

Final publishable summary report

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Executive summary

The practical implementation of the EC recommendation on a definition of nanomaterial (2011/696/EU) is an enormous analytical challenge especially for the material manufacturing industry that need to classify their materials according to this new definition but also for regulatory bodies that need to assess compliance with this new provision across various policy areas, including REACH, product labelling, food, cosmetics or biocides. Indeed, the recommended definition includes all particulate materials with a size distribution below 100 nm, which in turn means that a lot of conventional materials, such as pigments, fillers, additives etc., need to be assessed accordingly.

To support the implementation of the proposed definition, the EU FP7 NanoDefine projects was initiated by 29 partners from 11 European countries representing top expertise in material science, metrology, material and instrument manufacturing. The main goal of the planned work was to develop an integrated approach consisting of a continuous performance evaluation of relevant measurement techniques, improvement of available software and instruments, and of proper sample preparation methods, and finally the design of a new 2-tiered measurement strategy based on a decision-support system in the form of a method manual and an e-Tool to easily select the most appropriate (or combination of) methods to classify materials according to the EC definition. The concept underlying this new tool includes techniques of increasing complexity that complement and support each other by adequate procedures for sampling, sample preparation, instrument calibration and data analysis as well as plausibility checks with minimum performance requirements. The main outcome achieved in the various WPs¹ is highlighted in the following:

A well-selected matrix of industry-relevant substances (15) and products (4) has been provided in WP1 to the project. The matrix approach ensured the relevance of the project results for many different industries, material systems and applications. Homogeneity and stability of all materials were assessed. Certification and development of distinct substances into CRMs will set the basis of future relevance of NanoDefine. WP2 developed and optimized methods for the dispersion of NM from powders and the extraction of NM from products, as provided by WP1, and for further analysis by tier 1 and tier 2 methods developed in WP4 and WP5. The comprehensive evaluation and selection of techniques and methodologies in realized in WP3 and resulting in the sizing methods evaluation hub, has delivered for the first time a quantitative assessment of the achievable capabilities of almost all nanoparticle sizing methods as a result of their systematic testing on the NanoDefine QCMs² and RTMs³ defined in WP1. The achieved method performance parameters have been successfully transferred to WP7 and fed into the NanoDefiner e-tool as well as the NanoDefine Method Manual. Moreover, based on WP3 measurements, recommendations have been also made of methods to be further considered and/or developed for tier 1 (WP4) or tier 2 (WP5), or for method validation in WP6. WP4 established a set of validated, cost-efficient, robust, and easy implementable methods for the rapid distinction between nano/non-nano according to the definition. This included improvement of instruments and related methods (in cooperation with WP2) to increase the range of applicability and cost-efficiency, and the determination of individual methods' limitations and derivation of maximum application ranges with regard to the requirements of the definition (see also WP6). The following potential screening methods have been developed: Analytical Centrifugation methods (AC/CLS), Dynamic light scattering (DLS), Electric mobility analysis, MiniTEM, Particle tracking analysis (PTA/NTA), SingIe particle ICP-MS. These screening methods have been introduced as tier 1 in the decision tree of WP7 based on method selection criteria developed together with WP3, and on method validation developed together with WP6. An Auto-EM toolbox, including an automatic acquisition and analysis of transmission electron microscopy (TEM) micrographs has been

¹ WPs = Work Packages

² QCMs = Quality Control Materials

³ RTMs = Representative Test Materials

the major outcome in WP5. This novel EM technique allows classifying a material as a nanomaterial according to the EC definition with reduced user-bias. Another major outcome of WP5 consists in new standard operating procedures (SOPs) for determining the median diameter ($D_{50} / \times 50$) of the number-based particle size distribution of titanium dioxide (TiO_2) particles in sunscreen and aluminium oxide (Al_2O_3) particles in toothpaste, respectively, by asymmetric flow field-flow fractionation (AF4) coupled to multi angle light scattering (MALS) and inductively coupled plasma-mass spectrometry (ICP-MS).

In WP6, a common guideline for in-house validations was developed and agreed by all partners assuring a harmonized in-house validation over the various methods and work packages. Apart from this guidance, harmonized in-house validation was assured through common templates and continuous support from the JRC. A special project was started under VAMAS and an international call for inter-laboratory validation exercises launched. These validation studies were conducted and evaluated for 8 methods, some of them showing fitness for purpose, while others still need further optimisation. Liaison with the standardisation committee CEN/TC352 was realized and contribution to many standardisation committees provided. The NanoDefine Methods Manual was proposed to CEN/TC352 as a New Work Item Proposal.

WP7 pooled all findings from all other work packages to implement the project results for the primary interest groups: regulators and enterprises. Its main products are a decision support flow scheme, which is implemented also in an e-tool, a material classification system and the NanoDefine Methods Manual. WP7 also prepared recommendations on a revision of the EC Definition of nanomaterial, based on analytical possibilities.

Summary description of project context and objectives

One of the main purpose of NanoDefine is to develop a new and reliable measurement strategy and approach that can support potential end-users, such as the manufacturing industry and regulatory bodies to implement the EU Definition (2011/696/EU). To cover the vast spectrum of diversity and complexity of existing and newly emerging materials, the concept of NanoDefine has been built upon a new 2-tiered approach consisting of (1) rapid and cost-effective screening methods that rather easily identify nanomaterials (NM) in substances or product formulations, and of (2) confirmatory techniques that complement these screening methods and match more difficult materials with complex matrices, such as industrial or consumer products.

Although the EU definition of nanomaterial is solely based on determining the number based size distribution of the constituent particles of a material, including agglomerates and aggregates, without relating to any specific functional or hazard properties, it can be also used as reference across different policy areas and legislations such as chemicals (REACH) or product labelling, food, cosmetics or biocides. Accordingly, the 2-tiered approach developed by NanoDefine does not only help to classify materials as nano or non-nano according to the EU Nano-Definition, but also produces accurate and reliable size-related data needed for assessing hazard and risk associated with nanomaterials.

As a major outcome, a decision support flow scheme and method manual have been developed and designed as "NanoDefiner" e-Tool to guide manufacturers or enforcement laboratories to select the most appropriate measurement method(s) to determine if a material or product is or contains nanomaterial, or not. In addition, the vast knowledge base produced by NanoDefine on methods and their performance and applicability to correctly measure the size of materials will shed new light on still critical aspects of the EU definition, such as the question of the plausibility of the number size distribution threshold of 50 % and its replacement by a threshold between 1 and 50 % for complex and environmentally relevant materials, or the fact that implementing the EU definition will include all conventional particulate materials < 100 nm to be classified accordingly.

In the following, main objectives and results achieved within the various work packages are described within this overall project context:

WP1 assessed the availability of calibration standards and reference materials and provided the project with suitable calibration standards and test materials. As CRMs for size measurement are still lacking (see also WP6 below), a panel of in-house synthesized or externally procured stable and homogeneous, industry-relevant test substances and products covering a matrix of different chemical and physical properties, material systems, sizes, shapes and size-distributions were supplied to implement the planned project tasks. As one consequence, project outcome like dispersion protocols and analysis strategies can therefore be applied to a vast range of similar industrial substances with only minor adoptions of protocols. Also, the robustness of the developed tools, such as the decision-making framework (NanoDefiner e-tool) or the ParticleSizer software could be successfully tested by a broad range of different sample properties.

Main objective of **WP2** was the development and optimization of methods for the dispersion of NM from powders and the extraction of NM from products, as provided by WP1, for further analysis by the tier 1 and tier 2 methods developed in WP4 and WP5, respectively.

One main objective of **WP3** was the evaluation of a broad range of available techniques against quantitative quality criteria as defined in WP7 (see below) to establish unambiguous ranges of applicability. Based on this thorough quantitative evaluation of all available sizing techniques applied on the well-defined sets of Quality Control Materials

(QCMs) and real-world Representative Test Materials (RTMs) generated in WP1, a set of methodologies had to be developed that have the potential to be further developed into standard methods for the measurement of particle size distributions of nanomaterials according to the EC recommendation for a definition as nanomaterial (within WP4, 5 and 6). Further, the continuous benchmarking of the methods under development against the set of quality criteria established in WP7 had to be carried out, considering purpose, sample complexity and acceptable limitations – in agreement with WP7.

Another main objective in Wp3 addressing a particular aspect of the EC recommendation on a definition of nanomaterial was the evaluation of the relationship between the volume specific surface area (VSSA) and the number based particle size distribution for standards and representative powder materials from WP1. Hereby, a collaboration with the parallel EU/FP7 project NANoREG was realized. As a continuous dynamic activity involving also experts from outside of NanoDefine, the monitoring of analytical device developments and assessment of new or improved techniques using QCMs from WP1 against quality criteria as defined in WP7 was the final achieved objective in WP3.

Main objective in **WP4** was the establishment of a set of validated, cost-efficient, robust, and easy implementable methods for rapid distinction between nano/non-nano according to the definition. This included improvement of instruments and related methods (in cooperation with WP2) to increase the range of applicability and cost-efficiency, and the determination of individual methods' limitations and derivation of maximum application ranges with regard to the requirements of the definition (see also WP6). The following potential screening methods have been developed in WP4: Analytical Centrifugation methods (AC/CLS), Dynamic light scattering (DLS), Electric mobility analysis, MiniTEM, Particle tracking analysis (PTA/NTA), SingIe particle ICP-MS. WP4 delivered the screening methods for tier 1 in the decision tree of WP7 based on method selection criteria developed together with WP3, and on method validation developed together with WP6.

WP5 utilized established principles of analytical separation and high resolution imaging and analysis to overcome typical problem of sample polydispersity and heterogeneity. Established techniques are electron microscopy (SEM, TEM) with EDX and field flow fractionation hyphenated to multiple detection systems in conventional and single particle mode. Therefore, the objectives of WP5 were:

- Establishment of methods for the implementation of the EC definition in difficult samples, non-dispersible powders, complex matrices, products and the environment.
- Development and validation of an 'auto EM' toolbox for automated particle recognition, measurement, counting and analysis according to the EC definition.
- Validated reference methods for the classifications of materials with characteristics outside the applicability of the rapid screening methods (WP4).
- Evaluating and establishing reliable procedures to generate the number based distributions from raw data based on detection principles other than particle counting.
- Development of FFF coupled to particle counting detectors (liquid and aerosol based) to obtain true number based distributions after high-resolution separation.

To ensure harmonisation of the in-house validation in WP4 and WP5, a common guideline for in-house validations was developed in **WP6** and agreed by all partners. Continuous support from the JRC to the validation laboratories was given. This assured a harmonized in-house validation over the various methods and work packages. To conduct and evaluate interlaboratory validation studies of key methods according to international method evaluation standards, an international call for interlaboratory validation exercises was launched and conducted with VAMAS and results evaluated for eight methods. Some methods showed their fitness for purpose; other methods still need some optimisation. Another goal in WP6 was to establish links and be the contact point for standardisation organisations (CEN, ISO) and regulatory stakeholders (European Commission) and submit

validated methods as proposed New Work Items. For this, project liaison with the standardisation committee CEN/TC352 was achieved. The NanoDefine Methods Manual was proposed to CEN/TC352 as a New Work Item Proposal.

WP7 pooled results from other work packages and prepared output supporting parties which need to identify nanomaterials for regulatory purposes in practice. As a main objective the WP developed a comprehensive decision support framework, taking into account and integrating all relevant project results. A user who applies the framework is guided logically and uniquely through the decision steps for all reasonably expected cases in regulatory practice to come to a recommendation whether a specific material is a nano or a non-nanomaterial. A decision flow scheme derived from the framework is implemented in an e-tool for end-users. The decision support flow scheme was successfully tested in a number of case studies representing a wide variety of materials. Based on analytical capabilities, the WP also developed recommendations on a revision of the EC definition of nanomaterial.

Description of the main S&T results/foregrounds

NanoDefine has successfully completed an ambitious and comprehensive work plan with the ultimate goal to establish a new analytical measurement approach that supports the implementation of the EC recommendation on the definition of nanomaterial. To achieve this goal, a bottom up approach was used consisting of various consecutive tasks, deliverables and milestones organized around 7 vertical (scientific/technical) and 3 horizontal (managerial/dissemination) work packages (WP) that allowed a stepwise implementation of the planned work (see Figure 1).

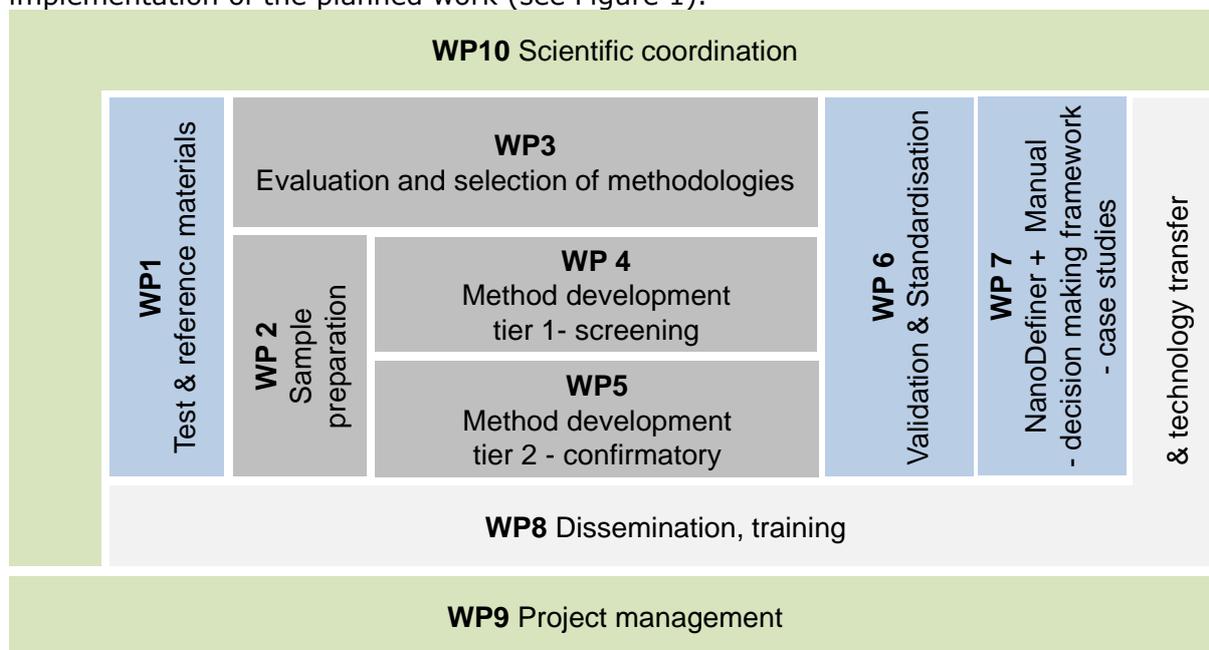


Figure 1: Structure of the NanoDefine work plan

Figure 2 is highlighting main paths of interaction, feedback loops and interdependencies between the various work packages ensuring a close cooperation among the various expert groups at different levels and emphasizing their interaction required to implement such a complex and large-scale project. Different partners came together from different European countries to mobilize the critical mass of expertise and the number of expert laboratories needed to successfully implement the comprehensive inter-laboratory method and materials testing and validation work.

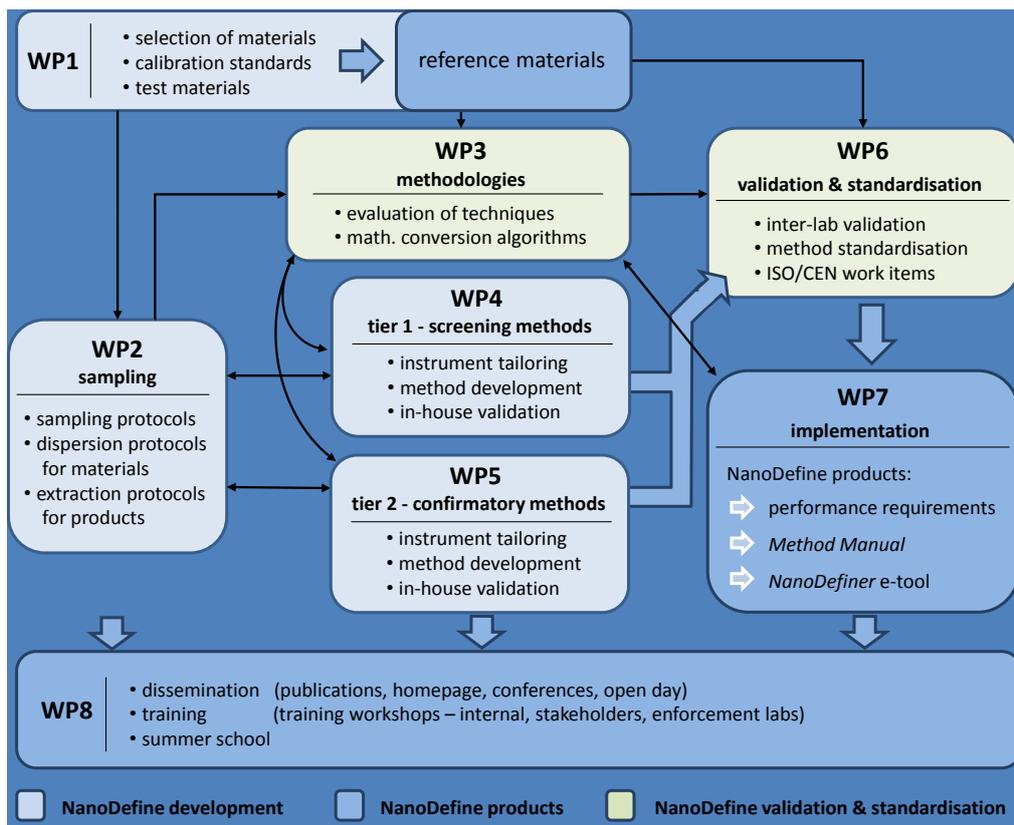


Figure 2: Overview on the coherence and interactions of the different WP, tasks and subtasks

A short description of the main work performed within each technical WP and across WPs is presented below, followed by a description of main S&T results/foreground achieved within each WP during the 4-year project duration.

Summary of main work implemented during the 4 years project duration

In **WP1** 15 representative test materials as well as 4 calibration standards have been provided to WP2-7, in addition to labelled nanomaterials for method development and verification of particle extraction protocols from matrices. Homogeneity and short-term stability of all test substances was tested before. **WP2** developed validated methods for sample preparation, dispersion and sampling. Standard operating procedures for the dispersion of all eleven substances have been established and sample preparation methods provided for NMs in three products (toothpaste, sunscreen, polyethylene) to be used in WP4 and WP5 for tier 1 and 2 methods. Also, different sample carrier pre-treatment methods and sample deposition methods have been tested for EM to be used in WP5 resulting in a compact electrospray deposition system for NM deposition on TEM grid. **WP3** performed a comprehensive evaluation and selection of key measurement techniques (MT) and performance parameters of the characterisation methods (CMs) for use in the NanoDefiner e-tool and NanoDefine Method Manual (in **WP7**) defined. A survey on ongoing developments in sizing techniques has been completed including more than 300 experts resulting in recommendations of the most suitable CMs to WP 4, 5 and 6 according to a quantitative ranking of available CMs. Potential new candidate CMs (miniTEM and DLS) were selected and recommended to WP4. Robust testing of the CMs performance characteristics has been achieved by using the NanoDefine quality control materials and real-world materials. In WP3, also evaluation of conversion algorithms has been completed. In cooperation with the EUFP7 NANoREG project, the applicability of VSSA as a rapid

screening tool for the determination of the number based particle size average has been assessed and a corresponding screening strategy developed.

WP4 has developed screening methods, including AC/CLS, DLS, miniTEM, spICPMS and PTA. A generic SOP has been established for analytical centrifugation (AC) techniques that can be used for material classification. Three AC techniques (cuvette-type and disc-type AC with turbidity detectors, cuvette-type AC with interferometric detectors) successfully passed intra-lab validation with representative test materials. Also, the applicability of DLS as screening technique was improved by testing poly-disperse materials with significant amounts of coarse micrometre-sized particles. Size analysis by miniTEM was shown to be fit for purposes when used as screening method (tier 1) as well as confirmatory method (tier 2 in **WP5**). For spICPMS, a new platform independent ICP-MS software was developed that facilitates size analysis of single particles and the new method successfully tested by intra-lab validation for nanomaterials in complex matrices. New instrument and software modifications resulted in a significant reduction of lower size limits and hence in more reliable measurement of particle number concentration by PTA. Also in WP4 a new prototype for size analysis of extremely fine (1-5 nm) aerosol particles by HRMS has been realized. Confirmatory methods have been developed in **WP5**, including (1) an auto-EM toolbox for automatic serial acquisition and analysis of TEM micrographs that is working for different EM instruments and is using a new automated image analysis software, incl. algorithms for agglomerate/aggregate deconvolution and size based classification, and (2) SOPs for the determination of the median diameter of the number-based particle size distribution of TiO₂ particles in sunscreen and Al₂O₃ particles in toothpaste by asymmetric flow field-flow fractionation (AF4) coupled to multi angle light scattering and inductively coupled plasma-mass spectrometry. In addition, interfaces for coupling FFF with particle counting detectors, a new software and needle valve to improve AF4 performance and reduce costs and an intra-lab validation of the measurement of the minimal external dimension of the primary particles of particulate materials from TEM images by the NanoDefine ParticleSizer software have been also realized in **W5**. A common guideline for in-house validations was developed in **WP6** to ensure harmonized in-house validation across all methods and work packages. Liaison of NanoDefine with CEN/TC352 was achieved and continuous contributions to many other standardisation committees provided. The NanoDefine Methods Manual was proposed to CEN/TC352 as a New Work Item Proposal. Inter-laboratory validation of eight selected key methods was realized in cooperation with VAMAS, whereby some methods showed their fitness for purpose, while other methods still need some optimisation. In **WP7**, the NanoDefiner e-tool and method manual was developed and case studies performed to assess the feasibility of the developed approach to real-world materials. For this, a catalogue of performance criteria to assess the capabilities of characterisation methods was developed (see **WP3**). These criteria were used to develop templates for characterisation of tier 1 and tier 2 methods and the templates filled in in **WP3**. Both the methods-focused material classification and the criteria used to steer the decision process became the pillars of the decision support flow scheme that was established based on the experience with materials used in the project and the assessment of the performance and capabilities of CMs. The NanoDefine Manual was developed to provide all information necessary for the user to perform an assessment of any material against the EC nanomaterial definition. Finally, the decision flow scheme and manual were implemented in the NanoDefiner e-tool, an easy to use software that guides the user in a straightforward and semiautomatic way through all steps of assessment to a final traceable decision (nano, non-nanomaterial, or inconclusive based on the provided information) with extensive reporting. Both decision flow scheme and e-tool were tested and refined in case studies on real world materials involving industrial chemicals and also products. The experiences and findings resulted in recommendations on the revision of the EC definition of nanomaterials based on experimental capabilities.

Description of main S&T results/foreground achieved with the different WPs

WP1: Test and reference materials

The test materials selected, sourced, and analysed by WP1 are listed in Table 1. SEM images of the substances and products are shown in Figure 3 and 4.

Table 1: Selection of representative test materials

Material ID	material	Subst.(S) / product (P)	pre-selected	nano (n) / coarse (c)	link to NANoREG WP2	Origin: manufactured (m); natural (n); incidental (I)	chem. composition	shape	presence of different sized particles	trade form
IRMM-382	MWCNT	S	x	n	x	m	carbon	fiber	nano	powder
IRMM-380	Organic pigment	S	x	n		m	organic	particle	nano+(non-nano)	powder
IRMM-386	Organic pigment	S		c		m	organic	particle	(nano)+non-nano	powder
IRMM-384	CaCO ₃ (fine grade)	S	x	c		m	inorganic	rod / cigar	(nano)+non-nano	powder
IRMM-383	Nano Steel	S	x	n		m	metal	platelets	nano+(non-nano)	powder
IRMM-387	BaSO ₄ (ultrafine grade)	S		n	x	m	inorganic	particle	nano+(non-nano)	powder
IRMM-381	BaSO ₄ (fine grade)	S		c		m	inorganic	particle	non-nano	powder
IRMM-385	Kaolin	S		c	x	n	inorganic	multiscale platelets	(nano)+non-nano	powder
BAM-11	Zeolite powder	S		c		m	inorganic	particle	(nano)+non-nano	powder
IRMM-389	Basic methacrylate copolymer particles (BMA)	S		c		m	organic	particle	non-nano	powder
IRMM-388	coated TiO ₂	S		c		m	inorganic/composite	core-shell	non-nano	powder
BAM-12a/b	Food with SiO ₂ , (NanoLyse-material+ commercial dry pancake mix)	P	x	n		m	inorganic	particle	nano+(non-nano)	suspension /powder
BAM-13a	TiO ₂ +surface treatment with aluminum compound formulated in sunscreen (w/ Fe ₂ O ₃ , micro-TiO ₂)	P	x	n		m	inorganic/composite	particle	nano + (non-nano)	cream
BAM-13b	TiO ₂ +surface treatment with aluminum compound formulated in simplified sunscreen	P	x	n		m	inorganic/composite	particle	nano	cream
BAM-14 BAM-14-1 BAM-14-2	Fe ₂ O ₃ in HDPE (+ raw HDPE + raw Fe ₂ O ₃ powder)	P		n		m	inorganic	particle	nano	granulate
BAM-15	Al ₂ O ₃ in toothpaste	P		c		m	inorganic	particle	(nano)+non-nano	paste

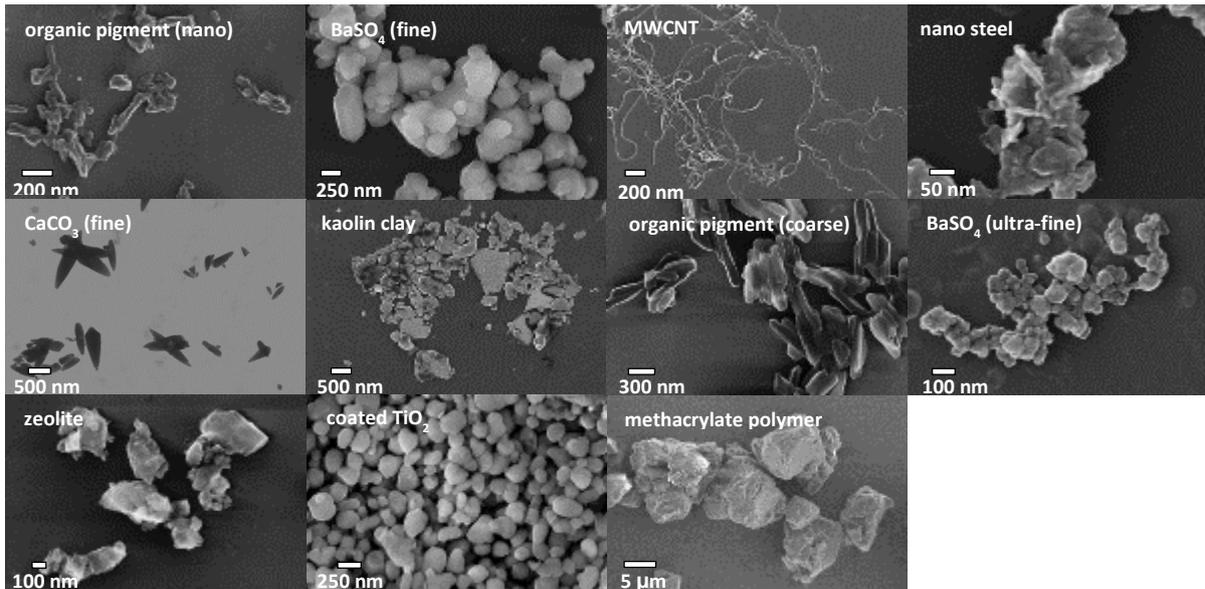


Figure 3: SEM images of the selected substances

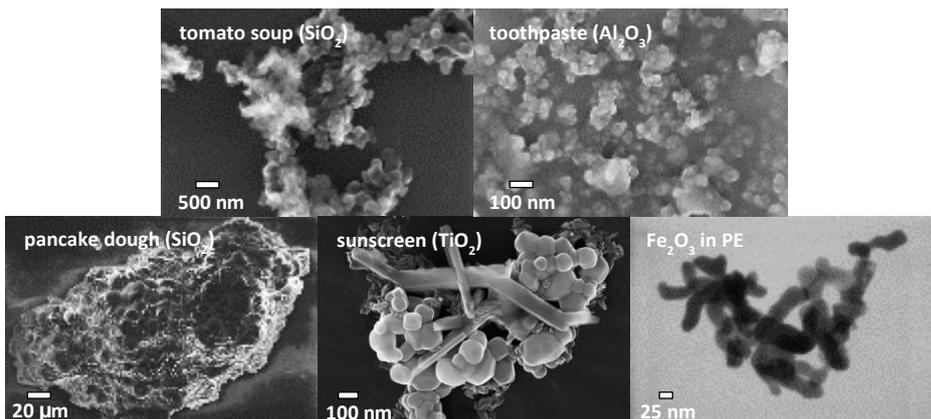


Figure 4: SEM images of the selected consumer products

In addition, calibration standards were provided to the project partners. Those comprised a 0.25% suspension of ~40 nm silica; a 1 % suspension of ~46 nm polystyrene; a suspension of 0.25% ~50nm, ~90 nm, ~120 nm silica; as well as a suspension of 0.5% ~46nm; 1% ~100 nm; 1% ~350 nm polystyrene. Labelled substances (project partners were provided with In₂O₃ nanopowder and coated TiO₂ particles) were synthesized and distributed to enable the various NanoDefine expert labs to evaluate the extraction efficiency of nanoparticles from complex matrices like in the provided products.

The physical and chemical properties of all samples were assessed and a summary of all characterization data was reported as overview table in D1.6. The characterization work also included studies on homogeneity and stability also reported in D1.6. Both homogeneity and stability studies demonstrate that the samples are of good quality and that no issues have to be expected. The shelf-life of some products might not exceed the time-span of the NanoDefine project.

WP2: Sample preparation, dispersion & sampling methods

A literature review was performed to evaluate suitable guidelines for sub-sampling. The number of publications dealing with the sampling or sub-sampling of nanomaterials was found to be limited. In general, the known procedures for coarser materials were applied. The norm ISO 14488:2007 was selected as guideline for the practical evaluation of sampling. A limited number of experimental studies was performed. The tested sub-sampling procedures were slurry, paste and heap sampling as well as dividing by spinning riffler. For the investigations three materials were selected: coated TiO₂ and fine and ultrafine BaSO₄. Dynamic light scattering (DLS) as a tier 1 method was applied (see WP4). A comparison of the expanded measurement uncertainties for all materials and sub-sampling procedures was made. It was observed that independent of the material the heap sampling led to the highest variance. The sub-sampling methods paste and slurry showed comparable influence on the measurement uncertainties. The spinning riffler seemed to have the smallest effect. A comparison of the two barium sulfate materials indicated a tendency: the expanded measurement uncertainties increased more for the ultrafine grade for all sub-sampling procedures than for the fine grade material. For further investigations on NM sampling scenarios, a combination of more sophisticated analytical techniques should be used. Additionally the use of a material with a known particle size distribution (PSD) or with labeled particles of defined sizes should be considered in order to be able to better understand changes in the PSD due to sampling/sample preparation.

To find and define suitable methods for the dispersion of powders, a literature survey of publically available dispersion protocols was made. The results of this survey showed that 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 generic 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, can contain only compounds which are compatible with biological system. It was concluded that the quality of dispersion achieved with these generic protocols would likely be sub-optimal for many of the priority materials in the NanoDefine project and consequently it was necessary to adapt or design individual protocols tailored for each material based on the outcome of the literature study. The dispersion protocols for the 11 priority materials were optimized for each material in terms of sonication energy, sonication time, dispersant liquid, stabilizers and stabilizer concentration. To allow the maximum transferability across the measurement techniques and laboratories, emphasis was placed on producing aqueous based dispersions containing only the minimum quantity necessary of commonly available stabilizing agents. The dispersion efficiency was evaluated qualitatively and where possible quantitatively by transmission electron microscopy (TEM) for two independently prepared suspensions to confirm repeatability. All priority materials have an individual dispersion protocol describing their use with probe sonication while selected materials were, in addition, also provided with data for use with a vial sonicator. A stability of the NM suspension of at least 30 min was assured, which was identified as the maximum relevant time for instrumental measurements. For the majority of substances, the dispersion protocols allowed the dispersion of the material up to the level of single primary particles and some small aggregates/agglomerates (2-10 primary particles). To improve the inter-laboratory transferability of the dispersion protocols, a calorimetric method of measuring the relative power of the sonication probes was used in the method development to allow an instrument independent value of absorbed ultrasonic power to be reported as part of each protocol. This procedure allows each laboratory to independently determine the performance characteristics of their own sonicator and adjust its power settings to match the values recommended in the dispersion protocols. The dispersion protocols were adapted by the tier 1 and tier 2 method developers in WP4 and WP5. Modifications were especially necessary in the case of TEM analysis where problems with some chemicals/dispersion agents were observed.

In total, sample preparation protocols for three consumer products were developed: sunscreen containing titanium dioxide (TiO_2) particles, toothpaste containing aluminium oxide (Al_2O_3) particles and solid polyethylene (PE) matrix containing Fe_2O_3 particles. The studied sunscreen formulae were specifically designed for the project and were not commercially available products. The attempt of developing a sample preparation procedure for pancake dry mix containing silicon dioxide (SiO_2) was not successful. The sample preparation methods were specifically tailored towards tier 1 (sp-ICPMS) and tier 2 (FFF or TEM) methods, which have different requirements in terms of sample preparation. As a starting point, the most relevant sample preparation methods for the specific material/matrix combinations were identified on the basis of previous experience from the involved partners, including the EU FP7 project NanoLyse. Two sample preparation approaches for analysis of SiO_2 in food by field flow fractionation (FFF) were tested: a) acid digestion (based on NanoLyse method for liquid tomato soup) and b) enzymatic digestion. For approach a) the "NanoLyse method" was successfully improved towards lower limit of detection for powdered tomato soup based on additional pre-digestion and acid evaporation steps. The improved acid digestion method was then applied to pancake mix. Several problems were observed when transferring the method to this distinct food matrix. For approach b) several enzyme combinations, buffers, and digestion durations were tested, but no complete digestion of pancake mix was possible and the obtained sample was too complex to be injected in FFF. For TiO_2 in sunscreen, a rapid approach of sample preparation for sp-ICPMS analysis based on matrix dilution was developed. Mixing with a detergent plus sonication gave the best results for FFF analysis after testing several approaches. For Al_2O_3 in toothpaste, dilution of the toothpaste in water was selected as the final sample preparation method for analysis by sp-ICPMS. For analysis of Al_2O_3 present in toothpaste by FFF, a more complex sample preparation strategy based on chemical oxidation and later dispersion in surfactant was required for FFF-ICPMS. Focused ion beam (FIB) and ultramicrotomy techniques were evaluated for the preparation of Fe_2O_3 particles embedded in a solid polyethylene matrix for EM analyses. Best results were obtained by UM followed by a gentle thermal treatment of the thin sections. Sample preparation for NM in products remains a challenging and time-consuming task and needs to be developed for each NM / matrix combination. Whereas sp-ICPMS is less sensitive to matrix residues, these residues can cause problems in FFF and EM analyses.

Sample preparation for TEM requires special attention. Various functionalization methods of TEM grids prior to NM deposition were studied. The methods included glow discharging, high vacuum baking, coating with Poly-L-Lysine (PLL) and coating with Alcian blue. Glow discharge renders carbon coated TEM grids hydrophilic with a low negative surface charge. Hence, NPs with positive zeta-potential can be deposited immediately onto the grid. For NPs with negative zeta-potential, additional chemical treatment is recommended. High vacuum baking of the TEM grids has a similar effect as glow discharging, giving a hydrophilic and low negatively charged carbon film with the addition of cleaning the surface from contaminants. This is especially useful if elemental specific information has to be recorded, e.g. by energy dispersive X-ray spectroscopy, where the beam induced contamination can be severe. A low cost high vacuum baking station was built and the beam induced contamination quantitatively tested using several TEM grids from different vendors. It was shown that the contamination varied greatly between the grids from the same vendor, but was overall reduced after high vacuum baking. There exist many different chemicals for the pre-treatment of the TEM grids to provide a monolayer of molecules with an opposite charge than the NPs zeta-potential. Alcian Blue and PLL were chosen here because of their wide usage, and ease of preparation. The different functionalization methods (including untreated TEM grids) were studied for three different NMs: 60 nm gold NPs dispersed in ultrapure water, ultrafine grade BaSO_4 NPs dispersed in bovine albumin serum and tri-modal SiO_2 NP dispersed in bovine albumin serum. For deposition, centrifugation was used. TEM grid functionalizations with PLL or glow discharging prior to PLL were found to be the best methods in terms of contamination,

deposition quality and coverage. The results from Alcian blue were also satisfactory showing little or no contamination with sufficiently good deposition quality and coverage. Three different deposition methods, drop deposition, electrospray ionization and centrifugation, were evaluated for their suitability to deposit NM evenly on TEM grids. A SOP based on drop deposition, which was originally developed in the context of the NANOREG project, was applied and evaluated for the NanoDefine materials. Sample dilution and charge of the grid were optimized for each NanoDefine material. The SOP was shown to be efficient for the preparation of EM specimens. The prototype of an Electrospray Deposition System for EM sample preparation was designed, manufactured and tested. For performance evaluation of the prototype, several NMs were tested. It was demonstrated that electrospray deposition of particle suspensions on a TEM grid is a very promising option for achieving even particle distributions. To achieve good and reproducible results, the suspension needs to be appropriate for electrospray deposition: it should not be stabilized with a surfactant that generates precipitates upon drying and the suspension concentration should not be too high. Experiments with a tri-modal polystyrene latex suspension demonstrated high deposition efficiencies for all three modes, which enabled allowed short sampling times. The spatial distribution of the deposited particles, however, strongly depended on the particle size. Hence, the entire deposition area needed to be evaluated to gain representative information, which unfortunately more than counterbalanced the short sampling times. Finally, the advantages and disadvantages of the three TEM grid deposition methods were identified and summarized, and protocols for all grid functionalization and deposition methods provided. A generalization of the most suitable grid functionalization and deposition method was not possible, as these strongly depend on the studied NM and the planned EM analysis.

A feasibility study showed that different direct aerosolisation techniques tested in various experimental conditions did not allow obtaining primary particles from NM powders in the aerosol form. Mainly agglomerate/aggregate airborne particles were measured. Further work beyond the NanoDefine project is required, including optimization of the aerosol generator operating parameters, the generation protocol as well as the scanning mobility particle sizer (SMPS) characterization protocol in order to improve the results. Moreover, systems improvements must be carried out in order to increase the stability over time.

The reproducibility of selected dispersion protocols (for fine and ultrafine BaSO₄ as well as coated TiO₂) was evaluated. These materials were selected for use in either intra-laboratory (instrument/method) validations or subsequently for inter-laboratory ring-trials and, thus, there was a clear added value to assessing the reproducibility of the associated dispersion protocols. For the materials subjected to the evaluation of repeatability the method chosen to measure particle weight-size distribution was analytical centrifugation (Line-start Disc-AC) (see WP4). It was found that the combination of the standardized sample preparation methods with systematic analysis by Disc-AC was able to produce median particle sizes of the cumulative number or mass size distribution with relative measurement uncertainty values below 11% and 5%, respectively, for all three materials. Considering that these uncertainty values are a combination of the variability of both the analytical method and dispersion step, it was concluded that strict adherence to the detailed dispersion protocols permits the preparation of samples which have reproducible PSDs.

WP3: Evaluation and selection of techniques and methodologies

The first task was a comprehensive study on current MTs which may be relevant for the reliable analysis of the number based size distribution of NM according to the EC recommendation. Based on the performance criteria already established in WP7 (D7.1), the potential MTs available in the literature were evaluated also by following the expertise available within the NanoDefine consortium. The specific advantages and disadvantages of each MT with respect to its applicability to the scope of NanoDefine were highlighted.

A CM evaluation table was produced so that the mostly suited MTs with respect to the EC definition can be recommended to the corresponding NanoDefine WPs 4-6 for further improvement and adaption, or for direct validation and standardisation (see Table 2).

Table 2: Results of the evaluation of all considered MTs and recommendations of MTs for further specific development or direct validation and standardisation.

		Size range						Direct counting CM	Convertibility to number weighted 3D Quantitative accuracy	Access to the smallest dimension of each particle	Measurement of the material as it is	ISO standards available	Size Accuracy	Chemical selectivity	Access to primary particles?	Costs per sample	Analysis turnaround time	Widely used/ Availability	Hyphenation to other methods?	Potential for improvement in NanoDefine within 2014-2017?	Recommended to WP4	Recommended to WP5	Recommended to WP6
		nm		µm																			
		1-10	10-30	30-100	0.1-1	1-10	>10																
Counting	SEM	-	(+)	++	++	++	++	++	+	-	0	+	++	+	-	-	++	-	+	-	++	+	
	EM	+	+	++	++	++	++	++	+	-	+	++	++	+	-	-	+	-	+	-	++	++	
	TSEM (+)	+	++	++	++	++	++	++	+	-	+	++	++	+	-	-	+	-	+	-	++	++	
	TEM	++	++	++	++	++	++	++	++	+	-	+	++	++	+	-	-	+	-	+	-	++	++
	SFM	+	++	++	+	0	-	++	++	++	-	+	++	++	0	-	-	+	-	+	-	++	++
	PTA	-	0	+	+	+	+	+	+	+	-	+	0	+	+	+	+	+	+	+	+	+	+
Fractionating	TRPS	-	-	0	+	+	+	++	++	-	++	++	++	-	-	+	+	-	+	-	+	+	
	sp ICP-MS	-	0	+	++	++	++	++	++	-	++	++	++	-	-	+	+	+	+	+	+	+	
	FFF	+	++	++	++	0	-	-	-	-	-	+	+	0	0	-	+	++	+	+	+	+	
	AC	0	+	++	++	+	+	+	0	-	++	++	++	-	-	+	+	+	+	+	+	+	
	DMAS	+	++	++	++	-	-	+	+	+	++	++	++	-	-	+	+	+	+	+	+	+	
	USSp	0	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	
Ensemble	DLS	+	++	++	++	++	++	++	++	-	++	++	++	-	-	+	+	+	+	+	+	+	
	SAXS	+	+	+	+	+	+	+	+	-	++	++	++	-	-	+	+	+	+	+	+	+	
	USSp	+	+	+	+	+	+	+	+	-	++	++	++	-	-	+	+	+	+	+	+	+	
	PRD	+	+	+	+	+	+	+	+	-	++	++	++	-	-	+	+	+	+	+	+	+	
Integral	ALS	-	0	+	+	++	++	++	++	-	++	++	++	-	-	+	+	+	+	+	+	+	
	DS	+	+	+	+	+	+	+	+	-	++	++	++	-	-	+	+	+	+	+	+	+	
	PSS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	BET	+	+	+	+	+	+	+	+	-	++	++	++	-	-	+	+	+	+	+	+	+	

Recommended to WP4 (Screening methods)		Recommended to WP5 (Confirmatory methods)		Recommended to WP6 (Methods ready for direct validation or standardisation)	
Strongly recommended	recommended	Strongly recommended	recommended	Strongly recommended	recommended
PTA/DUM	TRPS	EM	SFM	EM	SFM
sp ICP-MS	FFF	FFF	sp ICP-MS ¹		PTA/DUM
AC	SAXS				sp ICP-MS
DMAS	USSp				FFF
DLS	ALS				AC
	BET ²				

The evaluation report including the recommended MTs and those under development in WP4 and WP5 (almost the same) has been updated at mid-term based on results from testing the MTs by means of NanoDefine WP1 real world materials. Further, potential new candidate MTs to be passed to WP4 for development were proposed by a set of four methods, i.e. Archimedes, miniTEM, PCCS and Taylor Dispersion, to be finally selected as Tier 1 MTs after a dedicated test on 3 well-defined NanoDefine materials (monomodal SiO₂, trimodal PSL and coated TiO₂). The proposed MTs and the selection procedure were discussed in detail in conjunction with a dedicated NanoDefine event, the 'Science Workshop' organized at BAM in November 2015.

Performance characteristics sheets were developed for those MTs which were tested systematically within task T3.2 with respect to their ability to identify a particulate substance as nanomaterial according to the EC recommendation. These results feed directly the NanoDefiner e-tool (D7.8) and the critical review manuscript on the real-world performance of existing MTs (D3.3).

The real-world performance tests (T3.2) are considered to reflect the pre-NanoDefine state-of-the-art for both potential screening and confirmatory MTs. For this purpose, 15 MTs have been selected and applied to the characterisation of 7 Quality Control Materials (QCMs) and 11 particulate substances as Representative Test Materials (RTMs). Also a small selection of NanoDefine products was included. The originally developed concept for the performance characteristics sheets comprised tables for the technical characteristics and tables for evaluating the method's performance. During refining the structure of the NanoDefiner e-tool (D7.8) it turned out that both kinds of tables should be converged into consistent data sheets for each MT. The structure and the content of the performance characteristics sheets were explained in detail in D3.2, including data sheets designed as supplementary xls files.

After evaluating the performance of MTs by both QCMs and real-world particulate materials, it was found that no single MT can be strongly recommended. Required was a tiered approach that combines different MTs and employs prior knowledge on the material (such as physicochemical properties, including general morphological properties of the particles). The tiered approach comprises screening techniques (Tier 1—powder and Tier 1—suspension) as well as confirmatory techniques (Tier 2—imaging). Tier 1 MTs are

intended to provide clear statements whether a material is a NM, or whether more profound analyses by Tier 2 techniques are required, because the number-weighted median $x_{50,0}$ is close to the borderline of 100 nm. They either test integral properties of the particle system (e. g. VSSA) or determine the distribution of equivalent diameters rather than the geometric lengths of the external dimension. In addition, the intrinsically measured size distributions are typically non-number-weighted. For this reason, the Tier 1 MTs are expected to perform well for low and moderate polydispersity. Moreover, most MTs that are relevant for Tier 1—suspension cannot resolve the internal structure of particles aggregates, instead they probe aggregate properties. Their ability to reliably assess particulate materials according to the EC definition is, therefore, restricted to materials that consist of particles as individual entities or of well dispersible aggregates. Based on our data, matching nano/non-nano classification by both Tier 1—suspension and Tier 1—powder indicates that this validity criterion is fulfilled. Otherwise, Tier 2—imaging can help, but even then, ambiguity remains. This approach yields recommendations for the MTs that can be attributed to the different tiers based on their proven performance for real-world materials.

Tier 1—powders can rely on BET, but only outside the borderline region, whose limits we explored in detail elsewhere (Wohleben et al., *J. Nanopart. Res.*, 2017).

Tier 1—suspension can be realised with spray-DEMA, all AC techniques or DLS, yet similar as for BET, they are inconclusive for a borderline region and certain particle morphologies. Further candidates for Tier 1, which we tested, provided no reliable classification (ALS, PTA, and SAXS), or were not ready for a final assessment (AF4 and spICP-MS).

Tier 2—imaging measurements can be conducted with TEM or SEM, which give access to the constituent particles of aggregates and to the smallest external dimension of particles for most materials. However, the preparation of representative samples constitutes a major source of uncertainty and ambiguity for Tier 2, and the determination of the smallest external dimension remains challenging (if possible at all) for several classes of morphology, e. g., for three-dimensional aggregates and two-dimensional platelets.

Inconsistent results occur with both Tier 1 and Tier 2 techniques for highly polydisperse samples: for most screening techniques because of their relative insensitivity towards the fine size fractions and for imaging techniques, because any particle deposition process on substrates is affected by particle size. We also observed ambiguity in Tier 1 and Tier 2 results when the materials were composed of indispersible aggregates comprising a large number of constituent particles. In these cases, the Tier 1 – powder might be preferable for a pragmatic implementation. Despite these challenges, our results suggest that reliable NM identification is possible for a broad range of real-world substances, provided they are not borderline cases (i. e. if their $x_{50,0}$ is outside the 50 to 150 nm range). In this size range, conflicting results are to be expected also from EM labs, and weight of evidence approaches might be required to combine evidence from all tiers.

Finally, it could be extrapolated that the classification of mixtures of different substances is probably rather difficult and prone to artefacts. The interpretation of particle sizing results for such materials can be critically misleading if, for instance, the turbidity of a mixture is solely determined by one light-absorbing component (e. g. in AC-turb), or when the scattering signal of a mixture is dominated by the component with highest optical or electron density contrast (e. g. DLS and SAXS).

All the results obtained from the systematic evaluation study carried out within T3.2 (an overview of the results is illustrated in Fig. 5) have been published by Babick et al. as an Open Access Review in *J. Nanopart. Res.*, 2016. The measurement guidance, the uniform reporting templates (in agreement with ISO 9276 1-6) and the dispersion protocols are also included as open access information.

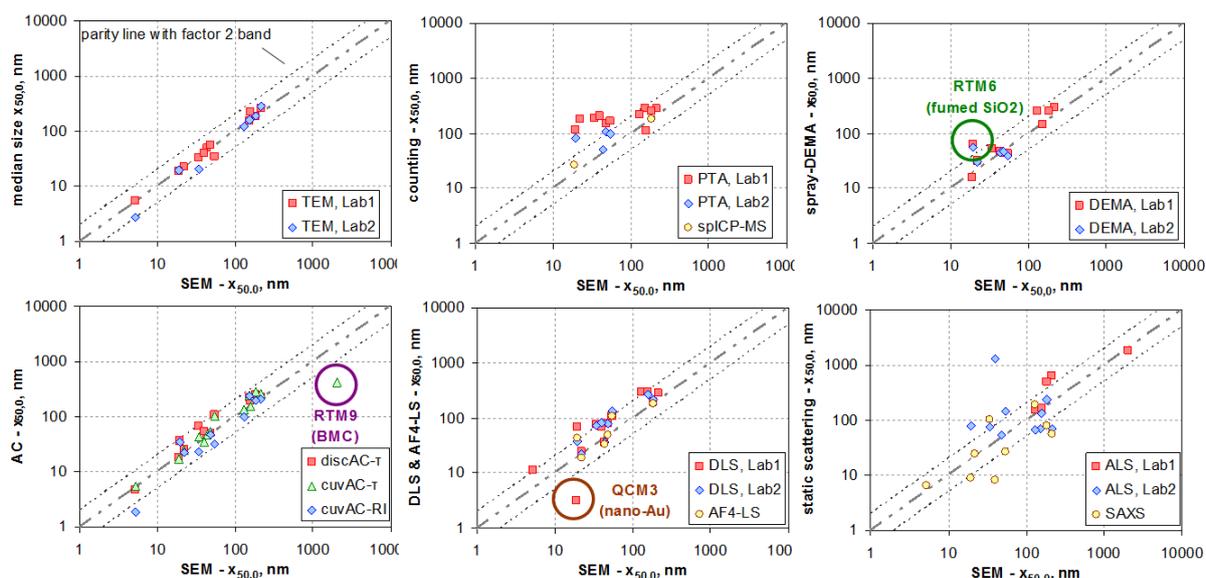


Figure 5: Parity plots of the number weighted medians $x_{50,0}$ as determined by the various MTs versus the SEM value; lines indicate parity and deviation from parity by a factor of 2.

The concepts of transforming size distributions, which refers to both size axis and weighting quantity, have been also reviewed. The basic mathematical procedures of converting known (continuous or discrete) size distributions and the implementation of analytical distribution functions have been discussed in D3.4. Attention was paid to the analysis of size measurements by means of spectroscopic ensemble techniques.

Formulae and references that can be used to feed the NanoDefiner e-tool and the principal limits of the different conversion schemes have been provided. The necessity of a-priori knowledge of the main morphology of the particles was highlighted. Such knowledge allows for selecting appropriate relationships between probed particle equivalent diameter and the quantity in which the size fractions are weighted.

VSSA of a particulate material is one of two apparently very different metrics recommended by the EC definition of “nanomaterial” for regulatory purposes: specifically, the VSSA metric may classify nanomaterials and non-nanomaterials differently than the median size in number metrics, depending on the chemical composition, size, polydispersity, shape, porosity, and aggregation of the particles in the powder. Within the public D3.5 the extent of agreement between classification by electron microscopy (EM) and classification by VSSA has been evaluated on a large set of diverse particulate substances that represent all the anticipated challenges except mixtures of different substances. EM and VSSA were determined in multiple labs to assess also the level of reproducibility. Based on the results obtained on highly characterized benchmark NanoDefine materials, we derived a tiered screening strategy for implementing the definition of nanomaterials. We applied the screening strategy also to additional industrial materials, which were classified correctly and left only borderline cases for EM. On platelet-shaped nanomaterials, VSSA is essential to prevent false-negative classification by EM. On porous materials, approaches involving extended adsorption isotherms prevent false positive classification by VSSA. We found no false negatives by VSSA, neither in Tier 1 nor in Tier 2, despite real-world industrial polydispersity and diverse composition, shape, and coatings. The VSSA screening strategy is recommended for inclusion in a technical guidance for the implementation of the definition.

Last WP3 task was dedicated to monitoring new developments of particle sizing techniques on the market and in research. For this, about 300 individual expert opinions, comments and suggestions on the proposed techniques were collected as part of D3.8 and have been discussed at the Science Workshop hold in November 2015 in Berlin. MiniTEM constitutes

an example of newly developed MT whose high potential was recognized by NanoDefine so that it was selected as a joker technique to be further evaluated under WP4.

WP4: Screening methods (tier 1 methods)

Main achievements addressed method developments and instrument tailoring and included the following outcome: 1) completion of the electrospray system prototype, 2) determination of the average LODsize on ICAP-Q by spICPMS for particles with 13 nm for Au, 15 nm for Ag, 44 nm for TiO₂, 3) design and manufacture of a HRMS prototype by RAMEM for the particle size analysis in the range of 1...5 nm showing good results for 2 nm Au reference suspension, 4) improvement of the accuracy for particle size analysis in particle tracking analysis also by means of a concentration measurement upgrade implemented in the software, 5) generic SOPs developed according to WP6 guidelines for all screening methods (CLS, HRMS, SMPS, particle tracking analysis and spICP-MS), 6) testing of additional screening methods "refined dynamic light scattering (DLS)" and MiniTEM showing clear advancements in comparison to the prior state of the art, 6) successful validation of key screening methods are according to international guidelines and in line with the default approach provided by WP6, 7) inter-laboratory validations of AUC and CLS methods and ParticleSizer software organised by WP6 in collaboration with VAMAS.

A set of validated, cost-efficient, robust, and easy implementable methods was established for the rapid distinction between nano/non-nano according to the definition. In cooperation with WP3 testing of substances and materials has been performed to determine the robustness, trueness and precision especially of the rapid screening methods. Using the reference materials (internal calibration standards) method specific limitations have been investigated, e.g. for SMPS the concentration of the particles, the composition and purity of the electrolyte for Electrospray. For Disk-CLS noise and baseline drift have been determined as limiting parameters and a strategy for specific validation, e.g. by cuvette centrifuge has been considered. All AC techniques and spICPMS were validated with respect to size analysis in the relevant size range, including the number-weighted median size. Moreover, DLS and MiniTEM successfully demonstrated their applicability as cost-efficient and robust screening methods at real-world materials.

Another goal in WP4 was the improvement of instruments and related methods (together with WP2) to increase the range of applicability and cost-efficiency. This included the improvement of existing instruments (NTA, CLS) and construction of prototypes (SMPS), and the development of software for the evaluation of spICPMS data. Dispersions procedures were exchanged with WP2 for the application of all methods and have been adapted for the application of spray-DEMA and of SMPS. Improvement in hardware and software of commercial PTA instrumentation allowed the determination of particle number concentration and reduced the lower size limit of the technique.

Concerning particle counting methods, both Dynamic Ultramicroscopy (NTA) and Single particle ICP-MS have been upgraded. The concentration upgrade is fully released and available to all NTA instruments matching required specifications. The upgrade has been incorporated into a final software version (NTA 3.1) and will be implemented by all future software developments. Besides, a protocol has been produced facilitating the upgrade process. This is intended to be completed by instrument users for field based instrument upgrades and Malvern production staff for build instruments ordered with the upgrade included. In addition, marketing and user support material has been produced with the aim to explain and promote the upgrade. The final product successfully reduces the influence of user selected settings (camera level, detection threshold) and sample properties on concentration measurements allowing greater reproducibility between measurements and a confidence in measurement accuracy across a range of settings. Also, improvements to

repeatability have been introduced through adopting a best practice protocol which optimises the amount of data captured along with incorporating sample flow and an automatic focus detection.

For spICPMS, a software has been developed that allows the determination of the size- and number distribution of nanoparticles in a sample. A complete package for the definition of suitable parameters for data acquisition and measurement control was established together with the required calculation algorithms to evaluate data sets according to the spICPMS calculations. Corresponding user interfaces were created. Preliminary evaluation of results obtained with this software have been compared to a widely accepted alternative calculation tool to provide a preliminary validation of the correct calculation of the particle size as well as the particle number concentration. The software development has been continued to be introduced as a commercially available part of the Qtegra ISDS software platform, so that users of different ICP-MS instrumentation are able to use it for the determination of size distribution and number concentration of nanoparticles in routine laboratories.

A High-Resolution Mobility Spectrometer (HRMS) was developed to characterise nanomaterials in the size range 1-5 nm, and performance validated to be adequate to the NanoDefine requirements regarding the smallest nanometric range 1-5nm. Instrument design was aimed at minimizing particle losses by diffusion. The particle path was minimized by using planar DMA geometry. The particle generator and detector were coupled to the classification zone. The pneumatic circuit was dimensioned to high sheath flow rates in order to minimize particle dwelling time in the system. The DMA manufacture was very difficult because of its extraordinary complexity and strict geometrical and precision requirements, but was completed successfully and in extraordinary agreement with the theoretical simulations. Despite the difficulties, all components were successfully manufactured. Engineering tests were realized in order to validate the instrument functionality. RAMEM carried out validation measurements with mobility standards, but could not accomplish an entire intra-laboratory validation over 5 days. This technical challenge is in accordance with the expert opinion in the new informative Annex G draft of the actually revised ISO 15900.

The performance of the two more commonly available centrifugation methods, disk-centrifugal liquid sedimentation and cuvette-centrifugal liquid sedimentation, have been considered in comparison with the more expensive, less common but more technically sophisticated and capable method of analytical ultracentrifugation (AUC).

The results of this evaluation done by using ideal particles (narrow size distribution, unaggregated and spherical) confirm that all three AC methods are very capable of reliably determining particle size. When applied to the problem of measuring a mass-based size distribution of bimodal and trimodal mixtures of approximately known composition, the results showed the AUC and cuvette-systems are more accurate than the disk-CLS.

The additional ("Joker") methods selected in WP3, "refined dynamic light scattering (DLS)" and MiniTEM, were tested and the advancements shown in comparison to the prior state of the art. In case of DLS, experimental studies showed that the quality of DLS data (autocorrelation function) can be improved by refining sample preparation and measurement protocol without changing the hardware configuration. The improvement aimed at an increased sensitivity towards the smallest size fractions. For this purpose, different approaches were examined: removing coarse particles from sample by filtering and depleting the measurement zone of coarse particles by expanding the equilibration time. This was successfully tested on four NanoDefine materials (kaolin, barium sulfate coarse grade, calcium carbonate and coated titania) in triplicates.

The state of the MiniTEM development before the beginning of the development process did not allow a validation but rather an evaluation of the MiniTEM analysis technique. The software was found to be in a "beta-state", and human interaction was frequently required.

Automated focusing and searching of suitable grid positions were optimised and tested for different sample systems at different magnification scales causing occasionally problems during the evaluation. The number-weighted medians determined by MiniTEM are close to the results generated by standard TEM. Moreover, the MiniTEM software evaluates TEM similarly well as the ParticleSizer software developed in NanoDefine. Image quality and resolution of MiniTEM is lower than in standard TEM due to lower acceleration voltage. Yet this appeared irrelevant for material classification according to the EC definition of nanomaterials. The parameters for the image evaluation module of the MiniTEM system can be optimised for each material as e.g. proposed in the ParticleSizer SOP.

Another objective in WP4 was the determination of individual methods' limitations and derivation of maximum application ranges with regard to the requirements of the definition. For this, the actual working range, detection and quantification limits have been established by means of an intra-laboratory validation process that started after finalisation of method development according to the guidelines generated in WP 6. Generic SOPs meet the defined requirements and have been approved by WP6. The determination of the actual working range, detection and quantification limits has been finished for all AC techniques and spICP-MS. Using at least two real world materials or substances, these key screening methods are successfully validated according to international guidelines and in line with the default approach provided by WP6. After finishing the evaluation, validation reports have been prepared by the laboratories in charge and sent to WP6 for review. Main results can be summarized: (i) spICPMS validation was carried out for analysis of titania nanoparticles in suspension, titania in sunscreen and alumina in toothpaste. The method produced repeatable results, especially for particle size. (ii) validation of the cuvette-type AC technique with interferometric optic showed its ability to properly distinguish between nano and non-nano materials. For the test material already in suspension (trimodal SiO₂), there is an excellent match between x50,3 and x50,0 values generated at the two participating labs with less than 6 % and 2 % difference, respectively. Differences are much higher in case of BaSO₄ powders: for the coarse grade, they amount to 56 % for x50,0, although the material is still correctly identified as non-nano-material. (iii) For the cuvette-type AC with turbidity detector, the working range regarding sample content is from 0.6 g/kg – 2.6 g/kg for (BaSO₄, coarse grade) and 0.6 g/kg – 10 g/kg for (BaSO₄, fine grade). The lower limit can be expanded until 0.1 g/kg by choosing 10 mm cuvettes. The intermediate precision was below 10 % for both BaSO₄ powders. (iv) The repeatability of the Disc-type AC with turbidity detector including ultrasonic dispersion and AC measurement is fairly high for both BaSO₄ powders – provided that appropriate instrument parameters are used. The method was found to be robust with respect to the sedimentation gradient composition, choice of calibrant and moderate changes in rotation speed.

WP5: Confirmatory methods (tier 2 methods)

Software for automated particle size analysis of recorded EM images.

The development of a new software for automated particle size analysis of recorded EM images (off-line batch processing) was one of the confirmatory methods developed. The splitting of touching / overlapping nanoparticles is a very challenging task with a trade-off between automatization, detection quality and user-input. Software based on ImageJ which is capable to handle different image qualities, backgrounds and polydisperse particle mixtures was developed. For that purpose, a pipeline was designed, which removes different noise-levels automatically by using a non-local-means filter, subtracting the image back-ground and binarizing the image using local adaptive thresholding technique. Finally, the software detects and removes false positive segmentations by defining a minimal feret diameter and a minimal object-to-background intensity difference. The ParticleSizer software successfully handled overlapping particles, various image qualities (intensities) and inhomogeneous backgrounds.

The performed intra-lab validation study showed that the ParticleSizer software succeeds in obtaining accurate and precise characterization results (Table 3). The method is considered to be feasible to 4 types of materials in the size range [1nm, 1µm], which can be brought representatively on a EM grid: stable aqueous colloids of non-aggregated particles, aggregated/agglomerated materials with spherical or ellipsoidal touching or slightly overlapping primary particles, aggregated and agglomerated materials with irregular touching or slightly overlapping primary particles and aggregated/agglomerated materials with highly overlapping primary particles. It was demonstrated that to apply the ParticleSizer correctly in Irregular watershed mode and in Ellipse fitting mode, user experience and training in optimization of settings are necessary. In Default mode and in Single particle mode, less optimization of settings is required, so user experience in optimization of settings is not required but assists in obtaining precise results. The effect of running the ParticleSizer in a different mode (Default, Irregular watershed, Ellipse fitting or Single particle mode) on the median Feret min value is material dependent, but is generally not larger than a few nm.

Table 3: U_{IP} obtained for the measurement of the median Feret min of the different materials by P1, P2, P3, TP1 and TP2.

U_{IP}	P1	P2	P3	TP1	TP2
ERM-FD100 18500x	1.8%	1.9%	1.7%	1.7%	2.0%
ERM-FD100 68000x	1.9%	2.3%	2.4%	Test 1: 31.7% Test 2: 1.9%	5.3%
ERM-FD304 18500x	1.3%	3.0%	1.4%	1.4%	1.4%
ERM-FD304 68000x	1.5%	2.2%	1.8%	6.1%	6.1%
Au nanorods	3.4%	7.4%	4.4%	Test 1: 29.5% Test 2: 23.8%	7.2%
NM-100	2.1%	2.6%	2.3%	3.9%	15.8%
NM-103	2.5%	3.1%	2.1%	6.3%	2.3%
NM-212	6.7%	9.0%	6.3%	4.4%	4.5%

Development of full automation of EM operation (NanoDefine ‘auto-EM’ toolbox) allowing rapid particle sizing and elemental identification.

A number based particle size distribution (PSD) of particulate materials with reduced user-bias was accomplished by developing a novel software (Auto-EM toolbox) that automatically acquires and analyses images using TEM. The only user input consists of a selection of a large area to be imaged in more detail and a set of specific input parameters such as an image overlap or particle number limits. The Auto-EM toolbox was developed as open-source program (MIT license) and uses the Particle Sizer software (see above), which analyses the acquired images online using the Fiji program. The Auto-EM toolbox was tested successfully at four different TEMs: FEI TF20, JEOL JEM-2800, FEI T12, and FEI TF20. Two examples are given where the software has been applied during recording NanoDefine materials at JEOL JEM-2800 (Figure 6)

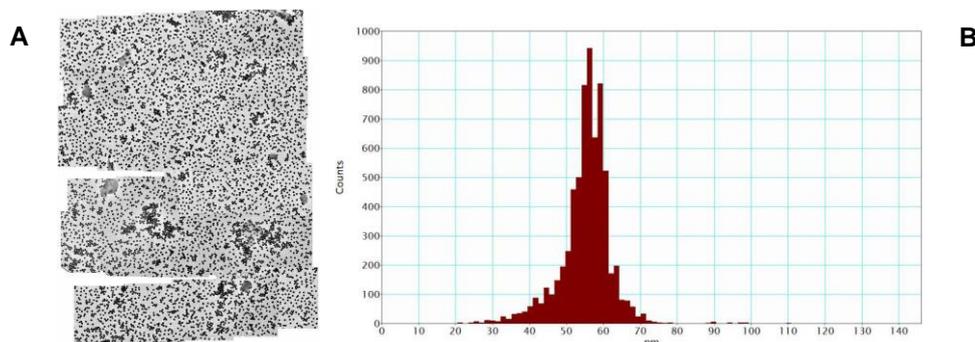


Figure 6: (A) A mosaic image (5x6) of Au NIST NPs (JEOL JEM-2800); (B) PSD of NIST Au NPs (X_{50} value is 56 nm and 6636 particles were analysed).

Measurement methods for complex materials based on particle separation (FFF) and conventional multi-detection techniques

SOP established for analysis of Al_2O_3 particles from toothpaste by AF4-ICP-MS

The AF4-ICP-MS method was developed and optimized directly on the BAM-15 toothpaste samples, which contained a mixture of Al_2O_3 , TiO_2 and SiO_2 particles. The experimental samples were prepared using chemical oxidation with hydrogen peroxide and dispersion in surfactant. ICP-MS was used for selective detection and quantification of the fractionated Al_2O_3 particles (Figure 7). The 90° light scattering signal from coupled MALS detector (sensitive to all 3 constituent particles) was exploited for optimizing the AF4 separation method.

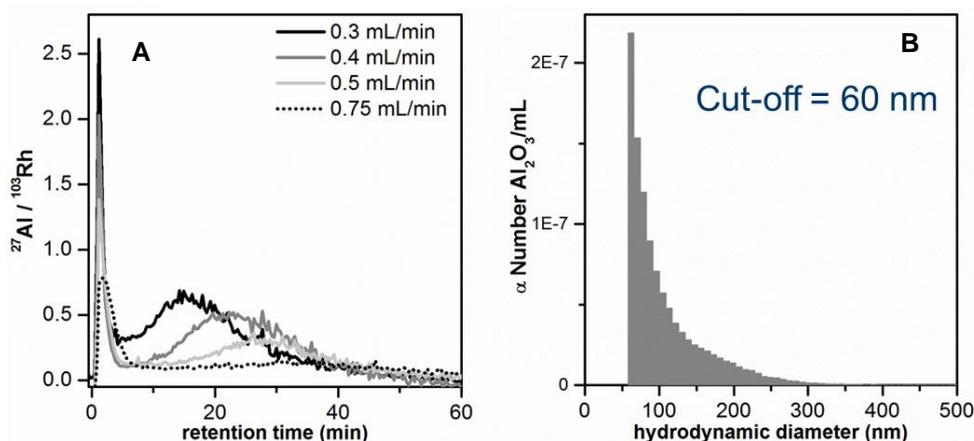


Figure 7: Fractogram of toothpaste with Al_2O_3 particles after AF4-ICP-MS (A) and conversion to number based particle size distribution (B).

SOP established for analysis of TiO_2 particles from sunscreen by AF4-MALS-ICP-MS

A quick and straightforward sample preparation method to directly analyse TiO_2 particles in BAM-13a/BAM-13b included: 1) dilution step using specially prepared extraction fluid, and 2) particle stabilization step using the anionic surfactant. TiO_2 particles in BAM-13a were detected and further characterized in terms of mass-based and number based PSD (Figure 8).

The intra-lab validation study demonstrates that the analysis of TiO_2 particles present in sunscreen by conventional AF4 separation-multidetector produces quantitative data (mass-based particle size distribution) after applying the sample preparation method developed within WP2 (see D2.6) with uncertainties based on precision of 3.9-8.8% and can be considered as a method with good precision. Including uncertainty due to bias coming from spiking experiments it is evident that the good method precision (17.1-25.3%) is obtained for the particle size in the region of 100 nm and smaller. The largest effects calculated for mass based PSD uncertainties were due to the higher values expected at the edges of a distribution.

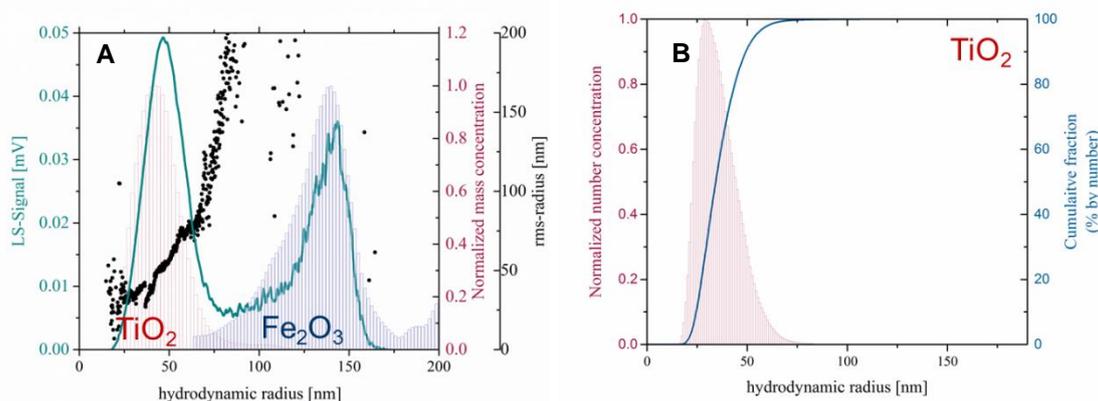


Figure 8: TiO₂ mass-based PSD (A) and number-based PSD (B) of BAM-13a (complex sunscreen) after dilution method. LS-signal (A) is referred to MALS data for a detector angle of 90°.

Measurement methods for particle separation (FFF) coupled to true particle counting techniques

An extensive feasibility study on the coupling of FFF to the aerosol based counting technique (ESI-CPC) demonstrated that such an approach was incompatible due to the huge difference between the required sample flow rates for FFF (1 ml min⁻¹) and ES (<100 nl min⁻¹), as well as different eluents used in FFF that are not appropriate for ES analysis. In order to achieve particle number concentrations suitable for spICPMS from diluted FFF eluents higher dilution factors that can be reliably varied during the analysis are required. This is especially the case for real samples that contain smaller particle sizes and/or are polydisperse. The current prototype interface cannot achieve this and for this reason collecting fractions and subsequent dilution after FFF and further spICPMS analysis manually is recommended.

Cost-efficient FFF separation instrument

The development of a more cost-effective FFF system was split into a hardware and software part. On the hardware side, the most desirable solution with significantly optimized technical characteristics at lower prices and substantively reduced service costs was achieved. The customers can decide themselves which peripheral hardware may fit best into their budget and may apply best the needs of the application. The connective centrepiece between hardware and software is the Rapid Control Interface built-in new data acquisition software VOYAGER CDS®. This allows the use of every RC.Net compatible HPLC module and the FFF at the same time. Because VOYAGER CDS® is made according to the moderate demands of FFF, a small software solution with a very interesting price compared with the well-known standard platforms was created. VOYAGER CDS® as pure data acquisition software works seamlessly together with the new data processing solution SCOUT DPS®. This program guides customers through all steps of method development by its easy-to-use interface, chromatogram prediction in real time and saving of the optimized method, ready to be instantly executed by the hardware.

Beside of all the improvement, a full hydrodynamic radius distribution analysis into SCOUT DPS® was implemented. Once, the system is calibrated by a nanoparticle standard, this evaluation does not need any extra work ahead of the separation, but calculates the Rh-distribution even for extremely low concentrated samples or for samples smaller than 10 nm. In many cases, this feature is capable to compensate a QELS detector, with a sales price of approximately 40k€.

WP6: Validation and standardization

The first task was to support and guide the in-house validation of tier 1 + 2 methods. As envisaged in the DOW, a guide for in-house validations was prepared that should support

the laboratories in charge of the in-house validation exercises. The aim of the in-house validation guideline was to ensure the compliance with international standards based on the IUPAC "Harmonised Guidelines for Single-Laboratory Validation of Analytical Methods", the Eurachem Guide "Fitness for Purpose of Analytical Methods" and Commission Decision 2002/657/EC on the reliability of methods for residues and contaminants in food of animal origin. As an outcome, the most promising methods should be subjected to inter-laboratory validation.

The guideline is divided in several parts that address general aspects; the workflows of preparing documents and their review; the listing of possible validation parameters and templates, and examples for the preparation of respective documents.

In the generic part, the aspect of pre-defined criteria and the importance of routine conditions for the validations are stressed. An important clarification is the definition of a method: it is understood that for substances and products, the scope of the individual method validation is a combination of a sample preparation protocol, the measurement technique (e.g. TEM) and a specific type of nanomaterial(s).

It is emphasized that a good method validation is not an ad hoc exercise, but a planned process based on three steps: the description of the underlying work instruction (standard operating procedure, SOP) describing all the details and operations that are needed to successfully perform a measurement; the validation plan describing all the validation parameters and experiments together with acceptance criteria; and a validation report that evaluates and summarizes the obtained results and assesses if the method is fit for purpose.

The proposed workflow included a reviewing step of each document by the JRC. Together with the provided document templates, this assured the harmonised approach over different validations and actors. In order to assure the harmonized approach in different laboratories, templates for SOPs, validation plans and validation reports are annexed to this document, together with an example of a typical SOP for nanoparticles. The in-house validation guideline was discussed and reviewed by project partners and finally accepted by all partners in the project.

As proposed in the guideline, a validation list was prepared summarizing all important information about the planned validations. Apart from more technical aspects (description of method, measurement technique, samples), also organisational aspects (laboratories in charge of the validation, contact persons, time frames) and the current status of activities are documented which gives an up-to-date overview on the whole exercise and allows a proper management and coordination of the studies. This document served as a backbone for regular WP6 discussions on the progress of the validations, typically carried out in frequent telephone conferences. JRC assisted in the preparation of the SOP, the validation plans and the in-house validation reports. Critical reviews were given on the respective documents involved. This ensured that the agreed, harmonised approach was followed. In the end, 8 methods/SOPs based on 6 measurement principles covering 22 parameters were assessed in the in-house validations. All of them were selected for the subsequent inter-laboratory validation studies (see below).

The second task was the inter-laboratory validation of key measurement methods by JRC-IRMM and all trained laboratories. The DOW specified that the most promising methods based on the outcome of the in-house validation studies should be selected. As a target, four methods should be selected to be studied in the inter-laboratory validations. In practice, more methods were chosen. First of all, at least four measurement principles are involved (the ParticleSizer software, spICPMS, AF4-MALS-ICPMS, CLS). In the case of the centrifugal methods, three subtechniques had been validated (Disc-CLS, Cuvette-CLS, Analytical Ultracentrifugation). Instead of selecting only one of them, it was decided that all three of them should be studied in the inter-laboratory validation exercises. Although

the measurement techniques are very similar, the principles do vary which leads to different measurands that per se cannot be compared directly. All CLS methods were developed for BaSO₄ and had shown a similar performance in the in-house validation. However, it was unclear whether all CLS techniques would be able to obtain the needed 8 valid data sets (see MS38). As a consequence, all CLS methods were subjected to inter-laboratory validation studies, taking into account that not all of them would collect sufficient data. For the spICPMS, three different methods were available (TiO₂ in suspension, TiO₂ in sunscreen, Al₂O₃ in toothpaste) and all of them were selected as well. The AF4 method was developed for TiO₂ in toothpaste. Finally, all 8 methods were selected.

For every of these methods, more than one parameter (measurand) has to be determined, such as particle size expressed as number- or mass-based median diameters, median Feret diameters, hydrodynamic radiuses, or particle number/mass concentrations. In total, 22 parameters for the 8 selected methods had to be measured in the inter-laboratory validation studies.

Given the low number of potential laboratories, it was evident that successful interlaboratory exercises could only be organised with external participants from all over the world. For this reason, a project under the auspices of VAMAS was started (see below) that should give the inter-laboratory exercises also the necessary standing and visibility. The proposed project was approved as new project 9 (Assessment of a quantitative nanomaterial definition) of TWA34. Shortly after, an international call was launched that should not only include metrological institutes, but also all kinds of public or private laboratories. In addition, available networks of consortium partners were activated and in the end, the international call was sent to over 200 laboratories world-wide inviting to join one or more of the inter-laboratory method comparisons. As stated above, the selected methods included measurement principles such as spICPMS, the ParticleSizer (automatic analysis of EM images), Disc-CLS, Cuvette-CLS, Analytical Ultracentrifugation (AUC), and AF4-MALS-ICPMS. Given the extremely short time of a few weeks to respond to this call, the feedback was very positive: in total, 104 laboratories showed their interest in participation and 88 of them actually registered. Interested parties received the respective SOPs and the necessary test materials, together with clear instructions from JRC.

Despite the large global call and the high number of participants, the number of acceptable data sets per method was sometimes below the minimum number of eight. This could be due to time constraints or problems to implement the methods in a laboratory in the relatively short period available, as was the case for Disc CLS, AUC, and AF4. Strictly speaking, no quantitative conclusions on reproducibility can be drawn from these results, even if some results are promising, e.g. in the case of FFF. However, due to the lack of sufficient data, the inter-laboratory study cannot be considered successful for these methods.

The remaining five methods (three spICPMS methods, Cuvette CLS and the ParticleSizer Software) obtained however a satisfactory to high number of acceptable data sets. In the case of the spICPMS methods, the within-laboratory relative standard deviations were comparable or better than the standard uncertainties obtained in the in-house validations. As a rule of thumb, relative reproducibility standard deviations larger than 20 – 25 % are very often not deemed as fit for purpose which means that the observed RSD_R of these methods of around 40 % indicates a problem in different laboratories. Data from particle number concentrations (data not shown) suggest that it could be an issue of sample preparation in the different laboratories.

The results of the Cuvette CLS method for BaSO₄ are quite positive. The ParticleSizer Software was tested on images of four different materials with increasing difficulty in terms

of shape and agglomeration. For the most complex material (CeO₂), the reproducibility was somewhat higher, but overall quite good.

Summarizing, appropriate inter-laboratory validation studies were organised that attracted a lot of international laboratories. Only a fraction of these laboratories could provide acceptable results. In some cases, too few data sets were obtained for the inter-laboratory validation. For five methods, sufficient data were obtained. In the case of spICPMS, the methods showed good within-laboratory performance, however, the reproducibility between laboratories should be improved. The ParticleSizer software and the Cuvette CLS method can be considered as successfully validated in the inter-laboratory studies. It should be kept in mind that there was no time for a training of the laboratories and very little time for the implementation of the methods. With a better preparation of the participating laboratories and more data sets, possibly a better performance could be expected (see Figure 9).

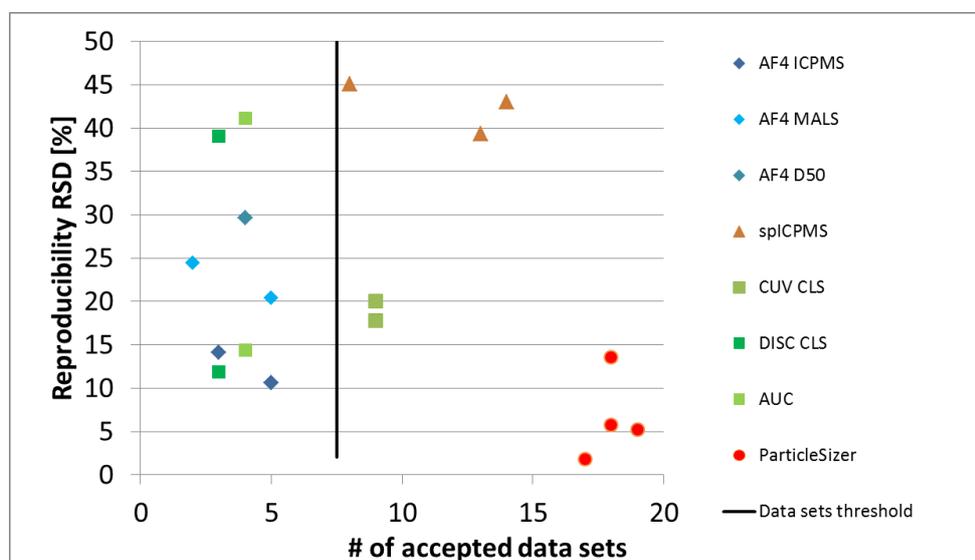


Figure 9: Selected relative reproducibility standard deviations vs. the number of accepted data sets in the inter-laboratory validation exercises. Only the most prominent parameters are shown (average particle sizes of number-based or mass-based distributions). The black bar represents the minimum number of valid data sets of eight. Identical symbols indicate where different test materials were available (CLS) or the particle distribution was bimodal (AF4).

To support relevant standardization efforts, it was decided that the two most appropriate standardisation committees would be the CEN/TC 352 and the ISO/TC 229 committees both named "Nanotechnologies" (see MS35). Together with support from DIN, the JRC drafted a letter that was sent by the project coordinator to CEN, asking for a liaison of NanoDefine with standardisation committee TC 352 "Nanotechnologies". After a lengthy internal procedure, an agreement was signed between the consortium and CEN-CENELEC and the liaison request was accepted by CEN/TC 352 in July 2015. As a representative for the project, Prof. Michael Stintz (TU Dresden) was nominated who is already active in the committee for a long time.

The DOW also envisaged the submission of the validated methods as new work item proposals (NWIPs) to standardisation committees. However, this is technically not possible for a series of reasons. Firstly, there is a timing problem. The organisation of the inter-laboratory validation was delayed due to the late finalisation of in-house validations. The finalisation of validations of test methods was delayed and a decision for the selection of three methods principles could only be taken in M42 (target: M41). As a scientific advisor

from the project SAB suggested to try everything to get also the FFF method selected, the deadline for the selection of the fourth measurement principle was postponed to M43, when the AF4 method was accepted. On top of this delay, the organisation of the VAMAS project also required some extra time that was not foreseen in the DOW. As explained above, the VAMAS project approval could only be obtained after the selection of methods for inter-laboratory validations. Secondly, there is a technical problem why the mentioned methods could not form the basis of a NWIP. The developed methods in NanoDefine are specific test methods that provide a protocol (SOP) for the quantitative analysis of a specific material which implies that the whole measurement procedure (as defined by the international vocabulary of metrology, VIM) is specified. This includes the definition of the measurand, the type of sample, its sample preparation, the actual measurement specifying the measurement technique with the detailed conditions, and the data evaluation that leads to the final result. From the point of view of standardisation committees, these methods are highly specific and developed and validated for a particular type of analysis. However, work items and published standards typically deal with more generic guidelines that result in a broad applicability of these standards. In other words, the methods developed in NanoDefine are rather over-specific in their scope when considering them as potential NWIPs.

There is also another (third) technical issue that should be considered. In the course of the NanoDefine project, there was limited time for the development and subsequent validation of the different methods. There was little or no time for optimisation, especially for the validation experiments. It is in the interest of the standardisation committees that only fully optimised methods are considered for their work programme. It is questionable if the methods provided by NanoDefine at the end of the project are already mature enough to pass a rigorous scrutinizing by the standardisation bodies before accepting them as new work items.

However, a proposal finally surfaced to consider the NanoDefine Methods Manual (D7.6 and D7.7) as a NWIP. This idea was taken up by M. Stintz, the project's representative in CEN/TC352. It basically proposes the content of the Methods Manual as a NWIP in the form of a technical report (TR). This is based on the work described in deliverable reports D7.6 and D7.7, but also in D3.1 and D7.12. The title of the proposed NWIP is *Eligibility of particle characterization methods for classification of a material as nanomaterial*. The proposal was put forward by DIN as it had to come from a CEN member body. One main feature of the Methods Manual is the decision support flow scheme, which is developed to logically guide the user through a sequence of tasks, decision nodes and options in order to decide whether a material is a nanomaterial or not according to the EC Recommendation for a definition of the term "nanomaterial". The Strategy Group of CEN/TC 352 has invited M. Stintz to its meeting on 18 October 2017 in Prague to comment the delivered outline and to answer questions from the other experts. If successful, at the next plenary meeting on 19 October 2017, CEN/TC 352 will ask DIN to launch the NWIP for the TR. A first discussion with experts could follow at the first CEN/TC 352 meeting in 2018 on 15 March in Brussels.

WP7: Implementation: NanoDefiner e-Tool, Manual & case studies

To enable authorities and enterprises to make the best use of the project findings, a decision support framework was developed, the pillars on which the framework is based and the tools for practical application (decision support flow scheme and e-tool). The pillars for the decision support framework are: (i) Knowledge base – methods evaluation, (ii) Materials Classification System, (iii) Data input – Methods Manual, and (iv) logical flow scheme – decision tree. The flow scheme is implemented in an e-tool (v). After completion, the decision support framework was tested in (vi) Case Studies, and in addition the WP developed (vii) recommendations on a revision of the EC definition of nanomaterial.

(i) Performance Criteria, Methods Evaluation and Knowledge Base. A catalogue of technical performance criteria was developed targeted at measurement requirements resulting from the EC NM definition of the term "nanomaterial". The capacities of each characterisation method (CM) was elaborated in detail at the beginning and at the end of the project, including

- Applicability to different groups of substances (chemical scope of the method)
- Applicability to polydisperse samples
- Measurement in terms of size, shape and test medium for different groups of substances
- Capacity to measure aggregates, agglomerates, primary particles and/or non-spherical particles.
- Accuracy of the results determined with the CM
- Standardization status (traceability of the measured values / availability of CRMs)

The results of these evaluations were collected in a Knowledge Base available as tabular database and in machine-readable form for the e-tool.

(ii) Materials Classification System. A method-driven materials classification system was developed which classifies a material according to criteria that are most influential for selecting appropriate characterisation methods. This enables the optimal match between a specific material and the most appropriate characterisation method to be used when going through the decision tree.

(iii) Method Manual – data input. The hands-on manual on the recommended use of methods specified by purpose and quality requirements, including performance characteristics was compiled based on filled methods templates and more in-depth information on specific methods added as annexes. The manual was updated and expanded based on findings from work packages 1-6, and according to the "state of the art" at the end of the project. It can be consulted by the user to give clear guidance on the CMs to be used and their application range as well as their limits throughout the entire nanomaterial characterization process. The manual also contains the complete material classification system and the decision support flow scheme with detailed instructions on all steps, including explanation of the decision process at each decision node, SOPs developed in the project and available standards. It is a stand-alone document to be consulted by the user throughout the entire decision process on how best to obtain and use experimental data necessary in the entire decision process. The manual is also integrated as online version into the NanoDefiner e-tool.

(iv) Decision tree – conceptual decision support flow scheme. The NanoDefine decision support flow scheme was developed to logically guide the user through a sequence of tasks, decision nodes and options in order to decide whether a material is a nanomaterial or not according to the EC Recommendation for a definition of the term "nanomaterial". The flow scheme implements and integrates the results obtained in the individual WPs into a comprehensive decision support framework and guides the user through the entire decision process. Step by step, based on the knowledge acquired within NanoDefine and depending on the nature of the material to analyse, the user is given a choice how to characterise the material in question with certain screening (tier 1) or confirmatory methods (tier 2). The flow scheme starts with a basic classification and then continues in two branches: one for powders and one for dispersions. The two branches go to a first tier (screening methods) and continue to tier 2 (confirmatory methods). For each step a dedicated node is proposed that indicates to the user a set of information required for proper classification of the material as nano or non-nano. The scheme is designed to allow economic and reliable decisions on whether a material can be identified as nano or non-nanomaterial.

(v) NanoDefiner e-Tool. The decision support flow scheme was implemented in the NanoDefiner e-tool. The NanoDefiner e-tool software (beta-version) was developed and successfully tested within the consortium. It was further developed into its final version which is available as standalone as well as downloadable version. The e-tool is an easy to

use software which guides the user through the entire decision process. The material classification system, the knowledge base with its quantitative and qualitative evaluation of tier1 and tier 2 characterisation methods and the logical decision support flow scheme, together with input of data at appropriate stages are the main elements ("pillars") of the e-tool. The work flow within the e-tool follows the decision support flow scheme. The e-tool is configurable via configuration files and therefore has the flexibility necessary to be adapted for technical progress in the CMs, modification of the decision criteria and revisions of the EC's nanomaterial definition.

(vi) Case studies. The decision work flow was tested in case studies with nine different materials. Three categories of case studies were conducted, which each take a distinct perspective:

- NanoDefine Materials already extensively tested in NanoDefine
- OECD sponsorship program materials from the JRC repository
- Materials with challenges not yet represented in the project

The case studies run also by external collaborators explored both tier 1 routes (powder and dispersion) and benchmarked against a tier 2 method (TEM/EM). They were carried out manually as well as using the NanoDefiner e-tool. There were no cases of inconsistent classification, two cases remained inconclusive regarding the decision nano or non-nano. The decision tree was optimized as a result of these studies to further reduce ambiguities. In all cases studied the two runs came to consistent results for a specific element regardless of whether the assessment was made by applying the flow scheme manually or whether the e-tool was used.

(vii) Recommendations on a revision of the EC nanomaterial definition. The project developed recommendations on a revision of the EC Definition of Nanomaterial based on analytical possibilities after project half-time and at the end of the project.

Potential impact, main dissemination activities and exploitation of results

NanoDefine was entirely devoted to develop a measurement strategy that can support the implementation of the EC recommendation on the definition of nanomaterial (2011/696/EU) by potential end-users, such as material industries, regulatory authorities of enforcement labs. This was mainly achieved by providing a set of representative test samples and reference materials (WP1), by testing and evaluating the most relevant particle size measurement methods (WP3), by establishing a variety of new and validated SOPs for sample preparation and dispersion (WP2), for particle counting, imaging and detection, including design of new measurement software (WP1-5), by developing a 2-tiered measurement approach based on the developed and validated measurement techniques, including rapid and cost-effective screening (WP4) and complementary and confirmatory methods (WP5), by using a stringent method validation and standardization process (WP6), and last not least by integrating all these methods into a decision support flow scheme and method manual that is applicable as the NanoDefiner e-Tool to guide potential end-users, such as material manufacturers or monitoring authorities, in the identification of nanomaterials as substances or in products, or in finding out if a material or product is or contains nano, or not (WP7).

NanoDefine has developed several exploitable results (i.e. foreground), as identified during a special Exploitation Strategy Seminar in March 2016 (see Annex II in D8.5). These include, among others, reference materials (some may be further developed to CRM), method protocols, new and/or improved measurement instruments and software for commercial use, e.g. by industry, testing laboratories or regulatory monitoring agencies.

Beside the development of a panel of well characterized reference tools, including reference materials and validated methods (SOPs) that will assist industry and regulatory bodies to classify particulate materials, NanoDefine also created an enormous wealth of new scientific knowledge and technical know-how on the physicochemical behavior of nanomaterials and associated products.

NanoDefine also produced a variety of commercially relevant foregrounds, such as new measurement instruments and software, or new material prototypes specifically designed to identify nanomaterials in complex real-world samples. In particular the development of a new electrospray deposition prototype for EM analysis, a novel ParticleSizer based on DMA with high resolution for particles < 5 nm, the improvement and further development of analytical centrifugation and of a new hard and software for commercial PTA/NTA, the creation of new interfaces between different particle counting and detection modules, such as the coupling of FFF and ESI for aerosol detectors, or a new software to determine the size- and number distribution of nanoparticles in a sample by spICP-MS, but in particular the NanoDefiner e-Tool offering proper analytical routes to classify a particular material or product, has to be emphasized.

In the following, a short summary is given on the potential impact, dissemination and exploitation of achieved results for each work package.

Summary on impact and dissemination/exploitation activities for each technical WP

WP1 was mainly an enabling work package to guaranty and enable a broad impact of the other WPs products. The main product (CRMs developed from 2 of the projects substances) is due after the end of the project. JRC-IRMM is committed to develop and provide this product after the project was finished.

WP2 provided the necessary sample preparation protocols for WP4, 5 and 6 and in this way contributed to the dissemination / exploitation activities in these WPs.

WP3: All results on the comprehensive method evaluation together with the raw analytical data obtained have been published in the following two review open access papers:

- 1 F. Babick, J. Mielke, W. Wohlleben, S. Weigel, V.-D. Hodoroaba, How reliably can a material be classified as a nanomaterial? Available particle-sizing techniques at work, *J Nanopart Res* (2016) 18: 158, and
- 2 W. Wohlleben, J. Mielke, A. Bianchin, A. Ghanem, H. Freiberger, H. Rauscher, M. Gemeinert, V.-D. Hodoroaba, Reliable nanomaterial classification of powders using the volume-specific surface area method, *J Nanopart Res* (2017) 19: 61.

While (1) includes for the first time results on the quantitative evaluation of all available sizing MTs as applied on the reference materials selected in WP1 but especially on the representative and challenging real-world particulate NanoDefine materials, (2) is also unique by demonstrating for the first time the reliability (together with applicability ranges) of the VSSA approach as extracted from BET measurements for various shapes of particulate materials. The latter was possible in the frame of inter-laboratory comparisons by both BET and electron microscopy applied on the same materials. Despite the recent publication (June 2016 and January 2017), both papers are very well cited, i.e. 19 times the first paper and 4 times the 2nd one (acc. to Google Scholar).

In addition to the first overview paper (1), another detailed report dedicated to the errors generated by conversion of measured quantity (i.e. intensity, volume or mass) into number-based particle size distribution has been published by WP3:

- 3 F. Babick, C. Ullmann, Error propagation at the conversion of particle size distributions, *Powder Technology* (2016) 301: 503.

A large number (23 x) of active participations in European and international workshops and conferences dedicated to the analysis of particle size distribution in support of the EC definition of nanomaterial has been organized by WP3 experts (see also Project Dissemination Activities and Publications at the EC Participant Portal). The presentation of the WP3 results as invited lecture at the Joint Workshop of ISO/TC 229 'Nanotechnologies'/ JWG 2 'Measurement and Characterization' & ISO/TC 24 / SC4 'Particle characterization': 'Frontiers of nanomaterial characterization' on 28th of May 2017 in Tokyo, Japan can be given as an example, in addition to the invited presentation of the WP3 evaluation study within the Metrology Study Group of ISO/TC 229 'Nanotechnologies'/ JWG 2 'Measurement and Characterization', on 29 May – 2 June 2017 in Tokyo, Japan. The necessity of transfer of knowledge gained in NanoDefine related to the complex as well as limited application of the sizing MTs for the specific implementation of the EU definition of nanomaterial was recognized by the WP3 experts early during the project. The valuable knowledge was disseminated diligently, but also debated intensively at the (NanoDefine) dedicated public workshops (in 2014 and 2017) and technology transfer events (in 2016 and 2017), where the relevant stakeholders were present, as well as at corresponding 'nano' conferences and standardization meetings.

WP4 generated substantial improvements of existing measurement procedures and data interpretation, which contributed to several standardization projects (see WP6) and commercially available devices and software tools.

WP4 selected the methods and materials for the inter-laboratory validation studies (in WP6) and developed the measurement specific SOPs. This resulted in the knowledge database required for the e-Tool and in various input to European standardisation efforts on Nanotechnology (e.g., TR project in CEN 352 and CEN open workshop, Malta 2017).

Another impact created in WP4 was the science based order of comparison and validation of number based distributions required before the intensity-based and mass-based distributions validation, which will contribute, e.g., to risk assessment and risk modelling (particle release, transport, exposure).

So far, there have been 2 peer-reviewed publications (1 submitted), 5 other publications (conferences, workshops), 1 contribution to proceedings and more than 8 talks and posters.

WP5: A key aspect of WP5 was the strong focus on industrial exploitation of the project developments/results. As one outcome, the ParticleSizer manual was developed to automatically measure the distributions of the characteristic size and shape properties of a nanomaterial and shared online: <https://imagej.net/ParticleSizer>.

Also the Auto-EM toolbox was developed as an open-source program (MIT license) and has been promoted at different events such as, the first and second NanoDefine industry-focused Workshop in 2016 and 2017, Frankfurt, Germany and the Final NanoDefine Outreach Event in Brussels, on 19-20 September 2017.

In terms of scientific training and education, 1 PhD thesis has resulted from WP5 and a variety of oral and poster presentations at relevant conferences, of which some are highlighted in the following:

PhD thesis, title: Methods in support of environmental risk assessment of ENMs by Fazel Abdolapur Monikh

Selected conferences:

253rd American Chemical Society NATIONAL MEETING & EXPOSITION: Advanced Materials, Technologies, Systems & Processes, 2-6 April 2017, San Francisco, CA, United States (oral presentation)

- TiO₂ nanoparticles in sunscreen lotion: asymmetric flow field-flow fractionation hyphenated to ICPMS for their characterization, Milica Velimirovic (Speaker)

18th International Symposium on Flow- and Field Based Separations (FFF2016), 22-26 May 2016, Dresden, Germany (oral presentation)

- Simultaneous on-line detection of SiO₂, TiO₂ and Al₂O₃ particles in toothpaste by asymmetric flow field-flow fractionation hyphenated to triple-quadrupole inductively coupled plasma mass spectrometry, Manuel Correia (Speaker)

RAFA 2015, 3-6 November 2015, Prague, Czech Republic (2 oral presentations)

- Application and future perspective of automated electron microscopy to quantify engineered nanoparticles in complex matrices. Ralf Kaegi (Speaker)
- Simultaneous on-line detection of Si, Ti and Al-containing particles in toothpaste by asymmetric flow field-flow fractionation coupled with ICP-QQQ-MS, Katrin Loeschner (Speaker)

2nd International Fresenius Conference, 9-10 September 2015, Cologne, Germany (oral presentation)

- An update on method development to analyse nanoparticles in complex samples, Stephan Wagner (Speaker).

WP6: Dissemination activities included:

- Two public outreach events for a general audience and for industry
- Publication of the material classification system, decision support flow scheme and the e-tool in peer reviewed journals
- Free access to the Manual and the e-tool
- Dissemination activities will continue after project end, e.g., by organizing training events for authorities and industry as requested

The CEN/TC352 committee accepted at its plenary meeting on 18 October 2017 in Prague, the NanoDefiner Method Manual as a NWIP. For this, CEN/TC 352 asks DIN to accordingly launch the NWIP for the TR. A first discussion with experts could follow at the first meeting of CEN/TC 352 in 2018 on the 15th of March meeting in Brussels. With this, an important deliverable of NanoDefine can have its impact in the standardisation work for nanotechnology in Europe.

As the validated test methods developed by NanoDefine are public, they can be used by laboratories from industries or regulatory bodies to analyse the nanomaterials in question, which will generate another relevant impact of NanoDefine on the need to meet the analytical challenges linked with the regulatory implementation of the EU definition.

Apart from this, these methods can also serve as a starting point for further method development for similar or even very different materials. Some of the method development work in NanoDefine dealt with very challenging, polydisperse material resulting in validated methods. This can encourage other laboratories to address this challenging task as well. The chosen approaches to standardise sample preparation or the approach how to validate methods in this field can be seen as role models.

Also, the harmonised in-house validation approach used by NanoDefine and the professional organisation of the inter-laboratory validation studies can encourage laboratories or organisations to follow this approach, as it could be demonstrated that the ambitious challenge to tackle complex, real-world nanomaterials can be successfully mastered. Indeed, previous inter-comparisons have often lacked this harmonised approach using common test samples of demonstrated homogeneity and stability and common, detailed method protocols.

As the external validation activities were completed during the final phase of the project, little dissemination apart from the public workshops could be achieved so far. However, the strong response from a vast number of analytical laboratories to the international call for the inter-laboratory validation exercises showed that there is considerable interest in this kind of activities. During their preparation, the respective method protocols have been distributed to more than 100 laboratories already. The results from the inter-laboratory validation study will be also distributed to many laboratories. The approach and the results of these studies will give sufficient material for future peer-reviewed publications.

As the project 9 of the VAMAS TWA34 launched by NanoDefine does not end with the project, it is possible to continue the optimisation of methods. Depending on the in-depth analysis of the inter-laboratory study results, laboratories could further optimise their methods which could be tested in a second inter-laboratory exercise, which would be very welcomed by VAMAS. However, such a follow-up would depend on the confirmation of the willingness and commitment from involved parties to continue this work, even without funding from NanoDefine.

WP7

Summarized, it can be stated:

- That the implementation of the EC's Recommendation on a definition of nanomaterial in a regulatory context will be considerably facilitated through the results described above, in particular the decision flow scheme, the NanoDefine Manual and the NanoDefiner e-tool.
- That the EC's Guidance on the implementation of the NM Definition will substantially benefit from the NanoDefine project results. It is expected that the uptake of these results by regulators and relevant industries will facilitate and harmonize the regulatory classification of nanomaterials.

- As the project provides the analytical tools to support implementation of the EC definition it can be expected that the amendments of Regulations covering nanomaterials will make progress, either by introducing the EC definition of nanomaterials across existing policy areas (such as REACH) or by replacing historical definitions of nanomaterial with the EC recommendation (e.g. within the Foods, Cosmetics, Biocides regulation).

In total, all obtained project results (foreground) have been disseminated to all relevant stakeholders (industry, regulatory, academia, EC, public) mainly by means of peer-reviewed publications, conference or workshop presentations/proceedings, exhibition stands, email shots, flyers, brochures, project-specific and NSC bulletins and newsletters and in particular via the project-specific website. Internally, a specific partner intranet has been installed at the website to allow the password protected uploading/downloading, sharing and exchange of all produced documents including publications among partners, such as deliverable or milestones reports, presentations and minutes from meetings or legal documents, such as amendments of the grant agreement etc.

All partners contributed to the dissemination of the results they produced both by preparing technical deliverable and milestone reports and by publishing in peer-reviewed scientific journals or presenting their results at conferences or workshops. A list of main dissemination activities and of peer-reviewed publications is presented at the EC Participant Portal and attached to the Final Report. So far, NanoDefine partners have been engaged in almost 140 different dissemination activities and produced 6 peer-reviewed publications (more publications are still in progress after the end of the project).

In the final project year, NanoDefine has engaged the wider community through a range of different dissemination activities and events, including those within the ENF2017 international conference and CEN Open Workshop in Malta June 2017, a special Summer School organized at RIKILT in June 2017, a 2nd Public Workshop in combination with the Final Outreach Event in Brussels, September 2017, and a 2nd Industry Focused Workshop in Frankfurt October 2017, but also by initiating and participating in minor meetings or workshops, such as the Nanosafety Workshop in Saarbrücken/Germany in October 2017.

In addition, a continuously updated project specific website with an integrated database of new results and new knowledge produced, and with relevant news and events sections and information streams made the wider community aware of the resources being developed within the project. Relevant initiatives, organizations and individuals could register and receive regular output, regular reports (targeted to different user and stakeholder groups, including citizens, scientists, policy makers and industry).

Part of the results (new knowledge, methods and materials) generated during the project have been also used to contribute to ongoing relevant standardization efforts, mainly through DIN, BAM, JRC and TUD, e.g., under CEN/TC 352 "Nanotechnologies", ISO/TC 229 "Nanotechnologies", or ISO/TC 24/SC 4 "Particle characterization, but in particular to provide input to the currently ongoing revision of the EC recommendation on the definition of nanomaterial. A project liaison was agreed in the beginning between CEN/TC 352 and NanoDefine.

Concerning exploitation of results, many instrument, test material and software prototypes have been produced by instrument and material manufacturers, such as Malvern, RAMEM, Superon, or BASF for further future commercialisation. Various types of reference and calibration materials can be used in future projects as benchmark for material characterization and classification as well as for biological testing, e.g. for risk and safety assessments (see Table 4).

Table 4: Overview of new results (foreground) with high potential for commercial exploitation

Exploitable product	Main producer	Innovation and commercialization potential
NTA Validation Protocol	Malvern	Describes a set protocol for in-house and inter-laboratory validation of NanoDefine samples. Offers a method for generating values for instrument validation. A technique for evaluating PTA technology.
NTA Validation SOP for sample preparation and measurement	Malvern	Describes a set protocol for sample preparation (including powder dispersion) and measurement setup for NanoDefine supplied samples. To be used in conjunction with NTA Validation protocol. A technique for evaluating PTA technology
HRMS for nanoparticle characterization	RAMEM	There is no commercial High-resolution DMA for particles in the range of 1-5 nm. High Resolution, detection of the smallest nanoparticles.
Electrospray deposition for EM	RAMEM	Sampling system to avoid aggregation and agglomeration of NP for EM. No other sampling system exists to avoid aggregation and homogeneous distribution of NP
Coupling FFF and ESI for aerosol detectors	RAMEM	Increase in the number and type of detectors for FFF. First development apart from FFF-ESI-ICP-MS
Concentration Calibration of NTA instruments	Malvern	Current NTA instruments use fixed values across all instruments and capture settings in determining concentration measurements. This innovation aims to calibrate individual system at a range of capture and analysis settings thereby removing concerns of low reproducibility and user dependence. Additionally, accuracy of measurements will be improved. Increases accuracy and robustness of concentration measurement producing a unique, high throughput, number based concentration tool
spICP-MS data evaluation software	RIKILT	The Single Particle Calculation tool version 2 (SPCTv2) allows to generate spreadsheet-based analysis data of spICP-MS measurements. Data can be imported into the NanoDefiner e-Tool for an automatic nano/non-nano/borderline decision for a specific material. http://www.wur.nl/nl/show/Single-Particle-Calculation-tool.htm

Auto-EM toolbox for automated operation and image analysis	EAWAG	The ParticleSizer is an ImageJ plug-in for TEM image analysis. Measures automatically distributions of characteristic size and shape properties of potential nanomaterials. Analysis results exportable in a text-based file format and imported into the NanoDefiner e-Tool for an automatic nano/non-nano/borderline decision on a specific material. https://imagej.net/ParticleSizer
The NanoDefiner e-tool	FHDO	a Standardised automated procedure for method selection and NM classification for the most economic implementation of the definition. https://labs.inf.fh-dortmund.de/NanoDefiner/ and http://www.nanodefine.eu/index.php/nanodefiner-e-tool
The NanoDefine Method Manual	JRC	Technical guidance on the use of available methodologies
New Standard operation procedures (SOPs) for dispersion control and various particle size measurement and imaging methods	WP1-7 partners	SOPs described in WP1-7 deliverable reports developed and validated by intra- and/or inter-laboratory comparison studies

Impact on employment and education

NanoDefine has provided new job, training and education opportunities for both senior, junior and young female and male researchers in support of their professional career within the safe development and application of nanomaterials (see Table 5).

Table 5: Number of females and male researchers/technicians as well as postgraduates, PhD students and postdocs involved in NanoDefine

Beneficiary name	Female	Male	Postgraduate	PhD students	Postdocs
RIKILT	5	6	0	0	0
NOMI	2	2	0	0	0
JRC	8	12	0	0	0
UNIVIE	2	4	0	1	4
DTU	4	2	0	0	1
BfR	2	2	0	0	0
EAWAG	0	3	0	0	2
CEA	1	1	0	0	0
TUD	0	3	1	1	2
CODA-CERVA	1	1	0	0	1
UoB	3	0	0	1	1
FHDO	1	5	0	2	0
BAM	6	3	1	1	1
DIN	3	2	0	0	0
BASF	0	4	0	0	0
CLARIANT	0	3	2	1	0
SOLVAY	0	2	0	0	0

MBN	0	6	3	2	0
L'OREAL	2	2	0	0	0
MALVERN	2	4	2	4	0
RAMEM	3	8	0	0	0
SUPERON	0	5	2	0	0
THERMO FISHER	1	4	1	0	0
EUROFINS	0	2	0	0	0
NIA	2	5	0	0	0
VdMi	2	0	0	0	0
Cosmetics Europe	0	1	0	0	0
LNE	1	2	0	0	0
TOTAL	50	94	12	13	12

Address of project public website and relevant contact details.

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