	140.3 ± 0.7	131.8 ± 2.5

10 SOP for production of an aqueous based dispersion of IRMM-388 (Coated TiO₂)

10.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a colloiddally stable water-based dispersion of IRMM-388 starting from dry powder form.

10.2 Scope

The scope of this SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant-stabilised, water-based colloidal suspension of IRMM-388, Titanium Dioxide TiO₂. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are suitable for use with the particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by DLS or CLS, does not significantly change (according to DLS or CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

10.3 Abbreviations

CLS	Centrifugal Liquid Sedimentation
DLS	Dynamic Light Scattering
EM	Electron Microscopy
NaDS	Sodium Dodecyl Sulphate
NEKAL-BX	Commercial Surfactant (Sodium Butyl Naphthalene Sulphonate (CAS No.25638-17-9))
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS)
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SHMP	Sodium hexametaphosphate (Calgon)
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy in Transmission Mode
TSPP	Tetra-sodium pyrophosphate
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator
VM	Vortex mixer
VS	Vial sonicator

10.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (2 mL or 6 mL) of a surfactant stabilised aqueous suspension (0.1 mgmL⁻¹ of IRMM-388) of IRMM-388, Titanium Dioxide (TiO₂). The procedure foresees starting from a dry powder sample

of IRMM-388 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water containing a the commercial surfactant Sodium hexametaphosphate (Calgon). The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjusting the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator. When this procedure is conducted using a probe sonicator the batch volume which is produced is 6 mL while the alternative method using a vial sonicator permits the production of 2 mL batches. The particle size distributions of the two methods have been evaluated by CLS and found to be comparable.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<1 hour). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 10 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 6 days.

10.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 μ L, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication
- Vortex mixer

10.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. DLS or CLS or MALS)

10.4.3 Material Supplies

- 22 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- 2 mL plastic microcentrifuge tubes with sealing lid (for use with vial-sonicator)
- Disposable plastic spatula for weighing of NM and any chemicals in powder form
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

10.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity $18.2 \text{ M}\Omega\text{cm}^{-1}$, $0.2 \mu\text{m}$ in-line filtration)
- Titanium Dioxide (TiO₂) distributed by IRMM with project ID no. IRMM-388
- Ice-water mixture for cooling the sample during sonication
- Surfactant: aqueous solution of Sodium hexametaphosphate (Calgon) (CAS No. 10124-56-8)

10.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with DLS or CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

10.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the primary method of applying ultrasonic energy was a vial sonicator. This system was operated with 2 mL Eppendorf vials containing 2 mL of the sample dispersion. In all cases the instrument was operated at 75 % amplitude and 50 % cycle time. To estimate the power absorbed a 2 mL aqueous sample was sonicated under these conditions and the temporal variation of the liquid temperature measured and used to determine the absorbed power as described in chapter 13. Under these conditions the mean power absorbed was 2.1 W corresponding to a specific power absorbed of 1.1 WmL^{-1} for 2 mL sample. In this case the power value is likely to be an underestimate as the experimental conditions would lead to higher thermal dispersion than in the standard calorimetric method with 500 mL of water.

To ensure that the method could be adopted by the other laboratories sonicator conditions based on a conventional probe sonicator system were also determined. In this case the system used was Hielscher UPS200S instrument whose power output characteristics were determined as described in chapter 13 and the results are shown in Figure 1.

In this procedure for dispersion of IRMM-384 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 % and a cycle time of 50 %. From a similarly prepared calorimetric calibration curve it was determined that under these operating conditions the

instrument was producing a mean total power output of 7.8 W which corresponds to 1.3 WmL^{-1} when treating a 6 mL sample. At 100 % cycle time and 75 % amplitude the peak power output was determined (chapter 13) to be 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator it is necessary that the operator determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what are the correct amplitude and cycle time settings required to produce peak and mean power outputs (50 % cycle time) of 18 W and 7.8 W respectively. The amplitude and cycle time settings of the sonicator should then be adjusted to approximate these values before proceeding with the dispersion procedure.

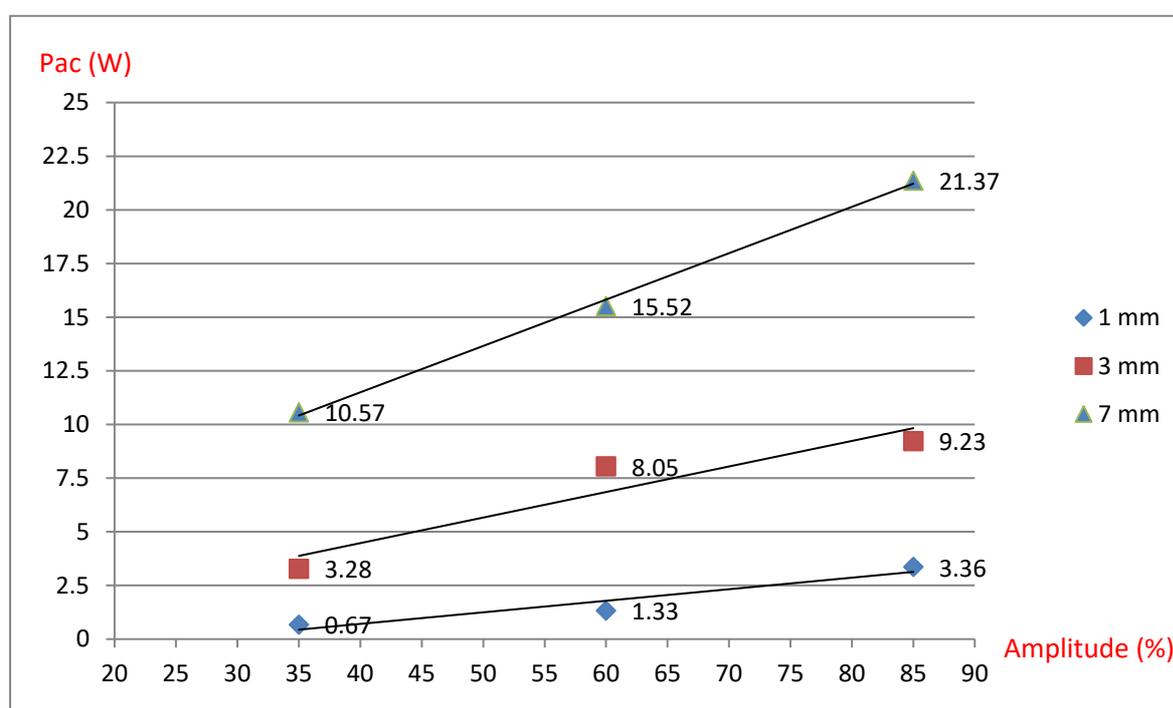


Figure 1: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time

10.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 10.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

10.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh an empty 22 mL glass vial and add approximately 20 mg of Sodium Hexametaphosphate (SHMP) using a spatula. Reweigh the vial and calculate by difference amount of SHMP before adding sufficient ultrahigh purity water to give a concentration of 2 mgmL^{-1} and vortex for 2 minutes. This solution will hereafter be referred to as solution A.

Weigh approximately 10 mg of IRMM-388 into a 22 mL glass vial and add sufficient solution A to give a concentration of 1 mgmL^{-1} . It is recommended that an Ionizer be used to neutralize electrostatic charge during weighing of fine powders. Homogenize the mixture by firstly vortexing (2') and then sonicating in USB (10'). This will hereafter be referred to as solution B.

Take an empty 22 mL vial and add 5.94 mL of pure water followed by 60 μL of solution B to give a final concentration of 0.1 mgmL^{-1} IRMM-388 in water. This will hereafter be referred to as solution C (6 mL Volume).

Sonication using probe sonicator: Take the 22 mL glass vial containing solution C and mount the probe sonicator head inside the vial as shown in Figure 2.



Figure 2: Photograph illustrating the mounting of probe sonicator in sample

The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is fully immersed in the cooling water. If using Hielscher UPS200S with 7 mm probe the sample should then be sonicated at an amplitude of 75 % and 50 % cycle for 20 minutes.

The correct sonication settings should be determined from the calibration curves determined by the method described in chapter 13. The resulting solution should now be suitable for testing.

If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low amplitude (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.

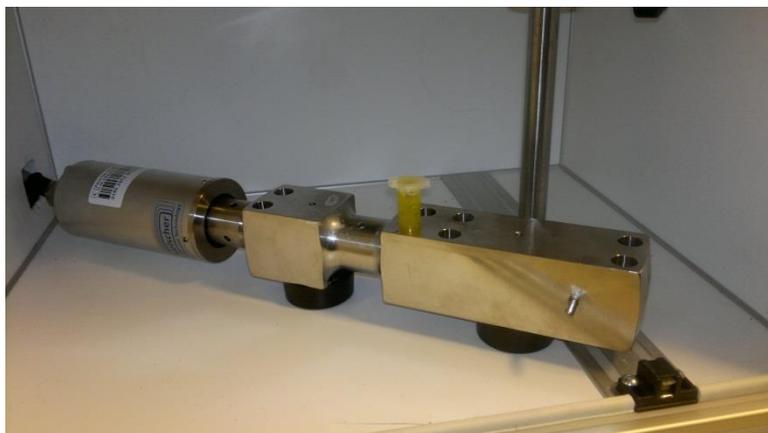


Figure 3: Positioning of sample in vial-sonicator

Alternative to step 5: Sonication using vial sonicator: Place 2 mL of solution C in a 2 mL plastic centrifuge tube and close the plastic lid. The tube should be fitted in one of the sample holder positions with highest energy input (see manufacturer's instructions and Figure 3). The vial should be sonicated for 15 min at 75 % amplitude with the cycle time set at 50 %. Cooling cannot be applied in the case of vial sonicator and the use of 50 % cycle time serves to maintain the maximum temperature of the sample below 50 °C.

10.4.6 Optional verification of dispersion quality

Where the operator has access to a DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that noted in section 10.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>20 %) than that shown in section 10.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

10.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepared in previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in section 10.4.5. In

the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-388, material aging of up to 6 days may be fully reversed if the sample vial is vortexed for 2 minutes and then treated for 10 minutes in a laboratory scale bath sonicator at room temperature.

10.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

10.5 Validation status

This method has not yet been subjected to validation

10.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

10.7 Information on expected particle size distribution

Table: 1: DLS size measurement of IRMM-388 (TiO₂) dispersed in SHMP solution

Mean Particle Size in nm of IRMM-388 by CLS (weight-size distribution)	Sonication Method		
		USP 7 mm 20' (*)	
	316	319	365

(*) results of two different analyses

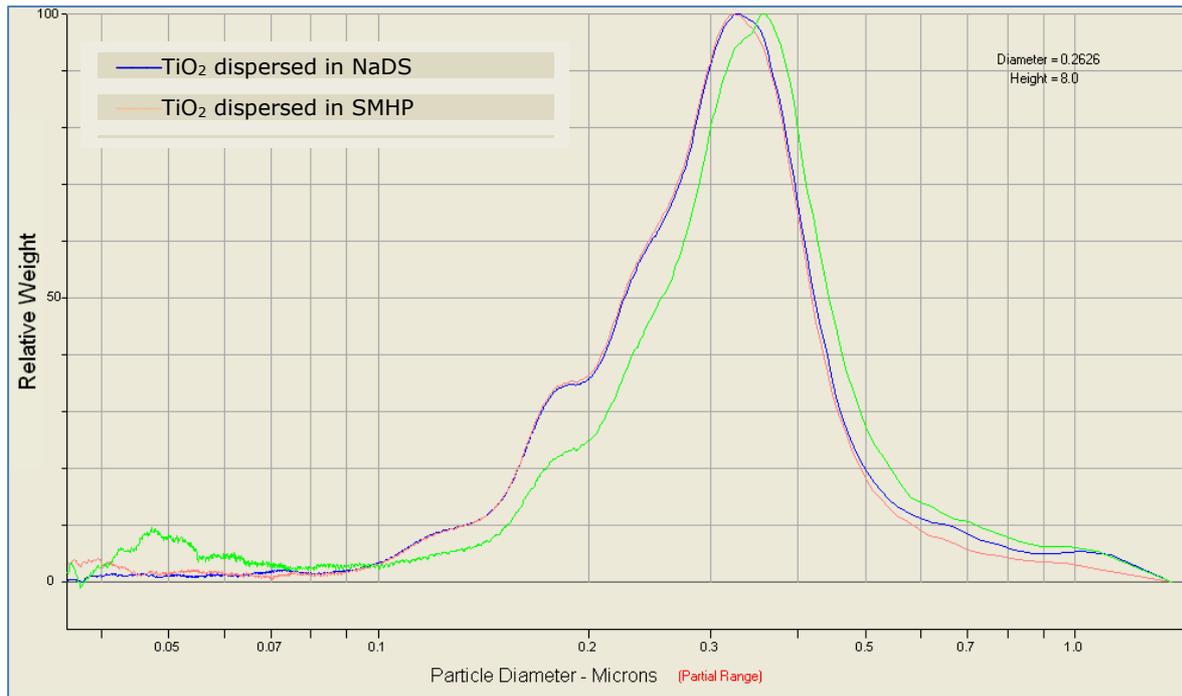


Figure 4: CLS measurement of IRMM-388 (TiO₂) dispersed in solutions pure water, SMHP and NaDS

11 SOP for production of an aqueous based dispersion of IRMM-389 (basic methacrylate copolymer)

11.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a stable water-based dispersion of IRMM-389 starting from dry powder form.

11.2 Scope

This scope of the SOP is to describe, in detail, a laboratory scale method able to produce small volume batches of a surfactant stabilised, water-based colloidal suspension of IRMM-389, basic methacrylate copolymer. The procedure has been designed specifically to produce samples with volumes, concentrations and temporal stability which are for use with suitable particle size measurement methods adopted in the NanoDefine project. Furthermore, the method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution. The method has been designed to produce samples whose particle size distribution, as determined by LD or AC-CLS, does not significantly change (according to LD or AC-CLS measurements) over a time period of at least 30 minutes from completion of the dispersion procedure.

11.3 Abbreviations

AC	Analytical Centrifugation
CLS	Centrifugal Liquid Sedimentation
LD	Laser diffraction
NaDS	Sodium Dodecyl Sulphate
NM	Nanomaterial
PSD	Particle Size Distribution
SEM	Scanning Electron Microscopy
SOP	Standard Operating Procedure
USB	Ultrasonic bath sonicator
USP	Ultrasonic probe sonicator

11.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (20 mL) of a surfactant stabilised aqueous suspension (10 mgmL⁻¹ of IRMM-389) of IRMM-389 basic methacrylate copolymer.

The procedure foresees starting from a dry powder sample of IRMM-389 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator or vial sonicator) to disperse the solid NM into high purity water containing a low concentration of the surfactant NaDS and the supporting wetting agent stearic acid. The procedure, as described here, may be conducted using either a probe sonicator or a vial sonicator with variable power output to supply the mechanical energy necessary.

The present SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value

which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document. When this procedure is conducted using a probe sonicator the batch volume which is produced is 20 mL while the alternative method using a vial sonicator permits the production of 2 mL batches.

The particle size distributions of the method has been evaluated by laser diffraction and found to be comparable. The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (< 35 min). The use of a shorter time may produce measurably larger mean particles size while longer treatment times will either degrade the quality (re-formation of larger aggregates) or will not provide a significant further reduction in the mean size.

To evaluate the stability of the dispersions, particle size distributions have been measured immediately after sonication (pristine) and again after a rest period of 30 minutes (aged) with the results showing no major variation in the means size distribution.

Dispersed solution stored for more than the 30 minutes time period may undergo moderate agglomeration or sedimentation but can be returned to the pristine state by treating the solution vial in a bath sonicator for 5 minutes. The effectiveness of this additional step has been verified with dispersions stored up to 1 day.

11.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.01 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W. Alternatively a vial sonicator may be used
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 μ L, 1 mL and 5 mL with disposable tips
- Heat-insulated box for ice-cooling of samples during sonication
- Stirring device

11.4.2 Recommended optional equipment

- Particle size measurement instrument (e.g. LD or CLS)

11.4.3 Material Supplies

- 500 mL glass bulk for the NaDS solution
- 50 mL glass beaker. The beaker should have an inner diameter of ca. 4 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing of NM and any chemicals in powder form
- Disposable nitrile gloves and other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

11.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 MΩcm⁻¹, 0.2 μm in-line filtration)
- Basic methacrylate copolymer distributed by IRMM with project ID no. IRMM-389
- Stearic acid powder (CAS No. 57-11-4)
- sodium dodecyl sulphate powder (CAS No. 151-21-3)
- Ice-water mixture for cooling the sample during sonication

11.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with LD or AC-CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

11.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was a Hielscher UPS200S with nominal maximum power of 200 W. This sonicator could be fitted with probe heads with diameters of 1 mm, 3 mm or 7 mm diameter. The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in in Figure 1.

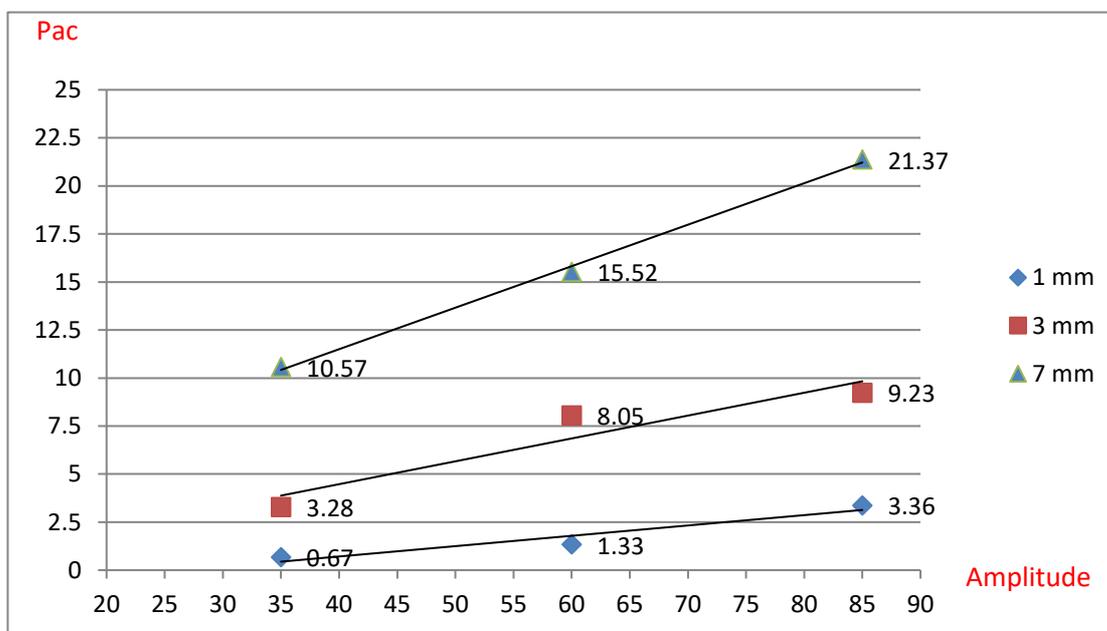


Figure 1: Power output calibration of Hielscher UPS200S probe sonicator with different probe sizes operating at 100 % cycle time

In this procedure for dispersion of IRMM-389 the sonicator was fitted with a 7 mm probe head and operated at a set amplitude value of 75 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 18 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator the operator must determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce an output of 18 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

11.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 11.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

11.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Prepare a stabilising agent solution (1 wt%) by dissolving the appropriate amount of sodium dodecyl sulphate powder (NaDS) into ultrapure water ($18.3 \text{ M}\Omega\text{cm}^{-1}$), e.g. 5 g NaDS to 495 g ultrapure water. Shake the glass bulb to ensure that all powder is solubilized. Subsequently filter the solution using a $0.2 \mu\text{m}$ filter to ensure that no particles are present.

Weigh sufficient substance to produce a final suspension with a mass concentration of about 10 mgmL^{-1} IRMM-389 (e.g. 200 mg of IRMM-389 powder for finally 20 mL suspension).

Weigh sufficient stearic acid (e.g. Sigma Aldrich 75366) to produce a final suspension with concentration of about 0.15 mgmL^{-1} (e.g. 30 mg of stearic acid powder for finally 20 mL suspension)

Add corresponding volume of the NaDS solution (e.g. 2 mL of NaDS solution for finally 20 mL suspension) to produce in the following step a suspension with a NaDS concentration of 0.1 wt%.

Add sufficient ultrapure water ($18.3 \text{ M}\Omega\text{cm}^{-1}$) to reach the mass concentration 10 mgmL^{-1} and a NaDS concentration of 0.1 wt%. (e.g. 17.7 mL ultrapure water for 200 mg IRMM-389). Take care on the floating particles and do not blow them away when an Eppendorf pipette is used.

Homogenize the suspension by brief stirring for at least 20 min. Take care that all floating particles were brought into suspension. Start with low rotational speed and increase the speed slowly that no floating particles were blown away.

Treat sample with high power ultrasonic (e.g. probe sonication). The Influence of sonication is rather low. Reproducible results were generated with sonication using a Hielscher UP200S (200 W source) equipped with a 7 mm probe operating 70 % amplitude (constant current input: 334mA) for 2 min. The probe tip shall be hold 1-2 cm below the liquid surface. Ice cooling during the sonication is required. The ice bath should be positioned such that the lower half of the beaker is immersed in a mixture of crushed ice and water. The beaker should be held at a depth sufficient that the liquid in the vial in the vial is full immersed in the cooling water.

The resulting solution should now be suitable for testing. If necessary the suspension should be diluted with ultrapure water for individual measurement.

The probe sonicator head used should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air. Avoid any contact between ethanol and the suspension as IRMM-389 will dissolve in ethanol.

11.4.6 Optional verification of dispersion quality

Where the operator has access to DLS or CLS instrumentation it is strongly recommended that the dispersion be evaluated by DLS or CLS and the results compared with that shown in section 11.7.

The DLS/CLS measurements should be made following the manufacturer instructions and it is recommended the physico-chemical parameters used in data analysis be taken or interpolated from those tabulated in chapter 0. The values quoted in chapter 0 are the recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within the NanoDefine project.

If the mean size (LD/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 11.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

11.4.7 Recovery of dispersions after aging beyond verified period of stability

The temporal stability of the dispersions prepare in previous steps have been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 30 minutes following completion of the primary dispersion procedure outlined in the previous section 11.4.5. In the case of materials which are allowed to age for longer than 30 minutes it is possible that some degree of agglomeration may occur but this may be reversed by the use of a single additional step of bath sonication. In the case of IRMM-389, material aging of up to 1 day may be fully reversed if the sample is treated with ultrasonic power for 5 minutes in a laboratory scale bath sonicator at room temperature.

11.4.8 Reporting of results

The main objective of the procedure does not foresee any specific metrological step and consequently it has not been deemed necessary to detail any step relating to the reporting of results.

11.5 Validation status

This method has not yet been subjected to validation

11.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) and related operating instructions of sodium dodecyl sulfonate and basic methacrylate copolymer to be aware of known hazards relevant in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

11.7 Information on expected particle size distribution

Figure 2 below shows an image of particles of IRMM-389 basic methacrylate copolymer taken by SEM using the drop-on-grid method. Clearly visible are larger particles with sizes of about 20 μm and smaller ones with diameters of approximately 3 μm .

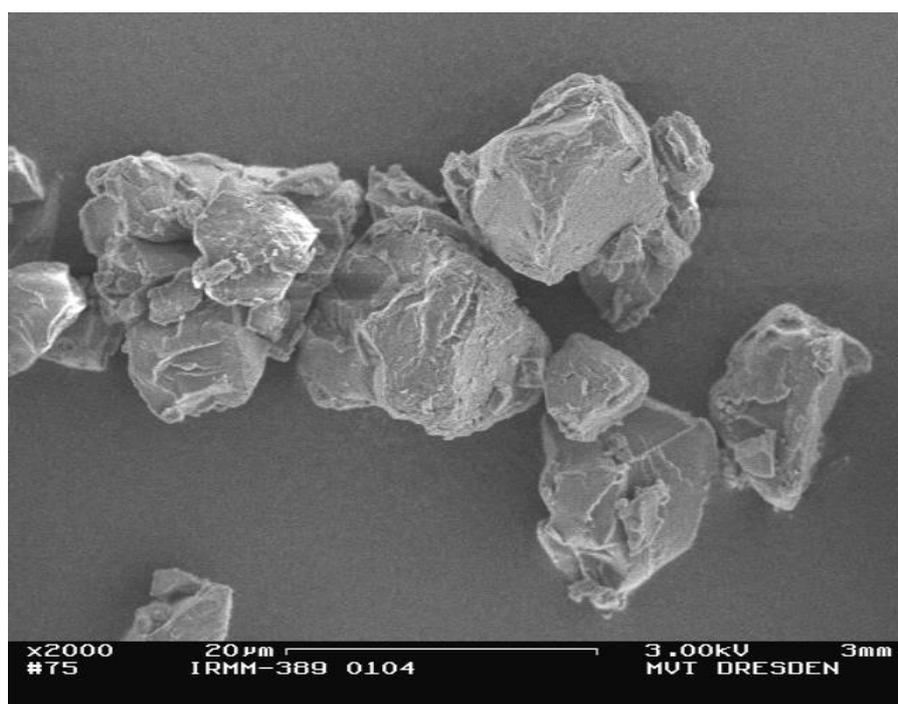


Figure 2: SEM image of IRMM-389

In table 1, values of measured particles sizes (modal value, median value) by laser diffraction are given to enable evaluation of results obtained when using this SOP. The suspension was achieved as described in section 11.4. The treating time for ultrasonic power input was varied but no significant shift was detected.

Table: 1 Expected particle size values after various ultrasonic power input times

Ultrasonic Probe Sonication	$X_{mod,3}$	$X_{10,3}$	$X_{50,3}$	$X_{90,3}$
	μm	μm	μm	μm
5 min USP	1.426	1.797	8.948	19.800
5 min USP	1.417	1.815	9.135	19.955
5 min USP	1.393	1.897	9.646	21.311
10 min USP	1.421	1.695	8.449	19.290
10 min USP	1.406	1.776	9.096	20.778
15 min USP	1.361	1.953	9.913	19.205
15 min USP	1.364	1.938	9.656	17.917
25 min USP	1.383	1.893	9.406	17.456
25 min USP	1.398	1.884	9.307	17.569
25 min USP	1.399	1.838	9.072	17.194

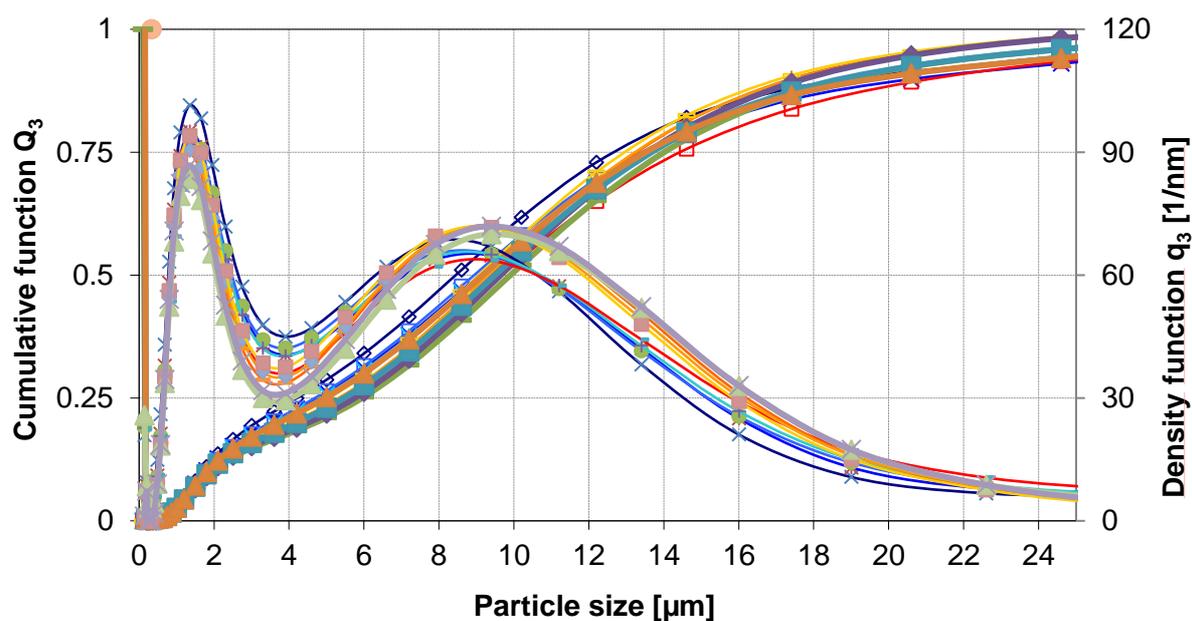


Figure 3: LD-analysis of aqueous dispersion of IRMM-389 with NaDS and stearic acid. No significant shift of peaks is visible in the mass weighted PSD by varying the ultrasonic power input

12 SOP for production of an aqueous based dispersion of BAM-11 Zeolite

12.1 Aim

The aim of the procedure is to describe a laboratory scale method to produce a water-based dispersion of BAM-11 (Zeolite powder) starting from dry powder form.

12.2 Scope

This scope of the Standard Operating Procedure (SOP) is to describe, in detail, a laboratory scale method able to produce small volume batches of a water-based suspension of BAM-11, Zeolite powder. The method has been developed with the scope of maximizing the proportion of free single particulates and minimizing the number of residual agglomerates and aggregates in the solution.

The physico-chemical properties of this material do not allow obtaining a stable dispersion: due to their relatively large size, the particles sediment rapidly. This instability is not compatible with several of the particle size measurement methods adopted in the NanoDefine project. The protocol remains useful for methods such as EM, where the sample is transferred to a solid carrier by sedimentation.

12.3 Abbreviations

AFM	Atomic Force Microscopy
DLS	Dynamic Light Scattering
EM	Electron Microscopy
NM	Nanomaterial
PdI	Polydispersity Index (a number calculated from a simple 2 parameter fit to the correlation data (the cumulants analysis) measured using DLS)
PSD	Particle Size Distribution
SOP	Standard Operating Procedure
TEM	Transmission Electron Microscopy
USP	Ultrasonic probe sonicator
VM	Vortex mixer

12.4 Description

The following Standard Operating Procedure (SOP) describes a method for the preparation of small volumes (6 mL) of an aqueous suspension (2.6 mg mL^{-1}) of BAM-11, zeolite powder. The procedure foresees starting from a dry powder sample of BAM-11 and utilizes a laboratory scale ultrasonic disruptor (probe sonicator) with variable power output to supply the mechanical energy necessary to disperse the solid NM into high purity water. The SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of the ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given in chapter 13 of this document.

The procedure, as described here, may be conducted using a probe sonicator and has been developed to produce a batch volume of 6 mL.

The sonication time and power values detailed in the procedure have been determined to produce the lowest mean particle size distribution in an experimentally relevant time (<60 minutes). The use of a shorter time may produce measurably larger mean particle size values. The described sonication procedure leads to the release of small particles from the zeolite material (as compared to the mean particle size value). It is unclear whether these particles can be considered as constituent particles or pieces of material broken off of the original particles. The release of these small particles increases with the extent of sonication time and power.

The stability of the dispersions after sonication is evaluated visually immediately after sonication (pristine). The physico-chemical properties of this material do not allow obtaining a stable dispersion for direct measurement in dispersion (e.g. by techniques such as DLS). Due to their relatively large size, the particles sediment rapidly and during measurement. The protocol remains useful for methods such as EM where specimens can be prepared by transferring a fraction of the sample to a solid carrier by sedimentation. The dispersion efficiency is evaluated based on the particle size distribution determined by TEM.

12.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Analytical balance (0.1 mg precision)
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a tip diameter of approximately 6-7 mm. The sonicator should have nominal power output of at least 100 W.
- Ultrasonic bath sonicator
- Temperature controlled hot-plate or water bath
- Adjustable volume pipettes of 100 μ L, 1 mL and 5 mL with disposable tips.
- Heat-insulated box for ice-cooling of samples during sonication.
- Stirring device

12.4.2 Recommended optional equipment

- Ionizer to neutralize electrostatic charge during weighing of fine powders
- Particle size measurement instrument (e.g. TEM)

12.4.3 Material Supplies

- 20 mL glass vial with screw top or other appropriate stopper. The vial should have an inner diameter of ca. 2 cm and an opening which is sufficiently large as to ensure that the probe head can be inserted and operated without risk of contact between the vial and probe
- Disposable plastic spatula for weighing of NM
- Disposable plastic weighing boats or similar for weighing chemicals in powder form
- Disposable nitrile gloves
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs

12.4.4 Chemicals

- Type (I) ultrahigh purity water (e.g. MilliQ from Millipore Corp.; resistivity 18.2 M Ω cm⁻¹, 0.2 μ m in-line filtration)
- Zeolite powder distributed by BAM with project ID no. BAM-11
- Ice-water mixture for cooling the sample during sonication

12.4.5 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for producing the dispersions. In particular it describes the steps necessary to harmonise the sonication power applied when operating with instruments and probes different from those utilized in the development of the procedures. For laboratories equipped with LD or AC-CLS instruments additional information is given on how to verify whether the resulting dispersions are comparable with that which would be routinely expected from a successful application of the SOP and, if necessary, how to further optimize the sonication conditions to achieve this.

12.4.5.1 Determination of suitable sonicator power settings

In the development of this procedure the probe sonicator used was Microson XL 2000 (Qsonica, LLC (Newtown, USA) with nominal maximum power of 100 W.

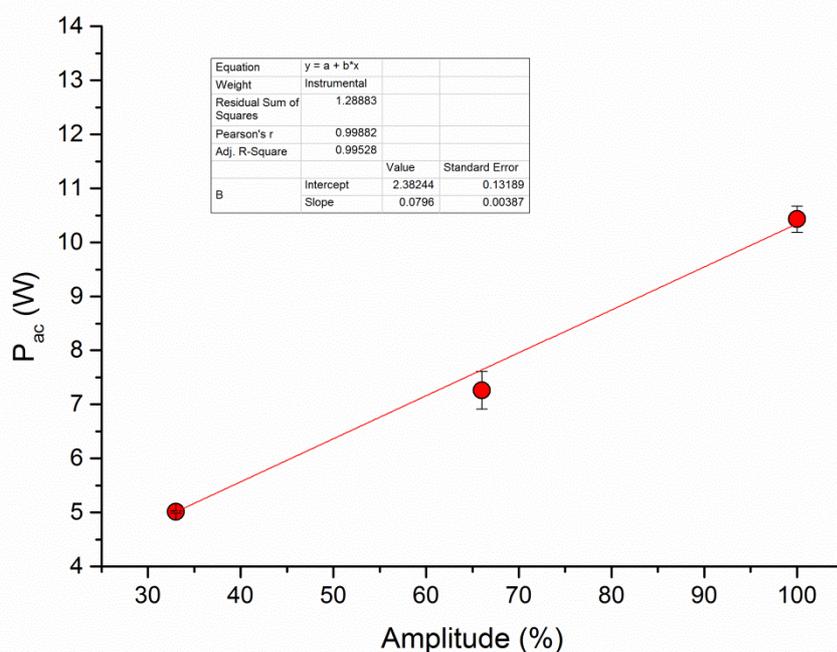


Figure 1: Calculated delivered output power P_{ac} of the probe sonicator at different amplitude settings. This calibration curve was used to determine the output setting value which corresponds to $P_{ac} = 10.3$ W (in this example: amplitude of 100 %)

The sonicator was fitted with a probe head with diameters of 6.4 mm (length of 117 mm and maximum peak-to-peak amplitude of 60 μm .). The output power characteristic of the sonicator with each of these types of head has been determined following the calorimetric calibration procedure detailed in chapter 13. The resulting calibration curves can be seen in in Figure 1. In this procedure for the dispersion of the BAM11 materials the sonicator was operated at a set amplitude value of 100 %. From the calibration curve it can be determined that under these operating conditions the instrument was producing a measured power output of 10.3 W.

Before attempting to use this dispersion procedure with any other type or configuration of probe sonicator the operator must determine a similar calibration curve for their own instrument following the procedure detailed in chapter 13. Once the calibration procedure has been completed an examination of the resulting amplitude-power curve must be done in order to determine what is correct amplitude setting required to produce an output of 10.3 W. The amplitude setting of the sonicator should then be adjusted to this value before proceeding with the dispersion procedure.

12.4.5.2 Verification of sonicator probe integrity

Prior to using a probe sonicator the integrity of the instruments probe head should be assessed. Visual inspection of the head should be made to evaluate whether damage or wear is evident. If there is any evidence of wear or damage the probe should be substituted or its operational integrity checked by operating the probe in pure water for a minimum of 15 min at the power settings determined in for use in the protocol (section 12.4.5.1). After 15 minutes of sonication the vial should be removed, closed with a suitable cap and allowed to stand untouched for at least 2 hours before being inspected visually for the presence of any fine sediment on the bottom of the vial. If any trace of sediment can be seen in the bottom of the vial the probe head should be substituted and not be used further in this procedure.

12.4.5.3 Detailed dispersion procedure for NM

The operator should verify that the sonicator probe is clean and free of any surface residue. If necessary the operator should follow the cleaning step noted below or alternatively follow a suitable cleaning method recommended by the instrument manufacturer.

Weigh approximately 15.6 mg of BAM-11 into a 20 ml glass vial. It is recommended that an ionizer be used to neutralize electrostatic charge during weighing of fine powders. Add the respective volume of ultrapure water to give a BAM-11 concentration of 2.6 mgmL⁻¹ (6 mL for exactly 15.6 mg of BAM-11, adjust volume to compensate for small deviations in the final weighed mass). Homogenize the mixture by vortexing (2').

12.4.5.4 Sonication using probe sonicator

Take the 20 mL glass vial containing the 2.6 mgmL⁻¹ BAM-11 suspension and mount the probe sonicator head inside the vial as shown in Figure 2. The probe head should be immersed in the solution to a depth approximately 1 cm. The ice bath should be positioned such that the lower half of the vial is immersed in a mixture of crushed ice and water. The vial should be held at a depth sufficient that the liquid in the vial is full immersed in the cooling water. The sample should then be sonicated at a constant amplitude setting corresponding to Pac of 10.3 W for 25 minutes. The correct power setting should be determine from calibration curve which was previously determined by the method described in chapter 13. The resulting dispersion should now be suitable for testing. If the probe sonicator has been used the probe head should be thoroughly cleaned by rinsing in water and ethanol. It is recommended that the probe be operated at low at low power (15 %) for 5 min in high purity ethanol then 5 min in water before being dried with a flow of clean compressed nitrogen or air.



Figure 2: Ultrasonic probe sonication setup for dispersion of NM powders

12.4.6 Optional verification of dispersion quality

Where the operator has access to a TEM instrument, it is strongly recommended that the dispersion be evaluated by TEM and the results compared with that shown in section 12.7.

TEM specimens can be prepared following the drop-on-grid method¹. This method includes pre-treating pioloform and carbon coated, 400 mesh copper grids (Agar Scientific, Essex, England) with 1 % Alcian blue (Fluka, Buchs, Switzerland) to increase hydrophilicity and rinsing 5 times with distilled water. Just after sonication, a droplet of 10 μL BAM-11 dispersion (2.6 mgmL^{-1}) is then placed on the grid and left for 10 minutes. Subsequently, the excess fluid is removed using a strip of filter paper.

The described laboratory scale method produces a water-based dispersion of BAM-11 starting from this material in a dry powder form. The presented protocol allows dispersing the material down to a combination of single constituent particles, and smaller and larger aggregates/agglomerates. The constituent particle size ranges from 20 nm to 3 μm (see section 12.7 of this SOP). The constituent particles are heterogeneous in shape. The morphology of the constituent particles of the NM can be irregular polygonal, rectangular or circular. Some of the apparent differences in constituent particle shape might be the result of projection of similar particles with different orientations. The surface of the constituent particles is generally rough. For the agglomerates, the size ranges from 100 nm to several μm , measured manually on the TEM images. In most cases, the agglomerates tend to have a complex 2D structure. Diffraction contrast, which indicates that the material is crystalline, can be observed in the constituent particles. During the development of this procedure it was observed that the sonication procedure resulted in the release of small particles from the zeolite material (as compared to the mean particle size value). It is unclear whether these particles have to be considered as constituent particles or pieces of material broken off of the original particle. The release of these small particles increases with the extent of sonication time and power.

If the expected mean aggregate/agglomerate size is significantly larger (>15 %) than that shown in section 12.7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

12.4.7 Recovery of dispersions after aging beyond verified period of stability

The physico-chemical properties of zeolite powder (BAM-11) do not allow obtaining a stable dispersion: due to their high density and relatively large size, the particles sediment rapidly.

12.4.8 Reporting of results

Reporting of results should be done in a way all measurements and analysis of results can be repeated.

12.5 Validation status

This method has not yet been subjected to validation

12.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) and related operating instructions of sodium dodecyl sulfonate and basic methacrylate copolymer to be aware of known hazards relevant in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

12.7 Information on expected particle size distribution

Quantitative TEM analysis is performed using methods described by Verleysen et al. and De Temmerman et al.²⁻⁴. Figure 3 shows representative TEM images of BAM-11. The corresponding size distribution is shown in Figure 4 and is determined by a semi-automated approach using imageJ software (National Institute of Mental Health, Bethesda, Maryland, USA). This approach can be briefly summarized as follows:

- To suppress background noise, a mean filter is applied before analysis. The use of other filters was not necessary for the examined material.
- A threshold for the detection of the particles based on mass-thickness contrast in the image is chosen manually.
- Particles are only detected in a pre-defined Region of Interest (ROI), which allows excluding border particles.
- For every micrograph, the 'Fill holes' option is switched on.

Descriptive statistical analysis of the Feret min of the particles is obtained using a home-made script in the python programming language. The raw data is represented as a histogram ('Number based distribution') (Figure 3, left panel). A log-normal curve is fitted iteratively to the scatter plot (Figure 3, right panel), and gives estimates for the mode, height, width and asymmetry of the distribution (Table 2). The errors on these parameters are determined as described by Wojdyr⁵ and Wolberg⁶. The median of the Feret min distribution is 82.41nm and the mode Feret min distributions is 46.24 ± 0.26 nm. A sub-fraction of smaller particles was visible when imaging the sample (size 10-50nm). It is unclear whether the smaller particles have to be considered as constituent particles or pieces of material broken off of the original particles.

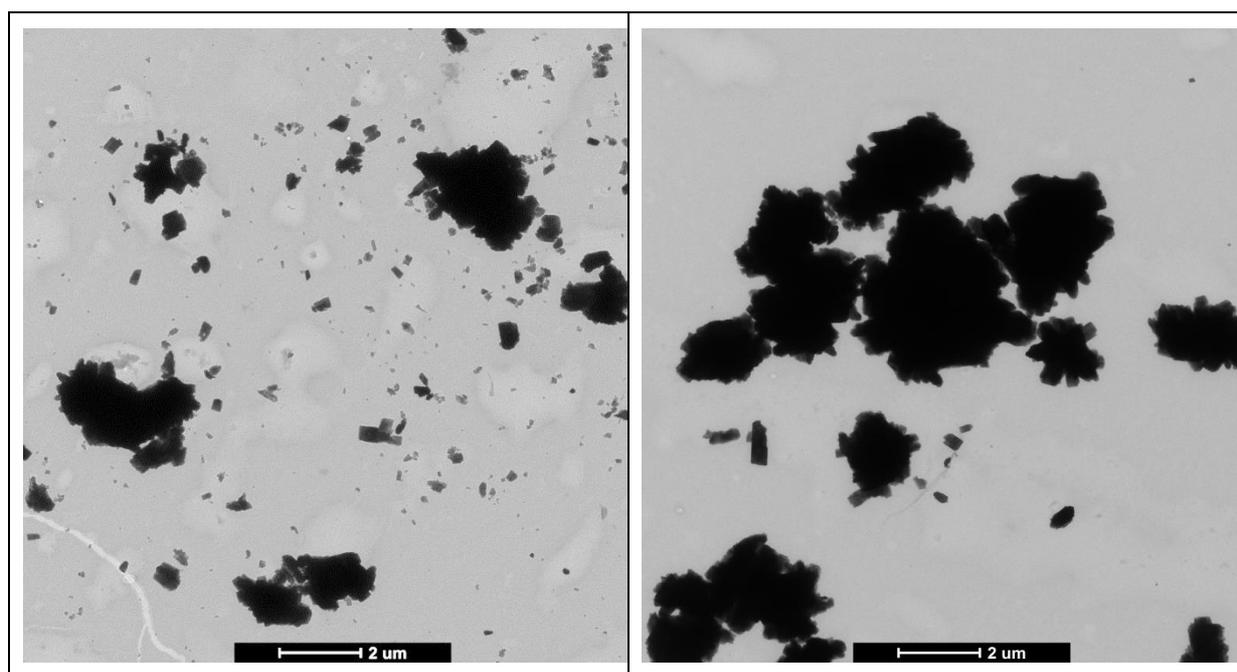


Figure 3: Representative TEM images of the BAM-11 Zeolite powder particles dispersed using the presented SOP

Table 1: Statistics on the Feret min distributions: best fit parameters (height, mode, width and asymmetry) of the log normal functions fitted to the distributions and median values of the datasets

Parameter	Median Value
Number of particles	2254
Height	924.65 ± 37.20
Height normalized	0.410 ± 0.016
Mode	46.24 ± 0.26 nm
Width	39.62 ± 3.74 nm
Asymmetry	1.47 ± 0.06
Median	82.41 nm

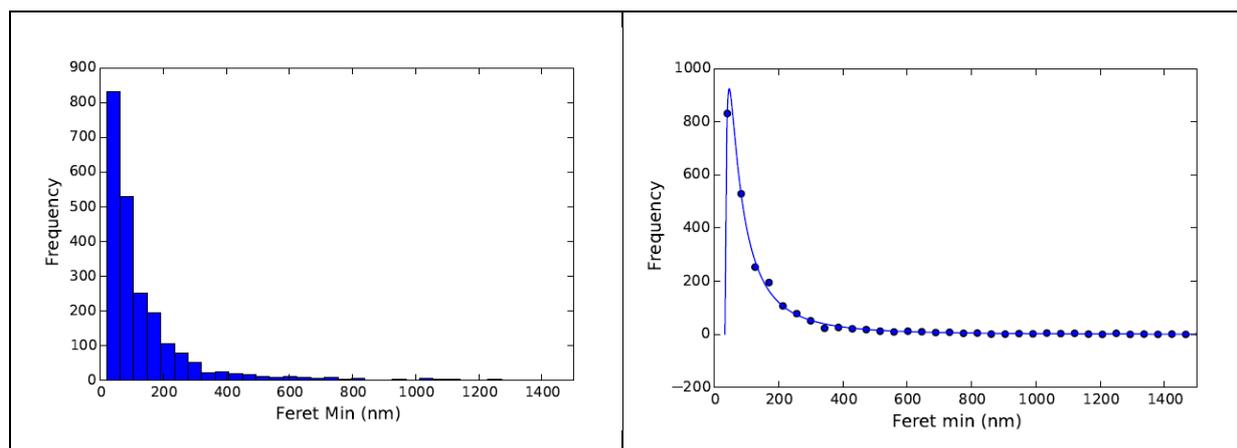


Figure 4: Left panel: representative size distribution (Feret min) of BAM-11 zeolite powder obtained by quantitative TEM. Right panel: scatter plot of the Feret min distribution of BAM-11, zeolite, fitted log normal function

References

- ¹ Mast, J., Demeestere, L., Electron tomography of negatively stained complex viruses: application in their diagnosis, *Diagn. Pathol.* 4 (2009)
- ² Verleysen, E., Van Doren, E., Waegeneers, N., De Temmerman, P.-J., Abi Daoud Francisco, M., Mast, J. TEM and SP-ICP-MS Analysis of the Release of Silver Nanoparticles from Decoration of Pastry. *J. agricultural and food chemistry*, 63(13), 3570-3578 (2015)
- ³ Verleysen, E., De Temmerman, P.-J., Van Doren, E., Abi Daoud Francisco, M., Mast, J. Quantitative characterization of aggregated and agglomerated titanium dioxide nanomaterials by transmission electron microscopy. *Powder Tech.* 258, 180-188 (2014)
- ⁴ De Temmerman, P.-J., Van Doren, E., Verleysen, E., Van der Stede, Y., Francisco, M., Mast, J. Quantitative characterization of agglomerates and aggregates of pyrogenic and precipitated amorphous silica nanomaterials by transmission electron microscopy. *Journal of Nanobiotechnology*, 10 (2012)
- ⁴ Wojdyr, M. Fityk: a general-purpose peak fitting program. *J. Appl. Cryst.*, 43, 1126 (2010)
- ⁵ Wolberg, J. *Data Analysis Using the Method of Least Squares: Extracting the Most Information from Experiments.* ISBN 978-3-540-31720-3. Springer. 2006.

13 Generic SOP for calorimetric calibration of an ultrasonic probe sonicator

13.1 Aim

The aim of this SOP is to describe a calorimetric based experimental method to determination the level of ultrasonic energy which a generic probe sonicator system can transfer into a liquid.

13.2 Scope

This scope of the SOP is to provide a general method for determination of delivered power of probe sonicators for use in harmonising the description and application of nanoparticles inter-laboratory comparison of nanoparticle dispersion. The results obtainable from this SOP may be used to improve the inter-laboratory transferability of Standard Operating Procedures for the dispersion of dry nanomaterials into liquids by allowing better harmonisation of the sonication conditions. The following protocol and recommendations have been based on the National Institute of Standards and Technology (NIST) publication by Taurozzi et al.¹.

13.3 Abbreviations

No abbreviations required

13.4 Description

When it is required, starting from a dry powder, to produce liquid dispersions of nanoparticles with a minimum amount of residual agglomerated material it is often necessary to use high intensity sonication as the main means of supplying mechanical energy to break-up agglomerates. The efficiency, effectiveness and speed which such agglomerates can be broken up into smaller particle assemblies depends on many instrumental and experimental factors including source frequency, probe size and shape, volume of liquid treated, temperature and treatment time. For the purposes of describing and harmonising suitable reproducible methods for dispersing specific materials it is desirable to be able to define the ultrasonic energy applied on the basis of some experimentally measureable values which can be defined independently of the instrument used. Using this procedure it is possible to obtain estimates of the effective acoustic power output from generic laboratory probe sonicators when operating at a variety of instrument settings. Once the power output characteristics of an instrument are known then it becomes possible to adjust these values to match those of a dispersion procedure developed using another type of probe sonicator but whose power output characteristic have been measured in the same manner.

13.4.1 Essential equipment

- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- Laboratory scale balance with maximum weight boundary greater than 700 g and a weighing accuracy of ± 0.1 g or better
- A variable power sonicator operating at 22.5 kHz (please report deviating frequencies) equipped with the probe tip which that will be used in implementing the nanoparticle dispersion protocols. For the purposes of dispersing nanomaterials it is recommended that the sonicator should have nominal power output in the range 50-500 W and be fitted with a 6-7 mm diameter probe

- Water; thermally equilibrated to fume-hood air temperature (Nanopure-filtered water or MilliQ-filtered water or similar; resistivity 18.2 M Ω cm⁻¹)
- 600 mL tall form borosilicate glass beaker with approximate dimensions of 150 mm in height and 80 mm in diameter
- Thermal insulation foam or similar to wrap beaker to reduce heat loss(optional)
- Digital thermometer with metal or glass sheathed thermocouple probe capable of a measurement accuracy better than ± 0.1 °C
- Digital timer capable of measurement accuracy better than ± 1 s

13.4.2 Materials and methods

This chapter describes the set-up and use of the equipment, necessary reagents and a detailed step-by-step procedure for undertaking the measurements necessary to characterise the power output of the sonicator probe.

13.4.2.1 Calibration of delivered acoustic power from calorimetry

1. Measure the weight of the empty 600 mL tall form beaker and then add 500 mL de-ionized water
2. Measure the total weight of the filled beaker and then calculate the amount of water by difference
3. Immerse the sonicator probe ca. 2.5 cm below the liquid surface
4. Immerse a temperature probe connected to a temperature meter in the liquid and position it approximately 1 cm away from the sonicator probe as shown in Figure 1
5. Use a hook/clamp to fix the beaker, so it cannot move during the measurement
6. Let the liquid temperature stabilise at room temperature and note the equilibrium temperature
7. Select a sonicator output setting (e.g. 'amplitude in μ m' or '% of amplitude'; usually set by a dial in the sonicator power module), operating in continuous mode and record the water temperature increase for the initial 5 minutes with minimum resolution of 30 s
8. Using the recorded temperature values, create a temperature vs. time curve (see Figure 2) and obtain the best linear fit for the curve using least squares regression
9. With the obtained slope $\left(\frac{\Delta T}{\Delta t}\right)$, the delivered acoustic power P_{ac} (W) can be calculated from the following equation:
10.
$$P_{ac} = \frac{\Delta T}{\Delta t} M C_p$$
where T and t are temperature (K) and time (s), respectively, C_p is the specific heat of the liquid (4.18Jg⁻¹K⁻¹ for water) and M is the mass of liquid (g)
11. Repeat steps 7-9 with new sonicator output settings after exchanging the water in the beaker for at least three power settings (2 repetitions for each setting). Plot the calculated delivered acoustic power values P_{ac} over the chosen output setting values (Figure 3)



Figure 1: Photograph of probe sonicator and thermocouple positioning for calorimetric calibration (thermal insulation removed for clarity)

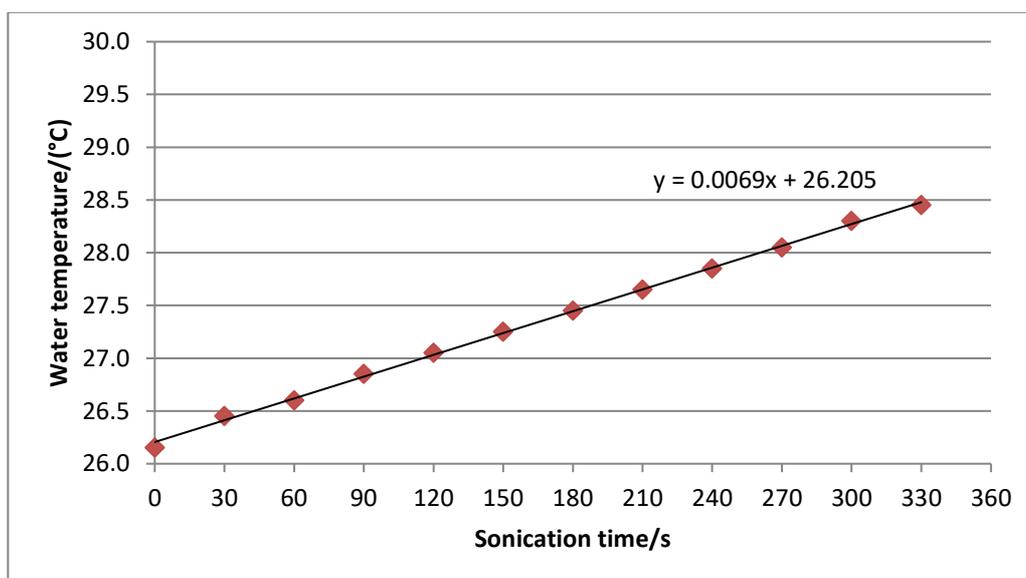


Figure 2: Temperature vs. time curve for 500 mL H₂O sonicated using a Hielscher UPS2005 ultrasonic disruptor fitted with 7 mm probe operating at 60 % amplitude

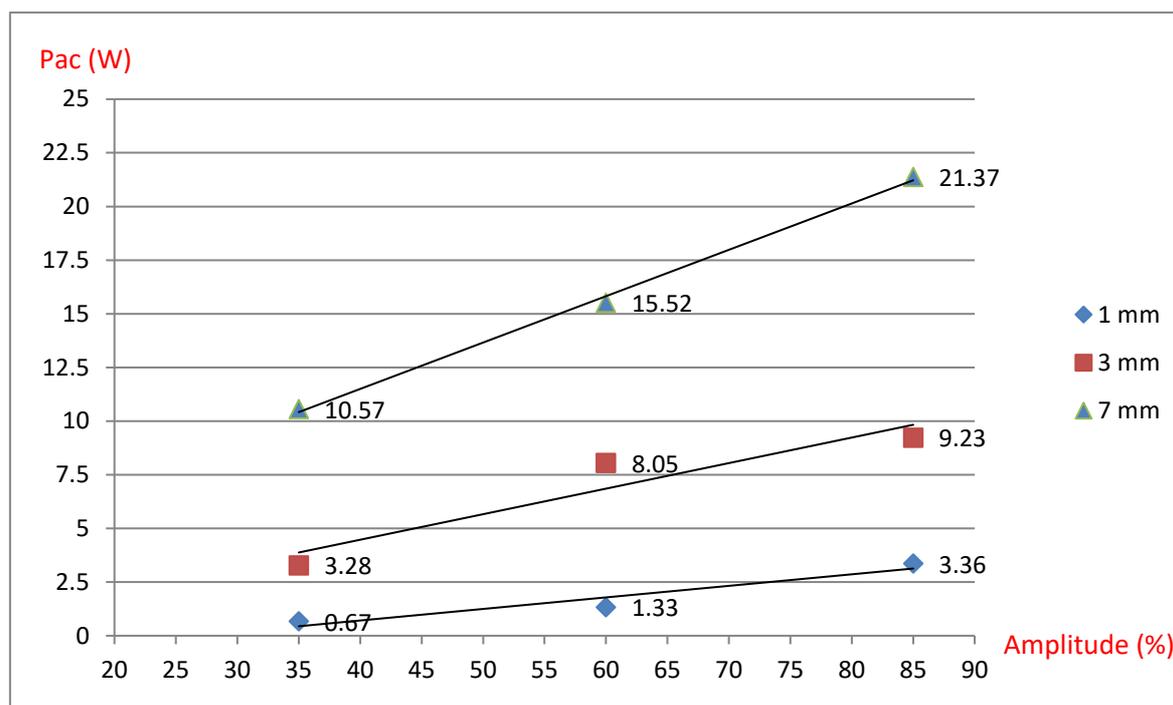


Figure 3: Example of calorimetrically determined sonication power

13.4.3 Reporting of results

No additional recommendations are currently available regarding the reporting of results.

13.5 Validation Status

This method has not yet been subjected to validation within the NanoDefine project

13.6 HSE Issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

13.7 References

¹ Taurozzi J, Hackley V, Wiesner M. Preparation of nanoparticle dispersions from powdered material using ultrasonic disruption. *National Institute of Standards and Technology*. 2012. Available at: <http://nvlpubs.nist.gov/nistpubs/specialpublications/nist.sp.1200-2.pdf>

14 Properties of recommended materials

Recommended material properties (skeleton density and refractive index at selected wavelengths, at 20 °C) for data analysis within NanoDefine.

Code material	ρ , gcm ⁻³	m_{405nm}	m_{470nm}	m_{530nm}	m_{633nm}	m_{670nm}	m_{865nm}
ID-19 (PSL mono)	1.05(x)	1.624	1.608	1.598	1.587	1.584	1.576
ID-20 (PSL 3-mod)	1.05(x)	1.624	1.608	1.598	1.587	1.584	1.576
ID17 (ERM-FD304, SiO ₂)	2.305	1.469	1.463	1.459	1.455	1.454	1.45
ID-18 (SiO ₂ 3-mod)	2.305	1.469	1.463	1.459	1.455	1.454	1.45
ID-16 (BAM-nano-Au)	19.3	1.46-1.96i	1.28-1.88i	0.569-2.26i	0.155-3.36i	0.140-3.74i	0.175-5.48i
ID-21 (BAM-nano-Ag)	10.5	0.170-2.03i	0.142-2.64i	0.140-3.15i	0.140-3.98i	0.140-4.27i	0.140-5.75i
IRMM-384 (CaCO ₃)	2.657	1.551	1.541	1.534	1.525	1.522	1.507
IRMM-387 (BaSO ₄ UF)	(4.4)						
RMM-381 (BaSO ₄ fine)	(4.4)						
IRMM-388 (coated TiO ₂)	3.99	2.955/2.737	2.735/2.567	2.639/2.493	2.554/2.429	2.536/2.415	2.480/2.373
IRMM-385 (kaolin)	2.61						
IRMM-383 (nano steel)	7.8*	1.85-3.07i*	2.29-3.27i*	2.58-3.31i*	2.85-3.39i*	2.91-3.44i*	3.11-3.82i*
IRMM-382 (MWCNT)	2.05						
IRMM-380 (Y83 nano)	1.484	1.47-0.457i	1.92-0.42i	1.93-0.07i	1.75-0.029i	1.73-0.023i	1.72-0.03i
IRMM-386 (Y83 opaque)	1.5	1.47-0.457i	1.92-0.42i	1.93-0.07i	1.75-0.029i	1.73-0.023i	1.72-0.03i
IRMM-389 (BMA)	1.13(x)	1.391	1.387	1.384	1.381	1.381	1.378
BAM-11 (zeolite)	2.07						
BAM 12a-1 (fumed SiO ₂)	2.2	1.469	1.463	1.459	1.455	1.454	1.45
water (H ₂ O)	0.997	1.343	1.338	1.335	1.332	1.331	1.328

Generic SOPs for methods

15 Generic SOP for DLS method for sample preparation and measurement of substances in suspension

15.1 Aim

This document describes the Standard Operating Procedure (SOP) to be used to determine the particle size and particle size distribution of diluted suspensions of nanomaterials. The principle is based on the estimation of the kinetics of the relaxation process by application of the principles of light scattering and Stokes-Einstein law.

15.2 Scope

This SOP describes the use of a dynamic light scattering (DLS) method to perform particle size measurements. The procedure is applicable for the determination of particles suspended in stable aqueous suspensions. Depending on the optical properties and effective density of the dispersed materials, particle sizes in a range from 1 nm to 5 μm in diameter can be measured at mass concentrations of 1mgL^{-1} to 1gL^{-1} . The SOP includes information on the sample preparation, method parameters, data evaluation and reporting of the number-based hydrodynamic diameter after conversion from intensity-based particle size distribution.

This SOP is intended for the determination for the NanoDefine materials given in Table 1. The operating conditions were chosen with respect to the mean particle sizes in the sub-micrometre range.

It can also be applied to comparable types of powders (e.g. inorganic insoluble salts), considering that adaptations for the sample preparation and measurement might be needed. Depending on the material properties (e.g. particle size, effective density, hydrophilic or hydrophobic behaviour), adaption have to be done regarding sample preparation, filtration, delay and measurement time.

15.3 Abbreviations

DLS	Dynamic light scattering
IRMM	Institute for reference materials and measurement ^c
NP	Nanoparticle(s)
RI	Refractive index
PDI	Polydispersity index
PTFE	Polytetrafluoroethylene
SOP	Standard Operating Procedure
SHMP	Sodium hexametaphosphate, Calgon, (CAS No. 10124-56-8)
TSPP	Tetra sodium pyrophosphate
USB	Ultrasonic bath

15.4 Description

DLS measurement is based on the principle that smaller particles are in faster Brownian motion than smaller ones. Dynamic light scattering allows the measurements of this translational diffusion. The hydrodynamic diameter can be determined by application of the Stokes-Einstein

^c Currently: Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, European Commission

equation and assuming that the sample being investigated consists of a set of non-interacting spherically shaped particles. This method is only applicable to dilute suspensions without particle-particle interaction¹. The DLS technique probes this particle motion in liquids by optical principles. A laser beam illuminates the particles. The light scattered from the particles has a time-dependent phase imparted to it from the time-dependent position. Measured over time the random particle motion forms a distribution of optical phase shifts or spectral frequency shifts. These shifts are determined by comparison with all scattered light (self-beating mode). The optical signals received from the particles are intensity weighted. ISO 22412² gives more information on this technique and more setups of DLS. Two types of data analysis algorithms have been established: Cumulants method (e.g. CONTIN algorithm) and distribution method (e.g. NNLS algorithm). The cumulants method is independent from the refractive index³ chosen and leads to a harmonic mean diameter of the intensity-based particle size distribution. The distribution method lead to intensity weighted size distribution and this distribution can be converted into the number-based size distribution applying Mie's theory. DLS is a calibration free system which does not require calibration. An already suspended reference solution shall be used to carry out the qualification of the instrument. This SOP gives information about how to apply DLS to the NanoDefine materials, but it can also be applied to chemically and optically comparable types of samples. The instructions for sample preparation are described briefly in Chapters 3 and 9.

Table 1: NanoDefine materials in the non-nano range analysed by DLS

Sample	Description	Sample identifier
S1	BaSO ₄ fine grade	IRMM-381
S2	CaCO ₃	IRMM-384
S3	Kaolin	IRMM-385
S4	Coated TiO ₂	IRMM-388

15.4.1 Materials and methods

This section provides information on the required chemicals, samples and analytical instrumentation needed for both sample preparation and DLS measurement (characterisation and quantification).

NOTE: The refractive index of the dispersed particles must be known for calculation of the number-weighted particle sizes and their distributions. For the NanoDefine substances (Table 1) the information on the refractive indices are given in Table 6

15.4.1.1 Chemicals

Chemicals required for sample preparation are detailed in Table 2 and substances for DLS measurement are listed in Table 3.

Table 2: Chemicals for sample preparation

Chemical	Description	Manufacturer/Provider
Deionised water	deionised water, filtered at 0.2 μm , final resistivity 18.2 $\text{M}\Omega\text{cm}^{-1}$ at 25 $^{\circ}\text{C}$	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 μm) Millipore, Billerica, MA, USA or equivalent
SHMP	sodium hexametaphosphate, analytical grade or better (CAS 10124-58-8)	e.g. Sigma Aldrich
TSPP	tetrasodium pyrophosphate, analytical grade or better (CAS 7722-88-5)	e.g. Sigma Aldrich

Table 3: Substances for DLS measurement

Chemical	Description	Manufacturer/Provider
Reference solution	e.g. polystyrene 46 nm suspension	e.g. JRC, Fisher Scientific

15.4.1.2 Instrumentation

Table 4 gives an overview on Instrumentation and accessories required for sample preparation. Table 5 names the instrumentation and software packages required for measurements by DLS.

Table 4: Instrumentation and accessories required for sample preparation

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance (readability at least 0.1 mg)	e.g. XPE205, Mettler Toledo, Vienna, Austria or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication device with a probe (e.g. with probe 7 mm and able to operate at 70 % amplitude) or a sonication device with similar energy input	e.g. Hielscher, Germany or equivalent
Calibrated pipettes	3 pipettes (20-200 μL , 0.1 - 1.0 mL, 1.0 - 5.0 mL), equipped with suitable plastic tips	e.g. Eppendorf, Hamburg, Germany or equivalent
Stirrer	Stirrer or magnetic stir bars and stirrer device	e.g. Mettler Toledo or equivalent
Filtration membranes and filtration equipment	Hydrophilic PTFE filtration membranes, nominal pore size 1.0 μm and equipment for filtration, e.g. holder, vacuum pump	e.g. Merck
Ice bath	Mixture of crushed ice and water	-

Table 5: Instrumentation, consumables and software packages for DLS measurements

Instrument	Description	Manufacturer/Provider
DLS instrument	Instrument for dynamic light scattering	e.g. Malvern, Sympatec, Anton Paar
Cuvettes	Transparent cuvettes/cells for samples, recommended by the DLS manufacturer	e.g. Sarstedt
Software	Description	Manufacturer/Provider
Instrument software	e.g. DTS, Windox, Kaliopé	e.g. Malvern, Sympatec, Anton Paar
Spreadsheet calculation programme	e.g. Excel	e.g. Microsoft

15.4.1.3 Reagents for sample preparation and measurements

The preparation of reagents needed for the sample preparation of samples in Table 1 can be found in Chapters 3, 6, 7 and 10 of this report.

Note: When choosing another liquid than aqueous solution, the user shall take care the material of the cuvette is inert to the liquid. Especially organic and acidic solvents should not be used if the user cannot ensure that the material of the cuvette is chemically resistant against the given solvent.

The suspension for qualification of the instrument is prepared according to the manufacturer's SOP of the reference material. In other cases, the suspension for qualification is prepared by giving one drop of a pre-dispersed suspension with a referenced particle's size (e.g. polystyrene 46 nm by Thermo Scientific) into a clean cuvette and adding with 2 mL 10 mM NaCl solution. After gently shaking the suspension for one minute, the suspension shall be treated in an ultrasonic bath for 30 seconds.

15.4.2 Preparation procedure

15.4.2.1 Suspension preparation

The preparation of suspension of the materials given in Table 1 can be found in Chapters 3, 6, 7 and 10 of this report. Suspensions suitable for DLS measurement must be stable at least in the period between sample preparation and the end of the measurement against sedimentation and formation of agglomerates. If there are doubts on the homogeneity, e.g. are visible supernatant or sediment, the measurement would be carried out on a not representative sample.

Using the HPPS by Malvern, the (calculated) derived count rate of the pure dispersion medium shall never exceed 100 kcps, whereas a value below 50 kcps is preferred. The critical value is depending on the laser intensity. In general, the signal to noise ratio should be at least 10.

NOTE: The user must ensure that the beakers and cuvettes are free of contamination or other particles that may contribute or interfere with the measurements.

15.4.2.2 Filtration of suspensions

Regarding the non-nano materials given in Table 1, filtration is recommended for IRMM-385 only. A volume of 20 mL with a sample content of 100 ppm kaolin should be prepared. The suspension should be filtrated through a hydrophilic PTFE membrane with a nominal pore size of 1.0 μm . The user shall not touch the membrane with hands.

NOTE: Filtration of suspensions leads to the deposition of a high quantity of particles much smaller than the nominal pore size of the filtration membranes. Only suspension with expected particle sizes close to 100 nm or below this value shall be filtrated. In other cases the impact of filtration is very high regarding the number-based median diameter and filtration could lead to false positive classification according to the EC NM definition of a nanomaterial.

15.4.2.3 Dilution of sample

The sample should have a concentration that leads to a detection signal with a derived count rate of at least 1000 kcps. Due to concentration effects that are expressed by multiple scattering and particle-particle-interactions, the maximum concentration must be determined empirically. The measured particle size shall be constant for different concentration levels (e.g. 100 ppm, 1000 ppm). If required, dilution of the sample must be performed with a particle-free diluent of the same refractive index, ionic strength, surfactant concentration, pH etc. as the original dispersion medium. A diluent of different physicochemical properties may change the surface chemistry of the particles.

NOTE: The working range (concentration) is varying on the instrument used, especially the light source and detector chosen, and the detection angle (90°, backscattering)

15.4.3 Performing measurements in DLS

In this chapter, the details of the generic measurement process are described. The user always has to follow the manual and manufacturer's guidelines.

NOTE: DLS instruments use a laser source, which can cause eye damage! Never look into the laser beam! DLS instruments have to be maintained according to the manufacturer's instructions.

15.4.3.1 Set-up

According to the manufacturer's guidelines the instrument has to be connected to the PC and communication between the instrument and the computer has to be ensured. The optical devices inside the instrument have to be clean and free of dust. The user shall not touch the surfaces of the cuvettes and inside of the instrument required for measurements. Typically, a period of 30min is required to stabilise the laser intensity.

15.4.3.2 Daily Performance Check

In general, DLS instruments are calibration-free. Most manufacturers advise the user to check the performance with a reference material annually. The user should take care that the optical components inside the measurement cell are clean and free of dust. If there is dust on the optical components a brush can be used to carefully clean the surfaces.

15.4.3.3 Typical measurement conditions

For selection of instrument settings, it is required to consider the scattering behaviour of the suspension that leads to visible turbidity of the suspension. For the NanoDefine materials given in Table 1 the recommended measurement conditions are given below, see Table 6.

The cuvette position recommended is for the HPPS instrument only. Using another instrument, the user shall check if the measurement volume is close to wall and reduce optical paths of incident and scattered light to a minimum if required. In other cases, the central position of measurement zone is preferred.

NOTE: If stability cannot be ensured (e.g. with polymers) or special material effects (e.g. hydrogel swelling) or any other reason (e.g. experiments in toxicology studies prefer a temperature of 37 °C), which require a certain temperature range; the user should reflect on temperature, known material properties at the temperature.

Table 6: Recommended measurement conditions

Operating temperature:	25 °C
Cuvette type:	acrylic cuvette 10 x10 x 45 mm
Equilibration time:	5 min
Cuvette position:	-1 mm
Intensity of illuminating light	automatic
Measurement duration:	60 s
Delay between measurements:	0 s
Setting of inversion algorithm/ mode for size analysis	general purpose

15.4.3.4 Delay time before measurement

Regarding the non-nano materials given in Table 1, a delay time between sample preparation and measurement is recommended for IRMM-384 and IRMM-385 only. A sample volume of 1.5 mL has to be taken from the recently prepared suspension and given into a cuvette. This cuvette shall be stored in the laboratory at a place protected from vibration and shocks and without direct sunlight. After a period of 24 h the cuvette can be inserted into the DLS instrument. The user shall take care not to homogenise the suspension.

15.4.3.5 Measurement description

The time between sample preparation and inserting the cuvettes has to be as short as possible to avoid the disintegration of the homogeneous particle concentration inside the samples before the measurement has started. The volume needed for measurement shall be looked up in the instrument manual. Typically a volume of 1.5 mL is used. During the preparation steps of filling the cuvette and inserting into the instrument, it is important that the user does not touch the surfaces foreseen for light transmission.

A typical sequence for preparation and measurement is as follows:

- Starting the instrument and stabilisation of the laser
- Equilibrating the temperature of the instrument
- Measurement of the deionised water used for preparation
- Measurement of a reference suspension in the expected size range, e.g. polystyrene 46 nm
- Measurement of blank samples with dispersing agents
- Measurement of the sample

Evaluation of results

The instrument software will guide the user and calculate most of the results. This section provides briefly information on the fundamental equations and the required parameters to calculate the results. DLS intrinsically determines intensity-weighted size distributions. Two data analysis algorithms have been established: Cumulants method and distribution method, e.g. CONTIN or NNLS algorithm:

The cumulants algorithm assumes a second-degree polynomial and leads to a material independent mean diameter and the polydispersity index (PDI). Both values need to be reported for every measurement.

The distribution method applies a multi-exponential fitting to the measured correlation function. This leads to a particle size distribution from which a median particle size can be determined (intensity-based hydrodynamic median diameter). Software typically also computes number- and volume-weighted size distribution. For this purpose, an optical model for scattering intensity is included in the inversion algorithm (Mie theory for electromagnetic scattering at spheres).

If the software provides the opportunity to calculate the number-based particle size distribution from the raw data, the user should follow this wizard. The standard deviation shall be calculated from the cumulants diameter and additionally from the number-based median diameter results.

Determination of intensity-based particle size

Please note that DLS does not determine the particle size in a direct way, but the translational diffusion coefficient due to Brownian motion that correlates to the particle diameter.

The investigated suspension is illuminated by a monochromatic and coherent light source with the wavelength λ_0 . The light is scattered by the particles and detected at an angle with respect to the incident radiation. The observed scattered intensity $I(t)$ will fluctuate with time correlated to the Brownian motion of the dispersed particles. Analysis of these intensity fluctuations as a function of time provides information on the motion of the particles. In correlation analysis, this analysis is carried out with a correlator which constructs the time autocorrelation function $G^{(2)}(\tau)$ of the scattered intensity

$$G^{(2)}(\tau) = \langle I_S(t) I_S(t + \tau) \rangle$$

Here $I_S(t)$ is the scattered intensity of beam at time t and $I_S(t + \tau)$ is the scattered intensity of beam at time $t + \tau$. For polydisperse samples, the correlation function of the scattered intensity is related to the normalised field autocorrelation function $g^{(1)}(\tau)$, where A is a factor reflecting the baseline of scattering light or a time-independent constant proportional to the square of the time averaged scattered intensity and B is an instrumental factor.

$$G^{(2)}(\tau) = A[1 + B|g^{(1)}(\tau)|^2]$$

The field autocorrelation function $g^{(1)}(\tau)$ is related to the normalized distribution function of decay rates $C(\Gamma)$:

$$g^{(1)}(\tau) = \int_0^{\infty} C(\Gamma) \exp(-\Gamma\tau) d\Gamma \text{ with } \int_0^{\infty} C(\Gamma) d\Gamma = 1$$

The decay rates Γ relate to the translational diffusion coefficients of spherical particles in Brownian motion:

$$\Gamma = Dq^2$$

Here, D is the translational diffusion coefficient of the set of illuminated particles and q is the modulus of the scattering vector, given by this equation:

$$q = 4\pi n \sin(\theta/2)/\lambda_0$$

In this equation, n is the refractive index of the dispersion medium and λ_0 is the wavelength of the laser in a vacuum. The particle diameter x is calculated by rearranging the Stokes-Einstein equation to give the following equation, assuming that the sample being investigated consists of a set of non-interacting spherically shaped particles. Here k_B is the Boltzmann constant; T is absolute temperature; η is the dynamic viscosity of the dispersing medium.

$$x = \frac{k_B T}{3\pi\eta D}$$

If any other temperature is chosen the values for liquid density ρ_F and viscosity η have to be adapted for Stokes' equation. The literature recommends a dynamic viscosity η of 0.890 mPa s at 25 °C for water.

The result is the median diameter of the intensity based particle size distribution ($x_{50,int}$).

15.4.3.6 Conversion into number-based particle size distribution

The instrument software will guide the user for calculation of the number-based particle size distribution following Mie light scattering theory. The software also will allow the user to calculate the volume-based size distribution too. The recommended values for the refractive indices (RI) of the NanoDefine materials and water are given in the table below. The refractive indices are given this way:

$$RI = \text{real part} \pm \text{imaginary part } i$$

Some templates may also call the imaginary part of the refractive index 'absorption'.

(NOTE: The adaption of Mie's solution is associated with assumptions, e.g. spherical particles)

Table 6: Recommended RI values for NanoDefine non-nano materials for a wavelength of 633 nm

Sample	Description	RI
S1	BaSO ₄ fine grade	1.64
S2	CaCO ₃	1.66
S3	Kaolin	1.56
S4	Coated TiO ₂	2.77
H ₂ O	Water	1.332

If the refractive index of the sample is unknown then approximate values may be found in many databases, e.g. the open source database at www.refractiveindex.info.

NOTE: If the investigated particulate material consists of core-shell-particles the user shall ask the material's supplier on the optical properties of the sample or use an open source data base.

After the step of data evaluation the data should be exported to a commonly used file format for spreadsheet calculation such as EXCEL[®]. The resulting diameter is the hydrodynamic median diameter of the number-based particle size distribution $x_{50,0}$.

15.4.4 Reporting of results

In order to allow full interpretation and reproduction of the measurement results, the analysis report shall include at least the following:

- The average particle size by cumulants method and the PDI
- Information on sample preparation, especially the concentration of sample, the suspending liquid and volume, and the dispersing agent and its concentration, the method of dispersion including the dispersion time, the amount of energy added and net power density
- Information on measurement instrument and applied settings, especially the instrument type, the temperature und the cuvette position
- Information on parameters for data analysis, especially the refractive index used of the sample and water
- Information on data fitting and correction

and information required by the guidelines of Good Laboratory Practice (date of analysis, laboratory, operator's name, identification of the sample, page numbering, name and signature of person authorising the analysis report, ...).

For validation purposes, the number-based median diameter shall be used and additionally the average particle size by cumulants method and the corresponding PDI need to be reported

15.5 Validation status

This method is not validated yet.

15.6 HSE issues

DLS instruments have to be maintained according to the manufacturer's instructions. Never use a DLS instruments if you are not sure that the laser is shielded properly.

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations. Each chemical/particulate material should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized.

Waste disposal: According to the safety regulation of the nanomaterials, the suspending liquid and the particulates and other waste from cleaning or preparations must be collected and eliminated in a manner respects all necessary norms relating to safety and environmental protection.

15.7 References

¹ Babick F. Suspensions of Colloidal Particles and Aggregates. Springer. 2016

² ISO 22412:2017 Particle size analysis - Dynamic light scattering (DLS)

³ <http://refractiveindex.info/>

16 SOP for Cuvette-AC method for sample preparation and measurement of BaSO₄ and comparable types of powders in suspension

16.1 Aim

This document describes the Standard Operating Procedure (SOP) to be used to determine the particle size and particle size distribution of diluted suspensions of nanomaterials based on the principles of centrifugal sedimentation and Stokes' law.

16.2 Scope

This SOP describes the use of a cuvette-based analytical centrifuge or a cuvette photocentrifuge (ISO 13318-2¹) with turbidity detection to perform particle size analysis measurements. The procedure is applicable for the determination of particles suspended in stable aqueous and a lot of non-aqueous suspensions. Depending on the optical properties^{2, 3} and effective density of the dispersed materials, particle sizes in a range from about 5 nm to 10 µm in diameter can be measured at mass concentrations of 0.1 gkg⁻¹ to 10 gkg⁻¹. The SOP includes information on the sample preparation, method parameters, data evaluation and reporting of the number based Stokes' median diameter after conversion from extinction based particle size distribution

This SOP is primarily intended for the determination for the NanoDefine materials BaSO₄ (Table 1) for measurements of instruments of the LUMiSizer® range. The operating conditions were chosen with respect to particles sizes distributions in the sizes range 30 nm – 1 µm.

It can also be applied to comparable types of powders and cuvette-type centrifugation instruments, considering that adaptations for the sample preparation and measurement might be needed. Depending on the material's properties (e.g. particle size, effective density, hydrophilic or hydrophobic behaviour) adaptations have to done regarding sample preparation, rotational speed, operating time and detection frequency.

16.3 Abbreviations

AC	Analytical centrifugation
CLS	Centrifugal liquid sedimentation
IRMM	Institute for reference materials and measurement
NP	Nanoparticle(s)
RI	refractive index
RRI	relative refractive index
SOP	Standard Operating Procedure

16.4 Description

Centrifugal liquid sedimentation is based on the simple principle that larger particles sediment faster than smaller ones if they have the same effective density⁴. Measurement of the sedimentation rate allows, through the application of a modified Stokes' equation (ISO 13318-1⁵), the determination of a spherical-equivalent Stokes diameter. The described method is only applicable to dispersion fluids at low Reynolds numbers and particles that sediment in an unhindered fashion.

The cuvette-AC technique, also referred to as the homogeneous incremental technique, measures the sedimentation rate of particles. Under influence of a centrifugal field, particles which are initially uniformly dispersed throughout the test sample, will segregate depending on their size and density. By means of a CCD line sensor, the intensity of the transmitted light is detected across the entire length of the sample cell as function of time and position. In a homogenous sample the initial transmission is at its minimum at each position, what corresponds to the homogeneous concentration of particles. During measurement, particles settle through the liquid, and the intensity of the transmitted light gradually increases. The progress of sedimentation is stored in the time- and space-resolved transmission profiles. In order to determine the particle size, the transmission values are converted into extinction values by using the maximum transmission value as obtained for the last profile (i.e. which should correspond to the particle-free medium/supernatant).

The cumulative light extinction-weighted particle size distribution is obtained through equations (10) to (13) of ISO 13318-1. Apart from the specified centrifuge type, instruments differ with regard to the measurement of particle concentration. There are also integrating detectors, which measure the total amount of particles above/ below a certain position. Cuvette-AC is a calibration-free system which does not require calibration for sedimentation rate determination. An already suspended reference substance shall be used to carry out the qualification of the instrument.

This SOP gives information about how to apply cuvette-AC to the NanoDefine materials, but it can also be applied to chemically comparable types of samples. The instructions for sample preparation described are in Chapters 3 and 9 of this report.

Table 1: NanoDefine representative test materials for analysis by cuvette-AC

Sample	Description	Sample type
S1	BaSO ₄ fine grade	IRMM-381
S2	BaSO ₄ ultrafine grade	IRMM-387

16.4.1 Materials and methods

This section provides information on the required chemicals, samples and analytical instrumentation needed for both sample preparation and cuvette-AC measurement (characterisation and quantification).

NOTE: The effective density and the refractive index of the dispersed particles must be known for calculation of the number-weighted particle sizes and their distributions. For the NanoDefine substances (Table 1) the information on density is given in Table 8. The refractive indices for 5 wavelengths are given in Table 9.

16.4.1.1 Chemicals

Chemicals required for sample preparation are detailed in Table 2 and chemicals required for measurement are detailed in Table 3.

Table 2: Chemicals for sample preparation

Chemical	Description	Manufacturer/Provider
Deionised water	deionised water, filtered at 0.2 µm, final resistivity 18.2 MΩcm ⁻¹ at 25 °C	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 µm) Millipore, Billerica, MA, USA or equivalent
SHMP	sodium hexametaphosphate, analytical grade or better (CAS 10124-58-8)	e.g. Sigma Aldrich

Table 3: Chemicals for Cuvette-AC measurement (see also Table 2)

Chemical	Description	Manufacturer/Provider
Reference solution	e.g. polystyrene 50 nm suspension	e.g. JRC, Fisher Scientific

16.4.1.2 Instrumentation

Instrumentation and accessories required for sample preparation is detailed in Table 4, instrumentation and software packages required for measurements by cuvette-AC are detailed in Table 5.

Table 4: Instrumentation and accessories required for sample preparation

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance (readability at least 0.1 mg)	e.g. XPE205, Mettler Toledo, Vienna, Austria or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication device with a probe (e.g. UDS probe 7 mm and able to operate at 70 % amplitude) or a sonication device with similar energy input	e.g. Hielscher, Germany or equivalent
Calibrated pipettes	3 pipettes (20-200 µL, 0.1-1.0 mL, 1.0 – 5.0 mL), equipped with suitable plastic tips	e.g. Eppendorf, Hamburg, Germany or equivalent
pH electrode	pH electrode range pH 0-14	e.g. Mettler Toledo or equivalent
Stirrer	Stirrer or magnetic stir bars and stirrer device	e.g. Mettler Toledo or equivalent

Table 5: Instrumentation, consumables and software packages for cuvette-AC measurements

Instrument	Description	Manufacturer/Provider
Cuvette-AC	Instrument for integral sedimentation technique	e.g. Beckman, LUM
Cuvettes	Transparent cuvettes/cells for samples, recommended by the cuvette-AC manufacturer	e.g. Beckman, LUM
Software	Description	Manufacturer/Provider
Instrument software	e.g. SEPVIEW	e.g. Beckman, LUM
Spreadsheet calculation programme	e.g. Excel	e.g. Microsoft

16.4.1.3 Reagents for sample preparation and measurements

The preparation of reagents needed for the sample preparation of samples in Table 1 can be found in Chapters 3 and 9.

Note: When choosing another liquid than aqueous solution, the user shall take care the material of the cuvette is inert to the liquid. Especially organic and acidic solvents should not be used if the user cannot ensure that the material of the cuvette is chemically resistant against the given solvent.

16.4.2 Procedure

16.4.2.1 Preparation

The preparation of reagents needed for the sample preparation of samples in Table 1 can be found briefly in Chapters 3 and 9. Suspensions suitable for sedimentation measurement must be stable at least in the period between sample preparation and the end of the measurement against formation of agglomerates. The time between sample preparation and inserting the cuvettes has to be as short as possible to avoid the disintegration of the homogeneous particle concentration inside the samples before the measurement has started.

16.4.2.2 Dilution of sample

The sample should have an optical transmission in the range 30 - 60 % with regard to the light source wavelength. Due to concentration effects, the concentration level shall not exceed 0.25 % (v/v). If required, dilution of the sample must be performed with a particle-free diluent of the same refractive index, ionic strength, surfactant concentration, pH etc. as the original dispersing medium. A diluent of different physicochemical properties may change the surface chemistry of the particles.

NOTE: The user must ensure that the beakers and cuvettes are free of contamination or other particles that may contribute or interfere with the measurements.

16.4.3 Performing measurements in Cuvette-AC

In this chapter, the details of the measurement process are described. The user always has to follow the manual and manufacturer's guidelines.

NOTE: Centrifuges have to be maintained according to the manufacturer's instructions. Never use a centrifuge if you are not sure that the centrifuge and the rotor are maintained properly.

16.4.3.1 Set-up

A runnable cuvette centrifuge consists of the cuvettes, an appropriate rotor and the instrument. According to the manufacturer's guidelines the rotor has to be connected to the instrument and communication between the instrument and the computer has to be ensured. The optical devices inside the instrument have to be clean and free of dust. The user shall not touch the surfaces of the cuvettes and inside of the instrument required for measurements.

16.4.3.2 Daily Performance Check

In general, these instruments are calibration-free. Most manufacturers advice the user to check the performance with a reference material annually.

The user should take care that the optical components inside the instrument (laser or LEDs, detectors) are clean and free of dust. If there is dust on the optical components a brush can be used to carefully clean the surfaces. The rotor should rotate easily without any obstacles.

16.4.3.3 Typical running conditions and tune settings for specific samples

Typical tune settings for all samples are reported below:

Operating temperature:	25 °C
Cuvette type:	thin (2 mm for LUMiSizer® cuvettes)
Light factor:	1

NOTE: If there is the possibility for negative stability effects (e.g. with polymers) or special material effects (e.g. hydrogel swelling) or any other reason (e.g. experiments in toxicology studies prefer a temperature of 37 °C), which require a certain temperature range; the user should reflect on temperature, known material properties at the temperature and should ensure stability of the temperature during measurement.

For selection of instrument settings it is required to have estimations on the upper and lower limit of the size distribution. The lower limit may correspond to the size of the constituent particles. The upper limit depends on the state of aggregation and agglomeration. The rotational frequency shall be chosen that it is possible to detect the largest assumed particles. The operating time shall be chosen that the smallest assumed particles are settled down. For NanoDefine the recommended running and measurement conditions are given in Table 6.

Table 6: Recommended running and detection conditions for BaSO₄ NanoDefine samples (IRMM-381 and IRMM-387) relevant for LUMiSizer® instruments

Sample	Description	Detection intervals	Rotational frequency
S1	BaSO ₄ fine grade	250 x 5 s, 250 x 10 s, 250 x 20 s, 250 x 45 s	2000 rpm
S2	BaSO ₄ ultrafine grade	250 x 5 s, 250 x 10 s, 250 x 20 s, 250 x 45 s	3000 rpm

If there is the opportunity of choosing the wavelength, it is recommended to use a wavelength which gives the substance the highest optical contrast to the liquid, which means a high relative refractive index. For many cases this means to measure at rather low values of the wavelength. In case of using the LUMiSizer, a wavelength of 470 nm is recommended.

16.4.3.4 Measurement description

Several prepared samples can be measured at the same time. Subsamples need to be measured at least in three replicates. Recommended are at least six replicates.

The volume needed for measurement shall be looked up in the instrument manual. During the steps of filling and closing the cuvette and inserting into the rotor, it is important that the user does not touch the surfaces foreseen for light transmission. The rotor has to be loaded symmetrically.

A typical sequence for preparation and measurement is as follows:

Equilibrating the temperature of the instrument

Normalisation

Inserting the sample

Measurement of the sample

Removing the sample

Evaluation of results: The instrument software will guide the user and calculate most of the results. This section provides information on the fundamental equations briefly and the required parameters to calculate the results. If the software provides the opportunity to calculate the number based particle size distribution directly from the raw data, the user should follow this wizard. The way of data evaluation is instrument specific. The user shall follow the instrument specific manual provided by the manufacturer. The standard deviation shall be calculated from the repeated extinction based and additionally from the number based median diameter results.

16.4.3.5 Determination of extinction based particle size distribution

For analysis of transmission the Lambert-Beer law shall be applied. For sedimentation, Stokes' law has to be applied to calculate the particle size x from the sedimentation coefficient.

$$x = \sqrt{\frac{18 \cdot \eta_F \cdot S}{\rho_P - \rho_F}}$$

The specific sedimentation coefficient, S , (Svedberg and Rinde 1924) is calculated from the sedimentation velocity, v , and the machine specific parameters of rotational frequency ω and distance r from the centre of rotation to the detection position. This parameter is given in the instrument's documentation.

$$S = \frac{v}{r \cdot \omega^2}$$

The recommended values for the NanoDefine materials and water are given in the table below. If any other temperature is chosen the values for liquid density ρ_F and viscosity η have to be adapted for Stokes' equation.

Table 7: Recommended properties of water at 25 °C

Property	value
Dynamic viscosity η	0.890 mPa s
Density ρ_F	0.997 gcm ⁻³

Table 8: Recommended values for density for BaSO₄ (IRMM-381 and IRMM-387) samples at 25 °C

Sample	Description	Solid density ρ_P [gcm ⁻³]
S1 / S2	BaSO ₄	4.4

NOTE: If it is a porous particle the density ρ_P chosen has to be averaged with the density of the medium ρ_F depending on the porosity. If it is a core-shell-particle the density ρ_P chosen has to be averaged between two materials depending on the mass ration of the materials.

NOTE: If the concentration of the sample in the suspension was chosen above 0.25 % (v/v), a correction by applying a hindrance function is needed, e.g. reported by Richardson and Zaki 1954.

The result is the median Stokes diameter of the extinction based particle size distribution ($X_{50,ext}$).

16.4.3.6 Conversion into number based particle size distribution

The instrument software will guide the user for calculation of the mass based particle size distribution following Mie light scattering theory. The software also will allow the user to calculate the number based size distribution too. The recommended values for the refractive indices (RI) of the NanoDefine materials and water are given in the table below for commonly used wavelengths. The refractive indices are given this way:

$$RI = \text{real part} - \text{imaginary part } i$$

Some templates may also call the imaginary part of the refractive index 'absorption'.

NOTE: The adaption of Mie's solution is associated with assumptions, e.g. spherical and optical isotropic particles.

Table 9: Recommended RI values for BaSO₄ (IRMM-381 and IRMM-387) at selected wavelength

Sample	Description	RI at					
		405 nm	470 nm	530 nm	633 nm	670 nm	865 nm
S1 / S2	BaSO ₄	1.697	1.668	1.652	1.634	1.63	1.617

If the refractive index of the sample is unknown then approximate values may be found in many databases, e.g. the open source database at www.refractiveindex.info.

NOTE: If it is a core-shell-particle the user shall ask the material's supplier on the optical properties of the sample or use an open source data base.

After the step of evaluation the data should be exported to a commonly used file format for spreadsheet calculation such as EXCEL®.

The result is the Stokes' median diameter of the number-based particle size distribution $x_{50,0}$.

Reporting of results

In order to allow full interpretation and reproduction of the measurement results, the analysis report shall include at least the following:

- Information on sample preparation, especially the suspending liquid and volume, and the dispersing agent and its concentration, the method of dispersion including the dispersion time and amount of energy added and net power density
- Information on measurement instrument and applied settings, especially the instrument type, the cuvette dimensions or cuvette identification and the centrifugal speed.
- Information on parameters for data analysis, especially the powder solid density and the refractive index used of the sample and water, temperature and viscosity
- Information on data fitting and correction

and information required by the guidelines of Good Laboratory Practice (date of analysis, laboratory, operator's name, identification of the sample, page numbering, name and signature of person authorising the analysis report, ...).

For validation purposes, the number based median diameter shall be used and additionally the extinction based median diameter reported

16.5 Validation status

The validation parameters of the method were determined successfully regarding the Stokes' diameters $x_{50,0}$ and $x_{50,3}$ for both grades of BaSO₄ particles IRMM-381 and IRMM-387. The working range regarding sample content is from 0.6 gkg⁻¹ – 2.6 gkg⁻¹ for IRMM-381 and 0.6 gkg⁻¹ – 10 gkg⁻¹ for IRMM-387. The upper limits were set with regard to the initial transmission and possible multiple scattering, whereas the lower limits were set due too large uncertainties (RSD) and too large deviations to the expected number-based median diameter. The lower limit is 0.1 gkg⁻¹ by choosing 10 mm cuvettes, but the user should also note that this could lead to other sedimentation flow conditions and use of thicker cuvettes is not recommended in general.

The intermediate precision was determined to 9.7 % for IRMM-381 and 9.4 % for IRMM-387: It should be noticed that the results of the f-tests led to a rejection of the null hypotheses for the

number-based diameter $x_{50,0}$ for IRMM-381 and for the volume-based diameter $x_{50,3}$ for IRMM-387. In the case of the nanomaterial IRMM-387, the average number-based median could be determined certainly and the number-based median results of the 15 measurements are randomly distributed. The determination of the related volume-weighted median diameters does not fulfil the null hypothesis the reason for this could be the presence of rare particles in the sub-micrometre size range caused by erosion of the ultrasonic device and the following sample taking. The user might take samples not the same way every day and therefore decreases the chance of catching the same amount of rare large particles. The user should keep this in mind and handle the results with care if the volume based median diameter is required for any reason. In the case of the non-nanomaterial IRMM-387 this effect changes. The volume-based median diameters of the 15 measurements are randomly distributed, whereas the null hypotheses for the number-based median determination was rejected. Reasons for this are probably related to the sample preparation and the data treatment. The user should take care that the volume specific energy input by de-agglomeration with ultrasonic devices needs to be constant even if different devices of one type are used. In the case of broadly distributed particle size distributions, e.g. IRMM-381, the user should take care to keep a very reproducible procedure on determining the amount of the smallest particles as this fraction is dominating the position of the number-based median.

Tests on trueness, linearity and selectivity could not be carried out for several reasons.

16.6 HSE issues

Centrifuges have to be maintained according to the manufacturer's instructions. Never use a centrifuge if you are not sure that the centrifuge and the rotor are maintained properly.

All laboratory personnel must comply with local safety regulations when working in the laboratory. All personnel must consult the Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in the procedure described here.

Personnel should utilize all necessary precautions to avoid exposure to chemicals and nanomaterials. Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses.

Ultrasonic devices can cause damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations. Each chemical/particulate material should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized.

16.7 References

- ¹ ISO 13318-2:2007 Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 2: Photocentrifuge method
- ² M. J. Weber, Handbook of optical materials. CRC Press, Boca Raton, 2003. (based on: J.H. Weaver, E. Colavita, D.W. Lynch, and R. Rosei, Phys. Rev. Sect. B, 19:3850, 1979) ³ <http://refractiveindex.info/>
- ⁴ Lerche, D., Sobisch, T., 2007. Consolidation of concentrated dispersions of nano- and microparticles determined by analytical centrifugation. Powder Technology 174:46-49
- ⁵ ISO 13318-1:2001 Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 1: General principles and guidelines

17 SOP for analysis of Fe₂O₃ in Polyethylene Matrix with Electron Microscopy methods

17.1 Aim

The aim of this SOP is to provide and determine the sample preparation protocols and quantitative methods for fully automatic particle size distribution (PSD) analysis for Fe₂O₃ nanoparticles embedded in high density polyethylene (PE) matrix.

17.2 Scope

This SOP describes the use of an ultramicrotome (Leica EM UC6) for sample preparation, a transmission electron microscope (TEM) operated in scanning mode (STEM, Hitachi HD-2700) for imaging and NanoDefine ParticleSizer for analysis. The Fe₂O₃ in PE matrix was manufactured by industrial partners and received as small cylinder shaped rods. The dimensions of the rods were ~ 2 mm x 5 mm. The mass ratio of the hematite nanoparticles was 5 % (g/g). The NPs were ~ 40 nm in diameter and were agglomerated into complex 3D structures. The scope of the sample preparation can be extended to any nanocomposite soft material that can be cut by an ultramicrotome; and the analysis guidelines are valid for any complex nanoparticle agglomerates.

17.3 Abbreviations

DF	Dark field
DMSO	Dimethyl sulfoxide
HAADF	High angle annular dark field
PE	Polyethylene
PSD	Particle size distribution
SOP	Standard operating procedure
STEM	Scanning TEM
TEM	Transmission electron microscope

17.4 Description

17.4.1 Materials and methods

Ultramicrotome: Leica EM UC6

Hotplate: Gerhardt Hotplate

TEM: Hitachi HD-2700 200kV

Software:

Digital Micrograph™ script for magnification calibration:

Find Cross grating distance

Online: <http://portal.tugraz.at/portal/page/portal/felmi/DM-Script/DM-Script-Database>

ImageJ plug-in for particle size distribution analysis:

Nanodefine ParticleSizer

Solvents for sample preparation:

MilliQ H₂O: Millipore Advantage A10

DMSO: Analysis Emsure® Acs from EMD Millipore

17.4.2 Performing measurements

17.4.2.1 Sample preparation

The Fe₂O₃ in PE rods were prepared using cryo ultramicrotomy (Leica EM UC6). The rods were first embedded in epon block and trimmed to get a smooth cutting surface. The diamond knife temperature was set to -30 °C and sample temperature -130 °C. Low cutting speed was applied 0.4 – 0.8 mmmin⁻¹. The sections were left floating in a mixture of H₂O/DMSO at 75 °C and then transferred to TEM grids. This procedure results in crushed sections with a wavy structure. The TEM grids were then placed on top of a clean pin mount SEM holder and heated (Gerhardt Hotplate) for 1 h at 120 °C to straighten the sections.

17.4.2.2 Measurement description

The most prominent TEM calibration related to PSD analysis is the magnification calibration. This was done using a standard cross grating sample and a custom written automatic software (Find cross grating distance) for the pixel size calculation. The eucentric height has to be accurately determined such that no large defocus deviation occurs. The microscope should be additionally well aligned (user alignments) for optimized imaging conditions. Dark field (DF) or high angle annular dark field (HAADF) mode is recommended. The magnification should be chosen such that the smallest estimated particles are at least 10 pixels across (here at least 40 kX). The number of images should be chosen such that the PSD contains at least 1000 particles.

17.4.2.3 Evaluation of results

Due to the complex 3D structure of the agglomerates it is recommended to use single particle mode of the NanoDefine ParticleSizer and irregular watershed with high convexity threshold (> 0.9). All images should be visually checked and possible agglomerates should be removed.

17.5 Validation status

This method is not yet validated.

17.6 HSE issues

DMSO: Flammable liquid and vapour. Keep away from hot surface, sparks and other ignition sources. Take precautionary measures against static discharge. Wear protective latex gloves, protective clothing, eyes and face protection.

TEM: The user should be trained and guided to proper and safe usage of a TEM.

Ultramicrotome: The user should be trained and guided to proper and safe usage of an ultramicrotome.

18 SOP for extraction of TiO₂ from Sunscreen for analysis with Electron Microscopy

18.1 Aim & Scope

The aim of this SOP is to describe a mineral charge extraction protocol using solvents applied to cosmetic matrix.

Although allowing the detection of nanoparticles, direct observation of the finished product by EM does not readily give access to particle size distribution. To circumvent this limitation, extraction of particles from the organic matrix, prior to the determination of particle size distribution, by means of EM or other techniques, has been investigated. Three samples were delivered to the NanoDefine consortium: *i* ID 13a is the actual representative sample, containing 4 % NanoTiO₂, with an aluminium salt based surface treatment, as a UV filter, plus: micro-Titanium and Iron oxides for colouring purpose; *ii* ID 13b, is a simplified formula, containing 4 % NanoTiO₂, with an aluminium salt based surface treatment, as a UV filter (same particles as for ID 13a); *iii* A blank formula, without mineral particles (noted ID blank) has been provided. ID 13b and ID blank were delivered to the Consortium for the purpose of helping for the extraction of Nano-particles from ID 13a. The organic components are identical for the three samples.

18.2 Abbreviations

Rpm	rounds per minute
QSF	quantity sufficient for
g	gravitational constant
FFP3	Filtering Facepiece Particles 3
Dry ice	frozen CO ₂
Vortex mixer	allow to mix liquid +solid with vibration
Centrifugal apparatus	allow to separate solid and liquid
Magnetic stirrer	allow to mix liquid and solid with mechanic movement
PTFE	Polytetrafluoroethylene (chemically inert)
SOP	Standard Operating Procedure

18.3 Description

18.3.1 Materials and methods

Centrifugal apparatus: SIGMA supplier/ SIGMA centrifuge 3 K 30/ controlled temperature system (set temperature to 23 °C) / max 28200 rpm / equipped with 12155 rotor 4x 85 mL - max radius 9 cm- min radius 2.1 cm-angle 30°-max speed 20000 rpm- max gravitational 40248 g / AUREAU VERITAS controlled every years

Vial for centrifugal: SIGMA supplier /Polycarbonate tube 85 mL standard screw cap diameter 38x104 mm

Vortex mixer apparatus: Heidolph supplier/ REAX2000 / 2400 x 1/min

Magnetic stirrer apparatus: 2mag magnetic^emotion supplier / MIX15

Rod for magnetic stirrer: PTFE coated ovoid shape in order to fit to the bottom centrifugal polycarbonate tube

Ultrasound bath apparatus: Branson supplier / US Branson 3510E-DTH, 100 W 42 kHz \pm 6 %

Solvents used:

- Absolute Ethanol - VWR Chemicals supplier/ AnalaR NORMAPUR –ref 20821.296

-Water for HPLC - CARLO ERBA supplier / filtered through 0.1 μ m membrane

Freeze dryer apparatus: Thermo Supplier / Lyolab A

Freezing mix: dry ice + acetone (normal quality)

Weighing apparatus: METTLER supplier / AT261 deltaRANGE scale / 205 g to 0.1mg or 62 g to 0.01mg

18.3.2 Sample preparation

18.3.2.1 Sample preparation

Particle extraction protocol:

In a centrifuge tube (60 mL /polycarbonate)

- Add magnetic stirrer (ovoid shape)
- Weigh 5 g of finished product
- Add absolute ethanol (QSF: 50 g)

Cycle 1

- Vortex mixer: 30 seconds
- Magnetic stirrer: 15 min (700 rpm)
- Ultrasonic bath: 15 min
- Centrifugation; 20 000 g (14026 rpm): 15 min
- Remove slowly the liquid phase
- Add absolute ethanol to solid phase residue (QSF: 50 g)

Cycle 2 = Cycle 1

Cycle 3 = Cycle 1

Cycle 4

- Vortex mixer: 30 seconds
- Magnetic stirrer: 15 min (700 rpm)
- Ultrasonic bath: 15 min
- Centrifugation; 20 000 g: 15 min
- Remove slowly the liquid phase
- Add 30 mL of water
- Magnetic stirrer: 5 min (700 rpm)

- Pour into a freeze drying flask (rinsing out with 10 mL of water)
- Freeze the dispersion with a bath full of a mix of dry ice and acetone
- Freeze drying during 12 h.

18.3.2.2 Measurement description

The insoluble fractions have been weighed for the three products, and corresponding percentage determined in mass:

ID 13a: the insoluble fraction corresponds to 11.1 wt% of the initial formula

ID 13b: the insoluble fraction corresponds to 7.7 wt% of the initial formula

ID blank (blank formula): the insoluble fraction corresponds to 3.3 wt% of the initial formula

18.4 HSE issues

Solvent absolute Ethanol: Highly flammable liquid and vapour:

- keep away from hot surface, sparks and other ignition sources - take precautionary measures against static discharge
- wear protective gloves/ protective clothing/ eyes protection/ face protection.

Extractions residue: as residues may contain nanoparticles

- Wear protective gloves/ protective clothing/ eyes protection/ face protection/ mask protective FFP3.
- Place weighing apparatus in a protective area such as an Erlab laboratory hood / Captair flex XLS 392 with 2 filter HEPA UP17

Ultrasound apparatus: use EAR protection

Freezing mix (dry ice + acetone):

- Dry ice: due to the low temperature (-78 °C) wear temperature protective gloves/ protective clothing/ eyes protection/ face protection.
- Acetone: Highly flammable liquid and vapour- keep away from hot surface, sparks and other ignition sources: take precautionary measures against static discharge and wear protective gloves/ protective clothing/ eyes protection/ face protection.

19 Size characterisation of suspended particles by AUC-RI with speed ramp option

19.1 Aim

The aim of this SOP is to determine the number and mass based median particle size, the particle size distribution (PSD) and the mass concentration (C) of suspended micro- and/or nanoparticles, based on the principles of Analytical (Ultra) Centrifugation with Rayleigh interference Refractive Index detection (AUC-RI).

19.2 Scope

This SOP describes the use of the AUC-RI to perform particle size and particle concentration analysis measurements by measuring the refractive index radial profiles during sedimentation by means of Analytical (Ultra) Centrifugation).

Specifically for polydisperse samples or samples of unknown size range, this SOP includes also the optional operation of the AUC-RI in rotor speed ramp mode, or g-sweep.

The SOP was validated for SiO₂ and BaSO₄. In case of BaSO₄, both nano and non-nano forms (median in number metrics either below or above 100 nm) were analysed. The method is applicable to all particles that do disperse but not dissolve in the suspension medium, and which have a density contrast >0.05 gcm⁻³ and refractive index increment >0.01 cm³g⁻¹ in the specific suspension medium.

The technique can also be used to measure the molecular mass of proteins or dissolved macromolecules. However, note that these types of measurements are outside the scope of the current SOP.

The data acquisition part of this SOP specifies parameters for the AUC-RI model 'XLI' from Beckman-Coulter (Palo Alto, USA), currently the only commercial provider of AUC-RI instruments. The basic principles of analytical centrifugation are described by ISO 13318, which covers methods for determining the particle size distributions of particulate materials, by centrifuges other than the AUC-RI, so that the ISO 13318 only covers the size range 0.1 µm to 5 µm, whereas the higher centrifugal acceleration of the Beckman XLI extends the range to smaller sizes down to 1 nm.

19.3 Definitions

As far as possible, terminology follows ISO 13318-1:2001(E) 'Determination of particle size distribution by centrifugal liquid sedimentation methods — Part 1: General principles and guidelines' and terminology developed in NanoDefine..

N = Centrifuge speed or rotational frequency, in units of '1/min'

ρ = skeleton density of the material, in units of 'gcm⁻³' (= true particle density in ISO 13318)

ρ₁ = liquid density of the suspension medium, in units of 'gcm⁻³'

η = viscosity of the suspension medium, in units of 'Pa*s'

s = sedimentation coefficient, in units of 'Svedberg', where 1 Svedberg = 10⁻¹³ sec

λ = wavelength of the RI detector, in nm

l = optical path length in the AUC cell, in m

C = cumulative (mass) concentration, in units of 'mgmL⁻¹'

C_{RI} = total (mass) concentration represented by PSD, in units of 'mgmL⁻¹'

x_{50,3} = Median diameter in volume metrics, in units of 'nm'

x_{50,0} = Median diameter in number metrics, in units of 'nm'

Q₃ = cumulative size distribution in volume metrics

Q₀ = cumulative size distribution in number metrics

PSD = Particle Size Distribution

DLS = Dynamic light scattering

CLS = Centrifugal liquid sedimentation

SHMP = Sodium hexametaphosphate

19.4 Description

19.4.1 Materials and methods

- Beckman XLI or other AUC with RI detector.
- 4-hole (An-60-Ti) or 8-hole (An-50-Ti) analytical rotor
- Double-sector cells, preferably with sapphire windows of 0° oriented optical axis.
- Standard laboratory equipment for sample preparation, filling and cleaning of cells.
- The suspension medium (e.g. water or sodium hexametaphosphate solution) should be of high purity and must be free of particles (e.g., passed through a membrane filter with appropriate cut-off)
- For BaSO₄ in water: Stabilising agent: sodium hexametaphosphate (SHMP)
- Sonication equipment able to deliver at least 30 W sonication power (as measured by calorimetry, Taurozzi et al.¹), for instance:
 - Preferably tip sonicator, e.g. Hielscher UPS200S operated at pulsed mode with an amplitude of 75 % and a cycle time of 50 %, and thus produces a mean absorbed power of 7.8 W, or 1.3 WmL⁻¹.
 - or vial tweeter, e.g. Hielscher 250 W Ultrasonic Processor UIS250v head fitted with VialTweeter sonotrode vial sonicator with an amplitude setting of 75 % and cycle time of 50 %.(Calorimetrically determined power input is 1.0-1.1 WmL⁻¹ mean energy absorbed) or equivalent.
 - or equivalent equipment.
- Software requirements: Sedfit, freeware, available at
 - <http://www.analyticalultracentrifugation.com/tutorials.htm>
- Computer (hardware) requirements to use Sedfit: PC with at least 500 MHz, 256 MB RAM, Pentium or Xeon processor (Cyrix 3 processors may not work). Multi-core processors are supported and can significantly speed up many computations.
- Windows NT, 95, 98, XP, 2000, or Vista, at least 2 MB of disk space.

19.4.2 Performing measurements

19.4.2.1 Operation of the equipment

- As described by AUC-RI operating instructions
- Use an empty double-sector cell, set N to the speed that is appropriate for the specific material (see below: measurement description), open the details menu, select laser setup, adjust laser delay and duration for optimum visibility of fringes. Stop the rotor.

- The recommended criterion for an 'appropriate' speed is a duration of sedimentation within approximately 30 minutes to 2 h. This ensures that the $x_{50,3}$ size is well within the limits of size range detection.
- If the size of the sample is unknown, the speed ramp option can save a lot of time for identification of the appropriate speed. Alternatively, the appropriate speed can be determined iteratively by repeated fixed speed measurements.
- Use 25 °C as measurement temperature.
- To avoid artefacts by sample sedimentation inside the AUC cells due to time-consuming temperature equilibration before data acquisition, ensure that the rotor and cells are at ± 0.1 °C the same temperature as the AUC before filling the cells.

- Only for the *optional* AUC-RI-ramp operation^d, to eliminate the need to select a speed that is appropriate for the specific sample, the speed ramp requires setup parameters. Specifically for the Beckman XLI, this SOP includes predefined setup files:
 - Copy the RampNanoDefine.EQU and RampNanoDefine.SCN files into the XLI software directory.
 - The RI laser delay has to be adjusted once for the local machine. This step is essential for the extended ramp that starts from N=1100 rpm, but is recommended also for the standard ramp from N=3,000 rpm.
 - Select File, select open, select the RampNanoDefine.SCN file.
 - Use an empty double-sector cell, set speed to N=3000 rpm, open the details menu, select laser setup, adjust laser delay and duration for optimum visibility of fringes. Check whether the selected laser delay and duration provide good visibility of fringes also at the highest rotor speed.
 - Select file, select save. The XLI operating software will recall this setting also after restart. Stop the rotor.

19.4.2.2 Sample preparation

The suspension medium (e.g. water) should be of high purity and must be free of particles (e.g., passed through a membrane filter with appropriate cut-off).

- Specifically for colloidal silica the suspension should be analysed without dilution.
- Specifically for BaSO₄, the materials under study are supplied as dry powders which require re-dispersion in aqueous media using an ultrasonic probe prior to use with the AUC. All information necessary to produce suitable samples of IRMM-381 and IRMM-387 for AUC analysis is documented Chapters 3, 9 and 13. Chapter 13 contains details of the procedure which must be followed to determine the correct power settings for a tip (probe) sonicator, while Chapters 3 and 9 of this report details the exact dispersion procedure, including sonication, to be followed for the materials IRMM-381 and IRMM-387. The liquid dispersions (6 ml batches of 2.6 mgmL⁻¹ suspensions) are prepared by mixing defined quantities of dry BaSO₄ powders with water containing 2.0 mgmL⁻¹ of Sodium hexametaphosphate (SMPH), homogenised by vortexing, de-agglomerated using high intensity probe sonication. The resulting dispersion should be diluted with further SMPH solution to produce a final analyte concentration of 1 mgmL⁻¹. The SMPH in the solution is present as an aid to de-agglomeration during sonication and later as a

^d Two files to represent the recommended speed ramp that is applicable to all commercial Beckman XLI instruments (from 3k rpm to 50k rpm, or adapted for specific materials with known size ranges):

- Speedsteps.equ (for data acquisition by import in XLI - EQU interface)
- Speedsteps.txt (for evaluation of data in Sedfit_ramp)

An extended speed ramp (from 1.1k rpm to 55k rpm) that extends the upper detection limit above 1 µm sizes requires an adaptation of the EPROM of the XLI, and is available upon request from BASF Material Physics.

stabiliser. The sonication steps were done using values of ultrasonic power and treatment times which were specific to each material. Table 1 shows the variations in sample preparation parameters for the two BaSO₄ materials.

- The suspensions are stable for at least 1 h.

Table 1: Variations in sample preparation parameters for the two BaSO₄ materials

Sample	SHMP conc.	Tip (probe) sonicator				Vial treater**		
		Temp.	Typical final volume	Power output*	Time	Temp.	Typical final volume	Time**
Non-nano BaSO₄ IRMM-381	2 mgmL ⁻¹	Ice bath	6 mL	10.3 W	20 min	Not controlled	1.7 mL	Time should be optimised for the individual equipment**
Nano BaSO₄ IRMM-387	2 mgmL ⁻¹	Ice bath	6 mL	7.6 W	5 min		1.7 mL	

* The sample preparation SOP requires that prior to conducting any dispersion procedure the operators must determine experimentally the power output characteristics of ultrasonic disruptor which will be used and using this data adjust the power settings of the sonicator to produce an output value which is specified in the procedure. A detailed SOP for the calorimetric determination of the sonicator power output is given Chapter 13 of this document.

** The operator has to have access to a DLS or CLS instrumentation. The dispersion has to be evaluated by DLS or CLS at various treatment times and the results compared with that shown in section 2.7. If the mean size (DLS Intensity based/CLS Mass based) obtained is significantly larger (>15 %) than that shown in section 7 of this SOP the sonication process should be repeated with a fresh sample but increasing the sonication treatment time by 5 min. This step should be repeated until additional increases in sonication time produce no further decrease (or increase) in mean size.

If applied to other materials with specific sample preparation protocols, these must fulfil the following requirements:

- The suspension medium can be water, optionally with dispersing agents, but can also be organic solvents.
- The resulting samples must be at least 0.5 mL volume of particle suspension in a medium of known density ρ_1 and known viscosity η . The particles should be suspended at a concentration in the range of 0.1 mgmL⁻¹ to 10 mgmL⁻¹. The validation showed that the widest size range can be covered at a concentration of 1 mgmL⁻¹.
-
- → fill 380 μ L \pm 10 μ L of sample into the sample sector of the double-sector cell.
- → fill 380 μ L \pm 10 μ L of suspension medium without particles into the reference sector of the double-sector cell
- Matching volumes will match the meniscus so that solvent compression is identical on both sides, thus reducing baseline tilt that would lead to artificial signal around s_{min} .
- For the conventional operation at fixed speed, it is possible to fill 400 μ L \pm 10 μ L into the reference sector of the double-sector cell. The solvent compression is not an issue at fixed speed and the mismatch of the menisci is advantageous in the data fitting to locate the position of the meniscus visually.
- *Optionally*, the medium in the reference cell is not pure water or pure solvent, but contains the same concentration of dispersing agents as the sample. The option can help to exclude any ambiguity of the assignment of components potentially observed

at low sedimentation coefficients, because the AUC-RI does detect surfactant micelles as separate component.

→ seal cells and insert the cells into the rotor, insert rotor into AUC instrument, activate evacuation and temperature equilibration.

- avoid times longer than 30 minutes until data acquisition due to potential sedimentation of large particles onto the lower cell window, potentially leading to artefacts.

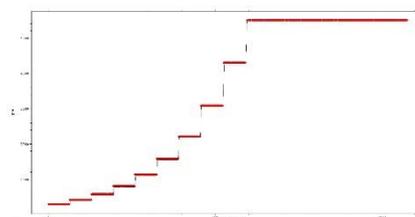
19.4.2.3 Measurement description

- AUC-RI is an absolute technique, requiring no signal response calibration by means of a particle size standard. As specified by the manufacturer, the magnification factor of the optical detection system must be calibrated by means of the counterbalance cell.
- Perform three measurements from three sample preparations, i.e. one analysis per prepared suspension.
- For the conventional operation at fixed speed, the appropriate speed needs to be verified by comparing the Q_3 (fixed speed) to a Q_3 (speed ramp): The $x_{50,3}$ values should match within 20 %.
 - Specifically for colloidal silica, the indicative speed is 15,000 rpm
 - Specifically for BaSO₄, the indicative speed is between 1,000 rpm (detection range 20 nm to 2 μm) and 6,000 rpm (detection range 2 nm to 200 nm).
 - The recommended criterion for an 'appropriate' speed is a duration of sedimentation within approximately 30 minutes to 2 h. This ensures that the $x_{50,3}$ size is well within the limits of size range detection. A complete fixed speed measurement generates between 50 and 500 scans, typically around 150.
- For the *optional* speed ramp operation: The present ramp is intended for the measurement of 2 samples in the same run, using either a 4-hole or 8-hole rotor.
 - The detection range is at least 5 nm to 1 μm for BaSO₄, or wider, depending on meniscus matching (19.4.2.2), g(s) truncation (19.4.3.3) and extended ramp (19.4.2.1).
 - In principle, up to 8 samples can be measured simultaneously in a single run, if the ramp is adapted accordingly with fewer RI scans per speed step.

→ Select *File*, select *open*, select the RampNanoDefine.SCN file. The software will now load the predefined ramp from the RampNanoDefine.EQU file. Verify by selecting *Method*

For *XL settings* → *Speed*, enter the final speed of the ramp (50,000 rpm).

→ *Start Method Scan*. Data acquisition is finished after 3 h. The long running time at the final speed ensures that particles down to 1 Svedberg (around 1 nm diameter) are detected. The power-law shape of the ramp is optimal for homogeneous information content across the entire size range.^e The specific ramp was developed for NanoDefine applications.



Data is stored in #.IPn files, with # the running number of scans, and n indicating the cell number. Each file represents the radially resolved interference fringe shift between

^e Ma J, Zhao H, Sandmaier J, Liddle JA, & Schuck P (2016) Variable field analytical ultracentrifugation: Gravitational sweep sedimentation velocity. Biophysical journal 110(1):103-112.

sample cell and reference cell. The RampNanoDefine.EQU file is designed to acquire a scan about every 15 seconds (depending on the specific machine), resulting in a number of 500 scans per cell per measurement.

19.4.3 Evaluation of results

19.4.3.1 Conversion from interference fringe shifts to sedimentation coefficient distribution

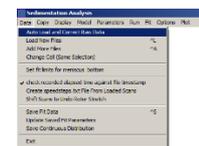
For generic introduction to AUC evaluation by the Sedfit software, refer to <http://www.analyticalultracentrifugation.com/tutorials.htm>

Specifically for evaluation of fixed speed measurements, use Sedfit Version 14 or later (download at <http://www.analyticalultracentrifugation.com/download.htm>)

- Choose -> *data* -> *Load new scans*; *Load* all scans 1 to 500
- Investigate raw data critically and load the appropriate number of scans (1 to n) again based on the remaining signal at the centre of the cell (0.05 fringes at mid-cell is considered as limit below which noise dominates). High number of measurements containing no information on sedimentation but contributing to noise decrease the quality of fit and increase analysis time.

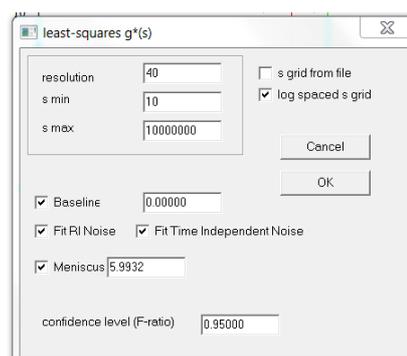
Specifically for evaluation of speed ramp measurements, the methodology is described in literature^e.

- Use version 'sedfit-lsgofs.exe'. The algorithm is described in the publication, but the specific version was developed for polydisperse distributions in NanoDefine cooperation.
 - Request a copy from Peter Schuck, NIH Bethesda, USA.
 - Unlike conventional XLI operation at fixed speed, the meniscus will shift between scans. This is not an indication of leaking cells, but is due to rotor stretching and solvent compression, and is corrected by the software.
- Choose -> *data* -> *Auto Load and Correct Raw Data*
- *Load* all scans 1 to 500; choose rotor (4-hole or 8-hole); close by ESC the ramp graphics. Investigate raw data critically and re-load the appropriate number of scans (1 to n) again based on the remaining signal at the centre of the cell (0.05 fringes at mid-cell is considered as limit below which noise dominates). High number of measurements containing no information on sedimentation but contributing to noise decrease the quality of fit and increase analysis time.



For both conventional fixed speed and the optional speed ramp, proceed to fitting:

- Choose *model* ls-g*(s) ('least squares fitting of sedimentation coefficient distribution')
- Set *parameters*
 - For fixed speed: start with range $s_{\min}=1$ to $s_{\max}=10^7$ Svedberg, resolution = 40. Reduce s_{\max} if SedFit alerts that the upper detection limit is exceeded.
 - For speed ramp: $s_{\min}=1$ Svedberg; $s_{\max} =$



- 10^7 Svedberg, resolution = 40
 - Select log spaced s grid
 - Set apparent meniscus and bottom, set meniscus fitting boundaries.
 - Set the fitting range to use the entire length of the cell, excluding approx. 0.3 mm from the apparent meniscus, and approx. 1 mm from the apparent bottom.
- Perform '*fit*'. It will result in a low-resolution $ls-g^*(s)$, only serves to find correct meniscus.
- Do not change the position of meniscus, but increase the *resolution* to 250
- Perform '*run*'. It will result in a high-resolution $ls-g^*(s)$ distribution.
- Do NOT close the program, but proceed to quality control checks.

19.4.3.2 Quality control checks (still within the Sedfit evaluation software)

- Select *Display*, select *Subtract all systematic noise*, critically check residuals.
 - If residuals of the early scans show jumps of interference fringes within the scan, the turbidity of the sample was too high for a useful interference pattern.
 - '*delete scan*', knowing that early scans represent larger particles.
 - Alternatively, repeat measurement at lower particle concentration.
 - If residuals diverge at the bottom, outside the fitting range, do not worry.
 - If residuals diverge at the meniscus, e.g. by an apparent baseline drift, this influences the distribution at lower s -values. Check meniscus settings. Check meniscus matching between sample and reference (only a single meniscus visible).
 - If residuals show a wavy pattern, this might be due to turbulence by insufficient temperature equilibration.
 - If residuals are 'jigsaw pattern exceeding 20 % of signal', the s_{min}/s_{max} range can be adapted for optimal resolution of the PSD (although the actual median is not influenced significantly):
 - *RUN* repeatedly, reducing s_{max} from 10^7 to 10^6 , 10^5 , 10^4 Svedberg until the $ls-g^*(s)$ distribution extends around $s_{max}/10$.
- Optionally, save a screenshot to document the residuals.
- Select *Data*, *Save continuous distribution*, default file name is 'newdat.dat', can be changed.

The resulting file is the distribution of sedimentation coefficients $g^*(s)$ in volume metrics, which is the intrinsic metric of the RI detector.

19.4.3.3 Conversion from sedimentation coefficient distribution to number metric distribution

- Load $g(s)$ distribution from the 'newdat.dat' file into a suitable software, e.g. excel.
- Optionally, truncate the lowest range of s values as appropriate. This serves to remove artifacts from baseline drift, but of course it reduces the detectable size range.
 - Appropriate truncation ranges can be identified by measuring pure suspension medium with zero particle concentration.

- Often, truncation of the lowest s-decade can be appropriate, e.g. if $s_{\min}=1$ Svedberg, delete g(1 Svedberg) to g(10 Svedberg).
- Convert each sedimentation coefficient s_i to particle diameter D_i . Typically (ISO 13318), this conversion assumes spherical shape with homogeneous density and applies the Stokes equation:

$$D_i = \sqrt{\frac{18\eta s_i}{\rho - \rho_1}}$$

- For other shapes, specific relationships between s and D can be calculated based on frictional drag and centrifugal forces, but are beyond the scope of the present SOP. (see Wohlleben, J Nanopart Res. 2012, 14:1300)
- To generate a size distribution, integrate the g_i values to C , knowing the refractive index increment dn/dc . Please note that if dn/dc is unknown, this has no influence on the median diameters!

$$C_i = C_{i-1} + g_i \frac{B}{dn/dc}$$

- Specifically for a wavelength of the RI detector of $\lambda=675$ nm and an optical path length of $l=12$ mm through the AUC sample cell (XLI standard parameters), the integration parameter $B=0.05625$. It is calibration-free and scales linearly with wavelength and inversely with optical path length.
- The cumulative size distribution in volume metrics is given by the D_i and C_i columns. Due to the measurement principle, D_i are the end-point, not mid-point intervals.
- The $C_{RI}=C_{\max}$ value is the actual concentration of particles represented by the PSD, in units of mgmL^{-1} .
- Normalize and read at $Q_3 = 50$ % the median diameter in volume metrics, $x_{3,50}$. Analogously, read $x_{3,10}, x_{3,90}$.

$$Q_{3,i} = \frac{C_i}{C_{RI}}$$

- As optional fitting-free cross-check, convert the raw data fringe shift Δj to absolute concentration.

$$C_{RI,fitfree} = \frac{\lambda \cdot \Delta j}{\frac{dn}{dc} \cdot l}$$

- By differentiation of Q_3 , obtain q_3 .

$$q_{3,i} = \frac{Q_{3,i} - Q_{3,i-1}}{\log(D_i/D_{i-1})}$$

- Convert to Q_0 , assuming a diameter – mass relation (typically spheres).

$$Q_{0,i} = Q_{0,i-1} + \frac{g_i}{\frac{\pi}{6} 10^{-21} \rho D_i^3}$$

- Normalize and read at $Q_0^{norm} = 50\%$ the median diameter in number metrics, $x_{50,0}$.
- $Q_{0,i}^{norm} = \frac{Q_{0,i}}{Q_{0,max}}$
 - By differentiation of Q_0 , obtain q_0 .

$$q_{0,i} = \frac{Q_{0,i} - Q_{0,i-1}}{\log(D_{0,i}/D_{0,i-1})}$$

19.4.4 Quality control checks based on concentration

- Does $Q_{3,max}$ match the concentration of particles in sample preparation?
 - If $Q_{3,max}$ is more than 10 % higher than the concentration of particles in sample preparation, either the dn/dc is incorrect (which has no consequences on the PSD or on the classification by the EC nanodefinition), or there is considerable adsorption of dispersing agent onto the particles (which might distort the PSD)
 - If $Q_{3,max}$ is more than 10 % lower than the content of particles in sample preparation, the PSD might not be representative for the material, because significant parts of the material have dissolved or have diameters larger than the upper detection limit.

19.4.5 Reporting of results

- PSD reporting:
 - plot Q_0^{norm} (unitless, normalized to 1) and q_0 in one graph over a log Diameter axis in nm.
 - Plot C (in $mgmL^{-1}$) and q_3 in one graph over a log Diameter axis in nm.
- Report $x_{50,0}$ in nm.
- Report $x_{50,3}$ in nm and C_{RI} in $mgmL^{-1}$

19.5 Validation status

Validation of the AUC-RI method showed that the method is able to properly identify as nano/non-nano materials the tested nano $BaSO_4$ and SiO_2 and non-nano $BaSO_4$ samples. Trueness of the method was not investigated because of the lack of appropriate reference material. Thus, relative measurement uncertainty was calculated as a combination of intra-day and day-to-day variation related uncertainties. The resulting standard measurement uncertainty values determined for $x_{50,0}$ and $x_{50,3}$ fall in the expected range (below the 20 %target uncertainty) and were below 12 % for all three test materials.

The total observed concentration C_{RI} (synonymously designated as $Q_{3,max}$) was significantly reduced by imperfections of sample preparations from powders. In contrast, for ID-18 as ideally pre-dispersed sample we observed $C_{RI} = 96\%$ of the specified concentration, with a relative measurement uncertainty of 5.9 %. This is an acceptable performance.

The method is robust for temperature changes in the +/- 1 °C range.

The working (particle size) range of the fixed speed experiments was appropriate for the characterisation of the samples. The extended ramp method improved the detection of both smaller and larger particles. The standard ramp program failed to detect larger particles in case of IRMM-381 showing that rotation speed is a very sensitive parameter of the AUC-RI method. The

option of the SOP (first ramp measurement to identify suitable speed, then decisive measurement at (this) fixed speed) is thus seen as most robust implementation.

19.6 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult Safety Data Sheets (SDS) to be aware of known hazards and exposure limits relevant to all chemical substance used in the procedure described here.

Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can produce damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

AUC Cells may be deteriorated in aggressive suspension medium, leading to leakage. The producer Beckman provides a compatibility table to verify that the cells are suitable for the solvent and pH.

The Beckman XLI is an *ultracentrifuge* (AUC), and as such some countries (e.g. Germany) require yearly inspection in disassembled state by qualified personnel (typically the Beckman service engineer). Rotors are recommended to be used only for a period of ten years by the manufacturers.

19.7 References

¹ Taurozzi, J. S., Hackley, V., Wiesner, M. Preparation of nanoparticle dispersions from powdered material using ultrasonic disruption. National Institute of Standards and Technology. 2012. Available at: <http://nvlpubs.nist.gov/nistpubs/specialpublications/nist.sp.1200-2.pdf>

20 Particle size distribution measurement of BaSO₄ using Line-Start Disc Centrifuge with Optical Detection

20.1 Aim

The aim of this SOP is to determine the number and mass based median particle size and the particle size distribution (PSD) of suspended micro- and/or nanoparticles, based on the principles of line-start Centrifugal Liquid Sedimentation (CLS) with optical detection.

20.2 Scope

This SOP details methods for determining the particle size distribution of particulate materials by means of centrifugal sedimentation in liquid using optical detection and quantification. Specifically, this SOP refers to the use of a line start disc centrifuge (CPS UHR24000 disc centrifuge) with optical detection by a light beam from a 405 nm laser diode light source. The methods are applicable to liquid dispersible powders in which all particles have the same density and comparable shapes and do not undergo chemical or physical change in the suspension liquid. It is necessary that the particles have a density higher than that of the liquid used in the density gradient. This SOP is primarily intended for the determination of the particle size distributions of the fine and ultrafine BaSO₄ materials IRMM-381 and IRMM-387. Depending in the chosen operating speed measurement size range for BaSO₄ particulates should be range from 2 µm to 70 nm (8000 rpm) and from 800 nm to 30 nm (16000 rpm).

20.3 Terms, definitions and symbols

As far as possible, terminology follows ISO standards listed in Table 4 below.

Table 4: ISO standards relative to centrifugal liquid sedimentation methods

Reference	Title
ISO 13318-1:2001	Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 1: General principles and guidelines
ISO 13318-2:2007	Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 2: Photocentrifuge method

Abbreviations

AC	Analytical centrifuge
AUC	Analytical Ultra Centrifuge
CLS	Centrifugal liquid sedimentation
Disc-AC	Line-start Disc-centrifuge
DLS	Dynamic Light Scattering
EtOH	Ethanol
NM	Engineered Nanomaterial
NP	Nanoparticle(s)
PQ	Performance qualification

PQ	Performance Qualification Test
PS	Probe Sonicator
PSD	Particle Size Distribution
PVC	Polyvinylchloride
RI	Refractive index
SHMP	Sodium HexaMetaPhosphate
SOP	Standard Operating Procedure
VS	Vial-sonicator also referred to as Vial-tweeter
USB	Ultrasonic Bath
USP	Ultrasonic Probe

20.4 Description

The general measurement principles of determining the particle size distribution by line-start centrifugal liquid sedimentation (CLS) are described in ISO 13318 which is applicable to powders that can be dispersed in liquids, colloidal suspension of solid particles and some emulsions. In particular the use of disc centrifuge with optical detection is detailed in ISO 13318-2. The method is applicable to powders in which all particles have the same effective density and comparable shapes and do not undergo chemical or physical change in the suspension liquid. It is usually necessary that the particles have a density higher than that of the liquid of the density gradient.

Depending on the density of the particles, and at a rotational speed of 18000 rpm to 20000 rpm, the working range of the disc centrifuges and cuv2ette centrifuges as considered in ISO 13318-2 covers the size range of approximately 0.1 μm to 5 μm . At higher rotational speeds (e.g. 24000 rpm) the lower limit of the working range can be 20 nm or lower.

In the disc-sedimentation version of CLS, the instrument is based on a hollow transparent rotating disc containing a liquid of increasing density into which a small volume of dispersed particles is injected and then undergo forced sedimentation through centrifugal force. By the use of suitable detectors it is possible to measure the sedimentation rate and to determine the particle size distribution. In the Disc-AC method, which is more correctly known as 'Line-start CLS', particles are injected into the centre of the rotating disc. Once the particles enter the density gradient they sediment radially outwards at a speed which is a function of their density and Stokes diameter. At a certain point in time the particles pass through a narrow beam of light which shines through a region near the outside edge of the rotating disc. As the particles pass through the light beam, the amount of light transmitted to the detector decreases due to absorption and scattering (extinction) by the particles. From the time of sample injection the variation in light extinction is continuously recorded as a function of sedimentation time. During a measurement sequence the method parameters such as sedimentation distance, refractive index, density and viscosity of the density gradient do not always remain constant. Since the true values of these method parameters cannot be easily assessed manufacturers of the major Disc-AC instruments recommend performing a calibration measurement prior to each sample measurement. Such calibration must be done with monodisperse particles of which their size and effective density are accurately known. The light extinction-weighted particle size distribution can be converted by the operating software into a mass-weighted particle size distribution. This conversion, which is based on the application of Mie light scattering theory, requires that the complex value of the particle refractive index is accurately

known. Finally, a number-weighted distribution can be calculated from the mass-based distribution using the particle density and geometric and shape factors as input parameters.

The following procedure is primarily designed for the determination of particles size distributions of IRMM-381 fine and IRMM-387 ultrafine grade BaSO₄ materials of the NanoDefine project. The selection of the instrument parameters have been chosen to meet the following criteria.

- The rotational speed should be chosen such to ensure that the sedimentation time for the smallest particles expected in the sample does not exceed 30 minutes. At longer measurement times baseline drift can become significant and in that case a subtraction of the baseline may be needed to allow reliable determination of the PSD.
- Throughput times for the full measuring cycle should be approximately 60 minutes. The full measurement cycle includes the calibration step, the sample measurement step and an additional rest period of 20-30 minutes to allow the sedimentation of any potential residual fine particulates, of which their sizes are below the detection limit, and to re-stabilise the density gradient.
- The rotational speed should be chosen such to ensure that the sedimentation time for the largest particles in the sample is not less than 0.5 s.

On the basis of these criteria and the expected sample size ranges of the two materials, rotational speed values of 8000 rpm and 16000 rpm have been considered suitable for the IRMM-381 and IRMM-387 materials respectively as detailed in Table 5.

Table 5: Estimated particle sizes at selected sedimentation times and rotational speeds*

Speed	Particle size at t=0.5 s	Particle size at t=30 min	Particle size at t=60min
8000 rpm	2000 nm	30 nm	20 nm
16000 rpm	800 nm	15 nm	10 nm

* Assuming operation with sedimentation gradient as described in section 20.5.4.5

20.5 Materials and Methods

20.5.1 Instruments and Equipment

- Disc centrifuge with optical detection and line-start capability. e.g. CPS UHR24000, DC20000 or DC18000 disc centrifuges, or equivalent.
- Laboratory scale analytical balance with maximum load greater than 100 g and a readability of ±0.1 mg or better.
- Variable volume pipettes 1-20 µl, 20-200 µl, 100-500 µl, 100-5000 µl,
- A variable power probe sonicator operating at 22.5 kHz (please report deviating frequencies), and equipped with a probe with a recommended tip diameter of approximately 6-7 mm. The sonicator should have a nominal declared acoustic output at least 100 W. A protocol describing the recommended procedure for measuring the effective acoustic energy output characteristics of a probe sonicator is detailed in Chapter 13 of this document.
- A chemical fume hood or laminar air flow cabinet configured for sample and user protection
- High purity (resistivity 18.2 MΩcm⁻¹).filtered water (0.1 µm or 0.2 µm filter) water; thermally equilibrated to fume-hood air temperature.
- Digital thermometer with metal sheathed thermocouple probe capable of a measurement accuracy better than ±0.1 °C.

- Digital timer capable of measurement accuracy better than ± 1 s.

20.5.2 Chemicals and consumables

- 2 ml plastic microcentrifuge tubes with sealing lid for use with vial-sonicator
- Disposable graduated plastic syringes (1 ml) with flat ended needle
- Disposable plastic spatula for weighing of NM.
- Disposable anti-static plastic weighing boats or similar for weighing of NM and any chemicals in powder form.
- Disposable powder-free nitrile gloves.
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including NMs.
- High purity filtered water (e.g. MilliQ from Millipore Corp.; resistivity $18.2 \text{ M}\Omega\text{cm}^{-1}$ at $25 \text{ }^\circ\text{C}$, $0.2 \text{ }\mu\text{m}$ in-line filtration).
- Sodium hexametaphosphate powder (CAS No. 68915-31-1, purity $\geq 96 \%$, e.g. 305553 Aldrich).
- Sucrose
- Dodecane
- Aqueous particle size calibration standard suitable for use with Disc-AC

20.5.3 Sample dispersion

The BaSO_4 materials under study are supplied as dry powders which require re-dispersion in aqueous media prior to use with the Disc-AC. All information necessary to produce suitable samples of IRMM-381 and IRMM-387 for Disc-AC analysis is documented in Chapters 3 and 9.

The liquid dispersions (6 ml batches) are prepared by mixing defined quantities of dry BaSO_4 powders with water containing 2.0 mgml^{-1} of Sodium HexaMetaPhosphate (SMPH), homogenised by vortexing, de-agglomerated using high intensity probe sonication. The resulting dispersion should be diluted with further SMPH solution to produce a final analyte concentration of 1 mgml^{-1} . The SMPH in the solution is present as an aid to de-agglomeration during sonication and later as a stabiliser. The sonication steps were done using values of ultrasonic power and treatment times which were specific to each material.

Chapter 13 contains details of the procedure which must be followed to determine the correct power settings for a probe sonicator while Chapters 3 and 9 of this report details the exact dispersion procedure to be followed for the materials IRMM-381 and IRMM-387. The optimum sonication times quoted in Table 6 were determined experimentally by comparing Disk-AC measured particle size distributions of samples treated for times ranging from 1 to 60 minutes at the quoted power output values. The sonication times were chosen to correspond with the minimum time beyond which no further significant decrease in mean particle size could be detected by Disc-AC. As the efficiency of sonication can vary significantly with instrument type, probe geometry and sample volume it is strongly recommended that Disk-AC be used to verify that the particles size distribution (maximum in the weight distribution) achieved using the quoted sonication times is within 10 % of those noted in Table 6. In the case that the particle size obtained is significantly higher than the quoted values, other samples should be prepared using longer and shorter sonication times and analysed to determine the sonication time which produces the minimum mean particle size in the shortest time.

Table 6: Summary of dispersion parameters for each material

Material	Material concentration (sonication)	Material concentration (AC analysis)	Surfactant	Batch volume	Sonication power used*	Sonication time (approx.)	Disc-AC determined size**
IRMM-381	(2.6 mgmL ⁻¹)	(1 mgmL ⁻¹)	SMPH (2 mgmL ⁻¹)	6 mL	10.3 W (1.8 WmL ⁻¹)	20 min	480 nm
IRMM-387	(2.6 mgmL ⁻¹)	(1 mgmL ⁻¹)	SMPH (2 mgmL ⁻¹)	6 mL	7.6 W (1.26 WmL ⁻¹)	5 min	70 nm

* Chapter 13

** Particle diameter size corresponding to the peak maximum in the weight based particle size distribution.

20.5.4 Instrument Operation

General operation of the instrument is detailed in manufacturer's instruction manual.

20.5.4.1 Preparation

For maximum stability of the optical system it is recommended that the instrument is powered-up at least 1 hour before attempting to make any measurement. After having injected the density gradient solutions, an equilibration period of at least 30 minutes must be applied. Furthermore, as the interior of the instrument, including the disc and its density gradient, heats up due to the friction between the rotating disc and the air. the instrument should be allowed to operate for sufficient time as to allow a stable temperature to be reached in the enclosure of the disc. This temperature should be verified and any temperature sensitive parameters used by the instrument software adapted to the observed temperature.

20.5.4.2 Choice of the rotational speed

The rotational speed shall be chosen so that it is possible to detect the smallest expected particles, and respect maximum and minimum times as noted above in section 20.4. This may be done using either the instrument software or from first principle using Stokes' law to estimate the sedimentation time. For measurements undertaken using disc-centrifuges by CPS instruments (e.g. CPS UHR24000, DC20000 or DC18000 disc centrifuges) rotational speeds of 8000 rpm and 16000 rpm are recommended for IRMM-381 and IRMM-387 respectively. For alternative instruments the operator should choose speeds appropriate to the particle size ranges noted in Table 7.

20.5.4.3 Instrument input variables and limitations

The correct operation of the instrument and subsequent data treatment requires the preparation of measurement procedures containing a series of parameters relevant to the sample materials and the measurement condition. For the two materials, IRMM-381 and IRMM-387, the input variables used for the two materials and their respective procedures are listed in Table 7 together with the expected approximate upper and lower size limits.

Table 7: Instrument procedure parameters and range limits

Parameter	IRMM-381 BaSO ₄	IRMM-387 BaSO ₄
Preferred rotational speed	8000 rpm	16000 rpm
Particle density [g/cm ³]	4.4	4.4
Particle refractive index	1.697	1.697
Particle absorption value	0	0
Non-sphericity factor	1	1
Sedimentation gradient	Aqueous sucrose gradient (a) 8-24 wt% or (b) 0-8 wt%	Aqueous sucrose gradient a) 8-24 wt% or (b) 0-8 wt%
Mean density of gradient	(a) 1.045 gmL ⁻¹ or (b) 1.007 gmL ⁻¹	1.045 gmL ⁻¹ or 1.007 gmL ⁻¹
Refractive index at optical detector	(a) 1.357 or (b) 1.344	(a) 1.357 or (b) 1.344
Viscosity of gradient	(a) 1.2 cps or (b) 1.0 cps	(a) 1.2 cps or (b) 1.0 cps
Estimated measurement range limits		
Upper size limit (t = 0.5 s)	2 µm	0.8 µm
Lower measurement limit (t = 30 min)	30 nm	15 nm
Minimum diameter (t = 60 min)	20 nm	10 nm

20.5.4.4 Calibration

A particle size standard is used to perform a calibration run before each unknown sample is analysed. This calibration step is an integral part of the material specific measurement procedure which must have been pre-defined in the instrument software before starting any measurement. When the measurement procedure is initiated the instrument software obliges the operator, as a first step, to perform a calibration run using a suitable particle size standard. The data obtained from the calibration run is then automatically used by the instrument software to elaborate the particle size distribution of the unknown sample which is measured in the second step of the pre-defined material specific procedure.

When using aqueous liquid gradients, a PVC standard of known particle size and particle density can be used for calibration. The particles of the calibration standard should be spherical and have a narrow monomodal size distribution. The instrument procedure requires that the following parameters (typically from the calibration standard certificate) be included in the material specific measurement procedure.

- a) Peak diameter
- b) Half height peak width
- c) Particle density

20.5.4.5 Preparation of the aqueous sucrose gradient (8-24 % or 0-8 %)

The disc-AC operates with fluids that can be used to form a density gradient inside the disc. These fluids can be dilute solutions of sucrose in high purity water (resistivity $18.2 \text{ M}\Omega\text{cm}^{-1}$ at $25 \text{ }^\circ\text{C}$, $0.2 \text{ }\mu\text{m}$ in-line filtration) possibly with a very low concentration of surfactant (SHMP). A density gradient is built-up in the rotating disc by sequentially injecting a series of sucrose solutions with different, decreasing sugar concentrations as described below. Finally, a small volume of a water immiscible, low density, oil (e.g. dodecane) is injected into the disc to produce a thin film on top of the aqueous layer which acts as barrier to evaporation of the water. The density gradient created in this fashion serves to stabilise sedimentation of particles improving accuracy and reproducibility of the results. To prepare a gradient the following procedure can be used.

- prepare two solutions of sucrose with the maximum and minimum concentration to be used in the gradient (typically 8 wt% to 24 wt% or 0 wt% to 8wt%).
- prepare a rack of 9 empty centrifuge vials of volume 2 mL
- add suitable volumes of the two sucrose solutions to the vials so as to produce in each a volume of (typically) 1.6 mL and concentration which are evenly distributed between the maximum and minimum e.g. 8 wt%, 10 wt%, 12 wt%,20 wt%, 22 wt%, 24 wt%.
- insert the disc closure cap correctly and close the security door of the disc centrifuge
- inject the 1.6 mL of the highest density solution into the disc
- Set the desired rotational speed and start the instrument. Wait till the instrument reaches constant speed. (If the disc is accelerating while injecting the sucrose solutions, the gradient will be disrupted.)
- Inject 1.6 mL of each different sucrose solution in order of decreasing concentration. i.e. 22 wt%, 20 wt% ... 8 wt% etc.
- inject 0.5 mL of dodecane in the disc as a cap fluid to reduce water evaporation
- leave the gradient to stabilise for 30 minutes before injecting samples

20.5.4.6 Performance Qualification (PQ) Test

It is recommended that each time a new sucrose gradient is prepared at least one measurement of a known material be undertaken to check that the sedimentation gradient has been correctly formed and is stable. Comparison of the size distribution with previous performance evaluation measurements should be made to verify the correct operation of the system. The choice of test materials for use in the PQ is at the discretion of the test laboratory provided it is compatible with the test material (BaSO_4) and stabiliser (SMPH) being used in the trial.

20.5.4.7 Operation of CPS Disc centrifuge

- Select a predefined operating procedure for the material and gradient to be used.
- Select 'Operate Analyser', and follow the on-screen instructions as follows
- Introduce sample ID, then click on Start
- Wait till instrument completes measurement of background and requests the injection of the calibration standard
- Inject an appropriate, known, volume of the of the standard ($100\text{-}200 \text{ }\mu\text{L}$) with a 1 mL syringe in the disc and simultaneously press the space bar to start the data acquisition for the calibration standard.

- After completion of the calibration run, inject an appropriate, known, volume (100-200 μL) of sample with a 1 mL syringe in the disc and simultaneously press the space bar to start the data acquisition for the sample. The measurement must be left to run until either the procedure is completed or the measurement is terminated manually by the operator
- Once the measurement is finished, click on Next Sample before starting a new measurement.
- Before starting a new sample it is important to ensure that sufficient time passes that all the particles from the previous sample have passed through the field of the optical detector.
- If no new measurement has to be carried out, stop the disc by clicking on STOP on the main menu and wait until the safety interlock opens confirming the disc has stopped rotating and the disc may be accessed for cleaning.
- The gradient fluid in the disc may then be carefully removed by suction through a thin plastic tube using either a syringe or a suitable liquid pump.
- After removal of the disc closure cap, the liquid chamber should then be thoroughly rinsed using clean water (with trace or surfactant) while rotating the disc by hand. The rinsed water should be removed by suction and a piece of soft non-abrasive paper tissue (optical wipe) should then be inserted into the chamber until contacting the outside edge and slowly rotated to remove any residual liquid in the chamber. The above step of rinsing and wiping should be repeated at least once with pure water and finally done using ethanol or ethanol/water mix before final drying with a soft tissue.
- The exterior front and back faces of the disk should be carefully wiped using a moistened soft tissue or cloth to ensure that the surfaces is clean of dust or other residues such as sucrose particularly in the region where the detector light beam passes.

20.5.4.8 Reporting

The data shall be presented in graphical and tabular form. The report should contain at least the following data

- identification of the sample
- the date of test,
- identification of the operator and the testing institute and a unique report identification
- Information on sample preparation, especially the suspending liquid, its temperature, density, viscosity and volume, and the dispersing agent and its concentration, the method of dispersion including, where used, the sonication time and specifications of the sonication device.
- Information on measurement instrument and operational settings, especially the gradient used and the rotational speed.
- Information on the calibration and PQ materials used
- Information on parameters for data analysis, especially the effective density and the complex refractive index
- Information on any instrument defined software operations for data smoothing or compensation of baseline shift.
- Measurement data files: Where the instrument software permits, the participant laboratories are requested to make available data files (in electronic format) containing the measured particle size distributions according to one of the following 2 options

Data Export Option 1: Export the data of each sample analysis data files as ASCII text file containing the following fields (Sedimentation Time, Optical Signal, Diameter, Weight, Number). If any field is not available this should be stated in the report.

Date Export Option 2: For users of CPS disc-centrifuge instruments a copy of the complete sub-directory of the measurement procedures used should be made available in the original electronic format or alternatively as a single zipped file.

20.5.4.9 Evaluation of results

The instrument software will calculate the majority of the results. In cases where the instrument software is unable to provide the data in an appropriate tabular or graphical form, ASCII data files will be exported and elaborated in suitable excel files. The calculation of each cumulative mass and number base size distribution should be done over the specific size ranges for the different materials under examination, see Table 8.

Table 8: Size ranges to be used in calculating cumulative size distribution

Limit	IRMM-381	IRMM-387
Minimum size	70 nm	30 nm
Maximum size	2000 nm	400 nm

PSD reporting

1. Plot $Q_3(x)$ (norm) (unitless, normalized to 1) and q_3 in one graph over a log diameter axis in nm.
2. Plot $Q_0(x)$ (norm) (unitless, normalized to 1) and q_0 in one graph over a log diameter axis in nm.
3. Plot instrument optical signal vs particle size
4. Report $X_{50,0}$ in nm. Report $X_{50,3}$ in nm.
5. Report $X_{90,0}$ in nm. Report $X_{90,3}$ in nm.
6. Report $X_{10,0}$ in nm. Report $X_{10,3}$ in nm.
7. Report particle size at peak maximum in the weight, number and optical density distributions.
8. Report X_{max} , X_{min}

Measurement data files

Where the instrument software permits, the participant laboratories are requested to provide data files containing the measured particle size distributions according to one of the following 2 options:

Data Export Option 1: Export the data of each sample analysis data files as ASCII text file containing the following fields (Sedimentation Time, Optical Signal, Diameter, Weight, Number). If any of these fields is not available this should be stated in the report.

Date Export Option 2: For users of CPS disc-centrifuge instruments the complete sub-directory of each measurement procedure should be made available in the original format or alternatively as a single zipped file.

20.6 Validation Status

This method has been subjected to an intra-laboratory validation within the NanoDefine project.

20.7 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult Safety Data Sheets (SDS) to be aware of known hazards and exposure limits relevant to all chemical substance used in the procedure described here.

Chemical safety hoods or equivalent should always be used when manipulating dry nanopowders.

Ultrasonic devices can produce damage to hearing and personnel should wear ear protectors and/or operate the instruments within sound damping cabinets.

Residues and waste materials must be disposed of according to local environmental and safety regulations.

21 Measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software

21.1 Aim

This SOP describes an off-line method for automated image analysis of particulate materials imaged by electron microscopy, which has been developed in the NanoDefine project, referred to as the 'NanoDefine ParticleSizer'.

21.2 Scope

The off-line method for automated image analysis allows determining automatically the distributions of the characteristic size and shape properties (section 21.7) of constituent particles in aggregates and agglomerates, or present as single particles, from EM images. This application is specifically designed in the scope of implementing the EC-definition of a nanomaterial. The median value of the number-based distribution of the minimal external dimension of the constituent particles is assessed.

21.3 Application domain

This SOP describes an off-line method for automated image analysis of particulate materials, and requires that the material has been representatively brought on an EM grid e.g. by following the SOP 'SOP/NANoREG/D2.10/TEMSpePrep' entitled 'Preparation of EM-grids containing a representative sample of a dispersed NM'^f. In addition, the SOP requires that representative images of the material have been recorded by an electron microscopy based imaging technique such as TEM, SEM or STEM, e.g. following the SOP 'SOP/NANoREG/D2.10/TEMIma' entitled 'Transmission electron microscopic imaging of nanomaterials'^g. The ParticleSizer can also be applied on representative EM micrographs obtained in an alternative way.

The SOP can be used to characterise particulate materials (single particles and aggregated/agglomerated particles) and measure the size and shape properties of the electron microscopic projections of the constituent particle of the material.

In the context of implementing the EC definition of a nanomaterial³, it produces a number based distribution of the minimal external dimension of the constituent particles, assessed as the minimal Feret diameter or short axis length of fitted ellipse. The median value of these parameters allows classifying a material as a nanomaterial according to the EC NM definition. The other measured size and shape parameters (Section 21.7) allow a detailed characterisation of the materials required for e.g. risk analysis, batch and process control.

The SOP is tested on a series of certified reference materials (CRMs), such as ERM-FD304^h and ERM-FD100ⁱ and representative test materials (RTMs), such as NM-100, NM-103

^fhttp://www.nanoreg.eu/images/D2.10_Protocol_for_size-distribution_analysis_of_primary_NM_particles_in_air_powders_and_liquids_-_approvedpublic_for_website_final09.11.pdf

^g <https://www.rivm.nl/sites/default/files/2018-11/NANoREG%20D2.10%20SOP%2002%20Transmission%20electron%20microscopic%20imaging%20of%20nanomaterials.pdf>

^h <https://crm.jrc.ec.europa.eu/p/40456/40487/By-analyte-group/Particle-pore-size/ERM-FD304-COLLOIDAL-SILICA-40-nm-nominal/ERM-FD304>

ⁱ <https://crm.jrc.ec.europa.eu/p/40456/40487/By-analyte-group/Particle-pore-size/ERM-FD100-COLLOIDAL-SILICA-20-nm-nominal/ERM-FD100>

and NM-212^j, and other materials such as Au Nanoparticles and Nanorods.

21.4 Principle of the method

The SOP finds suitable configurations of the ParticleSizer for different types of materials (Table 1).

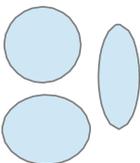
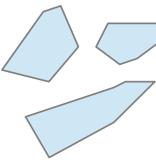
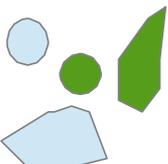
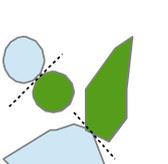
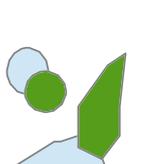
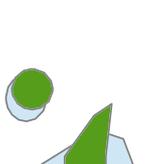
In a general procedure, the SOP helps to find suitable configurations of the ParticleSizer for different types of materials based on the amount of overlap between constituent particles (type of overlap) and the shape of the constituent particles (type of particle) (Table 1). Once a suitable configuration is selected, the images are automatically analysed.

During an automatic analysis, the software automatically detects and analyses particles on an image. The grey value is the criterion for the recognition of a particle. Therefore, in order to have successful particle detection, the particles must clearly stand out from the background. All detected particles can be automatically measured. A wide array of measured parameters can be chosen (Section 21.7).

An overview of the processing pipeline implemented in the ParticleSizer is presented in Section 21.9.

^j <https://ec.europa.eu/jrc/en/scientific-tool/jrc-nanomaterials-repository>

Table 1: ParticleSizer classification of materials based on A) the shape of the constituent particles (Type of particle) and B) amount of overlap between constituent particles (Type of overlap)

A) Type of particle	Name	Description
	Ellipsoidal	The outline of the particles can roughly be approximated by an ellipse in 2D images.
	Irregular	The outline of the particles is irregular and cannot be approximated by an ellipse in 2D images.
B) Type of overlap	Name	Description
	None	No overlap of the particles.
	Touching	These particles touch and do not overlap.
	Slightly overlapping	The particles show a low degree overlap.
	High or complete overlapping	These particles have a high degree of overlap.

21.5 Definitions, abbreviations and norms

CRM	Certified Reference Material
EM	Electron microscopy
NP	Nanoparticle
NRBS	noise reduced and background subtracted
OTB	Object-to-Background
RTM	Representative test material
SOP	Standard Operating Procedure
TEM	Transmission electron microscopy

21.6 System requirements and installation

The ParticleSizer couples image analysis by Fiji⁸ with data analysis by R Development Core Team⁷. Online manuals can be found on the following websites: <http://fiji.sc/> for Fiji and <https://www.r-project.org/> for R.

To install the ParticleSizer software, the following procedure has to be followed:

1. Download the latest fiji <http://fiji.sc/#download>
2. Activate the Biomedgroup update site^k
3. Add a new update site^l with the name 'psizer' and url <http://sites.imagej.net/Ndef-psizer/>. By adding the psizer update site, the ParticleSizer software is updated automatically when Fiji is up- dated.
4. Do the update. Now the particle sizer should be installed.
5. (Optional): It is recommended to install R to get better plots. When this point is skipped, a stripped-down plot will be shown.
 - 5.1 Download the latest R: <https://cran.uni-muenster.de/>
 - 5.2 Download the Rserver package: https://rforge.net/bin/windows/contrib/3.0/Rserve_1.8-0.zip
 - 5.3 Download the MASS package: https://cran.r-project.org/bin/windows/contrib/3.2/MASS_7.3-45.zip
 - 5.4 Start R and select the packages downloaded in 5.2 and 5.3 via 'Start Packages -> Install packages from local zip files'

21.7 Procedure to analyse sample images

21.7.1 Start Fiji

To start Fiji, simply open your Fiji folder and click on Image-J.exe

To open the ParticleSizer, the following procedure has to be followed:

1. Open Fiji

^k A manual how to follow a update site could be find here: [http://fiji.sc/How to follow a 3rd party update site](http://fiji.sc/How_to_follow_a_3rd_party_update_site)

^l A manual how to add a new update site could be find here: [http://fiji.sc/How to follow a 3rd party update site#Add update sites](http://fiji.sc/How_to_follow_a_3rd_party_update_site#Add_update_sites)

2. Go to plugins
3. Select nanodefine
4. Select particlesizer

21.7.2 Determine a basic suitable configuration

The software allows the user to set up a configuration suitable for a specific 'type of particle' and 'type of overlap' combination. The default configuration can be observed by activating the Settings Manager: Plugins → NanoDefine → SettingsManager and is illustrated by Figure 1. A default configuration is readily provided and can be applied on simple models with 'none' or 'touching' particles (Figure 2).

The default configuration can be split into 5 categories: mode selection, segmentation, Ellipse shape constraints, shape constraints and miscellaneous (Misc). For each option a default value is defined. The user has the option to optimize the settings for the images that will be analysed. A detailed description of each of these settings is given in Annex (Section 8).

The default values are as follows, see Figure 1A and 1B.

In a typical image analysis, the default settings in the Settings Manager have to be optimized for a certain 'type of particle' and 'type of overlap' combination.

	<p>Mode selection: Use watershed for irregular structures: off Irregular watershed convexity threshold: 0.7 Use single particle mode: off Use ellipse fitting mode: off</p> <p>Segmentation: Circular window radius: 1.5 % of the image width Rolling ball radius: 15 % of the image width Min. OTB intensity difference: 16</p> <p>Shape constraints: Minimal area: 0 px Minimal Feret min: 10 px Minimal convexity: 0 Minimal solidity: 0</p> <p>Ellipse shape constraints: Minimal long axis length: 5 px Minimal short axis Length: 5 px Maximal Aspect ratio: 100</p> <p>Misc: Smoothing factor: 1 Show binary result: off Ask me to select a region: off</p>
<p>Figure 1A: Settings Manager of the ParticleSizer with default configuration</p>	<p>Figure 1B: Default settings of the ParticleSizer</p>

Figure 2 shows the general procedure to determine a suitable configuration. The first step is to determine the type of overlap based on visual inspection of the particles. A distinction is made between 'none', 'touching', 'slightly overlapping' or 'high or complete overlapping' particles. The second step is to determine the type of particle based on visual inspection of the particles. A distinction is made between 'ellipsoidal' or 'irregular' particles. Besides the image quality the combination of overlapping type and particle type determines the configuration.

Depending on the selected 'type of particle' and 'type of overlap' combination, a suitable algorithm is selected for image analysis. These can be 'Default', 'Irregular Watershed', 'Ellipse fitting' or 'Single particle mode'.

For 'Irregular watershed', 'Ellipse fitting', 'Single particle mode', the boxes 'Use watershed for irregular structures', 'Use ellipse fitting mode' or 'Use single particle mode' have to be checked, respectively. The method can be readily applied on 'none', 'touching' or 'slightly overlapping' particles. For difficult materials, different modes can be combined. For particles with a high degree of overlap, single particle mode can be used on the condition that the single particles have the same physical properties as the constituent particles in aggregates/agglomerates. If this is not the case, the large aggregates/agglomerates have to be separated by optimizing the sample preparation.

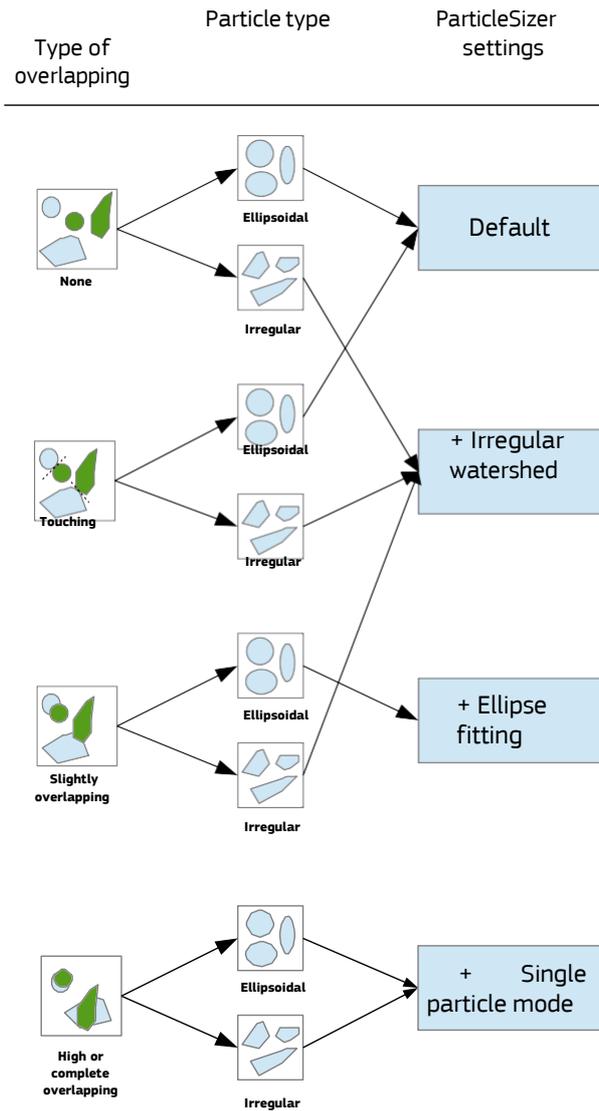


Figure 2: Procedure to determine suitable mode

The following general rules should be ensured: If the image contains a scale bar, this could interfere the analysis process. Therefore the largest possible region which does not contain the scale bar has to be selected. To do this, please check the option 'Ask me to select a region'.

The software expects bright particles on a dark background. If this is not the case, please check the option 'Use inverted images'.

21.7.3 Image analysis

To apply the ParticleSizer to your sample data, select 'Plugins → NanoDefine → ParticleSizer'.

A single image can be opened by 'File → Open'. If a whole image series (stack) should be analysed it can be opened by 'File → Import → Image Sequence'. The option 'Convert to 8-Bit Grayscale' should be checked. If the whole sequence does not fit in the memory, check 'Use virtual stack' in the sequence options.

21.7.4 Optional: Optimize the analysis

Certain artefacts might occur during the image analysis, which can have an influence on the results. The following strategies give advice how to proceed in those cases.

21.7.4.1 Some low contrast particles are not detected

Open the configure dialog by 'Plugins → NanoDefine → Settings Manager', decrease the 'Min. OTB intensity difference' by 2, and apply the ParticleSizer again. Repeat this until all particles are detected. By lowering the OTB difference it could happen that some small parts of the background are detected as particles. Experience shows that these objects are rather small and could be filtered out by increasing 'Minimal area' or 'Minimal Feret min'.

21.7.4.2 Background is detected as particles

IF the artefacts are as large as the smallest particles, then increase the 'Min. OTB intensity difference' by 2, press OK and apply the ParticleSizer again. Repeat this until you get reasonable results.

IF the artefacts are smaller than the smallest particles, then increase the 'Minimal Feret min' diameter until they are removed. The minimal Feret diameter can be estimated by measuring the smallest dimension of the largest artefact with the line tool.  If the images are scaled, than press 'ALT' while using the line tool to get it in pixels.

21.7.4.3 Use 'record process' to analyse the segmentation processing

If you check the option 'record process' every single interim image (segmentation step) is recorded. In the top left of the corner is written which step was recorded. This option can be used to determine which settings have to be optimized. Please note that the 'record process' option only works for a single image, not for a complete stack.

21.8 Output of results

There are several geometrical features which are calculated and saved into a results table by the Particle - Sizer software. The table could be exported by the user. The geometrical features are defined as follows:

- **Area (A):** The area enclosed by the outer contour of the particle.

- **Area convex hull (C):** The area enclosed by the convex hull of the outer contour of the particle.
- **Perimeter (P):** The perimeter of the outer contour of the particle.
- **Perimeter convex hull (H):** The perimeter of the convex hull of the particle.
- **Maximum Feret diameter:** The maximum distance between the two parallel tangents touching the particle outline in all directions.
- **Minimum Feret diameter:** the minimum distance between the two parallel tangents touching the particle outline in all directions.
- **Long side minimum bounding rectangle (L):** The larger side of the minimum bounding rectangle.
- **Short side minimum bounding rectangle (S):** The smaller side of the minimum bounding rectangle.
- **Aspect ratio:** Defined as L/S
- **Area / Perimeter ratio:** Defined as A/P
- **Circularity:** Defined as P^2/A
- **Elongation:** Defined as $1-S/L$
- **Convexity:** Defined as H/P
- **Solidity:** Defined as A/C
- **Number of holes:** The number of holes inside a particle.
- **Thinness ratio:** Inversely proportional to the circularity and normed. It is defined as $4\pi A/P^2$
- **Contour temperature:** It has a strong relationship to the fractal dimension, defined as $(\log(\frac{2P}{P_H}))^2$
- **Fractal dimension:** Estimated fractal dimension by the box count algorithm. The default box-sizes are '2,3,4,6,8,12,16,32,64'.
- **Maximum inscribed circle diameter:** Computes the largest inner circle of a particle.

The default plot is the minimum Feret diameter (Figure 3).

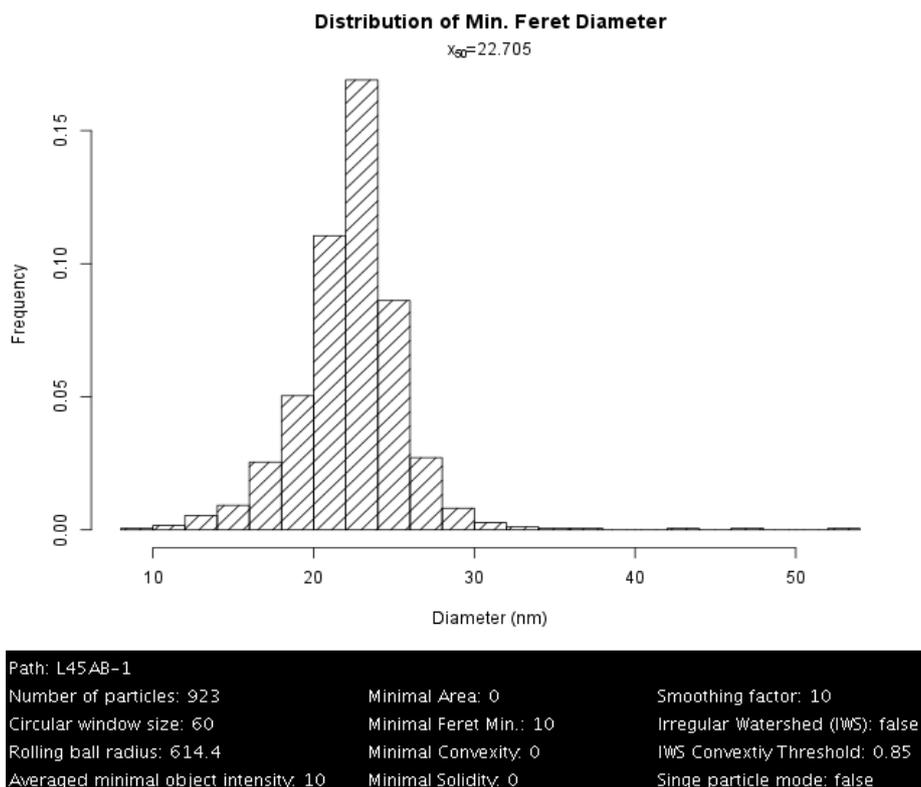


Figure 3: Size distribution of the minimal Feret diameter for a polystyrene sample

However, if the ellipse fitting mode is used, the short axis length is reported which is equivalent to the minimum Feret diameter. Directly below the plot, all used settings are documented for easy reproduction. Below the title the median value is outputted. By right clicking on the histogram and selecting 'Modify Plot', the X-axis and Y-axis labels and the number of bins can be altered. In addition, a function selected in the Fit Distribution field can be fitted through the histogram. Other geometric features could be plotted by selecting in Results table 'Results → Distribution'. Finally the plot could be exported by 'File → Save as'. Furthermore the segmented particles are visualized by an overlay (red lines) on top on the input image (Figure 4)

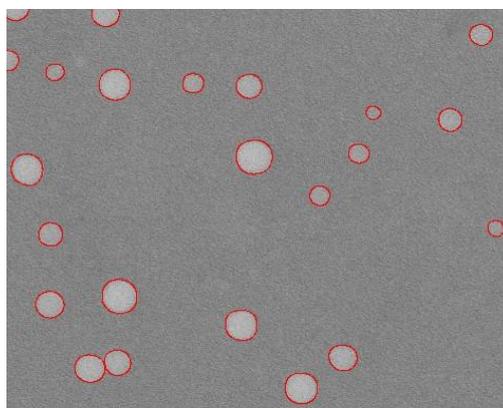


Figure 4: Segmented particles shown as overlay (red circles)

21.9 Description of adjustable settings in the Settings Manager

Mode selection:

- Use watershed for irregular structures: If selected, the mode for irregular structures is used. See section 0 for details.
- Irregular watershed convexity threshold: A threshold, which determines the amount of splitting up agglomerates. If the convexity of a particle is larger than this threshold, the splitting is stopped for this object. If the convexity is smaller than this threshold, the Software tries to split the particle again.
- Use single particle mode: If selected, the single particle mode is used. See section 21.10.5 for details.
- Use ellipse fitting mode: If selected, the ellipse fitting mode is used. See section 21.10.4 for details.

Segmentation:

- Circular window radius: This is a parameter of the local thresholding technique. The ParticleSizer does not use a global threshold to binarize the image. Instead it uses a local threshold which is estimated for a specific circular region with the configured radius.
- Rolling ball radius: The background is removed by rolling a ball with this radius over the surface (intensity interpreted as height) of the image. It should be at least as large as the largest object in image which does not belong to the background.
- Min. OTB intensity difference: Objects which have an object-to-background (OTB) intensity difference in the noise-reduced and background subtracted image (see section 9.2) lower than this threshold are considered as artefacts and are removed.

Shape constraints:

- Minimal area: Minimum area in pixels. Particles smaller than this threshold are removed.
- Minimal Feret min: Minimal Feret diameter in pixels. Particles smaller than this threshold are removed.
- Minimal convexity: The convexity is defined as the ratio of the perimeter of the convex hull of the particle and the perimeter of the particle. It lies between 0 and 1. The convexity increases with larger values. Particles smaller than this threshold are removed.
- Minimal solidity: Defined as the ratio of the particle area and the area of the convex hull of the particle. It lies between 0 and 1. The solidity increases with larger values. Particles smaller than this threshold are removed.

Ellipse shape constraints:

- Minimal long axis length: The length in pixels of the major direction of the fitted ellipse. Ellipses smaller than this threshold are removed.
- Minimal short axis length: The length in pixels of the minor direction of the fitted ellipse. Ellipses smaller than this threshold are removed.
- Maximal aspect ratio: Ratio of the length of major and minor axis. Ellipses with an aspect ratio larger than this value are removed.

Misc:

- Smoothing factor: It sometimes occurs that the estimated standard deviation of the noise is lower than the true value. The smoothing factor is a multiplicative factor for the estimated standard deviation.
- Use inverted images: The ParticleSizer expects bright objects on a darker background. If images show the opposite, then this option should be checked.
- Show binary result: If selected the ParticleSizer shows the binary result.
- Ask me to select a region: If selected, the software allows you to select a specific region to analyse.

21.10 The ParticleSizer pipeline

Sections 21.10.1 and 21.10.2 give an overview of the processing pipeline implemented in the ParticleSizer. The methods 'watershed for irregular structures', 'single particle mode' and 'ellipse fitting' in the category segmentation are most important for this SOP and will be described more detailed in the sections below.

21.10.1 Segmentation pipeline

The flow scheme of the segmentation pipeline is given in Figure 5. The standard deviation σ of the noise of recorded EM images is estimated using Immerkaer's method⁵ and used to adapt an efficient, parallelized noise filter called 'non local means'^{1,2}. To identify particles on EM images, the background is removed using the rolling ball algorithm (parameterization depends on the image size) implemented in ImageJ. If the noise standard deviation is higher than the threshold T_1 a small median filter is applied to homogenize the particles. The result is the noise reduced and background subtracted (NRBS) image. The NRBS image is then binarized by a local adaptive threshold technique⁶ and saved as 'Pre-Watershed image'. If single particle mode (SPM) is selected, all particles with a convexity smaller than T_2 are removed. When the SPM is deactivated the agglomerates are split into constituent particles by a user selected technique which provides an initial identification of particles (segmentation). In post-processing steps possible artefacts introduced by the segmentation procedure are removed before geometrical features and size distributions are extracted. The post-processing steps are described in the next section.

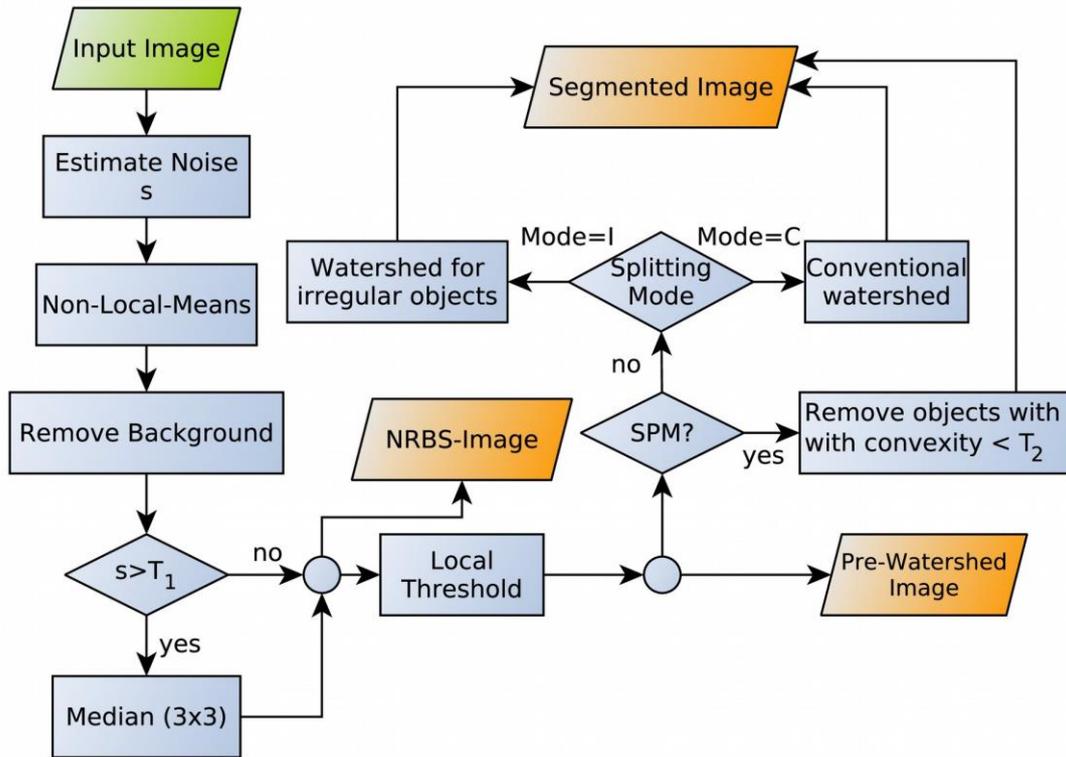


Figure 5 Pre-processing pipeline

21.10.2 Post-Processing pipeline

The flow scheme of the post-processing pipeline is given in Figure 6.

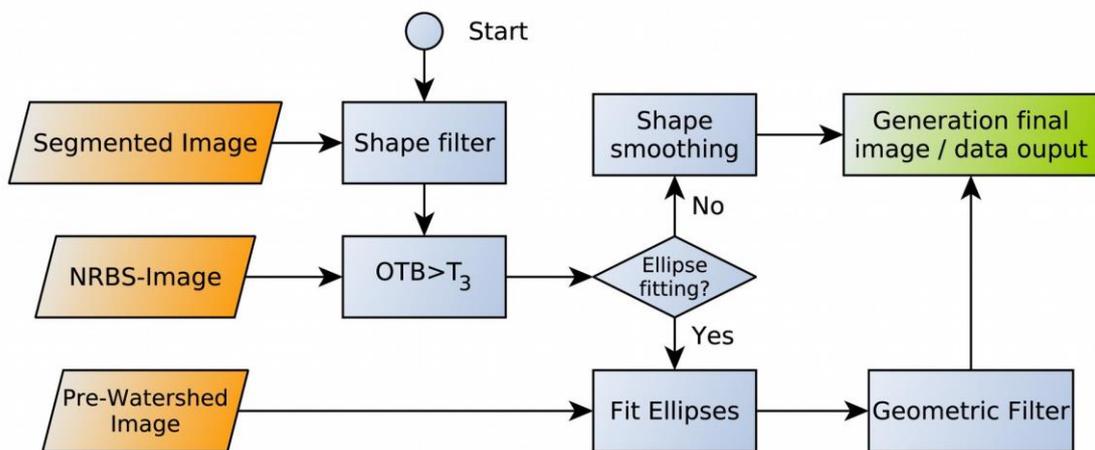


Figure 6: Post-processing pipeline

Starting from the segmented image resulting from the segmentation pipeline, all objects which are outside of user-defined limits of the geometrical features are removed. Default values are provided but the software also allows for an individual adaption of the limits to account for non-standard images. In a second step objects which have an object-to-background (OTB) intensity

difference in the NRBS Images lower than T_3 (as configured in the settings manager) are considered as artefacts and are removed. When ellipse fitting is activated the ellipses are fitted to the particle boundaries to expose potential overlapping. However, the segmentation process often results in objects showing rough boundaries, which is corrected by applying a shape smoothing algorithm using Fourier descriptors when ellipse fitting is deactivated. Geometrical features extracted from the remaining objects are listed in a results table, and a particle size distribution based on the minimal Feret diameter (including also X50 values) is displayed in a graphical format. The complete results can also be exported for more detailed processing, if required.

21.10.3 Irregular watershed

The irregular watershed technique combines a conventional watershed splitting with a morphological erosion. The procedure starts with the binary image I. The image L with watershed lines is calculated the following way:

$$L = I \cap W(I)$$

where $W(I)$ is a conventional watershed splitting based on the euclidean distance map and \cap the logical AND operation.

In a next step connected components of image I are eroded. The erosion of a connected component is stopped when a convexity larger than a user defined threshold (0.7 is used by default) is reached or when the component is fully eroded. This results in image E which contains those connected components which fulfil the convexity condition. In the final step watershed lines in L which are crossing objects in E are rejected. This method successfully splits overlapping irregular objects but prevents over-segmentation.

21.10.4 Ellipse fitting

The combined approach couples a conventional watershed splitting based on the euclidean distance map with the direct ellipse fitting method. Figure 7 illustrates the principle of the method: The objects in the input image (Figure 7a) are split by the watershed technique (Figure 7b).

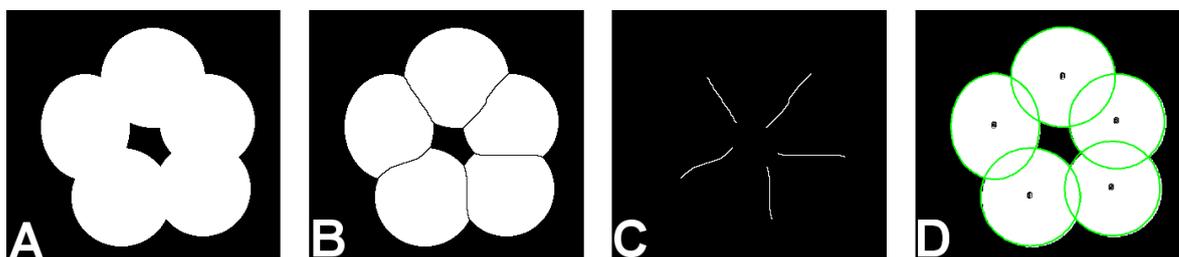


Figure 7: Ellipse fitting method (A) Input image of 5 overlapping circles, (B) splitted objects by conventional watershed technique, (C) the extracted watershed lines and (D) the ellipses fitted to the contours of the objects in image B rejecting all contour points which have a watershed line (image C) in the direct neighbourhood

By combining Figure 7a and Figure 7b by a logical XOR operation the watershed lines are extracted (Figure 7c). Contour lines of the objects in image B are then extracted and all

contour points which have a watershed line in the direct neighbourhood are rejected. Finally an ellipse is fitted⁴ to the remaining contour data of the objects (Figure 7d).

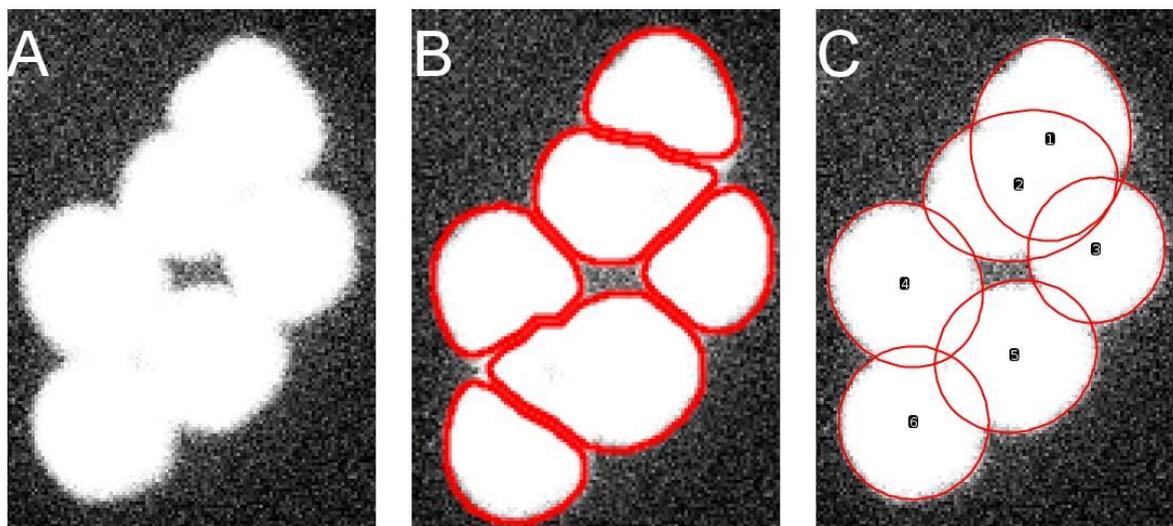


Figure 8: Au nanoparticle agglomerate analysed by the ParticleSize software in (B) default mode and (C) ellipse fitting mode

Figure 8 shows a comparison of the conventional watershed method with the ellipse fitting approach using an Au nanoparticle agglomerate. The results demonstrate that the ellipse fitting provides a better estimate of the effective size of ellipsoidal overlapping particles compared to the conventional watershed approach.

21.10.5 Single particle mode

In cases, where agglomerates cannot be well dispersed, the proper segmentation of the agglomerates may not be possible. For such cases, 'single particle mode' (SPM) is implemented in the ParticleSizer software. In the SPM, agglomerates are rejected and only constituent particles - defined as particles with a high convexity

- are included in the analyses. In the context of the ParticleSizer software the term 'high convexity' is defined as follows:

A particle has a high convexity when the ratio of the perimeter of the convex hull and the perimeter of the outer contour is larger than 0.7.

21.11 References

- ¹ Buades, A., Coll, B. & Morel, J.-M., 2005. A Non-Local Algorithm for Image Denoising. In *2005 IEEE Computer Society Conference on Computer Vision and Pattern Recognition (CVPR'05)*. IEEE, pp. 60–65.
- ² Darbon, J. et al., 2008. Fast nonlocal filtering applied to electron cryomicroscopy. In *2008 5th IEEE International Symposium on Biomedical Imaging: From Nano to Macro*. IEEE, pp. 1331–1334.
- ³ European Commission. Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU). OJ L 275, pp.38-40

- ⁴ Fitzgibbon, A., Pilu, M. & Fisher, R.B., 1999. Direct least square fitting of ellipses. *IEEE Transactions on Pattern Analysis and Machine Intelligence*, 21(5), pp.476–480.
- ⁵ Immerkær, J., 1996. Fast Noise Variance Estimation. *Computer Vision and Image Understanding*, 64(2), pp.300–302.
- ⁶ Phansalkar, N. et al., 2011. Adaptive local thresholding for detection of nuclei in diversity stained cytology images. In *ICCSP 2011 - 2011 International Conference on Communications and Signal Processing*. IEEE, pp. 218–220.
- ⁷ R Development Core Team, 2008. *R: A Language and Environment for Statistical Computing*, Vienna
- ⁸ Schindelin, J. et al., 2012. Fiji: an open-source platform for biological-image analysis. *Nature methods*, 9(7), pp.676–82.

Generic SOPs for nanomaterials in products

22 SOP for analysis of TiO₂ particles from sunscreen by AF4-MALS-ICP-MS

22.1 Aim

The purpose of this SOP is to provide the protocols for the sample preparation of sunscreen material containing TiO₂ particles, and instructions for the quantitative determination of characteristic parameters of particle size distributions of TiO₂ particles using AF4-MALS-ICP-MS.

22.2 Scope

This protocol describes the sample preparation procedure for TiO₂ particles present in sunscreen and particle analysis by means of AF4-MALS-ICP-MS. The method allows determining $r_{h,mode}$ (MALS), $r_{h,mode}$ (ICP-MS), and mass-based PSD (d_{10} , d_{50} , d_{90}).

The described procedure is applicable to sunscreen samples studied in the NanoDefine project, as well as TiO₂ particles present in sunscreen samples containing other UV blocker (e.g. ZnO). All steps required for the analysis are summarized in Figure 1.

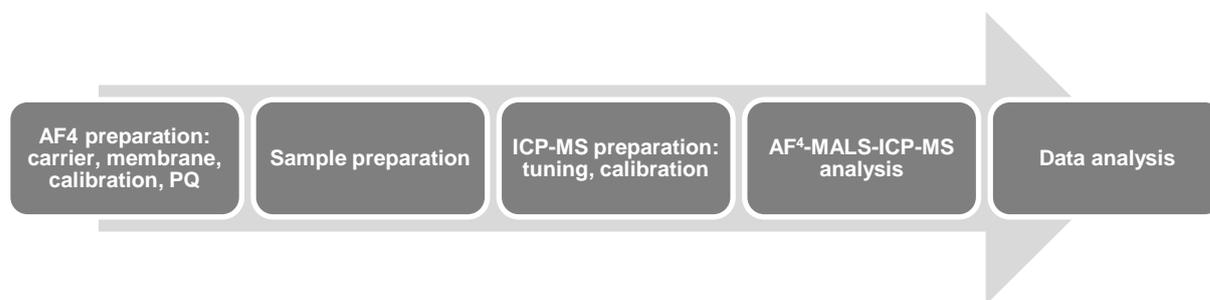


Figure 1: Overview of the different steps included in the measurement procedure for extraction of TiO₂ particles from sunscreen and AF4-MALS-ICP-MS analysis.

Definitions

AF4	Asymmetric flow field-flow fractionation
ICP-OES	Inductively coupled plasma-optical emission spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
MALS	Multi-angle light scattering
NaDS	Sodium dodecyl sulphate
NP	Nanoparticles
PQ	Performance qualification

22.3 Description

22.3.1 Materials and methods

22.3.1.1 Essential equipment

- Analytical balance (0.1 mg precision)
- Laboratory scale bath sonicator
- Shaker

- Vortex
- pH meter
- ICP-OES system
- AF4 system coupled to MALS detector
- ICP-MS system

22.3.1.2 Recommended optional equipment

- Vacuum pump for filtration of aqueous solutions.

22.3.1.3 Material supplies

- Glass/plastic vial (or similar, for ~20 mL sample volume) with screw top or other appropriate stopper
- Volumetric flask (e.g. 100 mL, 250 mL)
- Disposable plastic spatula for weighing
- Disposable plastic weighing boats or similar for weighing of chemicals in powder form
- Anodisc 0.02 μm nominal pore size membrane filters or similar for vacuum filtration of aqueous solutions (see section 22.3.2)
- Disposable (powder-free) nitrile gloves
- Adjustable volume pipettes of 100 μL , 1 mL and 5 mL with disposable tips
- Other personal protection equipment in accordance with the laboratory's safety rules for working with chemicals including engineered nanomaterials

22.3.1.4 Chemicals

- Ultrapure water (demineralised water of 18.2 $\text{M}\Omega\text{cm}^{-1}$ resistivity; purified by reverse osmosis and sanitization)
- Sunscreen samples
- Sodium dodecyl sulphate (NaDS) powder (purity $\geq 98.5\%$)
- FL-70TM (in the absence of FL-70TM NovaChem 100 might be used)
- Dishwashing detergent (Denk mit Ultra by DM, in further text cleaning agent)
- NaOH solution (0.01 M, 0.1 M and 1 M) - prepared from analytical grade NaOH and ultrapure water
- NaCl (0.03 M) prepared from analytical grade NaCl and ultrapure water

22.3.2 Additional chemicals/solutions to be prepared

0.2 % (m/v) NaDS solution

Prepare 0.2 % (m/v) NaDS solution by dissolving the appropriate amount of NaDS powder into ultrapure water (e.g. add 0.2 g of NaDS in volumetric flask and fill it by ultrapure water). Shake vigorously to ensure that all powder is solubilized. Adjust pH to 8.5-9 by using previously prepared NaOH solution and filter through a 0.02 μm nominal pore size membrane.

0.1 % (m/m) NaDS solution

Dilute 0.2 % (m/m) NaDS solution (pH=8.5-9) by a factor of 2 and filter through a 0.02 μm nominal pore size membrane.

0.5 % (v/v) FL70TM (or NovaChem 100)

Prepare 0.5 % (v/v) FL70TM by adding 0.5 mL of FL70TM solution in 99.5 mL of water. Filter the solution through the 0.02 μm nominal pore size membrane.

1 % (v/v) cleaning agent

Prepare 1 % (v/v) cleaning agent by adding 1 mL of dishwashing cleaning liquid (Denk mit Ultra by DM) in 99 mL of water.

22.3.3 Performing measurements

22.3.3.1 Sample preparation

- a. Weigh an empty glass/plastic vial, press tare and add approximately 10 mg of sunscreen using a spatula;
- b. Reweigh the vial and calculate by difference the effective amount of sunscreen added. Then add 1 % (v/v) cleaning agent to give a concentration of 1 mgmL⁻¹;
- c. Shake the sample for 10 min horizontally or until the sample has a homogeneous appearance;
- d. Sonicate the sample for 15 min in a laboratory scale bath sonicator;
- e. Slightly shake it before transferring 2 mL of the sonicated sample to an empty glass/plastic vial and add 2 mL of 0.2 % NaDS solution (pH = 8.5-9) (note: record the weight the mass of sample and surfactant added);
- f. Sonicate the sample for 5 min in a laboratory scale bath sonicator;
- g. Leave over night;
- h. Slightly shake the sample and sonicate for 15 min in a laboratory scale bath sonicator;
- i. Slightly shake the sample and dilute the sample further by taking 1 mL of sample and 4 mL of 0.1 % NaDS solution (note: record the weight of the mass of sample and surfactant added).
- j. Sonicate for 2 min in a laboratory scale bath sonicator.

22.3.3.2 Recovery of dispersions after aging beyond verified period of stability

Where the operator has access to DLS instrumentation it is strongly recommended that the dispersion state is evaluated by DLS before AF4-MALS-ICP-MS analysis.

The temporal stability of the dispersions prepared in the previous steps has been verified and no significant degree of re-aggregation or agglomeration has been found to occur during a period of 1 hour after completion of the final sample preparation step. Therefore, before each measurement homogenisation in a sonication bath for 2 minutes must be done.

The dispersion state of a sample aged for 1 day can be reversed to its original state by 10 min of bath sonication before measurements.

Additional, aging of 2 weeks is allowed for the sample after sample preparation step f. The aging can be fully reversed if glass vial with the sample is slightly manually shaken, treated for 15 min in a laboratory scale bath sonicator at room temperature. Final dilution step (step h-j) should be done before AF4-MALS-ICP-MS analysis.

22.3.3.3 Measurement description

The AF4 separation channel must have a length of 275 mm, and must be equipped with a 350 µm spacer and a 10 kDa regenerated cellulose membrane. For particle size characterisation (samples including quality control checks) the AF4-system has to be coupled online with light scattering

detection (e.g. MALS, DLS). Measurement conditions of AF4 analysis are presented in Table 9. A flow condition in the channel has to be set up as following: the detector flow rate to 1.0 mLmin⁻¹; the cross flow rate and the focus flow rate of 0.60 mLmin⁻¹; the injection flow of 0.1-0.2 mLmin⁻¹. As carrier liquid a mixture of 0.025 % (v/v) FL-70 has to be used.

The AF4 size-retention time calibration is performed using polystyrene (PS) latex beads standards (at least 4 calibrants from working range of 20 nm to 270 nm are selected for calibration purposes). Please note that the carrier liquid for the PS standards should be a mixture of 0.025 % (v/v) FL70™ (or NovaChem 100) and 0.003 M NaCl. Standards must be run under optimized run conditions every time when membrane is changed. After running the PSS, the data should be immediately analysed in order to construct the size-retention time calibration curve. The slope and the correlation coefficient (r value calculated using regression analysis) must be equal or higher than 0.990. For performance criteria monodisperse PS material (with a diameter in the range of 50 nm to 200 nm) should be run on weekly basis. If the obtained peak modes deviate strongly (e.g. deviation > 50 %) operator should change the membrane or reconsider troubleshooting (extensive flushing of the channel in focusing mode, changing tubing, adjusting focusing valve, etc.).

The r_h (mean of 3 independent replicate results for every sample) is determined from the size-retention time calibration curve. The retention times (e.g. 90° MALS signal), for every standard correspond to the mode of the largest peak present in the fractogram.

AF4-MALS-ICP-MS measurement conditions are summarized in Table 9. For Ti mass quantification during AF4 analysis an ICP-MS is coupled online to the outflow of the MALS detector. A splitter and a peristaltic pump provide the volumetric flow rate of 0.30 mL/min required by the ICP-MS system. The flow can be monitored by a flow meter. Ti mass quantification is done using the time resolved analysis mode. For calibration of the ICP-MS system solutions of 0 µgL⁻¹; 1.25 µgL⁻¹; 2.5 µgL⁻¹; 5 µgL⁻¹; 12.5 µgL⁻¹ and 50 µgL⁻¹ Ti are prepared using a dilution media of 0.025 % FL-70 and a Ti stock solution of 1000 mgL⁻¹. The sample uptake speed is adjusted to a flow rate of 0.3 mL/min. CSV-data files can be exported from ICP-MS software (Ti signal) into an Excel spread sheet. The correlation coefficient must be > 0.990.

Finally, all collected data sets (AF4 calibration, MALS signals, MALS fittings and ICP-MS data) can be copied into the spread sheet. The output parameters are:

- hydrodynamic size distribution based on AF4 calibration (mode),
- mass based particle size distribution (mode, d_{10} , d_{50} , d_{90}) and
- retention time.

Table 9: Measurement conditions for AF4-ICP-MS analysis

AF4 parameter [#]	unit	value
Tip to tip channel length	[mm]	275
Spacer thickness	[µm]	350
Focus flow rate	[mL min ⁻¹]	0.60
Injection flow	[mL min ⁻¹]	0.1-0.2
Focus time	[min]	2

Injection + focus time	[min]	10
Focus time	[min]	2
Elution time	[min]	50
Detector flow rate	[mL min ⁻¹]	1.0
Cross flow rate	[mL min ⁻¹]	0.60
Membrane		regenerated cellulose, 10 kDa
Carrier liquid #		0.025 % (v/v) FL-70™
Injection volume	[μL]	50-100 μL of sample suspension
ICP-MS parameters	unit	value
RF power	[W]	1600
Sample depth	[mm]	10
Gas flow rates		
Carrier	[L min ⁻¹]	1.06
Dilution	[L min ⁻¹]	0.40
Collision gas He	[mL min ⁻¹]	4.5
Sample uptake rate	[mL min ⁻¹]	0.3 (established by split flow)
Isotopes monitored		⁴⁷ Ti
Dwell time	[ms]	250-2000

Size-retention time calibrations of the AF⁴ channel are performed under identical run conditions, with the only exception being for a carrier composition 0.025 % FL-70 and 0.003 M NaCl.

22.3.4 Evaluation of results

Data analysis is performed by evaluating Ti bulk mass recovery, and particle size distribution.

Ti bulk mass recovery ($rec_{Ti,bulk}$; eq. 1) is defined as the ratio between Ti mass concentration after the sample preparation procedure ($c_{Ti,sample,ICP-OES}$) and initial Ti mass concentration ($c_{Ti,initial}$). The initial concentrations are calculated from the Ti mass concentration in the stock solutions and dilution caused by sample preparation. Ti mass concentration after preparation procedure is determined using ICP-MS or ICP-OES analysis directly after sample preparation, final dilution of 100x in 0.025 % FL-70 and addition of 200 μL of 5 M HNO₃ in 10 mL of sample.

$$rec_{Ti,bulk} = c_{Ti,sample,ICP-OES} / c_{Ti,initial} \quad (\text{eq. 1})$$

Particle size distribution obtained by AF⁴ separation and MALS analysis is derived from the measured fractograms. The spreadsheet is used for converting the retention times to hydrodynamic sizes using the calibration function determined in fractionations of the 50 nm – 200 nm diameter PS latex bead standards, after subtracting the time of the void peak. The ICP-MS is calibrated on daily basis for Ti (as representative element in sunscreen). In addition Fe and Al are also chosen as representative elements for the complex sunscreen samples (BAM-13a) which contains iron oxides in addition to TiO₂. Calibration functions for converting the ICP-MS signals to concentrations is set up by plotting the averaged intensities of standard solutions against the standard concentrations, after subtracting the background signal. The signal intensities in the

fractograms are then converted into concentration values. A size distribution is obtained based on particle mass.

22.3.5 Reporting of results

Reporting of the final results is presented below:

$rec_{Ti,bulk}$ [%]

$r_{h,mode}$ (MALS) [nm]

$r_{h,mode}$ (ICP-MS) [nm]

PSD (d_{10} , d_{50} , d_{90}) [nm]

22.4 Validation status

This method has been subjected to in-house validation.

22.5 HSE issues

All laboratory personnel must comply with local safety regulations when working in the laboratory.

All personnel must consult Safety Data Sheets (SDS) to be aware of known hazards relevant to all chemical substances used in this SOP.

Personnel should utilize all necessary precautions to avoid exposure to chemical and nanomaterials.

All residues and waste materials must be disposed of according to local environmental and safety regulations.

23 Sample preparation and spICP-MS analysis of TiO₂ particles in sunscreen products

23.1 Aim

This standard operating procedure (SOP) can be applied to determine the particle size and size distribution, and the particle number or mass concentration of TiO₂ nanoparticles in sunscreen products using single particle ICP-MS (spICP-MS).

23.2 Scope

The procedure is applicable for the determination of TiO₂ nanoparticles in sunscreen products. The procedure may also be applicable for other nanoparticles consisting of metal or metal oxides (e.g. Ag, Au, Al₂O₃, SiO₂, etc.) in aqueous suspensions or consumer products with a composition comparable with sunscreen products (e.g. cosmetic creams, toothpaste, etc.). In those cases additional quality control samples shall be incorporated. Depending on the type of nanomaterial, particle sizes (expressed as equivalent spherical diameter (ESD)) in the range of 10 to 1000 nm and mass concentrations in the range of 1 to 1000 ng L⁻¹ in the final extract/suspension can be determined. The mass concentration range can be extended by further dilution of the prepared extracts or suspensions.

23.3 Definitions

spICP-MS	Single particle inductively coupled plasma mass spectrometry.
Nanoparticle	A particle with at least one dimension in the range of 1 to 100 nm.
Dwell time	The time during which the ICP-MS detector collects and integrates incoming pulses. Following integration the total counts are registered as one data point, expressed in counts, or counts per second.

23.4 Description

The procedure consists of two parts:

- sample preparation
- spICP-MS measurement (detection and quantification of nanoparticles)

A sub-sample of the sunscreen product is collected and diluted in a diluting agent, which stabilises the nanoparticles in suspension. This first suspension is then diluted further in one or more steps before instrumental analysis.

spICP-MS is based on the measurement of diluted nanoparticle suspensions by an ICP-MS that is operated in time resolved mode and set at a pre-selected mass-to-charge ratio (m/z). When properly diluted, individual particles enter the plasma of the ICP-MS, are atomised and ionised, and produce a plume of element ions which travels through the mass spectrometer and reaches the detector. The discrete measurement intervals of the MS (the dwell time) are typically set at a value ≤10 ms. This allows the detection of the ion plume of single particles (hence the name 'single particle ICP-MS') resulting in a peak in the time scan which is proportional to the mass of the respective element in the particle. The particle size, expressed as ESD (equivalent spherical diameter), is calculated from the particle's mass. The number of peaks that are recorded during the

time scan is proportional to the particle number and mass concentration. Detailed characteristics of the instrumental method are reported in the NanoDefine Manual, Part 2: Evaluation of methods.

23.4.1 Materials and methods

23.4.1.1 Materials, chemicals and reagents

Chemicals required for sample preparation and measurement are detailed in Table 1.

Table 1: Chemicals for sample preparation and measurement

Chemical	Description	Manufacturer/Provider
Ultrapure water	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 μm)	e.g. Millipore, Billerica, MA, USA or equivalent
Triton X-100	Nonionic surfactant, laboratory grade	e.g. Sigma Aldrich, St Louis, MO, USA or equivalent
MelPers® 2450	Deflocculant for inorganic pigments (solid content 49-51 %)	BASF, Ludwigshafen, Germany
HNO ₃	Nitric acid (suprapure, 65 %)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
RM 8013	Au Nanoparticles, nominal diameter: 60 nm	NIST, Gaithersburg, MD, USA
Ionic Standard Solutions	Titanium ionic standards in 3 % nitric acid (1 g L ⁻¹)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Tune solution	Mix of elemental ICP-MS standards	Instrument specific, e.g. Tune B solution, Thermo Fisher Scientific, Waltham, MA, USA

Laboratory instruments and material are listed in table 2.

Table 2: Laboratory instruments and materials

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance	e.g. Mettler Toledo, Vienna, Austria
Mechanical homogenizer	Vortex Mixer, 20.000 to 30.000 rpm	e.g. VWR International, Radnor, PA, USA or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication probe with a CML-4 probe operated at 4 Watt	e.g. Misonix XL-2000, Qsonica, Newton, CT, USA or equivalent
Calibrated pipettes	3 pipettes (0.5-10 μL , 10-100 μL , 100-1.000 μL)	e.g. Eppendorf, Hamburg, Germany

Analytical instruments and software are listed in table 3.

Table 3: Analytical instruments and software

Instrument	Description	Manufacturer/Provider
ICP-MS	Quadrupole ICP-MS with quartz torch, spray chamber and injector, usage of nickel cones (nanoparticle suspensions) and platinum cones (products)	e.g. ICAP-Q, Thermo Fisher Scientific, Waltham, MA, USA or equivalent
Autosampler	e.g. ESI SC-4Q	e.g. Elemental Scientific, Omaha, NE, USA or equivalent
Qtegra npQuant	Qtegra software with integrated nano-application tool for nanoparticle measurements and data evaluation	Thermo Fisher Scientific, Waltham, MA, USA
RIKILT SPC spreadsheet ¹	Validated Excel spreadsheet for the data evaluation of nanoparticle measurements	RIKILT - Institute of Food Safety

The following reagents are needed:

Diluting agent. The diluting agent for sunscreen samples is prepared by weighing 5 g of MelPers® 2450, and 5 g of Triton-X 100 in a clean glass bottle and add 1 L of ultrapure water. Stir for at least 30 min. at room temperature until all material is dissolved and the liquid is fully homogenized. This stock can be stored at room temperature for at least 1 week.

Stock standard of 60 nm gold nanoparticles (50 µgL⁻¹). Pipet 50 µL of the gold reference standard RM 8013 to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 µgL⁻¹. Mix thoroughly and store at room temperature in amber glass screw necked vials. This intermediate standard is stable at room temperature for at least one month. Prior to use place the standard in an ultrasound bath for 10 minutes.

Working standard of 60 nm Gold nanoparticles (50 ngL⁻¹). Prepare the working standard by pipetting 50 µL of the stock standard to 25 mL of ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 ngL⁻¹. Mix thoroughly and store at room temperature in amber glass screw necked vials. This standard is prepared daily.

Stock standards of ionic titanium solutions (100 µgL⁻¹). Assuming the ionic standard solution has a concentration of 1 gL⁻¹, pipet 50 µL of the standard to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 1 mg L⁻¹. Repeat this step, pipetting 1 mL of the 1 mgL⁻¹ standard to 5 mL ultrapure water in a 10 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 100 µgL⁻¹. Mix thoroughly and store this intermediate standard in amber glass screw necked vials. Protected from light this intermediate standard is stable at room temperature for at least two weeks.

Working standards of ionic titanium solutions (0.2 – 10 µgL⁻¹). Prepare the calibration curve ionic standards according to table 1. Pipet the volumes of the stock standard of 100 µgL⁻¹ to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water. Mix thoroughly. Protected from light these working standards are stable at room temperature for the period indicated in Table 4.

(NOTE: When possible the composition of the ionic standard matrix shall be matched to the prepared samples)

Table 4: Volumes for the preparation of the working standards of the ionic stock solution

Volume of the stock standard diluted to 50 mL ultrapure water	Ionic concentration of the working standard	Stability of the ionic working standard in glass
5 mL	10 µgL ⁻¹	2 weeks
2.5 mL	5 µgL ⁻¹	2 weeks
1 mL	2 µgL ⁻¹	2 weeks
0.5 mL	1 µgL ⁻¹	2 weeks
0.25 mL	0.5 µgL ⁻¹	1 week
0.1 mL	0.2 µgL ⁻¹	1 week

23.4.2 Performing measurements

23.4.2.1 Preparation of products

The sample preparation of sunscreen samples consists of a matrix dilution. The following steps will be performed:

1. Weigh approximately 50 mg of the product in a 50 mL polyethylene tube and add 50 mL of the diluting agent.
2. Vortex the tube until the sunscreen is completely detached from the tube walls.
Sonicate the tube in a water bath for 10 minutes at room temperature.
3. First dilution. Vortex and shake the suspension before collecting a 50 µL subsample. Dilute the subsample of 50 µL in a 50 ml PE tube with approximately 25 mL of ultrapure water. Fill to the 50 mL mark with ultrapure water.
4. Sonicate the first dilution in a water bath for 10 minutes at room temperature
5. Second dilution. Vortex and shake the diluted suspension before a subsample is collected. Collect a subsample of 25 µL and dilute in a 50 ml PE tube with approximately 25 mL of ultrapure water. Fill to the 50 mL mark with ultrapure water.

(NOTE: This procedure was prepared for sunscreen products containing TiO₂ nanomaterial in a concentration of approximately 50 g Ti/kg product. For other nanomaterials or other matrices (e.g. facial creams, toothpaste etc.) the method may need adjustments.)

23.4.2.2 ICP-MS set-up and calibration

ICP-MS performance check

The instrument has a performance check and an autotune function which are designed to replace the manual checks and tuning procedures and the short term stability test. If the criteria of the performance check are not met, a tuning, autotune or manual tune, is performed to optimize the instrument.

A 3 % nitric acid solution is used to rinse the sampling system of the ICP-MS before and in between runs. Special attention shall be paid to the cleanliness of the sample introduction system of the ICP-

MS. If high nanoparticle concentrations have passed through the tubing this may result in continuous background levels is subsequent analysis leading to erroneous results. Analyse an ultrapure water sample and a blank matrix sample to determine the background signals. In neither of the two the number of observed particles shall exceed a number of 10.

Settings of the ICP-MS system

- Forward power : 1550 W
- Nebulizer : PFA
- Spray chamber : cyclonic, quartz
- Gas flows : plasma, 13 Lmin⁻¹
nebulizer, 1.1 Lmin⁻¹
- Rinsing liquid flow rate : 1 mLmin⁻¹
- Sample flow rate : 0.35 mLmin⁻¹
- Data acquisition : time resolved analysis (TRA) mode (npQuant)
- Dwell time : 3 ms
- Total acquisition time : 60 s
- Isotope monitoring : Au (m/z 197), Ti (m/z 48)

(NOTE: For elements with potentially polyatomic interferences the application of another measurement mode (e.g. KED) or reaction cell (CCT) can improve results)

In general the number of peaks in a time scan should not exceed 10 % of the maximum number of peaks based on the dwell time. Using a dwell time of 3 ms, the number of detected particles in the time scan shall not exceed 2000. If this number is exceeded, the aqueous sample extract shall be diluted and re-analysed. For the instrumental settings used in this procedure a particle number concentration in the range of 2×10^6 to 2×10^8 particles L⁻¹ results in useful measurement data.

23.4.2.3 Measurement description

Determination of transport efficiency

NIST RM 8013, a 60 nm Au nanoparticle is used to determine the transport efficiency on a daily basis. The number of detected of particle events depends on the ICP-MS setup, the sample flow and the type of nebulizer. To accurately determine the transport efficiency, 200-500 particles should be observed in the analysis of a 50 ngL⁻¹ standard.

(NOTE: With more efficient nebulizers the concentration of the 60 nm Au nanoparticles can be lowered to 25 or even 10 ngL⁻¹)

Determination of the response of the analyte

A mass calibration shall be performed using working standards in table 1 (6 concentrations in the range of 1 to 50 µgL⁻¹ and a blank) under the same measurement conditions as for spICP-MS measurements. Using linear regression the correlation coefficient of the calibration line will be determined. The correlation coefficient shall be >0.99.

(NOTE: When possible the composition of the size calibrant matrix shall be matched to that of the prepared samples)

Analysis of sample dilutions

Sunscreen and other facial creams containing TiO₂ NP also contain few large aggregates or agglomerates of these particles. The presence of such an aggregate or agglomerate may strongly influence the result of individual measurements. Therefore, each sample dilution shall be analysed in triplicate. Results will be calculated as the average of the three determinations.

Sample list

Blanks, ionic calibration standards and/or nanoparticle standards are included in the analyses sequence at the start, after every 10 samples, and at the end of the sample sequence to verify instrument performance over the course of the run. The calibration curve of the ionic standards is included only at the start of the sequence and at the end of the sequence if no more than 5 series of 10 samples are analysed. If uncertain about the quality or concentration of the samples, each sample may be followed by a blank with ultrapure water to check for memory effects or blank development. A typical sample sequence looks as follows:

- 1 Blank
- 2 Ionic standard 1
- 3 Ionic standard 2
- 4 Ionic standard 3
- 5 Ionic standard 4
- 6 Ionic standard 5
- 7 Ionic standard 6
- 8 Nanoparticle standard (NIST AuNP 60 nm)
- 9 Blank
- 10 Sample 1, rep 1
- 11 Sample 1, rep 2
- 12 Sample 1, rep 3
- 13 Sample 2, rep 1
- : :
- 17 Sample 10, rep 2
- 18 Sample 10, rep 3
- 19 Blank
- 20 Ionic standard 4
- 21 Nanoparticle standard (NIST AuNP 60 nm)
- 22 Blank
- 23 Sample 11, rep 1
- 24
- : :
- 40 Blank
- 41 Ionic standard 1
- 42 Ionic standard 2
- 43 Ionic standard 3
- 44 Ionic standard 4
- 45 Ionic standard 5
- 46 Ionic standard 6

(NOTE: When high particle concentrations are expected a ultrapure water sample can be placed after each sample to minimize and check on possible carry-over of analytes)

23.4.3 Evaluation of results

The raw data maybe processed with dedicated software from the ICP-MS supplier or from elsewhere. If not available, the raw data may be exported as a CSV file (intensities over time) and imported in a validated spreadsheet for data processing¹. This spreadsheet and a SOP how to use it are freely available from the RIKILT website². The spreadsheet calculates the ESD of the particles in the sample based on the detected elemental mass, and the particle's stoichiometry and density. The particles number and mass concentration is calculated from the number of particle peaks detected in the analysis, the transport efficiency, the sample flow and the acquisition time.

23.4.4 Reporting of results

The final results of the calculations within the spreadsheet are expressed as follows:

- Particle mass concentration (ngL^{-1})
- Particle number concentration (particleL^{-1})
- Particle size (nm) as ESD
- Ionic concentration (ngL^{-1})

In addition a graph of the particle's size distribution is presented.

23.5 Validation status

This method is validated.

23.6 HSE issues

Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses. Each chemical/particle shall be treated as a potential health hazard and exposure to these chemicals/particles shall be minimized.

23.7 References

¹ Peters, R., Herrera-Rivera, Z., Undas, A., van der Lee, M., Marvin, H., Bouwmeester, H. and Weigel, S. *Single particle ICP-MS combined with a data evaluation tool as a routine technique for the analysis of nanoparticles in complex matrices. J Anal. At. Spectrom.*, 2015, 30:1274-1285

² Single-Particle Calculation tool, SPC spreadsheet, RIKILT - Wageningen University & Research. <https://www.wageningenur.nl/en/show/Single-Particle-Calculation-tool.htm>

23.8 Performance characteristics

Table 1 gives the performance characteristics of the method for the detection and characterisation of TiO_2 NP in sunscreen product BAM-13A.

Table 1: Performance characteristics of the method for the detection and characterisation of TiO₂ NP in sunscreen

Linearity	0.5 to 50 µgL ⁻¹ based on ionic concentrations
Working range	size: from LOD _S up to 500 nm concentration: from LOD _C up when proper dilution is applied
LOD	LOD _S : 20 nm TiO ₂ LOD _C : 12 mgkg ⁻¹ product
LOQ	LOQ _S : 23 nm TiO ₂ LOQ _C : 30 mgkg ⁻¹ product
Repeatability	size: 2.4 % number concentration: 8.0 % mass concentration: 19.9 %
Intermediate precision	size: 4.0 % number concentration: 16.8 % mass concentration: 21.3 %
Trueness at 1.0VL and 0.5 VL*	84 % and 82 % for mass concentration
Ruggedness	determination of particle size not rugged for proper mixing (vortex) and setting of dwell time
Specificity/selectivity	yes/yes
Stability	the intermediate dilution is stable for at least 7 days
Measurement uncertainty, ux (Ux)	size: 5 % (11 %) number concentration: 20 % (41 %) mass concentration: 34 % (67 %)

24 Sample preparation and spICP-MS analysis of TiO₂ nanoparticles in suspensions

24.1 Aim

This standard operating procedure (SOP) can be applied to determine the particle size and size distribution, and the particle number or mass concentration of TiO₂ nanoparticles in suspensions using single particle ICP-MS.

24.2 Scope

The procedure is applicable for the determination of TiO₂ nanoparticles in suspensions. The procedure may also be applicable for other nanoparticles consisting of metal or metal oxides (e.g. Ag, Au, Al₂O₃, SiO₂, etc.) in aqueous suspensions. Depending on the type of nanomaterial, particle sizes (expressed as equivalent spherical diameter (ESD)) in the range of 10 to 1000 nm and mass concentrations in the range of 1 to 1000 ngL⁻¹ in the final suspension can be determined. The mass concentration range can be extended by further dilution of the suspensions.

24.3 Definitions

spICP-MS	Single particle inductively coupled plasma mass spectrometry.
Nanoparticle	A particle with at least one dimension in the range of 1 to 100 nm.
Dwell time	The time during which the ICP-MS detector collects and integrates incoming pulses. Following integration the total counts are registered as one data point, expressed in counts, or counts per second.

24.4 Description

The procedure consists of two parts:

- sample preparation
- spICP-MS measurement (detection and quantification of nanoparticles)

In case of suspensions dilution before instrumental analysis will often be required.

Single particle ICP-MS (spICP-MS) is based on the measurement of diluted nanoparticle suspensions by an ICP-MS that is operated in time resolved mode and set at a pre-selected mass-to-charge ratio (m/z). When properly diluted, individual particles enter the plasma of the ICP-MS, are atomised and ionised, and produce a plume of element ions which travels through the mass spectrometer and reaches the detector. The discrete measurement intervals of the MS (the dwell time) are typically set at a value ≤10 ms. This allows the detection of the ion plume of single particles (hence the name 'single particle ICP-MS') resulting in a peak in the time scan which is proportional to the mass of the respective element in the particle. The particle size, expressed as ESD (Equivalent spherical diameter), is calculated from the particle's mass. The number of peaks that are recorded during the time scan is proportional to the particle number and mass concentration. Detailed characteristics of the instrumental method are reported in the NanoDefine NanoDefine Manual, Part 2: Evaluation of methods.

24.4.1 Materials and methods

24.4.1.1 Materials, chemicals and reagents

Chemicals required for sample preparation and measurement are detailed in Table 1. Table 2 lists laboratory instruments and materials and Table 3 the analytical instruments and software.

Table 1: Chemicals for sample preparation and measurement

Chemical	Description	Manufacturer/Provider
Ultrapure water	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 μm)	e.g. Millipore, Billerica, MA, USA or equivalent
SHMP	Sodium hexametaphosphate (2 g L^{-1})	e.g. Fisher Scientific, Pittsburgh, PA, USA or equivalent
HNO_3	Nitric acid (suprapure, 65 %)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
RM 8013	Au Nanoparticles, nominal diameter: 60 nm	NIST, Gaithersburg, MD, USA
Ionic Standard Solutions	Titanium ionic standards in 3 % nitric acid (1 g L^{-1})	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Tune solution	Mix of elemental ICP-MS standards	Instrument specific, e.g. Tune B solution, Thermo Fisher Scientific, Waltham, MA, USA

Table 2: Laboratory instruments and materials

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance	e.g. Mettler Toledo, Vienna, Austria
Mechanical homogenizer	Vortex Mixer, 20.000 to 30.000 rpm	e.g. VWR International, Radnor, PA, USA or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication probe with a CML-4 probe operated at 4 Watt	e.g. Misonix XL-2000, Qsonica, Newton, CT, USA or equivalent
Calibrated pipettes	3 pipettes (0.5-10 μL , 10-100 μL , 100-1.000 μL)	e.g. Eppendorf, Hamburg, Germany

Table 3: Analytical instruments and software

Instrument	Description	Manufacturer/Provider
ICP-MS	Quadrupole ICP-MS with quartz torch, spray chamber and injector, usage of nickel cones (nanoparticle suspensions) and platinum cones (products)	e.g. ICAP-Q, Thermo Fisher Scientific, Waltham, MA, USA or equivalent
Autosampler	e.g. ESI SC-4Q	e.g. Elemental Scientific, Omaha, NE, USA or equivalent
Qtegra npQuant	Qtegra software with integrated nano-application tool for nanoparticle measurements and data evaluation	Thermo Fisher Scientific, Waltham, MA, USA
RIKILT SPC spreadsheet ¹	Validated Excel spreadsheet for the data evaluation of nanoparticle measurements	RIKILT - Institute of Food Safety

Reagents

Diluting agent. The diluting agent for the preparation of suspensions is prepared by weighing 2 g of sodium hexametaphosphate in a clean glass bottle and add 1 L of ultrapure water. Stir for at least 30 min. at room temperature until all material is dissolved and the liquid is fully homogenized. This stock can be stored at room temperature for at least 1 week.

Stock standard of 60 nm gold nanoparticles (50 µgL⁻¹). Pipet 50 µL of the gold reference standard RM 8013 to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 µgL⁻¹. Mix thoroughly and store at room temperature in amber glass screw necked vials. This intermediate standard is stable at room temperature for at least one month. Prior to use place the standard in an ultrasound bath for 10 minutes.

Working standard of 60 nm Gold nanoparticles (50 ngL⁻¹). Prepare the working standard by pipetting 50 µL of the stock standard to 25 mL of ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 ngL⁻¹. Mix thoroughly and store at room temperature in amber glass screw necked vials. This standard is prepared daily.

Stock standards of ionic titanium solutions (100 µgL⁻¹). Assuming the ionic standard solution has a concentration of 1 gL⁻¹, pipet 50 µL of the standard to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 1 mgL⁻¹. Repeat this step, pipetting 1 mL of the 1 mgL⁻¹ standard to 5 mL ultrapure water in a 10 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 100 µgL⁻¹. Mix thoroughly and store this intermediate standard in amber glass screw necked vials. Protected from light this intermediate standard is stable at room temperature for at least two weeks.

Working standards of ionic titanium solutions (0.5 – 50 µg L⁻¹). Prepare the calibration curve ionic standards according to table 1. Pipet the volumes of the stock standard of 100 µg L⁻¹ to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water. Mix thoroughly. Protected from light these working standards are stable at room temperature for the period indicated in Table 4.

(NOTE: When possible the composition of the ionic standard matrix should be matched to the prepared samples)

Table 4: Volumes for the preparation of the working standards of the ionic stock solution

Volume of the stock standard diluted to 50 mL ultrapure water	Ionic concentration of the working standard	Stability of the ionic working standard in glass
25 mL	50 μgL^{-1}	2 weeks
5 mL	10 μgL^{-1}	2 weeks
2.5 mL	5 μgL^{-1}	2 weeks
1 mL	2 μgL^{-1}	2 weeks
0.5 mL	1 μgL^{-1}	2 weeks
0.25 mL	0.5 μgL^{-1}	1 week

24.4.2 Performing measurements

24.4.2.1 Sample preparation

For the preparation of suspensions solid materials need to be suspended and the resulting suspensions need to be diluted prior to instrumental analysis.

24.4.2.2 Preparation of nanoparticle suspensions

The dispersion of nanomaterial powders is performed according to the following steps:

1. Prepare the initial stock by weighing 5 ± 2 mg of the nano-powder in a 50 mL glass vial. Add 5 mL of sodium hexametaphosphate (2 gL^{-1}) to produce a final concentration of $\sim 1 \text{ mgmL}^{-1}$.
2. Homogenize the dispersion by brief vortexing (30 seconds).
3. Sonicate the dispersion in ultrasonic bath for 10 minutes.
4. Dilute with sodium hexametaphosphate (2 gL^{-1}) to a total volume of 50 mL and a final concentration of $\sim 100 \mu\text{gmL}^{-1}$
5. Sonicate the second stock ($\sim 100 \mu\text{gmL}^{-1}$) for 15 minutes with an sonication probe (power: 4W) in an ice bath

Note: Sonication is a critical step in the preparation of the suspension. In case of doubt extend the sonication time (15 min) to 30 min.

6. For spICP-MS analysis dilute the sample 100,000 times in ultrapure water to a final concentration of $\sim 1,000 \text{ ngL}^{-1}$.

(NOTE: This procedure was written for the preparation of a suspension a TiO_2 nanomaterial. For other nanomaterials the method may need adjustments)

24.4.2.3 ICP-MS set-up and calibration

ICP-MS performance check

The instrument has a performance check and an autotune function which are designed to replace the manual checks and tuning procedures and the short term stability test. If the criteria of the

performance check are not met, a tuning, autotune or manual tune, is performed to optimize the instrument.

A 3 % nitric acid solution is used to rinse the sampling system of the ICP-MS before and in between runs. Special attention should be paid to the cleanliness of the sample introduction system of the ICP-MS. If high nanoparticle concentrations have passed through the tubing this may result in continuous background levels in subsequent analysis leading to erroneous results. Analyse an ultrapure water sample and a blank matrix sample to determine the background signals. In neither of the two the number of observed particles shall exceed a number of 10.

Settings of the ICP-MS system

- Forward power : 1550 W
- Nebulizer : PFA
- Spray chamber : cyclonic, quartz
- Gas flows : plasma, 13 Lmin⁻¹
nebulizer, 1.1 Lmin⁻¹
- Rinsing liquid flow rate : 1 mLmin⁻¹
- Sample flow rate : 0.35 mLmin⁻¹
- Data acquisition : time resolved analysis (TRA) mode (npQuant)
- Dwell time : 3 ms
- Total acquisition time : 60 s
- Isotope monitoring : Au (m/z 197), Ti (m/z 48)

(NOTE: For elements with potentially polyatomic interferences the application of another measurement mode (e.g. KED) or reaction cell (CCT) can improve results)

(NOTE: Use platinum cones when analysing samples containing fluorine)

In general the number of peaks in a time scan should not exceed 10 % of the maximum number of peaks based on the dwell time. Using a dwell time of 3 ms, the number of detected particles in the time scan should not exceed 2000. If this number is exceeded, the aqueous sample extract should be diluted and re-analysed. For the instrumental settings used in this procedure a particle number concentration in the range of 2×10^6 to 2×10^8 particles L⁻¹ results in useful measurement data.

24.4.2.4 Measurement description

Determination of transport efficiency

NIST RM 8013, a 60 nm Au nanoparticle is used to determine the transport efficiency on a daily basis. The number of detected particle events depends on the ICP-MS setup, the sample flow and the type of nebulizer. To accurately determine the transport efficiency, 200-500 particles should be observed in the analysis of a 50 ngL⁻¹ standard.

(NOTE: With more efficient nebulizers the concentration of the 60 nm Au nanoparticles can be lowered to 25 or even 10 ngL⁻¹)

Determination of the response of the analyte

A mass calibration should be performed using working standards in table 1 (6 concentrations in the range of 1 to 50 µgL⁻¹ and a blank) under the same measurement conditions as for spICP-MS measurements. Using linear regression the correlation coefficient of the calibration line will be determined. The correlation coefficient should be >0.99.

(NOTE: When possible the composition of the size calibrant matrix should be matched to that of the prepared samples)

Sample list

Blanks, ionic calibration standards and/or nanoparticle standards are included in the analyses sequence at the start, after every 10 samples, and at the end of the sample sequence to verify instrument performance over the course of the run. The calibration curve of the ionic standards is included only at the start of the sequence and at the end of the sequence if no more than 5 series of 10 samples are analysed. If uncertain about the quality or concentration of the samples, each sample may be followed by a blank with ultrapure water to check for memory effects or blank development. A typical sample sequence looks as follows:

1. Blank
2. Ionic standard 1
3. Ionic standard 2
4. Ionic standard 3
5. Ionic standard 4
6. Ionic standard 5
7. Ionic standard 6
8. Nanoparticle standard (NIST AuNP 60 nm)
9. Blank
10. Sample 1
11. Sample 2
- : :
24. Sample 9
25. Sample 10
26. Blank
27. Ion standard 4
28. Nanoparticle standard (NIST AuNP 60 nm)
29. Blank
30. Sample 11
- : :
44. Blank
45. Ionic standard 1
46. Ionic standard 2
47. Ionic standard 3
48. Ionic standard 4
49. Ionic standard 5
50. Ionic standard 6

(NOTE: When high particle concentrations are expected a ultrapure water sample can be placed after each sample to minimize and check on possible carry over of analytes)

(NOTE: If the material that is suspended has a wide polydispersity, or if large aggregates or agglomerates can be present, it is advised to analyse the final suspensions in triplicate to minimize the effect of these large particle structures)

24.4.3 Evaluation of results

The raw data maybe processed with dedicated software from the ICP-MS supplier or from elsewhere. If not available, the raw data may be exported as a CSV file (intensities over time) and imported in a validated spreadsheet for data processing¹. This spreadsheet and a SOP how to use it are freely available from the RIKILT website². The spreadsheet calculates the ESD of the particles in the sample based on the detected elemental mass, and the particle's stoichiometry and density. The particles number and mass concentration is calculated from the number of particle peaks detected in the analysis, the transport efficiency, the sample flow and the acquisition time.

24.4.4 Reporting of results

The final results of the calculations within the spreadsheet are expressed as followed:

- Particle mass concentration (ngL^{-1})
- Particle number concentration (particle L^{-1})
- Particle size (nm) as ESD
- Ionic concentration (ngL^{-1})

In addition a graph of the particle's size distribution is presented.

24.5 Validation status

This method is validated.

24.6 HSE issues

Protective clothing is required. Wear a lab coat, safety glasses, and gloves. Use reagents in an efficient fume hood. Handle acids wearing gloves and safety glasses. Each chemical/particle should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized.

24.7 References

¹ Peters, R., Herrera-Rivera, Z., Undas, A., van der Lee, M., Marvin, H., Bouwmeester, H. and Weigel, S. *Single particle ICP-MS combined with a data evaluation tool as a routine technique for the analysis of nanoparticles in complex matrices. J Anal. At. Spectrom.*, 2015, 30:1274-1285

² Single-Particle Calculation tool, SPC spreadsheet, RIKILT - Wageningen University & Research. <https://www.wageningenur.nl/en/show/Single-Particle-Calculation-tool.htm>

24.8 Performance characteristics

The Table 1 below gives the performance characteristics of the method for the detection and characterisation of TiO_2 NP in suspensions.

Table 1: Performance characteristics of the method for the detection and characterisation of TiO₂ NP in suspension

Linearity	0.5 to 10 µg/L based on ionic concentrations and based on other results extended to 0.5 to 50 µg/L
Working range	size: from LOD _s up to 500 nm concentration: from LOD _c up when proper dilution is applied
LOD	LOD _s : 25 nm TiO ₂ LOD _c : 250 pg/L in suspension
LOQ	LOQ _s : 30 nm TiO ₂ LOQ _c : 750 pg/L in suspension
Repeatability	size: 3.2 % number concentration: 23 % mass concentration: 20 %
Intermediate precision	size: 7.9 % number concentration: 24 % mass concentration: 21 %
Trueness	83 %
Robustness	robust sonication time and setting of dwell time
Specificity/selectivity	yes/yes
Stability	the suspension is stable for at least 2 days
Measurement uncertainty u_x (U_x)	size: 9 % (18 %) number concentration: 40 % (81 %)* mass concentration: 33 % (66 %)*

* Represents the sum of sample preparation and measurement, as performed in the NanoDefine project

25 Sample preparation and spICP-MS analysis of Al₂O₃ nanoparticles in toothpaste

25.1 Aim

This standard operating procedure (SOP) can be applied to determine the particle size and size distribution, and the particle number or mass concentration of Al₂O₃ particles in toothpaste products using single particle ICP-MS (spICP-MS).

25.2 Scope

The procedure is applicable for the determination of Al₂O₃ particles in toothpaste. The procedure may also be applicable for other particles consisting of metal or metal oxides (e.g. Ag, Au, TiO₂, SiO₂, etc.) in consumer products with a composition comparable with toothpaste. Depending on the type of nanomaterial, particle sizes (expressed as equivalent spherical diameter, ESD) in the range of 50 to 500 nm and mass concentrations in the range of 1 to 1000 ng L⁻¹ in the final extract/suspension can be determined. The mass concentration range can be extended by further dilution of the prepared extracts or suspensions.

25.3 Definitions

spICP-MS	Single particle inductively coupled plasma mass spectrometry.
Nanoparticle	A particle with at least one dimension in the range of 1 to 100 nm.
Dwell time	The time during which the ICP-MS detector collects and integrates incoming pulses. Following integration the total counts are registered as one data point, expressed in counts, or counts per second.
ESD	Equivalent spherical diameter (for particle size)

25.4 Description

The procedure consists of two parts:

- sample preparation
- spICP-MS measurement (detection and quantification of nanoparticles)

In case of toothpaste a sub-sample is collected and dispersed in a diluting agent, which stabilises the nanoparticles in suspension. This first suspension is then diluted further in one or more steps before instrumental analysis.

Single particle ICP-MS (spICP-MS) is based on the measurement of diluted nanoparticle suspensions by an ICP-MS that is operated in time resolved mode and set at a pre-selected mass-to-charge ratio (m/z). When properly diluted, individual particles enter the plasma of the ICP-MS, are atomised and ionised. Produced the plume of element ions travels through the mass spectrometer and reaches the detector. The discrete measurement intervals of the MS (the dwell time) are typically set at a value ≤10 ms. This allows the detection of the ion plume of single particles (hence the name 'single particle ICP-MS') resulting in a peak in the time scan which is proportional to the mass of the respective element in the particle. The particle size, expressed as ESD, is calculated from the particle's mass. The number of peaks that are recorded during the time

scan is proportional to the particle number and mass concentration. Detailed characteristics of the instrumental method are reported in the NanoDefine Manual, Part 2 : Evaluation of methods.

25.4.1 Materials and methods

25.4.1.1 Chemicals, equipment and instruments

The chemicals required for sample preparation and measurement are detailed in Table 1. Table 2 lists laboratory instruments and materials and Table 3 the analytical instruments and software.

Table 1: Chemicals for sample preparation and measurement

Chemical	Description	Manufacturer/Provider
Ultrapure water	e.g. Millipore A10 system equipped with a Milli-Pak Express 20 filter (0.22 µm)	e.g. Millipore, Billerica, MA, USA or equivalent
RM 8013	Au Nanoparticles, nominal diameter: 60 nm	NIST, Gaithersburg, MD, USA
Al ₂ O ₃ particles standard	e.g. powdered Al ₂ O ₃ particles, ESD 135 nm	e.g. US Research Nanomaterials, Houston, TX, USA
Dispersion agent	Sodium hexametaphosphate (2 gL ⁻¹)	e.g. Fisher Scientific, Pittsburgh, PA, USA or equivalent
HNO ₃	Nitric acid (suprapure, 65 %)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Ionic Standard Solutions	Aluminium ionic standards in 3 % nitric acid (1 gL ⁻¹)	e.g. Merck KGaA, Darmstadt, Germany or equivalent
Tune solution	Mix of elemental ICP-MS standards	Instrument specific, e.g. Tune B solution, Thermo Fisher Scientific, Waltham, MA, USA

Table 2: Laboratory equipment

Instrument	Description	Manufacturer/Provider
Analytical balance	Analytical balance	e.g. Mettler Toledo, Vienna, Austria
Mechanical homogenizer	Vortex Mixer, 20.000 to 30.000 rpm	e.g. VWR International, Radnor, PA, USA or equivalent
Ultrasonic water bath	Ultrasonic Cleaner	e.g. VWR International, Radnor, PA, USA or equivalent
Sonication probe	Sonication probe with a CML-4 probe operated at 4 Watt	e.g. Misonix XL-2000, Qsonica, Newton, CT, USA or equivalent
Magnetic plate	Magnetic plate used with magnetic stirrers to disperse the toothpaste	e.g. AG Germany or equivalent
Magnetic stirrers	Egg-shaped PTFE-coated magnetic stirrers used to disperse the toothpaste (L 15 mm, W 6 mm)	e.g. VWR Collection, Randor, PA, USA or equivalent
Calibrated pipettes	3 pipettes (0.5-10 µL, 10-100 µL, 100-1.000 µL)	e.g. Eppendorf, Hamburg, Germany

PE Tubes	PE centrifuge tubes used in sample preparation (50 ml) and measurement (12 ml)	e.g. VWR Collection, Randor, PA, USA or equivalent
----------	--	--

Table 3: Analytical instruments and software

Instrument	Description	Manufacturer/Provider
ICP-MS	Quadrupole ICP-MS with quartz torch, spray chamber and injector, usage of platinum cones	e.g. Perkin Elmer Nexion 350D or equivalent, Waltham, MA, USA.
Autosampler	e.g. ESI SC-4Q	e.g. Elemental Scientific, Omaha, NE, USA or equivalent
RIKILT spreadsheet ¹	SPC Validated Excel spreadsheet for the data evaluation of nanoparticle measurements	RIKILT - Institute of Food Safety

25.4.2 Methods

25.4.2.1 Stock and working solutions

Rinsing solvent (3 %). The ICP-MS system is rinsed after each measured sample therefore it is wise to prepared larger volume of the rinsing solvent. Fill the 8 L container with ultrapure water up to 2/3. Add ca. 370 ml of 65 % nitric acid. Fill with ultrapure water to 8 mL and homogenize. The rinsing solvent can be stored at room temperature for at least 1 week.

Dispersion agent (2 gL⁻¹). The dispersion agent for the preparation of toothpaste sub-sample is prepared by weighing 2 g of sodium hexametaphosphate in a clean glass bottle and fill to 1 L with ultrapure water. Stir for at least 30 min. at room temperature until all material is dissolved, and the liquid is fully homogenized. This stock can be stored at room temperature for at least 1 week.

Stock standard of 60 nm gold nanoparticles (50 µg L⁻¹). Pipet 50 µL of the gold reference standard RM 8013 to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 µgL⁻¹. Mix thoroughly and store at room temperature in amber glass screw necked vials. This intermediate standard is stable at room temperature for at least one month. Prior to use place the standard in an ultrasound bath for 10 minutes.

Working standard of 60 nm Gold nanoparticles (50 ngL⁻¹). Prepare the working standard by pipetting 50 µL of the stock standard to 25 mL of ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a final mass concentration of 50 ngL⁻¹. Mix thoroughly and store at room temperature in amber glass screw necked vials. This standard is prepared daily.

Stock standard of Al₂O₃ particles (2 gL⁻¹). If necessary the recovery of the Al₂O₃ can be approximated by spiking the diluted toothpaste dispersions with Al₂O₃ particles standard dispersed beforehand according to the modified NanoGenotox protocol². Briefly, weigh 20 mg of the powdered particle standard in 12 ml glass vial. Pre-wet the powder with 30 µL ethanol and vortex briefly. Add dispersion agent in two steps. First step, add 570 µL of dispersion agent followed by brief vortexing and second step add 4.4 mL of dispersion agent followed again by brief vortexing.

Sonicate the dispersion for 15 min using probe sonicator (4 Watt). Rinse the probe with 5 mL dispersion agent. This standard is stable for x. Vortex prior usage.

Stock standards of ionic aluminium solutions (1000 μgL^{-1}). Assuming the ionic standard solution has a concentration of 1 gL^{-1} , pipet 50 μL of the standard to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water resulting in a concentration of 1 mg L^{-1} . Mix thoroughly and store this intermediate standard in amber glass screw necked vials. Protected from light this intermediate standard is stable at room temperature for at least two weeks.

Working standards of ionic aluminium solutions (0.5 – 50 μgL^{-1}). Prepare the ionic working standards according to Table 1. Pipet the volumes of the stock standard of 1000 μgL^{-1} to 25 mL ultrapure water in a 50 mL glass measuring flask and fill to the mark with ultrapure water. Mix thoroughly. Protected from light these working standards are stable at room temperature for the period indicated in Table 4.

(NOTE: When possible the composition of the ionic standard matrix should be matched to the prepared samples)

Table 4: Volumes for the preparation of the working standards of the ionic stock solution

Volume of the stock standard diluted in 50 mL ultrapure water	Ionic concentration of the working standard	Stability of the ionic working standard in glass
2.5mL	50 μgL^{-1}	2 weeks
1 mL	20 μgL^{-1}	2 weeks
0.5 mL	10 μgL^{-1}	2 weeks
0.25 mL	5 μgL^{-1}	2 weeks
0.05 mL	1 μgL^{-1}	2 weeks
0.025 mL	0.5 μgL^{-1}	1 week

25.4.2.2 Sample preparation

1. Weigh approximately 0.5 g of the product in a 50 mL PE centrifuge tube and fill to 50 mL with dispersion agent. (dispersed sub-sample of toothpaste)
2. Place in the sub-sample of toothpaste magnetic stirrer and place the PE centrifuge tube on magnetic plate. Disperse the toothpaste for 15 min.
3. First dilution. Vortex 10 sec the suspension. Fill a 50 ml PE centrifuge tube with 25 ml ultrapure water and pipette 250 μL of dispersed sub-sample of toothpaste into the ultrapure water. Fill to the 50 mL mark with ultrapure water.
4. Second dilution. Vortex 15 sec and shake the diluted suspension. Fill a 50 ml PE centrifuge tube with 25 ml ultrapure water and pipette 100 μL of diluted dispersed sub-sample of toothpaste into the ultrapure water. Fill to the 50 mL mark with ultrapure water.

(NOTE: This procedure was prepared for toothpaste products containing Al_2O_3 in a concentration around 10 gkg^{-1} . For other nanomaterials or other matrices (e.g. facial creams, toothpaste etc.) the method may need adjustments)

25.4.2.3 Measurements

Settings and tuning of the ICP-MS (performance check)

Settings of the ICP-MS system

- Forward power : 1600 W
- Nebulizer : PFA
- Spray chamber : cyclonic, quartz
- Sample, skimmer cone : platinum
- Gas flows : plasma, 13 Lmin⁻¹
nebulizer, 1.1 Lmin⁻¹
- Rinsing liquid flow rate : 1 mLmin⁻¹
- Sample uptake rate : 0.35 mLmin⁻¹
- Data acquisition : time resolved analysis (TRA) mode
- Dwell time : 3 ms
- Acquisition time : 60 s
- Isotopes monitored : Au (m/z 197), Al (m/z 27)
- DRC parameters : 0.6 mLmin⁻¹ cell gas (ammonia), RPq 0.5

The ICP-MS instrument should be tuned and its performance check should be performed according to the manufacturer guidelines. A 3 % nitric acid solution is used to rinse the sampling system of the ICP-MS before and in between runs. Special attention should be paid to the cleanliness of the sample introduction system of the ICP-MS. If high nanoparticle concentrations have passed through the tubing this may result in continuous background levels in subsequent analysis leading to erroneous results. Analyse an ultrapure water sample after each sample and a blank matrix sample to determine the background signals should be implemented in the analysis sequence. In neither of the two the number of observed particles shall exceed a number of 10.

In general the number of peaks of the analysed diluted dispersed toothpaste sample in a time scan should not exceed 10 % of the maximum number of peaks based on the dwell time. Using a dwell time of 3 ms, the number of detected particles in the time scan should not exceed 2000. If this number is exceeded, the aqueous sample extract should be diluted and re-analysed.

(NOTE: In case of toothpastes containing fluoride, the fluoride can seriously damage the cones of the ICP-MS. This is certainly the case when non-platinum cones are used and therefore the use of platinum cones is advised)

Measurement description

a. Determination of transport efficiency

NIST RM 8013, a 60 nm Au nanoparticle is used to determine the transport efficiency on a daily basis. The number of detected particle events depends on the ICP-MS setup, the sample flow and the type of nebulizer. To accurately determine the transport efficiency, 200-500 particles should be observed in the analysis of a 50 ngL⁻¹ standard.

(NOTE: With more efficient nebulizers the concentration of the 60 nm Au nanoparticles can be lowered to 25 or even 10 ngL⁻¹)

b. Determination of the response of the analyte

A mass calibration should be performed using working standards of ionic aluminium prepared according to Table 1 (6 concentrations in the range of 0.5 to 50 μgL^{-1} and a blank sample) under the same measurement conditions as for splCP-MS measurements. Using linear regression the correlation coefficient of the calibration line will be determined. The correlation coefficient should be >0.99 .

c. Samples of interest

Blanks, ionic calibration standards and/or nanoparticle standards are included in the analyses sequence at the start, after every 10 samples, and at the end of the sample sequence to verify instrument performance over the course of the run. The calibration curve of the ionic standards is included only at the start of the sequence and at the end of the sequence if no more than 5 series of 10 samples are analysed. If uncertain about the quality or concentration of the samples, each sample may be followed by a blank with ultrapure water to check for memory effects or blank development. A typical sample sequence looks as follows:

1. Blank
2. Ionic standard 1
3. Ionic standard 2
4. Ionic standard 3
5. Ionic standard 4
6. Ionic standard 5
7. Ionic standard 6
8. Nanoparticle standard (NIST AuNP 60 nm)
9. Blank
10. Sample 1
11. Sample 2
- ...
25. Sample 9
26. Sample 10
27. Blank
28. Ion standard 4
29. Nanoparticle standard (NIST AuNP 60 nm)
30. Blank
31. Sample 11
-
- ...
44. Blank
45. Ionic standard 1
46. Ionic standard 2
47. Ionic standard 3
48. Ionic standard 4
49. Ionic standard 5
50. Ionic standard 6

25.4.3 Data processing and reporting of results

The raw data maybe processed with dedicated software from the ICP-MS supplier or from elsewhere. If not available, the raw data may be exported as a CSV file (intensities over time) and imported in a validated spreadsheet for data processing¹. This spreadsheet and a SOP how to use it

are freely available from the RIKILT website². The spreadsheet calculates the ESD of the particles in the sample based on the detected elemental mass, and the particle's stoichiometry and density. The particles number and mass concentration are calculated from the number of particle peaks detected in the analysis, the transport efficiency, the sample flow and the acquisition time.

The final results of the calculations within the spreadsheet are expressed as followed:

- Particle mass concentration (ngL⁻¹)
- Particle number concentration (particle L⁻¹)
- Particle size (nm) as ESD
- Ionic concentration (ngL⁻¹)

In addition a graph of the particle's size distribution is presented.

25.5 Validation status

This method was validated in-house.

25.6 HSE issues

Protective clothing (lab coat, safety glasses, and gloves.) is required. Each chemical/particle should be treated as a potential health hazard and exposure to these chemicals/particles should be minimized. If possible standards, chemicals and reagents should be prepared in a fume hood.

25.7 References

¹ Single-Particle Calculation tool, SPC spreadsheet, RIKILT - Wageningen University & Research.
<https://www.wageningenur.nl/en/show/Single-Particle-Calculation-tool.htm>

² NanoGenotox, Standard operating procedures for characterisation of the selected manufactured nanomaterials and dispersions thereof June, 2011

25.8 Performance characteristics

Table 1 below gives the performance characteristics of the method for the detection and characterisation of Al₂O₃ particles in toothpaste products using single particle ICP-MS (spICP-MS).

Table 1: Performance characteristics of the method for the detection and characterisation of Al₂O₃ nanoparticles in toothpaste

Linearity	0.5 to 50 µg/L based on ionic concentrations
Working range	size: from LOD _s up to 500 nm concentration: from LOD _c up when proper dilution is applied
LOD	LOD _s : 48 nm TiO ₂ LOD _c : 61 mg/kg product
LOQ	LOQ _s : 70 nm TiO ₂ LOQ _c : 180 mg/kg product

Repeatability	size: 5.3 % number concentration: 5.8 % mass concentration: 2.3 %
Intermediate precision	size: 15 % number concentration: 24 % mass concentration: 29 %
Trueness at 1.0VL and 2.0 VL*	81 % and 64 % for mass concentration
Ruggedness	the determination of particle mass concentration is not rugged for proper mixing (stirring)
Specificity/selectivity	yes/yes
Stability	the intermediate dilution is stable for at least 4 days
Measurement uncertainty, u_x (U_x)	size: 17 % (34 %) number concentration: 25 % (50 %) mass concentration: 33 % (66 %)

*VL was 10 g Al₂O₃ NP/kg product

26 General Conclusions

This report presents 23 Standard Operating Procedures, SOPs, developed in NanoDefine.

NanoDefine developed 11 detailed material specific protocols designed to produce liquid dispersions of the NanoDefine priority substances such that the resulting dispersions are stable and contain only or mainly constituent particles. The priority substances are: IRMM-380 (Pigment yellow 83 (transparent grade)), IRMM-381 (BaSO₄ (fine grade)), IRMM-382 (MWCNT), IRMM-383 (Nano steel), IRMM-384 (CaCO₃ (fine grade)), IRMM-385 (Kaolin), IRMM-386 (Pigment yellow 83 (opaque grade)), IRMM-387 (BaSO₄ (ultrafine grade)), IRMM-388 (Coated TiO₂ (pigment grade)), IRMM-389 (Basic methacrylate copolymer particles (BMC)) and BAM-11 (Zeolite powder).

An additional 13 SOPs have been developed:

- i) a generic SOP for calorimetric calibration of the sonicators,
- ii) the DLS method, which was developed for four of materials (IRMM-381 (BaSO₄ (fine grade)), IRMM-384 (CaCO₃ (fine grade)), IRMM-385 (Kaolin), IRMM-388 (Coated TiO₂ (pigment grade))). It can also be applied to comparable types of materials, considering that adaptations might be needed,
- iii) the Cuvette-AC method which was developed for two materials (IRMM-381 (BaSO₄ (fine grade)), IRMM-387 (BaSO₄ (ultrafine grade))), and which can also be applied to comparable types of materials, considering that adaptations might be needed,
- iv) a method for the analysis of Fe₂O₃ in polyethylene matrix with electron microscopy,
- v) a method for the analysis of TiO₂ in sunscreen with electron microscopy,
- vi) SOPs for size characterisation of suspended particles by AUC-RI with speed ramp option,
- vii) particle size distribution measurement of BaSO₄ using Line-Start Disc Centrifuge with Optical Detection,
- viii) measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software,
- ix) analysis of TiO₂ particles from sunscreen by AF4-MALS-ICP-MS,
- x) sample preparation and spICP-MS analysis of TiO₂ nanoparticles in sunscreen products,
- xi) sample preparation and spICP-MS analysis of TiO₂ nanoparticles in suspensions, and
- xii) sample preparation and spICP-MS analysis of Al₂O₃ nanoparticles in toothpaste.

26.1 Dispersion protocols

The dispersion procedure is a pivotal step in the process of making measurements of the particle size distribution. It is thus necessary that dispersion procedures are reproducible fit to the purpose of analysis/characterisation and lead to no/insignificant contamination. Of course it is also important that dispersion procedures are effective and efficient. The final dispersion obtained by these dispersion procedures should have a particle size distribution, which is as close as possible to the true distribution of constituent particles.

The issue of dispersion is particularly important in the evaluation of nanoparticle size as many nanomaterials are normally found in the form of dried powders which need to be brought into

stable liquid dispersions before they can be measured by many of the most common particle size measuring methods such as dynamic light scattering, angular light scattering, centrifugal liquid sedimentation and analytical centrifugation. To achieve comparable results, the procedures for bringing the materials into dispersion should as far as possible be harmonised and standardized. A first step towards this is to develop SOPs and NanoDefine developed 11 SOPs for dispersion protocols, which take into account material specificities and are based on aqueous dispersion. When developing these protocols it was found that agglomerated materials cannot be adequately dispersed by the use of low energy mixing (stirring/shaking) or by the use of ultrasonic bath (USB). Instead it was necessary to apply the high energy methods probe sonication (USP) or vial sonication (VS). Thus, the 11 SOPs all include a probe sonication dispersion protocol, as the probe-sonicator was the most common apparatus among the laboratories participating, and furthermore selected materials ((IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 (CaCO₃), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO₂)) have an associated protocol for vial-sonication.

The cup-horn sonicator was not available to the participating laboratories and is thus not included in the analysis.

Sonication is effective for dispersing the test material, but it introduces a significant variable in the process as a wide variety of different sonication instruments exists with different nominal power and probe size. To reduce that variability a SOP 'Generic SOP for calorimetric calibration of an ultrasonic probe sonication' was developed to allow ensuring a better harmonisation of the power output when using significantly different sonicator types or probe types/sizes for the dispersion compared to those used in the development of in the optimised protocols.

For the materials ((IRMM-380 (Pigment Yellow 83, Fine grade), IRMM-384 (CaCO₃), IRMM-386 (Opaque Pigment Yellow 83) and IRMM-388 (Coated TiO₂)) for which procedures using both probe and vial sonication were developed it was noted that the treatment times required (to achieve comparable levels of dispersion) were generally similar provided that the volume specific ultrasonic energy input were comparable. This indicates that it is not sufficient to consider only the power output of a sonicator, and that also the volume of sample which is being treated needs to be considered. For example to achieve a similar level of dispersion in a similar time (15-20 min) the vial sonicator supplied a much lower power of 2.2 W compared to the 7.8 W value output by the probe sonicator. However, when considering that in the first example the sample volume was 2 mL while in the second case it was 6 mL it can be seen that the actual specific power expressed as WmL⁻¹ are actually quite similar (1.1 WmL⁻¹ compared to 1.3 WmL⁻¹). This observation underlines the importance of respecting the technical details of dispersion protocols and in particular care should be taken to ensure that the sonication power used is appropriate to the volume and concentrations of the sample being treated. If it is necessary to deviate significantly from the volumes and concentrations specified in the protocols it would be critical that sonication times and energies be adequately adapted and optimised by the use of appropriate methods. It is also relevant to note that while insufficient sonication must be avoided it is equally important to avoid excessive treatment times or energies as this can lead to a loss in the number of the small and intermediate particulates/aggregates presumably by irreversible fusion of the particulates. This phenomenon has not been detailed in this report but it has been specifically observed to occur with TiO₂, BaSO₄ and CaCO₃ treated for periods of greater than 2 hours rather than the normal times of 20-30 minutes.

26.2 Possible contamination by probe sonicator

Despite being the most commonly available probes, sonication probes should be used cautiously, as probe material may be released due to wear of the probe, starting after only a few hours of use of the probe. Furthermore, this wear may not be easy to detect, as the residues (fine grey-white powder) may not be easily detected by visual inspection of the final dispersions.

If a laboratory regularly produces nanoparticle dispersions for metrological applications, such as those of NanoDefine, then it is advisable that, when appropriate equipment is available and sample volumes and temperature sensitivity permit, non-contact methods of sonication should be adopted to avoid any risk of this problem. Non-contact systems are e.g. vial or cup sonicators.

In cases where direct contact probe sonication is to be used then it may be preferable to use a probe sonicator which has an exchangeable tip so that this may be easily inspected and replaced whenever necessary. In this way regular substitution may be undertaken with a lower cost than in the case of substituting mono-block probes.

26.3 Material- and technique- dependent observations

For two of the priority materials which are platelets (IRMM-383 (nano steel) and IRMM-385 (Kaolin)), systematic optimisation of the dispersion protocols using DLS or CLS was not feasible and of limited relevance as these materials are likely to be limited to EM or BET analysis, which both require limited optimisation for colloidal stability. Consequently, the protocol development for these materials has mainly concentrated on achieving sufficient de-agglomeration in simple aqueous media to make them suitable for the preparation of TEM samples without the need for long term stability.

Also for IRMM-382 (MWCNT) the argument about analysis being limited to TEM applies, but as these materials are strongly hydrophobic and composed of tangled bundles which cannot easily be separated it was necessary to undertake a more detailed optimisation of the protocols as the use of a surfactant is critical. In this case, the commonly used 'universal' surfactant BSA was explored and found to be useable but the dispersions tended to re-agglomerate and consequently two other alternatives were examined. The first, Triton-X100, was probably the most effective in stabilising higher concentrations of the MWCNT in aqueous solution but a relatively high level of surfactant in solution was required and foaming during probe sonication may be produced. The second material, tannic acid, was able to stabilise only lower concentrations of the MWCNT but the relative concentration of additive need was also lower and the solutions did not produce problems of foaming.

For mineral type products (TiO_2 , CaCO_3 and BaSO_4) a single, commonly used dispersant, Sodium Hexametaphosphate (SHMP) was found to be generally applicable. More complex procedures using more exotic surfactants or combinations of surfactant were necessary only in the case of the organic materials (basic methacrylate co-polymer (IRMM-389) and the diarylide Pigment Yellow 83 (IRMM-380 and IRMM-386)).

Finally, BAM-11 (zeolite) was tested with a number of stabilising agents but no advantage was found over the use of pure water. Overall, this material could be dispersed in solution but the resulting particulates had sizes from the nanorange to the micrometre range.

26.4 Sample preparation for NM in products

A SOP for analysis of Fe₂O₃ in Polyethylene Matrix with Electron Microscopy methods was developed (Chapter 17). It illustrates protocols for preparation of products for microscopy methods and covers sample preparation and fully automatic particle size distribution analysis of Fe₂O₃ nanoparticles in high density polyethylene. The scope of the sample preparation can be extended to any nanocomposite soft material that can be cut by an ultramicrotome; and the analysis guidelines are valid for any complex nanoparticle agglomerates.

Three SOPs for analysing the presence of TiO₂ in sunscreen were developed, one by electron microscopy (Chapter 18), one by AF4-MALS-ICP-MS (Chapter 22), and one by spICP-MS (Chapter 23). Also for TiO₂ a SOP for sample preparation and spICP-MS analysis of TiO₂ nanoparticles in suspensions (Chapter 24) was developed.

One SOP for sample preparation and spICP-MS analysis of Al₂O₃ nanoparticles in toothpaste (Chapter 25) was developed.

Two SOPs for characterisation were developed: a SOP for measuring particle size distribution measurement of BaSO₄ using Line-Start Disc Centrifuge with Optical Detection (Chapter 20), and a SOP for size characterisation of suspended particles by AUC-RI with speed ramp option (Chapter 19).

Finally a SOP for measurement of the minimal external dimension of the constituent particles of particulate materials from TEM images by the NanoDefine ParticleSizer software (Chapter 21) has been presented.

SOPs for products were found to be highly targeted towards each product, and it is evaluated that a SOP would be needed for each combination of <product, NM, analytical technique>.

GETTING IN TOUCH WITH THE EU

In person

All over the European Union there are hundreds of Europe Direct information centres. You can find the address of the centre nearest you at: https://europa.eu/european-union/contact_en

On the phone or by email

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

- by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),
- at the following standard number: +32 22999696, or
- by electronic mail via: https://europa.eu/european-union/contact_en

FINDING INFORMATION ABOUT THE EU

Online

Information about the European Union in all the official languages of the EU is available on the Europa website at: https://europa.eu/european-union/index_en

EU publications

You can download or order free and priced EU publications from EU Bookshop at: <https://publications.europa.eu/en/publications>. Multiple copies of free publications may be obtained by contacting Europe Direct or your local information centre (see https://europa.eu/european-union/contact_en).

The European Commission's science and knowledge service

Joint Research Centre

JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.



EU Science Hub
ec.europa.eu/jrc



@EU_ScienceHub



EU Science Hub - Joint Research Centre



Joint Research Centre



EU Science Hub

