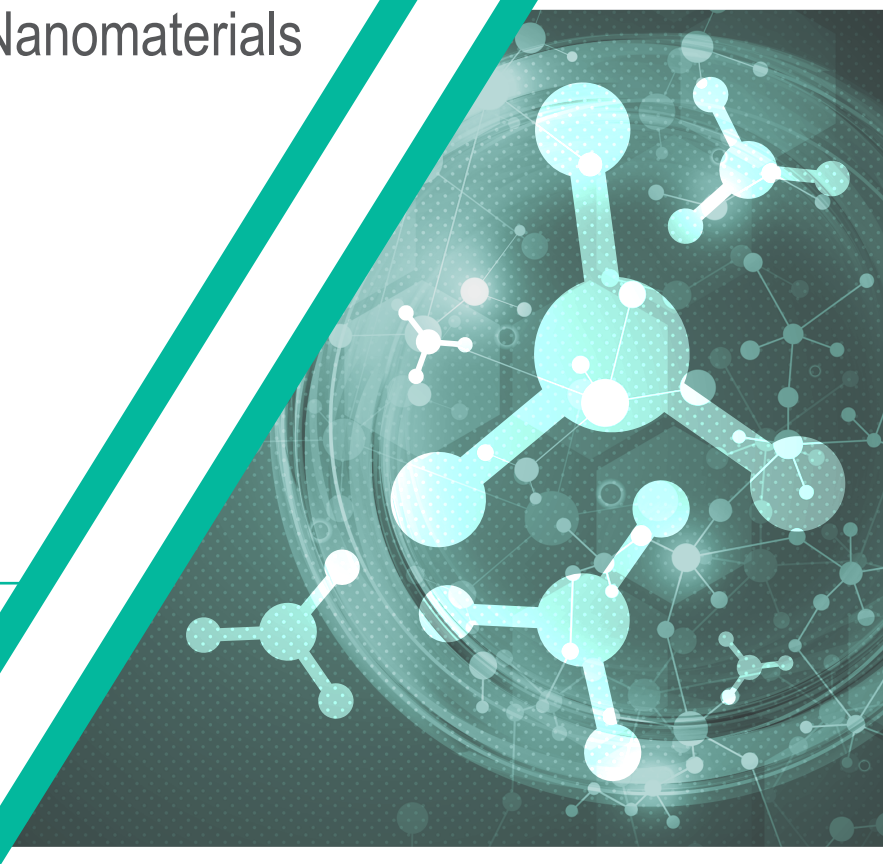
Section 1
Physical-Chemical properties

Test Guideline No. 125

Nanomaterial Particle Size and Size Distribution of Nanomaterials



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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Testing of Chemicals Particle Size and Particle Size Distribution of Nanomaterials

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1. Introduction

1. The OECD Working Party on Manufactured Nanomaterials (WPMN) has actively worked towards understanding possible safety issues for manufactured nanomaterials and has contributed significantly to resolving these by developing Test Guidelines, Guidance Documents, Test Reports and other publications with the aim of a safe use of manufactured nanomaterials. The OECD website (www.oecd.org/science/nanosafety) and the referenced publications [1-8] contain more background information.

2. To address the specific needs of manufactured nanomaterials, the OECD Test Guideline No. 110 "Particle Size Distribution/Fibre Length and Diameter Distributions" was identified as one of the test guidelines (TGs) to require an update. The current TG 110 (adopted in 1981) is only valid for particles and fibres with sizes above 250 nm. The WPMN prioritised to either update TG 110 to be applicable also to particles at the nanoscale or draft a new nanomaterial specific (TG).

3. Eventually, it was decided to develop a new TG that covers the size range from 1 nm to 1000 nm, intended for particle size and particle size distribution measurements of nanomaterials. Paragraph 11 provides further justification on the need for such measurements for nanomaterials. This TG overlaps with TG 110 in the size range from 250 nm to 1000 nm. When measuring particulate or fibrous materials, the appropriate TG should be selected depending on the size range of particles tested. In line with TG 110, the new TG for nanomaterials includes separate parts for particles and fibres.

4. For the part of this TG which addresses particles, several methods applicable to nanomaterials were reviewed and included to take into account developments since 1981 when the TG 110 was adopted. This TG includes the following methods: Atomic Force Microscopy (AFM), Centrifugal Liquid Sedimentation (CLS)/Analytical Ultracentrifugation (AUC), Dynamic Light Scattering (DLS), Differential Mobility Analysis System (DMAS), (Nano)Particle Tracking Analysis (PTA/NTA), Small Angle X-Ray Scattering (SAXS), Scanning Electron Microscopy (SEM), and Transmission Electron Microscopy (TEM). The method Single Particle Inductively Coupled Plasma Mass Spectrometry (sp-ICP-MS) could not be sufficiently validated within the interlaboratory comparison (ILC) carried out for the different methods in this TG (see also paragraph 6 for further details on the ILC). Applicability of sp-ICP-MS is strongly limited to nanomaterials with high mass values in combination with a sufficiently high particle size. However, the general method ICP-MS is widely used and the sp-mode for the size measurement of specific nanomaterials was successfully performed in ILCs elsewhere. The method is therefore included in the Appendix Part C of this TG, which further details the limitations of sp-ICP-MS.

5. For measuring the diameter and length of fibres, analysing images captured with electron microscopy is currently the only method available. This TG includes Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

6. To test the validity of this TG, an ILC was performed. Test materials were chosen to reflect a broad range of nanomaterial classes, e.g. metals, metal oxides, polymers and carbon materials. Where possible, well-characterised test materials were used. Additionally, the test materials were chosen to reflect a broad range of sizes representing the size range 1 nm to 1000 nm. Specifically for fibres, a broad range of aspect ratios was included (length/diameter of 3 to > 50). Some of the test materials used are

commercially available and further references are given in the validation report of the ILC [9].

7. Sample preparation for physical chemical characterisation is critical for all listed methods. Due to the differences between individual nanomaterials and due to the wide range of individual material properties it is impossible to have a generic protocol to obtain the best possible sample preparation for every nanomaterial. Therefore, a generic protocol on sample preparation is not part of this TG. Information on sample preparation is given in the paragraphs 25-29, 33, 34 and 39 for particles and in paragraphs 162) for fibres. Further information on sample preparation of nanomaterials for physical chemical characterisation can be found in the OECD Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials [10] and elsewhere, e.g. [11].

8. Further background information to this TG was developed in different publicly funded research projects. An example is the EU FP7 funded project NanoDefine [11-13].

2. Definitions

9. Definitions and units used in this TG can be found in Appendix Part A. For the regulatory context, please note that national and regional regulatory definitions for these terms can differ [14, 15]. When measuring potential nanomaterials within the scope of a regulatory context, it is recommended to consult the relevant regulatory body or guidance for specific advice.

10. In line with definitions by ISO (see Appendix Part A), this TG will use the term "particle" specifically for spherical and nearly spherical materials with an aspect ratio < 3 and the term "fibre" for fibrous, high aspect ratio (≥ 3) materials. In addition, the term "individual particle" and "individual fibres" are used for unbound (neither agglomerated nor aggregated) particles and fibres. The term "integral component" is used in this TG to describe the structures (either particles or fibres) within agglomerates and aggregates, e.g. being made of former individual particles or fibres.

3. Scope, significance and use

11. Nanomaterials are specific forms of chemical substances and are thus covered by chemicals legislation in the OECD member countries. Regulatory requirements may include information on their intrinsic properties and/or identity. Information on particle size and particle size distribution is generally needed to identify a nanomaterial as such. Particle size and particle size distribution may also be part of a general physical-chemical characterisation for subsequent regulatory risk assessment of nanomaterials [16, 17]. In this TG recommendations are given (in particular those on sample preparation) to focus on the particle size measurements as intrinsic property. Adaptations of these recommendations may be necessary for other purposes in risk assessment.

12. This TG specifies methods to determine the size and size distributions of nanoscale particles and fibres. The TG is subdivided into two parts: the description of the methods for particles and the methods for fibres. A more detailed description of the applicability to particles and fibres is given in paragraphs 15-17 and 18-19, respectively.

13. The TG covers particles and their agglomerates/aggregates with a size distribution between $d{\sim}1$ nm to $d{\sim}1000$ nm, where d is the particle diameter. Throughout the document *d* will be used when the size of particles are described. It also covers individual fibres and their agglomerates with a diameter between $x_d{\sim}1$ nm and $x_d{\sim}1000$ nm, where x_d is the fibre diameter, and a length $x_l \leq 20$ µm, where x_l is the fibre length, in general – for limitations see paragraph 18 and 167. Throughout the document *x* will be used when the size of fibres is described using the subscript *d* for fibre diameter and *l* for fibre length. The size range is chosen to cover the whole size distribution of nanomaterials. The upper size limit of 1000 nm allows to have an overlap in the measurement range with TG 110.

14. The methods in this TG are applicable to the measurement of spherical or nearly spherical (average aspect ratio < 3) particles with a size below 1000 nm. The measurement of non-spherical particles (average aspect ratio ≥ 3), i.e. fibres is limited to a few methods only (see paragraph 18). Non-spherical particles might have a preferred orientation during the measurement that can affect the obtained size distribution. Approaches to correct for such effects are beyond the scope of this TG and are discussed elsewhere [18-20]. It has to be noted that size is a method-dependent quantity or measurand, i.e. different measurement methods deliver different kinds of diameters which may also depend on the material and morphology of the measured particle. Nanostructured materials that are not based on nano-objects, e.g. nanocoated surfaces, as well as two-dimensional materials are not subject of this TG.

15. The TG is validated for the measurement of particle size and the particle size distribution of individual particles, agglomerates and aggregates in an, for the given purpose, optimally dispersed system obtained by careful sample preparation (see paragraph 25 and [9]).

16. Mixtures of particles consisting of different substances might be measured with selected specific methods. The separation of the size distributions of the different materials is difficult, but can sometimes be performed by a combination of different techniques for example by combination of EM with EDX (Energy Dispersive X-ray spectroscopy) [21, 22].

17. This TG explains how to utilise the following methods to determine the size distribution of particles in the size range from 1 nm to 1000 nm.

Atomic Force Microscopy (AFM)

Centrifugal Liquid Sedimentation (CLS)/Analytical Ultracentrifugation (AUC)

Differential Mobility Analysis System (DMAS)

Dynamic Light Scattering (DLS)

Electron Microscopy (EM)

Particle Tracking Analysis (PTA)

Small Angle X-ray Scattering (SAXS)

Additionally, the method single particle Inductively Coupled Plasma - Mass Spectrometry (sp-ICP-MS) – although not sufficiently validated within the ILC - is described in Appendix Part C, see also paragraph 4. To give generic information on the applicability of the methods, a short overview of the measurands and the main limitations of each method is given in ***Table 1***.

18. This TG explains how to utilise the techniques of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) for pairwise determination of the diameter and length of fibres (i.e. for each individual fibre, both length and diameter must be measured) and how to compile the number-based length and diameter distributions for fibrous materials. TEM and SEM are seen as the only two methods useable to obtain this information. AFM could be used to gain information on the fibre diameter for individual fibres but is not further considered in this TG as pairwise measurement of diameter and length is not possible with this method. A limitation applies to the use of TEM, because this method appeared less reliable for fibres with a median length of > 5 µm and a broad length distribution, see paragraph 21. The margins of error determined in the ILC were found to be not acceptable. Further details can be found in the Validation Report [9].

19. This TG specifies the pairwise determination of the diameter and length of individual fibres as a new standard protocol. Only pairwise measurement enables compiling diameter versus length distribution data that is required to quantify potentially harmful fractions in fibre ensembles. Such pairwise determination is of high relevance for reliable determination of fibre-related risks in toxicological and epidemiological studies. While the diameter controls the respirability of a fibre, its length is believed to affect its ability to be removed from lungs by phagocytosis by macrophages [2, 23].

4. Initial considerations and limitations

20. The particle size result is reported as an equivalent spherical diameter which depends on the measurement principle (e.g. aerodynamic, electromobility, light scattering) or an equivalent circular diameter for microscopy methods. The measured equivalent diameter is also influenced by the sample preparation, data analysis approach, distribution weighting and the type of averaging [18]. Additionally, methods might provide other diameters, such as minimum and maximum Feret diameter, see for example in [13, 19, 24].

21. The population of particles or fibres is characterised by the size distribution and by the geometric standard deviation σ_g of the size distribution. Depending on the measurement method, the obtained particle size distribution is determined based on particle numbers, equivalent surface area, equivalent volume or on the intensity or extinction of an average (arbitrary) measurement signal obtained by measuring many particles simultaneously. The size distribution of fibres is given as a function of the number of fibres analysed, and, for instance, the determined mean, median and modal diameter and length. For particle and fibre size distributions with different local maxima, the characteristic values of each local maximum should be determined. The geometric standard deviation of the measured particles and fibres is derived assuming a lognormal size distribution. It is a useful variable e.g. to characterise the width of the size distribution of a material according to the following definition [19].

a) A material with narrow size distribution is defined as material with $\sigma_g \leq 1.5$

b) A material with wide size distribution is defined as material with $\sigma_g > 1.5$

These values are calculated from ISO 13322-1 [20] with regard to error-limits and number of counted particles given in ISO 19749 and ISO 21363 [19, 24].

22. The different types of quantity for the size distribution – particle number, area, volume, mass or signal intensity based – are determined depending on the physical principle of the employed method. These types are specified by the index r [25, 26]: number $r = 0$, length $r = 1$, area $r = 2$, volume/mass $r = 3$, intensity $r = i$.

e.g.: The full nomenclature for the hydrodynamic diameter of a number-based size distribution measured with PTA is $d_{0,hyd}$. *An overview of the type of quantity and the respective index for the measured diameter for each method is given in* ***Table 1****.*

23. Comparing the outcomes of particle size measurements based on different measurement principles (see paragraph 22), the derived particle size has the following tendency $d_0 < d_2 < d_3$ [20, 26-29]. The mathematical transformation of one measure of size distribution to another is possible in very specific conditions (e.g. for ideal spherical and monodisperse particles). In most other cases the mathematical transformation is based on assumptions, leading to unknown uncertainties. Therefore, in general, it is not recommended to apply such transformations. See [25, 30, 31] for further information on the mathematical transformation of size distributions.

24. This TG addresses size measurements in the range from 1 nm to 1000 nm. However, each method and each corresponding instrument used have their own measurement range. Furthermore, the nanomaterial to be analysed may have an influence on the applicable measurement range e.g. due to contrast in EM. Hence, a combination of methods might be necessary to span the whole size range. Moreover, most real-life nanomaterials can only be characterised by a small number of methods, and only few nanomaterials may be well characterised by all methods. It is therefore strongly recommended to pay attention to the limitations and the applicability of each method to decide which method is optimal for the analysis of a potential nanomaterial. Calibration of the methods should be done with reference materials which are certified for that method and measurand. Due to the development of new or improved algorithms for the evaluation of the raw measurement results it is recommended to use up to date software versions.

5. Aspects of sample preparation

25. The TG can be applied for different types of samples, prepared via different routes:

a) from dry powder

b) from a suspension

c) from an aerosol

Please note that different, possibly all three, preparation routes should be tested to assess the reproducibility and robustness of the sample preparation. The choice of routes to be

tested may be influenced by the purpose of the size measurements and the material of interest.

26. Dispersions of particles in gases or liquids must be physically and chemically stable (over time), as described in paragraph 28 and 29, to obtain reproducible and representative measurements. As nanomaterials may be prone to agglomeration, dissolution or interaction with media compounds, special care has to be taken in the sample preparation [32]. It is recommended to gain prior knowledge on dissolution and interactions with media components, including their role in dispersion stability.

27. The test materials under consideration need also to be stable during measurement and under all the operating conditions of the measurement technique used. This may include

a) stability in vacuum (e.g. SEM, TEM)

b) stability under electron-beam radiation (e.g. SEM, TEM)

c) stability against deformation (e.g. AFM)

It is recommended to gain prior knowledge on these parameters.

28. The sample preparation is critical for the outcome of particle and fibre size measurement and distribution statistics. Detailed reporting of all preparation steps is crucial, see paragraph 180. Sample preparation has to be carried out with utmost care to achieve fit for purpose results that show a high reproducibility and avoid significant bias. This includes at least paying attention to:

a) An appropriate statistical sampling of the actual size distribution. The sample has to maintain homogeneity with regard to its size distribution. Any size fractionation has to be avoided. It is necessary to ensure representative sampling and to test the temporal stability and spatial homogeneity of a preparation by taking several subsamples at different ageing durations and sampling locations of the preparation [33, 34]. Further guidance for sampling can be found in [25, 31, 33-36].

b) Inadequate sample preparation may lead to modification of the size or size distribution, such as breakage of test materials due to excessive energy input, loss of smaller sample fraction when choosing porous filters with a too large pore size, dissolution of the test material in the test media, or insufficient disassembly of agglomerates (undergrinding) (see ISO 14887 [37]). The quantity of over- and undergrinded material can be estimated by an electron microscopy method. ISO 14887 provides general, not nano-specific guidance on how to quantify over- and undergrinded material and recommends a limit of 5% of the number concentration.

c) Concentration limits. The concentration of the final sample needs to suit the requirements of the chosen measurement method.

d) Particle sedimentation. The proper handling of rapidly sedimenting test materials and their agglomerates is important to minimise the effect of their sedimentation on the size distribution of sub-micrometre particles.

e) Large particles. Particles with d >1000 nm can either be removed before the measurement or excluded in the evaluation of the results. It should

be assessed and reported to what degree the removal of the larger particles influences particle size distribution of the remaining dispersed particles.

f) Depending on the purpose, specific (e.g. legal) requirements may apply on which particles of the sample have to be evaluated, either one or more of the individual particles, or agglomerates and/or aggregates, or their integral components, or a distinction between all of these. This may influence the sample preparation procedure.

Please note that in some cases the purpose of the measurement, and/or the properties of the test material may prevent the formation of any stable dispersion.

29. In general, for all measurement methods, an accurately established sample preparation protocol in accordance with GLP for each test material is needed. Due to the differences between individual nanomaterials and due to the wide range of material properties possible, a general protocol to obtain the optimal sample preparation for every potential nanomaterial cannot be defined here. This TG defines quality criteria for the preparation of particle (paragraphs 33-34) and fibre (paragraphs 158-160) samples instead. For each investigated potential nanomaterial, a specific sample preparation protocol has to be developed and tested to meet those quality criteria. Several protocols and standards exist which can be used as starting points [11, 33-35, 37].

6. Specific Part: Particles

6.1. Initial considerations and limitations

30. The particle size of particulate materials can be determined from a variety of measurands.

a) Some measurands for particle size rely on the assumption of spherical shape. The diameter is then given as the diameter of an equivalent sphere or of an area equivalent circle.

b) Each method measures a specific physical property and from this property, a diameter with the unit of length is derived. The diameters obtained by different methods may differ significantly due to different underlying physical principles and the way in which results are weighted. *e.g. Hydrodynamic diameters determined with DLS will normally be larger than the equivalent circular diameter obtained with TEM analysis, as the former measures the particle size including the surrounding solvent shell, while the latter measures the diameter of the projected circle of the dry particle (under the assumption that both values are weighted the same way).*

c) The particle size should be designated with the symbol d for the equivalent diameter of particles.

d) An index is used to mark the type of diameter. The methods included in this TG have the indices: hydrodynamic diameter d_{hyd}, equivalent circular diameter d_{ecd}, Stokes diameter d_{St}, electrical mobility diameter d_{emob}, mass equivalent spherical diameter d_{mass}, intensity of scattered light diameter d_{Iscat}, diameter based on particle height d_{hgt}.

31. Discrimination of individual particles from aggregates and agglomerates is challenging, especially if not using imaging methods.

e) If no prior knowledge of the particle size and dispersion state is available, an EM technique with a magnification sufficient to identify agglomerates/aggregates should be applied at least for qualitative screening of the sample. It is recommended to supplement the electron imaging by another method which is capable to measures a high number (> 4000) of particles for the particle size distribution [19, 38]. Nanostructured materials that are not based on nano-objects e.g. nanocoated surfaces, are not subject of this TG.

f) If prior knowledge of e.g. the particle size and dispersion state of the test material is available, an imaging technique might not need to be applied if the non-imaging measurements result in expected values (e.g. certified reference material (CRM), quality control of materials). Prior knowledge may include an expected value for the respective particle size and shape, or its behaviour regarding agglomeration and/or aggregation during manufacturing and sample preparation.

g) In all cases it is recommended to use two methods with independent measurement principles to ensure the consistency of the results.

32. No single measurement technique covers the entire range of available materials. This TG includes eight techniques for measuring particle size and compiling size distributions of sub-microscale materials. It is important to choose the appropriate technique(s). The measurands and ranges of applicability are described in paragraph 35.

6.2. Sample preparation

33. The long-term stability of dispersions is highly influenced by the test material properties and the dispersion medium. For a reliable measurement of the particle size and particle size distribution the prepared dispersion must remain stable until the measurements are finished. The particles have to be homogeneously distributed over the entire sample volume. The particles should not significantly sediment, agglomerate, or dissolve over this time span. The particles should be well separated with no or only a small amount of impurities that interfere with the quantity intended to be measured. If loss of dispersion stability occurs, it is only acceptable to an extent that it is still consistent with the requirements of the applied method and the purpose of the specific measurement.

34. In case the objective of the measurement is a size distribution for individual particles, specific quality of a particle dispersion is required. The quality can be checked during sample preparation, e.g. by repeated measurements with the same measurement method during the sonication procedure of the sample until a stable dispersion has been reached. For this objective, the quality of the dispersion has to fulfil one of the following criteria in accordance with the method requirements (see paragraph 35)

a) The dispersion contains only individual particles, or

b) The dispersion contains individual particles and aggregates and a low number of agglomerates (below 5% in number concentration of particles and aggregates is recommended, see paragraph 28.b).

6.3. Overview of methods and their applicability

35. Several methods are in principle appropriate to determine the size and size distribution of particulate materials. Each method has an applicable size range as well as technical and material limitations. An overview of the methods, including the size range in which they are applicable, the measurand, the corresponding type of quantity of the distribution, appropriate media and advantages/limitations associated with each method is summarized in ***Table 1***. Additional information for most of these methods can also be found in e.g. [12, 13, 22, 39, 40].

36. An overview of the applicability and abilities of the methods is given in ***Table 2***. Each of the methods is described in detail in the following paragraphs. Methods are listed in alphabetical order to facilitate navigating in this document.

Table 1: Summary of methods for particles with aspect ratios < 3

Method	Size range* (d in nm)	Type of quantity and index of diameter**	Type of diameter***	Measurement principle	Type of sample and medium
Atomic Force Microscopy (AFM)	1-1000	$d_{0,hgt}$	Height-based equivalent circular diameter	Height	Deposited particles
Centrifugal Liquid sedimentation (CLS) / Analytical Ultracentrifugation (AUC)	10-1000	$d_{3,St}$, $d_{i,St}$	Stokes equivalent spherical diameter	Detector dependent	Dispersion
Differential Mobility Analysis System (DMAS)	2-1000	$d_{0,emob}$	Electrical mobility equivalent spherical diameter	Electrical mobility	Aerosol
Dynamic Light Scattering (DLS)	1-1000	$d_{i,hyd}$	Hydrodynamic equivalent spherical diameter	Time resolved light scattering	Dispersion
Electron Microscopy (EM)	1-1000	$d_{0,ecd}$	Equivalent circular diameter	Projection (TEM) and imaging of signal electrons (SEM)	Vacuum, dry deposited particles
Particle Tracking Analysis (PTA)	10-1000	$d_{0,hyd}$	Hydrodynamic equivalent spherical diameter	Diffusion	Dispersion
Small Angle X-Ray Scattering (SAXS)	1-200	$d_{3,Iscat}$	Volume square equivalent diameter	Intensity of scattered light	Dispersion, powder
*Single particle Inductively Coupled Plasma – Mass Spectrometry (sp-ICP-MS)***	*10-1000*	$d_{0,mass}$	*Mass concentration equivalent spherical diameter*	*Mass concentration*	*Dispersion*

* Note: The given size range indicate the possible sizes to be measured within the size range of this TG, 1-1000 nm. The limitations, especially of the lower size limit are given in detail in the corresponding paragraphs of this TG.
** Note: See paragraphs 22 und d) for subscripts
*** Note: Other relevant diameters can be obtained with the methods too, e.g. minimum/maximum Feret diameter for EM ($d_{0,Fmin;}$ $d_{0,Fmax}$), but are not listed in order to not overload the table

**** Note: sp-ICP-MS is presented in Appendix Part C. Although literature indicates that the method might be valid to generate reliable, robust and reproducible data, an extended validation for consistency, comparability and instrumentation dependencies could not be successfully performed within the course of the development of this TG.

Table 2:Applicability and capabilities of the methods

Method is applicable …	AFM	CLS/ AUC	DLS	DMAS	PTA	SAXS	EM	*sp-ICP-MS**
to deliver directly a number-based particle size distribution	Y	N	N	Y	Y	N	Y	*Y*
to measure a high number of particles **	10^3	10^5	10^5	10^5	10^3	10^5	10^3	*10^3*
to determine the outer diameter	Y	Y	Y	Y	Y	O	O	*N*
to distinguish between individual particles and agglomerates/aggregates	O	O	N	O	N	O	Y	*O*
when a good resolution of the size distribution is needed	O	Y	N	Y	O	Y	O	*Y*
for mixtures of different particles (see paragraph 39.e)39.f)	N	O	N	Y	O	O	Y	*O*
for particles consisting of several substances (see paragraph 39.f)	Y	N	N	Y	O	O	Y	*O*

Method has the following capabilities:	AFM	CLS/ AUC	DLS	DMAS	PTA	SAXS	EM	*sp-ICP-MS**
Is prone to underestimate the corresponding diameters (see paragraph 37)	Y	N	N	N	N	Y	Y	*Y*

Is prone to overestimate the corresponding diameters (see paragraph 37)	N	N	Y	Y	Y	N	N	*N*
Can reliably obtain the modal diameter ***	Y	O	N	Y	O	Y	Y	*Y*
Can retrieve the equivalent circular diameter (see paragraph 38)	Y	N	N	Y	N	Y	Y	*Y*
Can retrieve the hydrodynamic diameter (see paragraph 38)	N	Y	Y	N	Y	N	N	*N*

O = case dependent
Y = Yes
N= No
*Note: sp-ICP-MS is presented in Appendix Part C. Although literature indicates that the method might be valid for reliable, robust and reproducible data, an extended validation for consistency, comparability and instrumentation dependencies could not be successfully performed within the course of the development of this TG.
** The numbers represent a rough approximation of the order of magnitude
*** The diameter of one mode within a multimodal size distribution.
Note: These tables display an idealised situation. There may be cases beyond this table.

37. Overestimation and underestimation

a) AFM might underestimate the height of a particle due to compression of the particle at the imaging force used [41]. SAXS might underestimate the diameter if the test material has a coating with no or very low scattering capability. SEM and TEM might underestimate the diameter due to particle shrinking in the vacuum and contrast problems due to material coatings. If a method is prone to underestimate the size it might be useful to evaluate whether the particles are bigger than a defined value. (This may be sufficient to determine that a test material is not a nanomaterial. For example, when the PSD has a measured mean or median diameter at 100 nm. In case the method is known to underestimate the particle size, it appears reasonable to assume the actual value will be rather higher (e.g. 120 nm) than lower than 100 nm. It may be concluded that the material is unlikely to be a nanomaterial which must be confirmed by measurements by a second complementary method.)

b) DLS and PTA might overestimate the diameter as the higher scattering intensity of bigger particles will blank out the smaller particles, DMAS might overestimate the diameter due to aerosol production from liquids. If a method is prone to overestimate the size and a particle size is measured to be higher than a defined value (e.g. 100 nm) it might be useful to evaluate whether the particles are smaller than the defined value. (This may be sufficient to determine that a test material is not a nanomaterial. For example, when the PSD has a measured mean or median diameter at 100 nm. In case the method is known to overestimate the particle size, it appears reasonable to assume the actual value will be rather lower (e.g. 80 nm) than higher than 100 nm. It may be concluded that the material is likely to be a nanomaterial which must be confirmed by measurements by a second complementary method.)

38. Direct measurement results are the preferred values to be reported.

a) The equivalent circular diameter can be obtained by imaging methods and SAXS/DMAS if the particles are nearly spherical. The particle diameters including a dry functionalisation layer (the outer diameter according to the definitions) can be obtained by DMAS directly and with imaging methods if the particles are nearly spherical.

b) Several types of image derived diameter are attainable by EM. For instance, the minimum and maximum Feret diameter can be obtained and may be of benefit if the particles are not ideally spherical (aspect ratio >1).

c) The equivalent spherical hydrodynamic diameter can be obtained by the three methods which measure in liquid: CLS, DLS and PTA.

In principle, different diameters (hydrodynamic, Stokes, etc.) can be calculated from a given measurement, but such calculations are discouraged as they increase uncertainties in the values obtained. If performed anyhow, both the measured and calculated values should be clearly reported, including the specific calculations used.

39. The following considerations have to be addressed in choosing the appropriate measurement method and data analysis approach.

a) Suitable medium and preparation method in which the particles will be measured – type of medium, whether powder, aerosol or liquid dispersion.

b) Requested information: diameter of typical particles in the medium.

i. If no prior knowledge of the individual particle size and dispersion state exists, an EM technique should be applied to get a first impression of the test material.

ii. Requested type of quantity of size distribution to be achieved, e.g. number, mass or volume based.

iii. Size range of the particles under consideration: choose a suitable method for the complete size range, also consider the dynamic size range of the technique, i.e. different settings for the instrument.

iv. If a mixture of particles consisting of different substances (e.g. 50% of particle A and 50% of particle B) is to be measured, the impact of the different properties of each particle type with respect to the applied sizing technique, mode of detection, resulting weighting, and purpose of testing should be considered carefully. For instance, the methods DLS, CLS and PTA will give particle size distributions of equivalent diameter and will not differentiate between the different particle fractions. In general, the distinction of the size distributions of different materials in one sample is difficult. In some cases it may be possible to do this by combining different techniques to enable simultaneous measurement of size and chemical identity, e.g. EM with EDX [21, 22], and advanced CLS methods.

v. If particles like core-shell particles or multi-shell particles, which consist of several substances, are to be measured, the impact of the properties of each shell or component with respect to the applied sizing technique, mode of detection, resulting artefacts and purpose of testing should be considered carefully. Misinterpretation of particle size may occur if it is ignored that the individual shells or components have different properties which defines their detectability (e.g., density, refractive index). Methods that are prone to give results that are easily misinterpreted for such particles are DLS, CLS, and sp-ICP-MS.

vi. Required prior knowledge of particle properties: e.g. complex refractive index for DLS and SAXS, density of the particles for CLS and sp-ICP-MS. More information on applicability of a method for different materials is listed for each method in the corresponding paragraph.

6.4. Atomic Force Microscopy (AFM)

6.4.1. Measurement principle AFM

40. A nanoscaled tip on a cantilever is used to scan the surface and detect the local height differences by measuring the deflection of the cantilever or the force between tip and sample when scanning the surface. An image is created from the height information and the equivalent circular diameter is determined from the height of the particles. Measurement of the particles immobilised on a surface can take place on the dry surface or in liquid. For further information on this method the relevant available standards are listed in the Appendix Part B, paragraph 177.

41. The resulting size distribution is the number-based size distribution of the diameter based on height $d_{0,hgt}$.

6.4.2. Applicability

42. This method can be applied for particles in the size range 1 nm to 1000 nm and even larger particles. The precise size range depends on the instrument used (refers to technical specifications of the equipment used).

43. This method can distinguish between individual particles and agglomerates/aggregates only under specific conditions i.e. for particles with a size distribution $\sigma_g < 1.2$, uniform morphology, and using prior knowledge of individual particle size. A careful case by case assessment needs to be performed to evaluate whether a distinction between individual particles and agglomerates/aggregates is possible or not.

44. AFM measures the size regardless of the particles chemical or structural identity (e.g. mixtures of two or more different particles).

45. As a result, determination of the size distribution of each type of particle in a sample that contains mixtures of particles will be difficult, if possible at all. Examples are samples that consist of overlapping (broad) particle size distributions, or samples that show differences in hardness (i.e. the measure of the resistance to localized plastic deformation).

6.4.3. Prerequisites

46. Prerequisites for performing the test

a) AFM apparatus equipped with an appropriate tip which should have an apex radius <10 nm.

b) The height response of the instrument should be calibrated (e.g. according the instruction manual). A cross check with a suitable (certified) calibration standard should be performed to demonstrate the correct performance of the instrument.

c) Computation unit and software for image evaluation.

d) Prepared sample with a sufficient number of particles (see paragraph 50 (prepared dispersions should be in accordance with paragraph 33 and 34)).

e) The investigated particles need to be immobilized on a sufficient flat surface for AFM to be applied.

6.4.4. Important influencing factors

47. When applying AFM to non-spherical particles, it has to be considered that only the height of the particles is determined accurately. The measured size then depends mainly on the orientation of the particle. Thus, the obtained diameter of a particle might differ from the geometric external dimensions of the particle.

48. The obtained measurement data in the lateral dimension does not directly represent the sample surface, it is a convolution of the sample and tip geometry. Thus, the measured data depend strongly on the tip quality. In the lateral direction the geometry of the tip has high impact on the measurement accuracy, and it is not recommended to take the data of the lateral direction into account for the particle size determination.

49. While scanning a sample, the roughness of particles and the subjacent substrate are measured collectively. Thus, the roughness of the substrate surface directly influences the measured size of the particles. Samples should be prepared on a flat surface (with variation in surface height being much smaller than the particle size, below 5% is recommended [42]) e.g. on a polished silicon wafer or mica.

50. The number of particles to be counted for the evaluation of a statistically representative sample depends on the width of the size distribution. For particles with narrow size distribution ($\sigma_g \leq 1.5$) a number of 300 particles are sufficient, for particles with a wide size distribution ($\sigma_g > 1.5$) at least 700 particles have to be measured. Generally, the minimum number of particles to be analysed mainly depends on the level of accuracy within a defined confidence interval (see also paragraph 119).

51. For the measurement, the intermittent contact mode (also called tapping mode or amplitude controlled mode) is the preferred mode to be used, in order to minimize damage to the sample. In this mode, the energy transfer to the particles is small and thus it may enable to measure also particles, which are not strongly adherent to the surface. If another mode than intermittent contact mode is used, reasons for this should be part of the reporting.

52. Vibrations, acoustic and electronic noise can influence the image acquisition; appropriate isolation should be used to reduce the total noise.

53. The scan size should be chosen in accordance to the particle size of the material to be analysed. In ***Table 3*** particle size and recommended scan parameters are listed [42]. The scan rate/scan size might be adjusted to the respective material, e.g. to control the noise level. The scan parameters shown below can be used as starting points.

Table 3: Recommended scan parameters for the corresponding particle size

Particle size (nm)	Scan size (µm)	Scan rate (Hz)	Pixel size (nm)
10	0.5x0.5	1	1
30	1x1	1	2
60	2x2	1	4

54. A larger pixel size might be selected, but in that case, it is mandatory to perform a close-up measurement to ensure that no height influence is observed when measuring.

6.4.5. Implementation of the measurement and data evaluation

55. Overview of steps to be performed for the measurement:

a) Setting of appropriate parameters of the cantilever to the sample for measurement (refer to the instrument's technical specification).

b) Image acquisition with a large scan size to gain an overview of the sample quality, to determine the scan size needed and to identify an area with a homogeneous distribution of the particles.Scanning of the sample in intermittent contact mode, an adequate quantity of images (see paragraph 50) in the appropriate scan size needs to be taken (see paragraph 54).

c) Data evaluation using the available instrument software or equivalent software, showing the size distribution obtained from the particle height maximum in a cumulative view. The maximum particle height can be derived by manual or automated analysis. When using automated analysis, it should be carefully checked that no agglomerates or artefacts (e.g. double features due to top contamination, oscillations due to vibrations) are measured.

56. Most obtained images show a tilt and/or bend, which results from the stage configuration or scanner. Background subtraction should be implemented to correct the level. Levelling can be done in different ways, most common are plane and line-by-line levelling. Plane levelling corrects the whole plane in one step to zero under the assumption that the surface is flat without any bending. Line-by-line levelling corrects the background of each single measured line of the image. Caution must be taken if line flattening is performed as it may alter the image. As the samples should be prepared on a flat surface for the size measurement, normally the use of plane levelling is sufficient.

6.5. Centrifugal Liquid Sedimentation (CLS) / Analytical Ultracentrifugation (AUC)

6.5.1. Measurement principle CLS/AUC

57. The centrifugal liquid sedimentation technique determines the particle size by use of the particle sedimentation rate in a centrifugal field based on the particles' effective density. Separation/fractionation of particles is caused by the size-dependent sedimentation velocities. The number of particles in the different fractions is quantified by integral or differential measurement of light extinction of the sample, by measuring the difference between the refractive index of the sample and a reference or by measuring the X-ray absorption. Different types of techniques for centrifugal liquid sedimentation are known, e.g. cuvette-CLS, disc-CLS, analytical ultracentrifugation (AUC). For further information on this method the relevant available standards are listed in the Appendix Part B, paragraph 178.

58. The resulting particle size distribution is based on Stokes diameter obtained from the difference of sedimentation velocity. The resulting measurand is either the light-extinction-based Stokes diameter, $d_{i,St}$, or the mass-based Stokes diameter, $d_{3,St}$, depending on the type of detector employed by the centrifugation instruments.

6.5.2. Applicability

59. This method can be applied for particles in the size range 10 nm to 1000 nm and even for larger particles. The precise size range, especially the lower size limit, depends on the properties of the material to be analysed (e.g. density, optical properties) and the instrument and settings used (refer to technical specifications of the equipment used).

60. This method can distinguish between individual particles and agglomerates/aggregates only under specific conditions i.e. for particles with a size distribution $\sigma_g < 1.2$, uniform morphology, and using prior knowledge of individual particle size. A careful assessment for each case needs to be performed to evaluate whether a distinction between individual particles and agglomerates/aggregates is possible or not.

61. The measured size is based on the sedimentation velocity. The sedimentation velocity depends on e.g. the chemistry, the effective density, and the structure of particles. Hence measurement results of particle mixtures (with different chemistry, morphology and/or structure) or unknown samples have to be handled with care.

6.5.3. Prerequisites

62. Prerequisites for performing the measurement:

a) Prepared sample dispersions (in accordance with paragraph 33 and 34).

b) Solutions with different densities for the density gradient (in accordance with paragraph 63) and a particle size calibrant (for line start CLS; particle size and effective particle density must be known accurately).

c) Information on

i. the refractive index of the particles at the wavelength of the light source used (if needed for the detection principle),

ii. light absorption coefficient of the particles at the wavelength of the light source used (if needed for the detection principle),

iii. effective particle density. Users should avoid using the bulk or skeletal density which is commonly available in literature. The effective density can be determined using methodologies described in ISO 18747-1 and ISO 18747-2 [43, 44],

iv. viscosity,

v. temperature of the dispersion medium.

a) Dispersion media: free of contamination, water freshly deionized and filtered.

6.5.4. Important influencing factors

63. The line-start disc-CLS uses a density gradient prepared by using solutions with different densities for the measurement for line start disc-CLS.

a) The density gradient has to be prepared before the measurement (e.g. aqueous sucrose solutions with different concentrations). The characteristics of the gradient for a certain particle system depend on the density, size and width of the size distribution of the particles.

b) The injection of the sample onto the gradient is a sensitive step in this procedure. Particle systems that need to be stabilised by a dispersing agent can exhibit instant agglomeration during injection, and any occurrence of this should be checked before measurement. For samples that agglomerate during the injection process, the density gradient should contain the solved dispersing agent in the same concentration as in the sample dispersion.

64. The need for calibration depends on the type of instrument:

a) Cuvette-CLS/AUC does not require calibration of the sedimentation time scale, but a regular reference measurement with a certified particle standard as verification should be done. For cuvette-CLS, two cuvettes containing a (certified) reference material might be run and analysed together with the sample cuvettes.

b) Disc-CLS: A size standard before each sample measurement for comparison and calibration is used, the quality of the density gradient and proper functioning of the instrument operation should be checked with a reference material at least once before measuring the material to be analysed. The disc-CLS instrument may be run without calibration. In that case, one must carefully and accurately determine all individual input parameters of Stokes' equation [45].

65. The optical parts of the instrument should be clean and dust free before use. The suspension media used should be free of any contamination. The rotor and/or the disc of the centrifuge always has to be balanced.

66. Test materials need to be dispersible in a suspension medium applicable for CLS. There should be a sufficient difference between the refractive indices of the suspension medium and of the particles. Partial absorption of the laser light used for measurement by the sample material can influence the result, as the intensity of the signal is reduced. In case X-ray absorption is used for detection, the absorption contrast between suspension medium and particles should be sufficient high (follow manufacturer guidelines).

67. For the AUC it is important to make sure that the centrifuge is able to rotate fast enough for the investigated particles. For all variants, the scattering intensity is very critical. It is recommended to evaluate the setup for the specific potential nanomaterial with a reference material of comparable density.

68. The suitable choice of the revolutions per minute (rpm) for the measurement depends on the size and density of the material to be analysed, the dispersion medium used and the available instrument, and should be chosen in accordance to the manufacturer manual. The chosen rpm should be in a range that ensures that a sufficient number of measurement points is observed to accurately trace the sedimentation of the particles. For particulate test materials with a wide size distribution ramped speed increase during the measurement should be used when available. With a ramped speed option for each particle size a suitable rpm can be chosen resulting in a better separation of the different particle size classes.

69. The background scattering of the instrument has to comply with the specifications given by the manufacturer to avoid measuring artefacts.

70. The actual concentration range of the sample depends on the analysed material and the available instrument. The particles should be investigated at different sample dilutions to exclude concentration dependency. At least two concentrations, which differ by factor of two, are to be measured.

6.5.5. Implementation of the measurement and data evaluation

71. Overview of steps to be performed for the measurement with a line-start disc-centrifuge instrument:

a) Initialisation and operational test of the instrument according to the manufacturer.

b) Preparation and stabilisation (approximately 30 min) of the density gradient. This gradient should be appropriate for the material to be measured (see paragraph 63) and the choice of suitable rotational speed (see paragraph 68).

c) A reference particle system should be measured to make sure the gradient is prepared correctly.

d) Measurement of the sample:

vi. verify that no concentration dependency is present by measuring at least 2 different sample concentrations, which differ at least by a factor of 2.

vii. measure at least 3 replicates of each of the two different concentrations.

viii. If systematic concentration dependency is obtained, further measurements with lower concentration are required until concentration independence is achieved.

a) Data evaluation using the available instrument software or equivalent software, showing the size distribution in a cumulative view.

72. Overview of steps to be performed for the measurement with a cuvette-centrifuge/AUC instrument/homogeneous start disc centrifuge:

a) Initialisation and operational test of the instrument according to the manufacturer. Choice of operational speed and wavelength. Make sure that the rotor of the centrifuge is balanced.

b) Blank measurement - it is recommended to measure a blank sample of the dispersion medium (if available) before or while performing the test.

c) Measurement of the sample

ix. verify that no concentration dependency is present by measuring at least 2 different sample concentrations, which differ at least by a factor of 2.

x. measure at least 3 replicates of each of the two different concentrations.

xi. If systematic concentration dependency is obtained, further measurements with lower concentration are required until concentration independence is achieved.

xii. For transmission measurement the transmission should be between 30-70% at the start of the measurement.

a) Data evaluation using the available instrument software or equivalent software, showing the size distribution in a cumulative view.

6.6. Differential Mobility Analysis System (DMAS)

6.6.1. Measurement principle DMAS

73. The differential mobility analysis system (DMAS) is a combination of a differential electrical mobility classifier (DEMC) with a condensation particle counter (CPC) or an electrometer. To obtain a defined charge distribution the aerosol passes a particle charge conditioner before the particles are classified by the DEMC. Classification occurs by drift of the charged particles in an electrical field in accordance to their electrical mobility. At a defined voltage, only particles with the corresponding electrical mobility reach the exit slit of the DEMC and the particles to the particle counter, CPC or electrometer. Different electrical mobility size fractions are transported to the particle counter by changing the applied voltage. A whole particle size spectrum is obtained by scanning the voltage over a

predefined range. For further information on this method the relevant available standards are listed in the Appendix Part B, paragraph 179.

74. An alternative setup is used in some cases where the detection of the particles is performed at the same time in parallel by a series of electrometers. This setup was not used in the ILC but might deliver better results in time critical cases. The use of a parallel detection system should be accompanied by a system consisting of DEMC and CPC.

75. The measured particle size distribution is the number-based particle size distribution of an aerosol based on the electrical mobility of the particles $d_{0,emob}$.

6.6.2. Applicability

76. This method can be applied for particles in the size range from 2 nm to 1000 nm. The precise size range, depends on the instrument and settings used (refer to technical specifications of the equipment used) and particle properties (e.g. density). The method is capable of direct measurement of an aerosol's particle size distribution, with almost instantaneous results. It does not require sample collection on suitable substrates for subsequent analysis (by e.g. EM).

77. This method can distinguish between individual particles and agglomerates/aggregates only under specific conditions for particles with a size distribution $\sigma_g < 1.2$, unique morphology, and with the use of prior knowledge of the individual particle size. A careful case by case assessment needs to be performed to evaluate whether a distinction between individual particles and agglomerates/aggregates is possible or not.

78. The size measurement is independent from the particles' chemical composition and the structural identity of the particles, and hence the size distribution of mixtures of two or more different particles can also be determined.

6.6.3. Prerequisites

79. Prerequisites for performing the measurement

a) A differential mobility analysis system consisting of a particle charge conditioner, differential mobility classifier and a particle detector with appropriate temporal and size related measurement efficiency.

b) Aerosolised sample. If the particles are not airborne generation of aerosol is needed (in accordance with paragraph 84) and a particle dispersion in liquid to generate an aerosol (dispersed in accordance with paragraph 33 and 34).

c) The effective density of the material to be analysed.

6.6.4. Important influencing factors

80. For the measurement, the particles must be airborne or the particles must be transferred into the gas phase with an aerosol generator before they can be measured with a DMAS. For specific purposes, knowledge on the presence of agglomerates and aggregates in the aerosol may be required. This may be determined by EM, for which EM samples should ideally be prepared by an electrostatic or impaction-technique.

81. The applicable concentration range of the inlet aerosol of the CPC is in general from 10^3 particles/cm^3 to 10^6 particles/cm^3 for time scanning systems and 10^4 particles/cm^3 to 10^6 particles/cm^3 for multiple detector systems. The actual limit depends on the available instrument and used settings (refer to technical specifications of the used equipment).

82. Humidity can influence the measurement of the aerosol. In case the aerosol is produced from a suspension and shows high humidity the use of a humidity control device (e.g. diffusion dryer) is recommended to avoid measuring droplets instead of particles. If possible, the humidity should be reported.

83. It needs to be ensured that the cut-off point of the inlet system (including sample point, tubing and inlet impactor) does not interfere with the particle size distribution. If the determination of the complete particle size distribution needs different measurement setups, the results from different DMAS setups can be combined to get a complete particle size distribution.

84. If the test material is not present as an aerosol, an aerosol generator is to be used to aerosolise the particles prior to the measurement. Electrospray aerosol generator, atomizer or acoustic dry aerosol generators should be used. Other generators are not recommended, due to the generation of an unstable aerosol or a high amount of agglomerates [46]. The particle size range within the generated aerosol depends on the aerosol generator used (refer to technical specifications of the equipment used). When using the atomizer, preconditioning by a dryer is mandatory to reduce the humidity of the sample flow and avoid measuring droplets instead of particles. It is important to only use ultrapure water (two times distilled water is recommended) for the aerosol generators. Untreated water contains salt and biological residuals which strongly influence the results by forming a contamination layer to the particles and generating artificial particles from the droplets.

85. The system should be calibrated according to the instrument operation manual. The manual needs to be in accordance with the relevant ISO standards ISO 15900 and ISO 28439 [47, 48], and for the CPC the instrument operation manual in accordance with ISO 27891 [49].

86. Choice of tubing: Due to electric surface charges the particles tend to deposit on the wall. The complete tubing and all connectors within the experimental setup must be of a conducting material, e.g. stainless steel or conductive rubber, and be kept as short as possible to prevent particle losses.

87. The separation of particles according to their electrical mobility is counteracted by Brownian motion. As the Brownian motion becomes more significant with decreasing particle size, the use of a high flow rate (≥10 L/min) in the DMEC is recommended to prevent losses of smaller particles.

88. For CPC systems the DMAS needs to be operated in the single counting mode. If the concentration of particles is high enough to exceed the photometric mode, a calibration of the CPC for the specific scattering intensity of the material is needed. Further guidance on calibration can be found in the corresponding ISO standard 27891 [49].

6.6.5. Implementation of the measurement and data evaluation

89. Overview of steps to be performed for the measurement:

a) Initialisation of instrument and equilibration of temperature.

b) Check the system to be operational and free of particles (Zero check).

c) Measurement of the background and/or the aerosolised particle free dispersion medium (if aerosol is generated from a dispersion).

d) For each sample the following routine should be performed

xiii. Particles measured as particle number per volume.

xiv. At least 5 scan repetitions and an overall measurement time of at least 15 minutes.

xv. Verify the concentration dependency with at least 2 sample replicates with different concentrations.

xvi. If systematic concentration dependency is obtained, further measurement with lower concentration is required until concentration independence is achieved.

a) Data evaluation using the available instrument software or equivalent software, showing the size distribution in a cumulative view.

90. Diffusion losses of nanomaterials occur due to deposition on vessel walls due to Brownian motion that increases in importance for smaller particles (especially <100 nm). This needs to be addressed either by the instrument software itself or by manual calculation [50]. Especially high diffusion losses require specific sampling and measurement device design in the particle size range smaller than 10 nm [29].

91. With increasing particle size, the probability of multiple charges on the surface increases. This affects the outcome of the measurement and needs to be corrected by the instrument software or manually as given in the instrument operation manual.

92. Aggregate analysis offered by some instrument software should be avoided since the implemented algorithms differ between instrument manufacturers and are not standardised.

93. Obvious background contamination from the dispersion medium should be mathematically subtracted (e.g. by fitting of the background) from the particle size distribution before performing the size analysis. The background can be determined from the measurement of the solution without the dispersed particles.

94. If a particle size distribution for individual particles is required and agglomerates are visible (multiple corresponding maxima) or suspected in the particle size distribution, the measurement has to be repeated with a higher dilution factor. It is recommended to supplement this measurement by a complementary method (see paragraph 35).

95. The counting efficiency depends on the CPC in use and the instrument setting. The CPC counting efficiency tends to decrease with decreasing particle size. This might lead to an underestimation of small particles with diameters d < 30 nm. Typically, the counting efficiency is corrected automatically with a reference curve from the instrument manufacturer. If it is not corrected automatically, it needs to be manually corrected. Furthermore, there can be a material dependency of the counting efficiency; e.g. a hydrophobic nanomaterial will not be counted correctly when using a water-based CPC.

6.7. Dynamic Light Scattering (DLS)

6.7.1. Measurement principle DLS

96. The particle motions are monitored by illuminating a diluted dispersion of particles with laser light and measuring the time-dependent fluctuations in the scattered light intensity. Assuming Brownian motion of the particles, the translational diffusion coefficient can be determined, and the equivalent spherical hydrodynamic diameter is calculated by use of the Stokes-Einstein equation [51]. For further information on this method the relevant available standards are listed in the Appendix Part B, paragraph 180.

97. The resulting particle size distribution is based on the intensity weighted time resolved light scattering. The resulting measurand is the intensity-weighted equivalent spherical hydrodynamic diameter $d_{i,hyd}$.

6.7.2. Applicability

98. This method can be applied for particles in the size range from 1 nm to 1000 nm and under some conditions even larger particles. The precise size range, especially the lower size limit, depends on the instrument used, settings used (refer to technical specifications of the equipment used), sample quality (e.g. size distribution: The measurement signal of large particles will overlay those from small particles leading to an underestimation of the number of small particles) and properties of the particles to be analysed (optical contrast to the medium).

99. Test materials need to be dispersible in a medium for DLS to be applied. The suspension should be free of dust particles or any other optical contaminants, and translucent without strong visible turbidity (in accordance with paragraph 33 and 34). Absorption of the used laser light by the sample can reduce the signal strength. A sufficient difference in refractive index (in accordance with the instrument manual) between suspension medium and sample is needed.

100. This method does not distinguish between individual particles, agglomerates and/or aggregates. The measured size is based on the scattering properties which is measured based on the physical structure and the chemistry of the particles. If the sample contains a mixture of particle populations with highly different scattering properties, the size distribution of the particles with lower scattering will in most cases not be determined correctly. In some cases, it might be possible to identify a smaller fraction of particles by mathematical methods, but this is not advised for a polydisperse distribution as it has a high uncertainty. The resulting uncertainty depends on the differences in size and scattering properties of the mixture's components.

6.7.3. Prerequisites

101. Prerequisites for performing the measurement

a) A DLS instrument (homodyne, heterodyne or cross-correlation (two simultaneous homodyne)), sample holder and a computation unit.

b) Prepared sample in suspension (in accordance with paragraph 33 and 34), all steps of the sample preparation should be reported. Ensure to insert the suspension air bubble free into the measurement cuvette.

c) Refractive index of the particles (only needed for transformation of size distribution, see paragraph 108), and refractive index, temperature, and viscosity of the suspension medium at used temperature.

d) Dispersion medium free of contamination, water freshly deionized and filtered (pore size: 0.2 μm [51]).

6.7.4. Important influencing factors

102. The proper functioning of the instrument's optical system has to be verified periodically using a high purity solvent (e.g., toluene). The intensity of the scattered light has to comply with the specifications given by the instrument manufacturer. Furthermore, the performance of the instrument and settings should be controlled by measuring a nanoparticle-based reference material certified for particle size by DLS, ideally in the same size region as the material to be analysed.

103. The concentration range of the sample depends on the available instrument; the scattered light-intensity of the sample should exceed the pure dispersion medium scattering by a factor of 10. The scattered light intensity should be investigated at different

concentrations of the sample to exclude a systematic concentration dependency (at least two concentrations, which differ by factor of two).

104. Too high sample concentration leads to multiple scattering that causes faster intensity fluctuation that results in a faster decay of the correlation function, smaller apparent diameters, and an increase of polydispersity. Furthermore particle-particle interactions become more substantial and collective diffusion instead of self-diffusion dominates. Those effects may also arise when the path length through the sample is too long. To avoid these effects, an unnecessary high concentration should be circumvented, the path length through the sample material should be kept short (e.g. use small sample cuvettes) and backscattering should be used if available.

105. The DLS method determines the equivalent spherical hydrodynamic diameter d_{hydr}. This diameter includes the electrical double layer around the particle surface in solution and any molecular species attached to the particle (e.g. dispersing agents, proteins). The thickness of this double layer can change as a function of the dispersing agent or dispersing conditions (e.g. pH, ionic strength), it is therefore important to always report all steps of the sample preparation to allow comparisons of different measurements.

106. Light scattering from large particles will dominate the measured spectrum, potentially resulting in missing or underestimating the presence of smaller particles as the light scattering intensity scales with the sixth power of the radius of the particles while not in the Mie-scattering region. The measurement signal of large particles will overlay those from small particles leading to an underestimation of the number of small particles. The complexity of samples with a wide or multimodal size distribution might be reduced by separating larger particles prior to the determination of small particles - e.g. via separation using field flow fractionation (FFF) prior to the measurement and successive measurement of fractions. However, these procedures are not part of this TG.

107. The hydrodynamic diameter of the investigated potential nanomaterial depends on the thickness of the double layer which can change with the dispersing conditions (e.g. dispersion medium, pH-value, dispersing agents, concentration, and ionic strength). Hydrodynamic diameters of different materials are therefore only comparable when using the same dispersing conditions.

108. Mathematical transformation of the resulting measurand to number- or volume-based distributions is not recommended because uncertainties in the analysis are greatly multiplied by this conversion process. If the obtained result of the particle size distribution is transformed, all steps of the transformation process have to be reported to enable quality assessment.

6.7.5. Implementation of the measurement and data evaluation

109. Overview of steps to be performed for the measurement:

a) Warm up of instrument to let the laser stabilise in a time range according to the manufacturer instructions (usually 15-30 minutes).

b) Measurement of particle free dispersion medium (thermal stabilisation at room temperature in the range $\Delta T = \pm 0.1$ °C) to verify that the background (if present) is particle free (e.g. low count rate).

c) Measurement of at least two different concentrations with at least three replicates of the dispersed sample (thermal stabilization at room temperature in the range $\Delta T = \pm 0.1$ °C). Record the average particle diameter with correlation or frequency analysis.

d) If systematic concentration dependency is obtained, further measurement with lower concentration is required until concentration independence is achieved.

e) In a first step after the measurement verify the sample visually for sedimentation of particles, if particles are sedimented the dispersion was not stable, and the measurement has to be redone with a stable dispersion. Furthermore, make sure that the quality criteria of the instrument are met in accordance with the manual. The manual should be in accordance with the relevant ISO standards (i.e. ISO 22412).

f) Data evaluation with the available software. A typical instrument offers a variety of algorithms for the evaluation of the results (further information see ISO 22412 [51] and ISO/TR 22814 [52]). The reporting of the applied algorithm and internal settings is crucial (see paragraph 176). The size distribution should be shown in a cumulative view.

g) For correlation analysis the cumulants method is only valid for samples with a polydispersity index (PDI) below 0.15 to determine average size values. For samples with a wider size distribution the distribution calculation algorithms (e.g. CONTIN, NNLS, Histogram [51]) should be used.

6.8. Electron Microscopy (EM)

6.8.1. Measurement principles

110. Scanning electron microscopy (SEM) is a method mainly used for the analysis of the morphology of the investigated material. The sample is scanned by a focused electron beam, and secondary or backscattered electrons are detected. This delivers an image of a two-dimensional projection of the sample. Secondary electrons are sensitive to the surface morphology; backscattered electrons are more sensitive to differences in material composition.

111. The SEM can sometimes be operated optionally in the transmission mode (STEM-in-SEM also known as TSEM), which provides an increased material contrast of structures in the nanoscale range. Furthermore, some SEM can be operated as an environmental SEM, i.e. in an operation mode enabling analysis of samples that are not stable in vacuum or electrically nonconductive; the use of this SEM variant leads to less sensitivity for this technique.

112. In transmission electron microscopy (TEM), electrons that pass through a sample are detected. This yields an image of a two-dimensional projection of the particles. The achievable lateral resolution in TEM is usually superior to that in SEM. For further information on the methods SEM and TEM the relevant available standards are listed in the Appendix Part B, paragraph 181.

113. With EM based techniques, the number-based particle size distribution of the equivalent circular diameter $d_{0,ecd}$ is determined from micrographs. Furthermore, the minimum and maximum Feret diameter can be determined.

6.8.2. Applicability

114. This method can be applied for particles in the size range from 1 nm to 1000 nm. The actual accessible size range depends on the instrument settings, technical specifications of the equipment used and also on the test material properties (e.g. contrast of the particles,

surface charging). To determine if the test material in question can be measured with EM, the following has to be considered:

a) Particles have to be stable under the measurement conditions (vacuum, electron beam irradiation).

b) A sufficiently high contrast is needed for the accurate delimitation of the particles from the background.

115. This method can distinguish between individual particles and aggregates/agglomerates. In addition, mixtures of particles of different substances can be identified by accompanying the measurement with a technique to determine chemical identity (e.g. EDX). In principle the identification and sizing of individual particles as integral components within agglomerates/ aggregates is only possible in cases where the physical boundaries of the integral components are fully visible.

6.8.3. Prerequisites

116. Prerequisites for performing the measurement:

a) A SEM or TEM with the required minimal pixel size for the expected particle diameter (see paragraph 121).

b) Prepared sample: The sample can be prepared according to one of the three routes described in paragraph 25.

117. Calibration and maintenance of the instrument has to be performed periodically and in accordance to the instruction manual prior to the measurements. The used manual needs to be in accordance with the relevant ISO standards. Further information can be found in [19, 53] for SEM and in [24, 54] for TEM.

6.8.4. Important influencing factors

118. Prior to the quantitative image evaluation, the quality of the sample preparation has to be visually examined by means of EM. The following preparation quality classes may be distinguished (in accordance to paragraph 15):

a) Best preparation quality

Images can be evaluated visually; eventually automated evaluation is possible:

i. Individual particles are well separated in a manner that they do not mutually touch or overlap.

ii. The number of impurities and artefacts in the images are small and distinguishable in size, shape, texture or grey value from the signal of the particles.

b) Acceptable preparation quality

Images can be evaluated visually:

i. Particles are not fully separated and touch mutually; however, most particles are distinguishable as separated.

ii. A few agglomerates/aggregates (see paragraph b) may exist in which the visual distinction between the individual particles as integral component is impossible.

iii. The number of impurities and artefacts in the images is small and distinguishable in size, shape, texture or grey value from the signal of the particles.

c) Insufficient preparation quality

Particles cannot be measured if one of the following points is fulfilled:

i. Individual particles are highly agglomerated and a distinction between the individual particles, aggregates and their agglomerates is impossible.

ii. The test material or coating is highly sensitive to the electron beam and/or vacuum, which leads to particle degradation.

iii. Artefacts or impurities are not distinguishable from the particles to be evaluated.

119. A sufficient number of individual particles needs to be available in the specimen. The number of particles to be counted for the evaluation of a statistically representative sample depends on the width of the particle size distribution. For particles with a narrow size distribution ($\sigma_g \leq 1.5$) 300 particles are sufficient, whereas for particles with a wide size distribution ($\sigma_g > 1.5$) at least 700 particles have to be measured, captured in one or multiple images. Generally, the minimum number of particles to be analysed mainly depends on the level of accuracy within a defined confidence interval.

a) If a maximum error (δ) is desired at a certain confidence level, the number of particles (N) to be measured can be estimated according to $logN = -2log\delta + K$, where K is a constant numerically determined by the confidence level, the width of the distribution, the type of quantity of the particle distribution and the exponent of the process variable (see [20, 55, 56] for further information).

120. The contrast between the particles and the substrate has to be high enough to allow a reliable image acquisition, processing and analysis.

a) If organic or polymeric particles are to be measured and a low contrast is detected, staining of the test material beforehand to enhance the contrast is recommended.

b) In non-conducting materials electrostatic charging of particles might occur. Resulting contrast problems can be counteracted by the following steps (with decreasing priority):

i. Reduction of electron-beam intensity.

ii. Enhancement of the conductivity between sample and substrate, e.g. by optimization of preparation routine.

iii. Application of a thin conductive coating to the sample prior to the measurement. This increases the size of the particles and thus leads to a bias in particle size determination. The thickness of the coating depends on the used sputter coater, sputtered material and settings (consult the instrumentation manual). The thickness of the coating has to be reported and subtracted from the resulting particle size.

121. To accurately determine the size of particles a certain minimal resolution is necessary. An overview of the required resolution (in nm/pixel) depending on the size range of the particles to be measured is given in *Table 4* [57].

Table 4: Overview of particle size and required resolution depending on the size range of the particles [57]

Particle size range (nm)	Resolution (nm/pixel)	Uncertainty in diameter for 1 pixel in image %
14 to 21	1.5 to 2.0	10.0 to 11.0
22 to 26	2.0 to 2.5	9.0 to 10.0
27 to 37	2.5 to 3.0	8.0 to 9.0
38 to 49	3.0 to 4.0	8.0
50 to 62	4.0 to 5.0	8.0
63 to 100	5.0 to 6.0	5.0 to 6.0
101 to 199	6.0 to 12.0	6.0
200 to 1000	12.0 to 20.0	5.0 to 6.0

Great care has to be taken to determine a multi-modal size distribution in a mixture of particle populations, where the particle sizes between each population differ significantly, i.e. two size steps (e.g. between row 1 and row 3) in the first column of *Table 4*.

122. For multimodal distributions, it is required to make image acquisition at different resolutions (see *Table 4*), and to evaluate the images for the respective ensemble of particles only. The evaluated area of the images with the different resolutions should be equal.

Note 1: This is just a recommendation and was not part of the interlaboratory comparison.
Note 2: It is recommended to accompany measurements by a complementary method.

6.8.5. Implementation of the measurement and evaluation of results

123. The images should be taken according to the following procedure:

a) Set instrument parameters to obtain the optimum electron beam conditions for high resolution imaging at the required magnification. Switch off dynamic focus and tilt correction to prevent defocussing.

b) Take several large field-of-view images with different resolutions to gain an overview of the quality of the sample and to determine the appropriate pixel size for diameter evaluation (see paragraph 121).

c) If the sample preparation quality is acceptable, images are to be taken in adequate quantity and with an appropriate resolution to allow evaluating the necessary number of individual particles dependent on the width of the size distribution (see paragraphs 119, 122).

d) If the sample preparation quality is insufficient, preparation needs to be optimized and SEM or TEM analysis to be redone.

124. Specification of particle counting. (More information in [19, 24])

a) Depending on the purpose of measurement the specification of particle counting for individual particles, agglomerates and aggregates may differ. Examples for specification for particle counting by e.g. ISO can be found

in ISO 21363 [24], or ISO 19749 [19]. A publication by Bresch et al. (2022) [89] provides an overview of four different counting rules and compares them. The applied specification for particle counting has to be reported. For regulatory purposes the specification for particle counting of the corresponding legislation have to be employed.

b) Only particles that can be clearly distinguished from other particles and which are fully visible are counted. Where no clear identification of the particles or integral compounds is possible, the size is not determined, and they are excluded from the analysis. The number of excluded agglomerates and aggregates has to be reported.

c) The determination of the particle size from images is prone to uncertainties due to subjective evaluation of the individual performer. The use of different image magnification during particle size evaluation is critical here. The highest reproducibility can be achieved if the diameter of the particles is evaluated automatically from typical grey value traces (see Appendix Part D paragraph 207). The reliability of the automatic detection depends on the used algorithm and is often not as good as manual evaluation, due to dependence of the threshold on acceleration voltage, material and particles size [58]. Additionally, a complex surface morphology influences the results. Where a comparable certified reference nanoparticle to the test material is available, this should be used to cross-check the results under the same analysis conditions. If diameter evaluation is done manually by using tools for tracing and marking the outer diameter of the particle, images have to be evaluated using high magnification levels for reproducible results. A high level of image magnification is reached once individual pixels in the image are visible. For the measurement the particles are delimited, and the minimum Feret, maximum Feret and equivalent circular diameter should be determined. The results should be cross-checked by different persons.

d) The minimal number of particles to be counted is N=300 (narrow size distribution $\sigma_g \leq 1.5$) or N=700 (wide size distribution $\sigma_g > 1.5$), see paragraph 119.

e) In principle it is possible to measure particles (integral components) within aggregates and agglomerates if the projected particle borders can be completely identified. The possibility to identify the boundaries of touching particles in EM images depends on the image quality. The image quality can be influenced by the properties of the test material and other factors, e.g. the chosen settings, the subjective perception of the operator, and sample preparation quality. Generally, the performance of the electron microscopy varies between instruments. The resulting size distribution of integral components within an agglomerate/aggregate may have a higher uncertainty than for individual particles, since the physical boundaries may be not as distinguishable as for the individual particle. The counting of the integral components within an aggregate/agglomerate might be possible on the surface of the aggregate/agglomerate (SEM) or in the image plane (TEM). Further information can be found in [11, 13, 24, 59, 60].

125. The measurements of the particle diameter can be automated to semi-automatically recorded. By using suitable image processing software, the diameter can be semi-automatically recorded in a spreadsheet during the visual evaluation of the images. Semi-

automatic detection means that the particles are identified by manual visual measurement and the respective diameters are calculated by the programme and given out in a spreadsheet. However, a validated method for a full automated detection of particles does not yet exist.

6.9. Particle Tracking Analysis (PTA)

6.9.1. Measurement principle PTA

126. The particle dispersion is illuminated with a laser and the scattered light is detected by a semiconductor sensor (e.g. CCD, CMOS). Light scattered by individual particles is tracked in videos and the 2-dimensional diffusion is measured and converted to the equivalent spherical hydrodynamic diameter by use of the Stokes-Einstein equation. For further information on this method, the relevant available standards are listed in the Appendix Part B, paragraph 182.

127. This method measures the number-based particle size distribution of the equivalent spherical hydrodynamic diameter $d_{0,hyd}$.

6.9.2. Applicability

128. This method can be applied for particles with diameters in the size range from approximately 10 nm to 1000 nm and even larger particles. The actual working range in terms of particle size, and in particular its lower size limit of detection, depends primarily on the optical properties of the analysed material, the Brownian motion and on the instrument used (refer to technical specifications of the equipment used).

129. The measured size is based on the scattering properties which is measured based on the physical structure and the chemistry of the particles. If the sample contains a mixture of particles with significantly different scattering properties, the contribution to the size distribution of the particles that scatters the light most weakly might not be determined. This method does not discriminate between individual particles and aggregates/agglomerates.

6.9.3. Prerequisites

130. Prerequisites for performing the measurement

a) A particle tracking analysis system and computation unit.

b) Prepared samples (in accordance with paragraph 33 and 34), all steps of the sample preparation should be reported.

c) Known viscosity and stable temperature of the test material suspension during analysis including repetitions.

6.9.4. Important influencing factors

131. Measurement of a material with a wide size distribution is problematic, as the light scattering intensity scales with the sixth power of the radius of the particles. The measurement signal of large particles will overlay those from small particles leading to an underestimation of the number of small particles.

132. The materials under investigation need to be dispersible in a dispersion medium for PTA to be applied. This method can be applied in the concentration range from 10^6 particles per ml to 10^9 particles per ml. The highly diluted particle dispersion can possibly affect the

results due to partial dissolution of particles. Prior knowledge of solubility and/or dissolution rate is crucial.

133. The hydrodynamic diameter of the investigated potential nanomaterial depends on the thickness of the double layer which can change with the dispersing conditions (e.g. dispersion medium, pH-value, dispersing agents, concentration, ionic strength). Hydrodynamic diameters of different materials are therefore only comparable when using the same dispersing conditions.

134. Number of detected particles: For particles with narrow size distribution ($\sigma_g \leq 1.5$) a number of 300 particles is sufficient, for particles with a wide size distribution ($\sigma_g > 1.5$) at least 700 particles have to be measured (see paragraph 119).

135. In general, this instrument does not need to be calibrated by the operator as the image size calibration has been done by the instrument manufacturer. Nevertheless, a performance qualification measurement with a reference nanomaterial certified for particle size in the same size range of the sample particles should be performed periodically, to ensure that the instrument is working correctly. If the instrument has been modified or the optics has been changed, the image size calibration should be done in accordance with the specifications given by the instrument manufacturer.

136. The optical parts of the instrument should be clean and dust free before use. Dispersion medium should be free of contamination. Water has to be freshly deionized and filtered (pore size: ≤ 0.2 µm) before use to reach a very pure state.

6.9.5. Implementation of the measurement and data evaluation

137. Overview of steps to be performed for the measurement:

a) Warm up of instrument and stabilizing of laser intensity and temperature (usually 15-30 minutes) according to the instrument manufacturer.

b) Optimization of xyz coordinates via optics or sample chamber adjustment.

c) Background check of the instrument, if the particle free dispersion medium is available it should also be measured and used for background check.

d) Measurement of the sample:

iv. Injection of the sample in the measurement chamber manually or automated.

v. Adjustment of the instrument focus – particles should not be blurred or show excessive amounts of diffraction rings.

vi. Adjustment of contrast to the given system.

vii. When not using flow mode whilst measuring, the dispersion should be immovable. A small constant drift can be corrected. In case a drift is observed, the system might not be entirely free of air bubbles or might have a leakage.

viii. Sample illumination with a laser wavelength suitable for the nanomaterials and an optimum contrast.

ix. Measurement of dispersed sample (thermally stable $\Delta T = \pm 0.1°C$) - at least 5 times (5 injections) with 90-120 seconds record time each.

x. Test of a potential concentration dependency with at least two sample replicates with different concentrations.

xi. If systematic concentration dependency is obtained, further measurement with lower concentration is required until concentration independence is achieved.

e) Data evaluation using the available instrument software or equivalent software, showing the size distribution in a cumulative view.

138. The processing software excludes intersecting particle tracks and particle tracks that are too short. Some instrument manufacturers allow a threshold to be set manually; this should be done in accordance with the manufacturer´s recommendation.

6.10. Small Angle X-Ray Scattering (SAXS)

6.10.1. Measurement principle SAXS

139. Elastic scattering of X-rays by contrasting particles results in an angular intensity distribution. The strength of the intensity distribution is determined by the electron density contrast between the phases, and the angle defined by the length scales in question: large objects scatter strongly to small angles, small objects to wider angles. The intensity distribution thus contains information about the size and structure of the particles. This scattering can be used to determine the size and size distribution of the particles. The scattering angle is usually given as the scattering vector q. Linearisation-based methods [61] such as the Guinier method and the Porod method have been used to determine some morphological parameters, other methods use a full fit to the collected, corrected scattering data. This allows a more accurate description of real-life materials, also accounting for inter-particle interference and size distributions, and allows for the weighting of data points by their uncertainties. Practically, the information from the scattering pattern closely represents surface- or volume-weighted size distribution information and provides a basis to determine volume-weighted monomodal or multimodal distributions accurately. Using appropriate data correction, intensities can be measured with an uncertainty of 1% [62] between data points, and 5-10% for the absolute intensity scaling factor [61]. For further information on this method the relevant available standards are listed in the Appendix Part B, paragraph 183).

140. The measured particle size distribution closely represents a volume-weighted particle size distribution based on the angular distribution of scattered X-rays by the particles $d_{3,Iscat}$.

6.10.2. Applicability

141. This method can be applied for particles in the size range approximately from 1 nm to 200 nm. The precise size range depends on the instrument used (refer to technical specifications of the equipment used), instrument settings, and potential nanomaterial (e.g. its electron density) to be analysed.

142. This method can distinguish between individual particles and agglomerates/aggregates only under specific conditions, i.e. for particles with a size distribution $\sigma_g < 1.2$, unique morphology, and with the use of prior knowledge of the individual particle size. A careful case by case assessment needs to be performed to evaluate whether a distinction between individual particles and agglomerates/aggregates is possible or not.

143. It is possible to take particles of a mixture of two or more structurally and chemically different particles into account if the electron densities do not differ significantly, but it is not possible to separate the scattering from the two without further information about the particle properties.

6.10.3. Prerequisites

144. Prerequisites for performing the test

a) A small angle x-ray scattering instrument typically consisting of X-ray source, optics, collimation system, sample holder, beam stop, detector, computation unit.

b) Prepared samples (in accordance with paragraph 33 and 34 when measuring particles in dispersion), all steps of the sample preparation should be reported.

c) Information on density and approximate atomic composition of particles and dispersion medium.

6.10.4. Important influencing factors

145. The optical parts of the instrument should be clean and dust free before use. Dispersion medium should be free of contamination. Water has to be freshly deionized and filtered before use.

146. The actual concentration range of the sample depends on the scattering contrast (atomic composition and density of the analyte and dispersing agent), and the concentration of the sample, as well as the noise floor of the available instrument. The sensitivity of the method depends on the electron density of the material to be analysed, the higher the electron density the more sensitive the method. It is recommended to investigate the particles at different concentrations to understand whether there is a systematic concentration dependency. At least two concentrations, which differ by factor of two are to be measured to exclude concentration influence on the resulting particle size distribution.

147. A potential nanomaterial with a wide size distribution can only be fully characterised if the particle sizes fall within the applicable q range of the instrument, and if a volume-weighted size distribution is sufficient.

148. In a mixture of particles with significantly different electron densities the particles with the lower electron density will, except in specific cases, not be visible. This also applies to core-shell particles, when highly different electron density materials are used. If the electron densities do not differ drastically, the method can in some cases, measure both the outer shell and the inner core. (e.g. citrate stabilizer around silver nanoparticles in water is invisible, but a PEG stabilizer around polymer nanoparticles can be observed).

6.10.5. Implementation of the measurement and data evaluation

149. Overview of steps to be performed for the measurement:

a) Warm up of instrument: stabilizing of x-ray source and temperature according to the manufacturer.

b) Background check, measurement of particle free dispersion medium (if available).

c) Measurement of sample (thermally stable $\Delta T = \pm 0.1$ °C).

d) Data evaluation using available software, showing the size distribution in a cumulative view.

150. The raw data has to be corrected before the data evaluation starts. Each step of the correction process has to be performed and reported [62].

a) Detector correction: Due to imperfections and physical limitations the detected signal may vary from the true signal. Intensity and geometric distractions should be corrected.

b) Transmission, time and thickness correction: Correction of absorption of radiation from the sample; correction of measured intensity in relation to measurement time; normalisation of the sample thickness.

c) Absolute intensity correction, after calibration of the intensity using a calibration standard.

d) Background correction: The background should be subtracted after the measurement dependent corrections have been done.

e) Flatfield correction: Correction for inter-pixel sensitivity.

f) Solid angle and angular efficiency correction: Correction of variations in the detector efficiency.

g) Polarisation correction: Correction for differences in probability in scattering events for polarised and unpolarised beams.

h) Displaced volume correction: Correction for sample dispersion with a high-volume fraction of particles (>1%).

151. The resulting measurement curve should be fitted with spherical approximation or, if the shape is known, in accordance to the morphology of the particles.

7. Specific Part: Fibres

7.1. Initial considerations and limitations

152. The fibre diameter and length distributions are obtained from electron microscopy methods as number-based distributions of the geometric size (diameter and length): x_d and x_l. Currently the method for size and size distribution determination of fibres is limited to electron microscopy.

153. For individual fibres and fibre agglomerates the ends and the course of the fibre should be visibly identifiable. If such visible identification is not possible, the discrimination of individual fibres in their entire length in fibre aggregates will be challenging. Measuring three-dimensional fibres on two-dimensional images is a further challenge as those fibres may not be straight but wound in two or three dimensions, see paragraph 158 for more details.

7.2. Electron Microscopy (EM)

7.2.1. Measurement principle

154. The working principle of the electron microscopy methods is sketched in paragraphs 110 and 111.

155. The number-based particle size distribution of the geometric diameter x_d and length x_l of fibres are determined with EM. In principle a Feret diameter can be determined as well but is not within the scope of this document because it would mainly be governed by the fibre length. As fibres are described by the fibre length and its diameter, the Feret diameter is not further considered.

7.2.2. Applicability

156. This method covers the size range in fibre diameter of 1 nm to 1000 nm and in fibre length up to 20 µm in general. The minimum pixel size that can reliably be achieved by the electron microscope in use, its resolution, will set the lower limit of this method in a particular instrument. The upper limit for the fibre length measurement will be influenced by the aspect ratio of the fibre. Further considerations on maximum aspect ratios can be found in the Appendix Part D in paragraph 203. For TEM the upper limit for measuring the fibre length for fibres with a wide length distribution is set to 5 µm for the mean length as a result of the ILC. This and further limitations are detailed below.

157. The method can only be applied to materials that are stable under vacuum and electron beam.

7.2.3. Prerequisites

158. Sample for electron microscopy have to be prepared using one of three routes described in paragraph 25. The final specimen needs to be stable under vacuum and electron beam.

159. The observation of three-dimensional objects in electron microscope carries the inherent problem of interpreting two-dimensional projections of three-dimensional objects. Thus, care has to be taken to obtain fibres lying flat on the substrate. E.g. sampling using electrical precipitation is not recommended for the size determination of fibres, as charges will accumulate at the fibre ends causing them to stand on the substrate leading to an underestimation of the fibre length.

7.2.4. Important influencing factors

160. The following preparation quality classes are to be distinguished to yield evaluable images if the aim of the measurement is the determination of fibre diameter and length:

a) Best preparation quality:

i. Fibres are separated without self- and inter-fibre crossings.

ii. The aspect ratio of the fibres is such that the full length of the fibres can be evaluated from images acquired with a pixel size of ¼ of the diameter of the fibres.

iii. The number of impurities and other artefacts in the images is small and distinguishable in size, shape, texture or grey value from the signal of the analysed fibres.
→ Fully automated (see paragraph 168a) and/or visual fibre measurement allows pairwise determination of length and diameter for the majority of the fibres.

b) Acceptable preparation quality:

i. Not all fibres are fully separated and may exhibit several crossings, most of them at an angle large enough to determine the route of each individual fibre.

ii. Only few fibres are agglomerated, tangled or attached to each other.

iii. The aspect ratio of the fibres is such that the full length of the fibres can be evaluated from images acquired with a pixel size of ¼ of the diameter of the fibres.
→ Visual fibre measurement combined with low level automation (see paragraph 168b) allows pairwise determination of length and diameter for the majority of the fibres.

c) Fair preparation quality:

i. As in b) but the aspect ratio of the fibres is larger than allowed by the limitation set through the instrument in use (defined in Appendix Part D, paragraph 203). The length and diameter can in this case be determined at different resolutions, as described in paragraph 167d). → Visual image evaluation of the image is possible but for the majority of the fibres length and diameter can only be determined separately, not pairwise.

d) Insufficient preparation quality

i. Fibres are highly tangled or aggregated, attached to each other.

ii. Fibres are sensitive to the electron beam or vacuum. Impurities and other artefacts are not sufficiently discernible from the fibres. →
Visual image evaluation is not possible. Sample preparation has to be reconsidered.

161. Prerequisites for performing the test

a) A scanning electron microscope or transmission electron microscope with a minimal pixel size of ¼ of the expected fibre diameter or smaller.

b) The fibre sample prepared by one of the three methods detailed in paragraph 25 is examined in an electron microscope (either scanning electron microscope or transmission electron microscope).

c) In a pre-examination step, the optimal pixel size has to be determined. It must allow to microscopically resolving the fibre diameter with a required minimum number of pixels, as specified below (see paragraph 162).

d) This pixel size is used to acquire and save images from a number of randomly selected sample positions.

e) After image acquisition, the images are to be evaluated by the help of image analysis software. The diameter and length of at least 200 fibres are to be determined pairwise, i.e. for each individual fibre, both length and diameter must be measured.

f) From the ensemble of fibre diameter and lengths, the cumulated number based distributions are calculated [25].

162. In order to accurately determine the diameter of the fibres, a minimal number of 4 pixels per mean diameter is necessary. Thus, the minimum accurately quantifiable fibre diameter is 4 times the minimal pixel size of the instrument.

a) Fibres thinner than the pixel size are generally visible at least up to a pixel size of twice the diameter of the fibre, but the actual diameter of a fibre is then masked by the size of the pixel. This leads to quantization-related experimental errors that let the fibres appear thicker than they are. Nevertheless, using a pixel size of twice the mean fibre diameter allows tracing fibres that are 8 times longer than using the correct pixel size for diameter determination. For cases where such long fibres are to be analysed the procedure according to paragraph ii can be followed.

163. Fibres with a very high aspect ratio may be too long to be pictured in one image. The maximal aspect ratio to be reproduced in one image depends on the size of the acquired image. Typical limits of aspect ratios for typical image sizes are given in the Appendix Part D, paragraph 203. In the ILC fibres having a median length > 5 µm and at the same time a broad length distribution with a $\sigma_g > 1.5$ (see paragraph 21), TEM could not be validated and thus it cannot be recommended for use in their size measurement. Only SEM should be used in those cases.

164. Fibres with an aspect ratio ≥ 3 shall be included in the statistical analysis of the fibre size distribution.

7.2.5. Implementation of the measurement and data evaluation

165. Calibration and maintenance of the instrument has to be performed in accordance to the instruction manual prior to the measurements. Further information can be found in [19, 53] for SEM and in [24, 54] for TEM.

166. Specification for Fibre counting:

a) Only fibres with both ends clearly detectable are counted. Fibres that cross or bundle are only counted if it is clearly distinguishable which of the visible fibre ends belongs to a fibre.

b) The length of the fibre is evaluated by tracing the course of the central line of the imaged fibre and determining its length.

c) The determination of the diameter of an imaged fibre is prone to experimental uncertainties and to uncertainties due to subjective assessment of fibre-to-substrate contrast differences.

i. The highest reproducibility is achieved if the diameter of the fibre is evaluated automatically from typical grey value traces (see Appendix Part D, paragraph 207).

ii. If evaluation of the diameter of a fibre is done manually, using tools for tracing and marking the outer edge of the fibre, images have to be evaluated using high levels of zoom for reproducible results. A high level of zoom is reached once individual pixels in the image are visible.

iii. The diameter of at least 3 positions along the fibre should be evaluated and averaged. The chosen positions should thereby reflect the variation in diameter along the fibre length.

a) The minimal number of fibres to be counted are N=200, captured in one or multiple images. From this number an uncertainty is obtained that depends on the width of the distribution as shown in ***Fig. 2*** of the Appendix Part D.

i. If the requirements for performing the test are that the median or mean of a distribution has to be determined within a certain uncertainty, the measurement of additional fibres has to be continued until the bootstrap error is within the required uncertainty interval.

a) If the majority of fibres are resolved with more than 4 pixels per diameter, fibres that are recorded with a resolution smaller than 4 pixels per diameter can nevertheless be measured. For the calculation of the median the overestimation in size of the latter leads only to minor deviations.

b) Entangled fibre agglomerates or aggregates, in which the course of the individual fibres cannot be traced visually, are excluded from this analysis. Each evaluated image has to be inspected whether such agglomerates/aggregates are present. From those inspected images the number of images in which non-evaluable agglomerates/aggregates are present has to be reported as a qualitative information on the degree of sample aggregation/agglomeration. In cases where, despite all preparation efforts, no individual fibres are found, it can be useful and feasible to measure a diameter distribution from fibres within the aggregates.

167. The measurement should be performed according to the following procedure.

a) Setting the correct instrument parameters to obtain the optimal beam conditions for high resolution and switch off dynamic focus and tilt correction.

b) Acquisition of several large field-of-view measurements with different resolutions to gain an overview of the quality of the sample according to the classes defined in paragraph 160 and to determine the appropriate pixel size for fibre diameter and evaluation.

c) If the sample quality is fair or better:

i. The necessary number of images to evaluate at least 200 fibres for their dimensions is to be acquired with a pixel size being approx. ¼ of the expected mean fibre diameter.

ii. The images are to be evaluated with an image evaluation software following the specification for fibre counting defined in paragraph 166.

d) If a fair sample preparation quality with fibres longer than the maximal aspect ratio of the instrument but shorter than 20 µm does not allow for concomitant determination of fibre diameter and length at one resolution, proceed as follows:

i. Acquire sufficient images with a pixel size of ¼ of the predominant fibre diameter to evaluate the diameter of 200 fibres.

ii. Acquire a second set of images with a pixel size of 2 times the fibre diameter (this pixel size is 8 times the pixel size of the first measurement) and evaluate the length of 200 fibres.

iii. Add to the report the maximal measurable length (can be calculated according to paragraph 203) and note that the distributions of diameter and fibres were determined in independent measurements as this method is a deviation from the standard protocol.

a) If the sample quality is insufficient, optimise the preparation step of the sample and re-measure.

168. The measurements of length and diameter of fibres may be automated at different automatisation levels (more information in [20]).

a) The highest level of automatisation is a fully automatic segmentation of the picture, which means, that the fibres are separated automatically from the background. From the objects detected as fibres the length and the diameter can be determined automatically as well. If this level of automatisation is available a larger number of fibres can be evaluated.

b) The lowest level of automatisation is to automatically record the pairs of length and diameter in a spreadsheet during the visual evaluation of the pictures, by using a suitable image processing software.

7.2.6. Data evaluation and uncertainty evaluation

169. From the list of paired values of length and diameters, the mean and median of the length distribution and the diameters distributions have to be calculated. Furthermore, the paired values can be used to calculate an aspect ratio for each fibre. Consequently, an aspect ratio distribution can be obtained and the corresponding mean and median values calculated.

170. As most of the distributions of diameter and length closely follow a logarithmic distribution, it is useful to calculate the geometric standard deviation σ_g as a parameter to characterise the width of the size distribution for diameter and length individually. Typical values are between 1.5 and 3.

171. The cumulated distribution function should be used to evaluate the distribution of fibre sizes. More information on the graphical representation of a cumulated size distribution can optionally be found in [25].

172. In order to check the reproducibility two cumulated distributions of two samples or two evaluators can be compared by use of a non-parametric statistical hypothesis test (e.g. Smirnov-Kolmogorov-two-sample-test, see Appendix Part D, paragraph 202). The name of the test and the result has to be reported in the test report.

173. Measurement uncertainty: The following contributions to the uncertainty of the mean and median of the size distribution for diameter and length should be evaluated and reported. The indicated values for the different contributions are quality criteria and should be met or undercut for a valid measurement.

a) Uncertainty of sampling during sample preparation (reproducibility of sample preparation).

b) Uncertainty associated with the method. This uncertainty is estimated to be $2\sigma_P < 45\%$ based on results of the interlaboratory comparison.

c) Subjective uncertainty of the operator $2\sigma_{SF} < 25\%$ (determined for SEM from the evaluation of the images obtained with one instrument by two different evaluators).

d) Uncertainty on the mean or median value due to limited statistical sampling. This uncertainty can be assessed with the bootstrap method (see Appendix Part D, paragraph 200). It depends on the number of fibres counted as well as on the width of the distribution. For wide distributions with $\sigma_g = 3.0$, it is $2\sigma_{Bt} < 25\%$.

e) The combined measurement uncertainty of the measurement can be calculated by summing up all uncertainty contributions:

f)

$$\sigma_C = \sqrt{\sum_i \sigma_i^2}$$

The combined measurement uncertainty is dominated by the number of fibres counted and the subjective evaluation. More on that topic can be found in [63].

a) The expanded measurement uncertainty u is defined as $u = k\sigma_C$. For a coverage factor of $k = 2$, the expanded measurement uncertainty is approximately equal to a confidence level of 95%. More on the topic can be found in [64].

8. Recommended materials for the validation of the tests

174. The methods described here were validated in an international ILC study [9]. In case that no experiences with the measurement method or the nanomaterial under investigation exists, it is recommended to perform the test at least once with one of the nanomaterials used in the ILC study or a certified reference material.

175. References to the test materials used within the ILC are given as an overview in Appendix Part E of this TG and in further detail in the validation report of the ILC.

9. Test report

176. The test report has to include the following information, when applicable, of which point e) is specific to nano-scaled particles and f) is specific to nano-scaled fibres;

a) Name of the testing laboratory

b) Information about the tested potential nanomaterial and sample preparation:

- Chemical composition, CAS-number, batch identifier, or other applicable chemical substance inventory number (if applicable)
- Surface modification, morphology, possible contaminations
- Amount of sample
- Preparation method
  - i. if a dry powder was prepared/used: type of substrate used, description of preparation procedure
  - ii. if a dispersion was prepared/used: dispersion medium, filtering procedure, concentration, dispersing agents, sample dilution, final sample concentration, dispersing procedure
  - iii. if an aerosol was prepared/used: type of substrate used (for EM), method of aerosol preparation (e.g. type of aerosol generator, concentration, humidity conditions, use of diffusion dryer)
- Time between sample preparation and performance of the measurement
- If applicable: additional information about the sample preparation as specified in the method section – particles, see paragraphs 33 and 34; fibres, see paragraph 158).
- If applicable: sonicator brand/type, calibration of delivered power (guidance on the calibration can be found in [28], Appendix 2), energy input, indicated power, amplitude and pulse time
- Further method specific information:

CLS	Sample volume and concentration injected (disk)
DMAS	Aerosol generator type and used settings; specifics on the dispersion medium used (e.g. water purity)

*sp-ICP-MS**	*Actual particle concentration; standard/reference particle system*

c) Information about measurement instrument and basic instrument settings

- The brand name and type of the used instrument
- Used software and version number, specific software routines (if used)
- Specific information about the instrument as specified in the method section
- Further technique specific information:

AFM	• Used sample substrate • Tip • Pixel size • Type of detection mode (with reasoning when not using tapping mode)
CLS	• Mode of operation (line start, homogeneous) • Measurement radius, inner disc radius, inner disc thickness (if available) • Mie correction (if performed and in which way) • Light source (photocentrifuge) • Detection type
DLS	• Used sample holder • Laser wavelength
DMAS	• DMA-Type / Specifications • Detector brand name and type • Inlet system: Type and specifications of impactor/cyclone • Type of dryer • Measurement range / number of channels per decade • Scan time / number of scans • Air flow rates (DMA aerosol flow, DMA sheath flow, CPC/EM aerosol flow) • Medium of CPC • Temperature setting of CPC condensation chamber • Pipe length and diameter between aerosol source and DMAS inlet
PTA	• Camera type • Laser wavelength

	• Frame rate
SAXS	• X-ray source • Wavelength, brilliance (if applicable) • Distance between sample and detector • Detector (type, area, possible angles)
*sp-ICP-MS**	• *Transport efficiency*
SEM	• Used sample holder • Electron beam energy • Working distance • Pixel size • Type of detector (SE, BSE, STEM, etc.) • Microscope resolution
TEM	• Mesh size of the grid • Electron beam energy • Diffraction aperture • Beam current settings • Objective lens current • Pixel size • Type of detector • Imaging mode • Condenser lens aperture size • Objective lens aperture size • Exposure/dwell time • Microscope resolution • For energy filtered images: i. Energy limiting aperture ii. Energy window iii. Energy shift

d) Experimental conditions (method specific)

CLS	• Centrifuge speed • Used density gradient and buffer layer (disc) • Viscosity of dispersion medium • Refractive index of sample and dispersion medium

	• Temperature
DLS	• Temperature • Viscosity of dispersion medium • Refractive index of sample and dispersion medium
PTA	• Temperature of sample • Viscosity
SAXS	• Temperature of sample • Particle density • Refractive index of sample and dispersion medium
SEM	• Thickness of coating (if relevant)

e) Specific points only for particles: Report of the measurement

- Analysed size range, type of quantity as measured by the according method, mean & median diameter, and if applicable the modal diameters and the selected size range for the determination of the different diameters (further guidance in ISO 9276-2 [65]).
- If the purpose of the measurement requires transformation of the results into a type of quantity other than measured, the reporting has to include: original results, transformed quantity including formula and underlying assumptions.
- For non-EM methods: In case that the determination of the individual particle size is the aim, evidence should be provided that the sample preparation procedure has resulted in the disintegration of the majority of agglomerates (e.g. report expected particle size with reasoning).
- Typical results obtained with the method: e.g. images, plots.
- The cumulated particle size distribution and differential size distribution (including a description on how larger particles were handled).
- The geometrical standard deviation as a measure of the width of the log-normal distribution.
- Number of individual measurements that were performed.
- Further method specific information:

AFM	• At least 3 typical images
DMAS	• Applied corrections (multiple charge, diffusion, agglomerate analysis)
DLS	• Depending on the used instrument (i.e. cumulants version): • Z-average and Polydispersity index • Attenuator setting • Measurement position

PTA	• Number of processed frames • Number of valid tracks • Actual particle concentration
SAXS	• Applied model • Used form factor • Range of q
SEM/TEM	• At least 3 typical images • Number of particles counted • Particle size distribution as minimum and maximum Feret diameter and the equivalent circular diameter • Number of agglomerates/aggregates excluded from analysis • Way of counting for the particle size distribution • For comparability: Particle size distribution of individual particles only ($d_{0,ecd}$; $d_{0,Fmin}$; $d_{0,Fmax}$) • Informative: Particle size distribution of the excluded aggregates/agglomerates ($d_{0,ecd}$; $d_{0,Fmin}$; $d_{0,Fmax}$) • Information if manual or automated analysis was performed

f) Specific points only for fibres: Report of the measurement

- At least three typical images.
- Information about the degree of automation of the image evaluation.
- List of paired values of length, diameter and aspect ratio for at least 200 fibres.
- Number of fibres counted.
- Plot of diameter vs. lengths of the evaluated dataset.
- The cumulated number distribution of length and diameters of the fibres, respectively.
- Mean and median length, diameter and aspect ratio (further guidance on how to calculate the mean and median from a size distribution is given in [65]).
- σ_g as measure of the width of the log normal distribution, as well as the expanded measurement uncertainty u for the distributions of length, diameter and aspect ratio, respectively.

g) Report on the deviations of the measurement for particles and fibres

- Accuracy calculated from bootstrap method (c.f. paragraph 200 for median and mean (AFM, SEM, TEM)).
- Deviation of the median and mean as reported by the instrument software for all other methods.
- Repeatability deviation of the obtained diameters.
- Uncertainty in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) (if available) [64].

h) If applicable: results obtained with reference materials when used for calibration or comparison

i) Deviations of the performed test from the description in this TG

j) Results of current calibration and its uncertainty

k) All information and remarks relevant for the interpretation of the results

*Note: sp-ICP-MS is presented in Appendix Part C. Although literature indicates that the method might be valid for reliable, robust and reproducible data, an extended validation for consistency, comparability and instrumentation dependencies could not be successfully performed within the course of the development of this TG.

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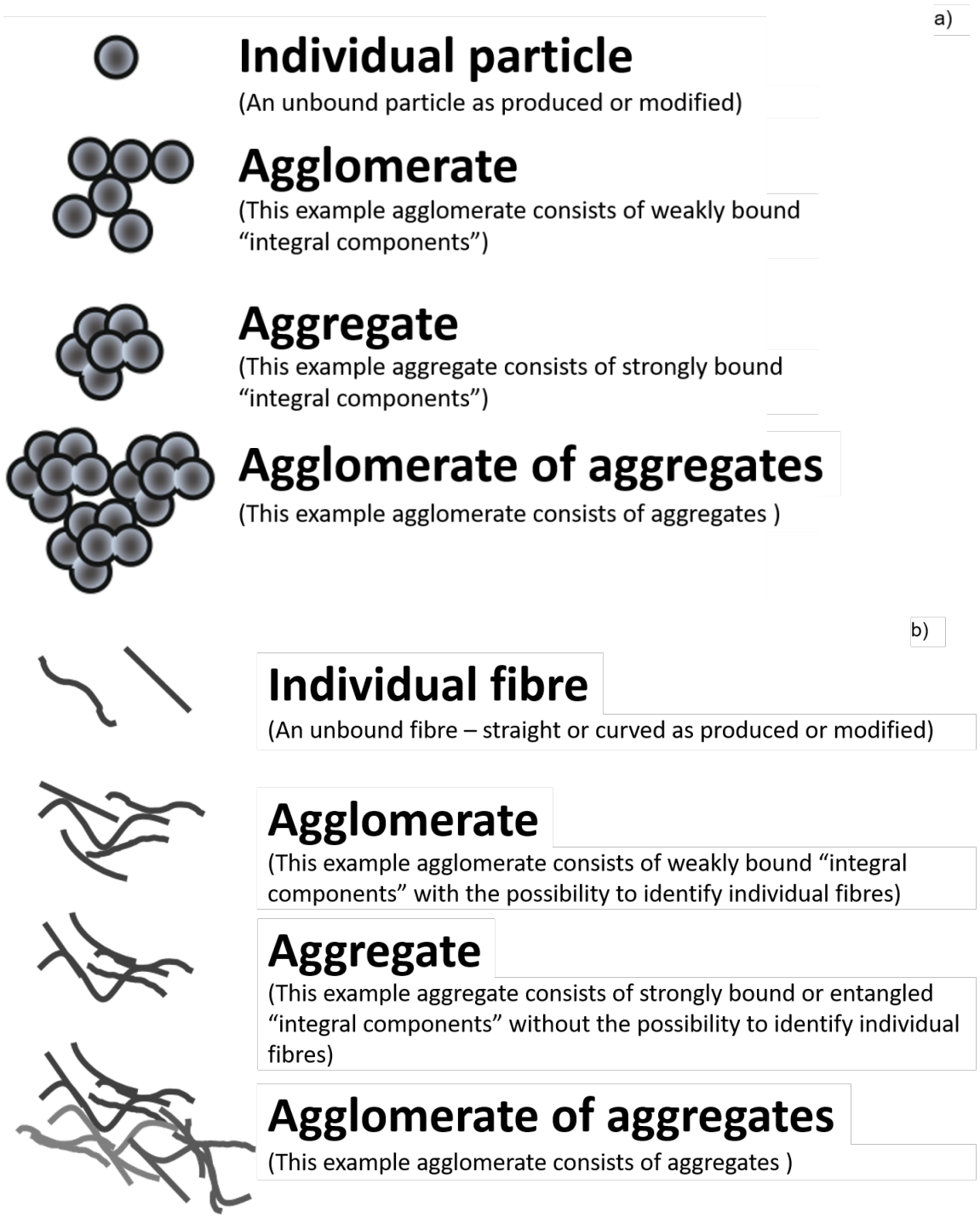
Appendix

Part A: Definitions

Figure 1 presents the general understanding of the terms used in this TG, referring to particles and fibres.

Fig. 1: Visualisation of used particle and fibre terminology in this TG: a) for the particles and b) for fibres. (Not shown: agglomerates/aggregates can contain a mixture of particle and fibres as well)



The TG uses the term "**individual particle**" for unbound particles. Individual particles can become integral parts of agglomerates and aggregates (see Fig. 1), then termed integral component. Besides that, agglomerates can also comprise smaller aggregates. When discussing unbound fibres, the term "**individual fibres**" is used within the TG. Fibres can also appear as fibres that are attached to each other. For that case, one can differentiate between agglomerates and aggregates by analysing whether individual fibres are identifiable integral components (agglomerate) or not (aggregate).

aerodynamic particle diameter [ISO 16000-18 Technical Corrigendum 1:2011-12] diameter of a sphere of density of 1 g/cm^3 with the same terminal velocity, due to gravitational force in calm air, as the particle under the prevailing conditions of temperature, pressure and relative humidity

aerosol [ISO 80004-6]
System of solid or liquid *particles* suspended in gas

agglomerate
collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components

aspect ratio
ratio of length of a particle (the longest dimension) to its width (the shortest dimension)

dispersion
heterogeneous system in which a finely divided material is distributed in another material

equivalent circular diameter d_{ecd} [ISO 21363:2020; 3.4.7]
diameter of a circle having the same area as the projected image of the particle
EXAMPLE: The ecd is:

$$ecd = \sqrt{\frac{4 \cdot A}{\pi}}$$

where A is the area of the particle.

equivalent diameter [ISO 80004-6:2013; 3.1.5**]**
diameter of a sphere that produces a response by a given particle-sizing method, that is equivalent to the response produced by the particle being measured

Feret diameter [ISO 10788:2014, 2.1.4]
distance between two parallel lines which are tangent to the perimeter of a particle

Feret diameter, minimum/maximum [ISO/TR 945-2:2011, 2.1]
minimum/maximum length of an object whatever its orientation

fibre
particulate object with two approximately equal lateral dimensions $x_d \coloneqq x_1 \cong x_2$ (i.e. the diameters of the fibre) and a third longitudinal extension x_l (i.e. the length of the fibre) which exceeds x_d by at least a factor of 3. In other words, a fibre is an object with an aspect ratio of

$$\frac{x_l}{x_d} \geq 3$$

integral component
an individual particle or fibre which is part of an agglomerate or an aggregate

measurand
quantity intended to be measured

nano-object
discrete piece of material with one, two or three external dimensions in the nanoscale

nanoparticle
nano-object with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

nanoscale
length range defined e.g. by legislation or standardisation bodies for their specific uses

particle
minute piece of matter with defined physical boundaries

particle size
size of the particles, aggregates or agglomerates is given in micrometres (μm) or nanometres (nm). The measured particle size depends on the measurement method used.

particle size distribution
distribution of the quantity of particles (for example, number, mass or volume based) as a function of particle size

pixel [ISO 12640-2:2004; 3.6**]**
smallest element of an image that can be uniquely processed, and is defined by its spatial coordinates and encoded with colour values

specimen
sample immobilised on a substrate for further analysis

suspension [ISO 4618:2014-01]
heterogeneous mixture of materials comprising a liquid and a finely dispersed solid material

Part B: Documentary standards that are available for the methods used in this TG

Atomic Force Microscopy

177. ASTM E2859 -11[42]; ISO 11039:2012[66]; ISO 27911:2011[67]; ASTM E2382 - 04 [68]

Centrifugal Liquid Sedimentation (CLS)/Analytical ultracentrifugation (AUC)

178. ISO 13318-1:2001[69]; ISO 13318-2: 2007[70]; ISO 13318-3:2004[71]

Differential Mobility Analysis System (DMAS)

179. ISO/TS 12025:2012[29]; ISO 15900:2009[47]; ISO/TR 19601:2017[46]; ISO 27891:2015[49]; ISO/TS 28439:2011[48]

Dynamic light scattering (DLS)

180. ISO 22412:2017[51]; ASTM E2490-09[72]; ISO 22814:2020[52]

Electron microscopy

181. ISO 10797:2012 [73]; ISO 10798:2011[74]; ISO 16700:2017[53]; ISO 19749[19]; ISO 21363[24]; ISO 21383 [75]; ISO/TS 22292[76]; ISO 22493:2014[77]; ISO/TS 24597:2011[78]; ISO 29301:2017[54], ISO 13322-1:2014[20]

Particle Tracking Analysis (PTA)

182. ISO 19430:2016[79]; ASTM E2834-12[80]

Small Angle X-ray Scattering (SAXS)

183. ISO 17867:2015[61]

Single particle Inductively Coupled Plasma–Mass Spectrometry (sp-ICP-MS)

184. ISO/TS 19590:2017[81]; ISO/TS 13278:2017[82]

Part C: Single particle Inductively Coupled Plasma Mass Spectrometry (sp-ICP-MS)

185. The method is known to operate for very specific materials. Good applicability is shown for gold [83] and silver particles [84] Also reasonable results for the analyses of titanium oxide are known [85]. Based on these indications from literature, the technique might be valid for reliable, robust and reproducible data generation on particle size distribution for the mentioned particles. However, it has to be proven that the results are not influenced by the used instrumentation. It is highlighted that for this method an extended validation for consistency, comparability and instrumentation dependencies could not be successfully performed within the course of the development of this TG. Only few results were submitted within the ILC. Still, regarding the importance of the method for several use cases and the validated applicability for specific nanomaterials the method was kept in this Appendix Part C.

Measurement principle sp-ICP-MS

186. A liquid dispersion is sprayed into an inductively coupled plasma where the particles are vaporised, atomised, and ionised and the ionised atoms are detected in a mass spectrometer. Transient signals are recorded using fast signal processing (short dwell times), yielding spikes that represent individual particle events. In sp-ICP-MS a highly diluted dispersion of the test material is introduced into the ICP-MS, such that statistically only one particle during a given time interval enters the ICP. For further information on this method the relevant available standards are listed in the Appendix Part B, paragraph 184.

187. The resulting size distribution is the number-based size distribution of the mass equivalent spherical diameter $d_{0,mass}$

Applicability

188. The accompanying ILC to this TG revealed that the results of this method strongly depend on the used instrumentation. More research effort is needed to clearly determine the most sensitive points in sp-ICP-MS analysis. Currently, there is a lack of precise and accurate standards to guide users in obtaining comparable and reliable results on particle size distribution. Use of the sp-ICP-MS for particle size distribution requires further validation.

189. This method can be applied for metal/metalloid-containing particles in the size range from 10 nm to 1000 nm and even larger particles. The precise size range, especially the lower size limit, depends on the instrument used (refer to technical specifications of the equipment used) and the test material to be analysed (e.g. atomic mass). Sensitivity also depends on the isotopic natural abundance. An isotope which is rare will only give a small signal compared to a common isotope.

190. The investigated material need to be dispersible in an aqueous based dispersion for sp-ICP-MS to be applied. The applicable concentration range depends strongly on the elemental composition and particle size of the investigated material.

191. This measurement method does not distinguish between individual particles, agglomerates and/or aggregates. It is possible to take particles of a mixture of two or more structurally and chemically different particles into account if suitable detectors are available and both can be detected with a sufficiently signal-to-noise ratio.

192. The range of materials that can be analysed with sp-ICP-MS is limited to materials composed of chemical elements with a sufficiently high atomic mass for the respective size to obtain good signal-noise ratio e.g. inorganic nanomaterials. The density of the material must be known.

Prerequisites

193. Prerequisites for performing the measurement

a) An inductively coupled plasma mass spectrometer with dwell times ≤ 10 milliseconds, with a software which can return the result of the analysis per dwell time (for example transient mode or single particle mode if available).

b) Prepared samples (in accordance with paragraph 33 and 34). All steps of the sample preparation should be reported.

c) Information about the density of the particles to be analysed.

d) A reference nanomaterial (e.g. Au-particles) for the determination of the transport efficiency.

e) A calibration standard for the linearity test.

Additional considerations

194. The transport efficiency has to be determined with a reference nanomaterial (e.g. Au-particles) and should be higher than 1%. When the transport efficiency is lower the nebulizer, its position and the nebulization gas flow should be reviewed.

195. The instrument has to be calibrated using a calibration standard in accordance with the analysed potential nanomaterial. The calibration curve should have a correlation coefficient > 0.99. An appropriate calibration strategy is required to link the intensity of the spike of single particles to mass information. Two common approaches exist using either calibration by nanomaterial standards or employing standard solutions and determining transport efficiencies.

196. Number of detected particles: For particles with narrow size distribution ($\sigma_g \leq 1.5$) a number of 300 particles are sufficient, for particles with a wide size distribution ($\sigma_g > 1.5$) at least 700 particles have to be measured (see paragraph 119).

197. Measurement of particles with a wide particle size distribution: Small particles could be allocated to the background. Prior to measurement the sample should be fractionated - e.g. by the help of Field Flow Fractionation /Asymmetric Field Flow Fractionation (FFF/AF4).

198. Due to the needed high dilution of the dispersions, dissolution and partial dissolution of particles is problematic. As the technique is very sensitive, contamination of the dispersion medium results in a poor signal-noise ratio.

Implementation of the measurement and data evaluation

199. Overview of steps to be performed for the measurement:

a) Performance check of the instrument.

b) Determination of the transport efficiency – based on standard of known size and concentration or standard of known size.

c) Determination of linearity of the response.

d) Determination of blank level.

e) Measurement of the sample material.

f) Data conversion.

g) Data evaluation using the available instrument software or another appropriate validated external software, showing the size distribution in a cumulative view.

Part D: Details of the uncertainty determination with the bootstrap method and fibre part related supplemental information

Calculation of bootstrap uncertainty

200. To calculate the statistical uncertainty of the median of the size distribution in microscopy methods, the bootstrapping method is used. This method is a resampling method and allows estimating the 95% confidence interval of the median without prior assumption of any specific shape of distribution. Many statistical data evaluation software implemented the method and require only the input of the parameters defined below.

201. It is recommended to perform the methods in the following steps

a) Drawn boot = 1000 samples by resampling with replacement the sample of the size N (e.g. N = 300 or 700 for particles, N = 200 for fibres).

b) Calculate the median in diameter and length (if applicable) of each sample.

c) Reject the 2.5% smallest and the 2.5% largest values. The remaining values define the 95% confidence interval.

Comparison of the size distribution of two samples

202. In order to test whether two size distributions obtained from measurements of the same material exhibit the same size distribution, a non-parametric hypothesis test is required. Applying the Kolmogorov-Smirnov-two-sample-test is recommended. This test allows for comparison of two differently sized lists of size values and tests the hypothesis that both lists origin from the same distribution. If the probability that the hypothesis is true, is below 1% ($p < 0.01$) the hypothesis is rejected ($\alpha < 0.01$). The test is implemented in many statistical data evaluation software.

Maximal aspect ratio

203. Fibres can in principle be visualized in an image if their length is shorter than the longest mapped length of a picture. If the position of images is chosen in a random manner, it is however, improbable to capture both ends of a fibre if its length is of approximately the size of the longest side of the image. Therefore, here it is estimated that fibres are typically captured with good statistical soundness, if the size of the fibre is at max 1/3 of number of pixels on the smallest side of the image n_pix_s. Taking into account that the fibre diameter is pictured with 4 pixel the maximal aspect ratio of a fibre can be estimated from the following formula

$$max\ aspect\ ratio = \frac{n\_pix_s}{3 \times 4}$$

204. Examples for typical rectangular images of format 4:3 are given in ***Table*** 5.

Table 5: maximal aspect ratio of a fibre for the pairwise determination of length and diameter of a fibre depending on the size of the image

Size of the image	Aspect ratio
20 Mpix ($n\_pix_s = 3840$)	320
5 Mpix ($n\_pix_s = 2560$)	213
1 Mpix ($n\_pix_s = 1280$)	107

Dependence of the bootstrap uncertainty on the width of the distribution, if number of fibres counted is 200

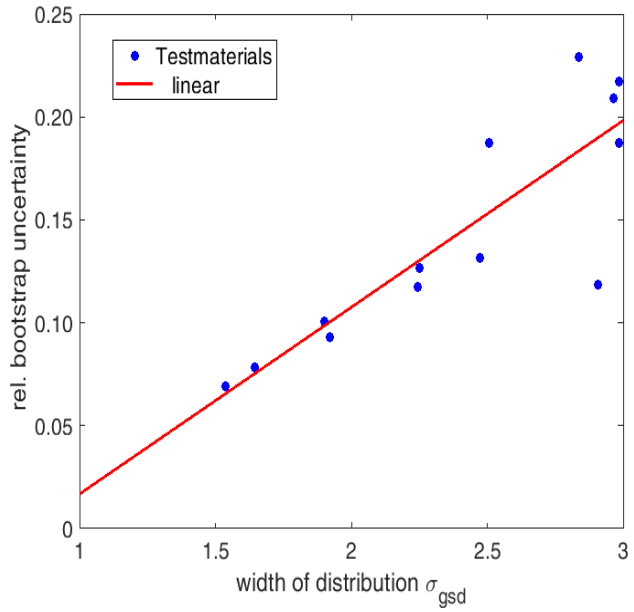


Fig. 2: Bootstrap uncertainty of the median value of a log normal distribution from 200 values with different widths of the distribution.

205. From a number of test measurement of materials with different size distributions, the uncertainty with the bootstrap method as a function of the distribution width was calculated. The red line is a guide for the eye. It is seen that an approximate linear increase of the statistical uncertainty with the width of the size distribution is to be expected if N = 200 fibres are counted.

Dependence of the measured diameter on the pixel size

206. The resolution of the images (i.e. the number of pixels per nanometre) influences the diameter measured. In order to verify the visibility of fibres, images with pixel sizes larger than the diameter of the fibre were taken. As an example, images of 28 nm thick test material Ag nanowires at different pixel sizes were captured and the diameter of N = 200 fibres was evaluated as a function of the pixel size. The result is presented in ***Fig. 3***. The fibres can be detected even at pixel sizes twice the diameter of the fibre, but the measured diameter increases with the used pixel size, resulting in overestimation of the diameter. To

prevent the overestimation of the fibre diameter and ensure reliable results, the pixel size chosen for the measurement should allow a minimal number of 4 pixels per mean fibre diameter, see paragraph 162). The validation report [9] provides further support for this value.

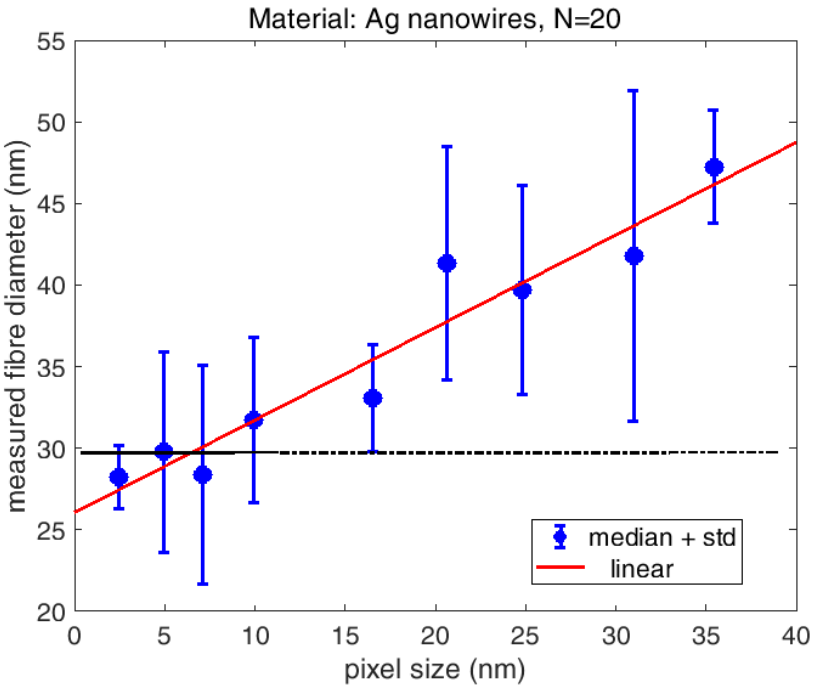


Fig. 3: Dependence of the measured diameter of a fibre on the pixel size of the image

Evaluation of the fibre diameter from grey value traces

207. In order to reproducibly measure the diameter of the particle/fibre while minimizing the influence of the evaluator it is recommended to evaluate the diameter of the particle/fibre from the grey value traces through the particle. A typical trace is shown in ***Fig. 4***. It is crucial to define the height at which the width of the peak in the grey value profile is evaluated. The optimal parameter depends on the material and size of the material [27, 86, 87]. A pragmatic approach is to measure the width of the peak at half height between the noise level (blue line) and the mean peak height (red). This line is shown as green arrow in ***Fig. 4***.

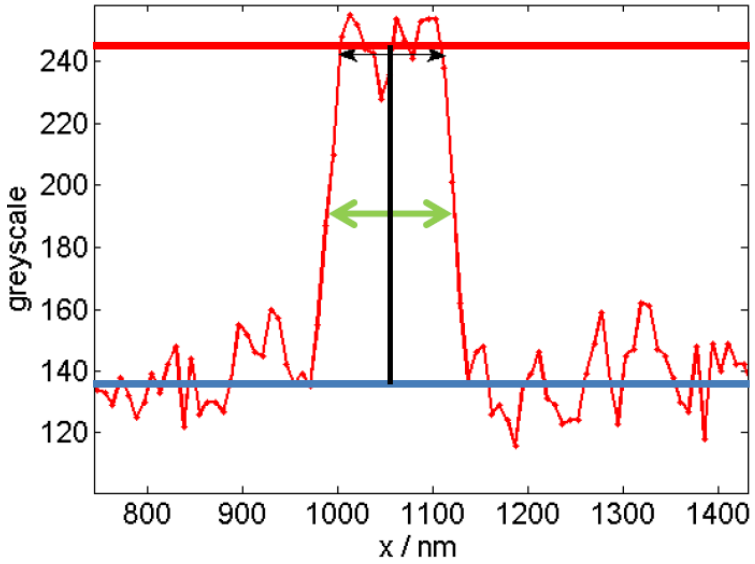


Fig. 4: Typical grey value obtained from a SEM image. The fibre diameter is defined as the width of the fibre at half peak height. Noise level (blue line), Peak height (red line), diameter of the particle/fibre (green line)

Part E: List of Test Materials used in Validation Report

Table 6: Overview of the materials tested (particles) with the respective method.

Method	Ag[1]	SiO_2[2]	SiO_2[3]	ZnO[4]	PSL Mix[5]	TiO_2[6]	PSL Mix[7]
diameter (nm)	17 nm	20 nm	50 nm	~100 nm	90/125 nm	~250 nm	80/800 nm
DLS	X	X	X	X	X	X	
CLS	X	X*	X*	X	X	X	X
PTA	X			X	X	X	
SAXS	X	X	X		X		
AFM			X	X	X		
TEM	X	X	X	X	X	X	X
SEM	X	X		X	X	X	X
DMAS	X	X	X		X	X	X

Table 7: Overview of the materials tested (fibres) and their size related properties, with σ_{GSD} being the Geometric Standard Deviation, given by manufacturer information (*) and own pre-validation for median values (+) performed with SEM.

Method	Au[8]	Ag[9]	MWCNT[10]	ZnO[11]	SiC[12]
length (µm)	0.045*	0.8^+	<15* 1.0^+	5-50* 5.4^+	50-100* 7.2^+
diameter (nm)	10*	20* 25^+	10-30* 30^+	50-120* 90^+	100-600* 140^+
SEM	X	X	X	X	X
TEM	X	X	X	X	X

208. Furthermore, actual valid and available certified reference (nano-)materials can be found in the international database on certified reference material database COMAR, a

[1] Manufacturer: BAM – Product labelling: Not purchasable
[2] Manufacturer: KRISS – Product labelling: CRM 301-01-002
[3] Manufacturer: KRISS – Product labelling: CRM 301-01-001
[4] Manufacturer: JRC – Product labelling: JRCNM62101a
[5] Manufacturer: Polysciences – Product labelling: Custom made mixture of Cat. 64009 and Cat. 64011 (1:1)
[6] Manufacturer: JRC – Product labelling: IRMM 388
[7] Manufacturer: Polysciences – Product labelling: Custom made mixture of Cat. 64008 and Cat. 64025 (2:1)
[8] Manufacturer: Sigma-Aldrich – Product labelling: 716839
[9] Manufacturer: Novarials – Product labelling: NovaWire-Ag-A20
[10] Manufacturer: Arry International Group Ltd. – Product labelling: ARIGM001
[11] Manufacturer: ACS – Product labelling: NWZO01A5
[12] Manufacturer: ACS – Product labelling: NWSC0202

cooperation of 16 international institutes [88]. The database is accessible by the permanent link www.comar.bam.de.

Part F: List of Abbreviations

AFM	Atomic Force Microscopy
AUC	Analytical Ultracentrifugation
CLS	Centrifugal Liquid Sedimentation
CPC	Condensation Particle Counter
CRM	Certified Reference Materials
d	Diameter
DLS	Dynamic Light Scattering
DMAS	Differential Mobility Analysis System
EDX	Energy Dispersive X-ray Spectroscopy
EM	Electron Microscopy
FFF/AF4	Field Flow Fractionation / Asymmetric Field Flow Fractionation
GUM	Guide to the Expression of Uncertainty in Measurement
ILC	Interlaboratory Comparison
ISO	International Organization for Standardization
min	Minute
µm	Micrometre
N	Number of particles
nm	Nanometre
NNLS	Non-Negative Least Squares
NTA	Nanoparticle Tracking Analysis
PTA	Particle Tracking Analysis
PSD	Particle Size Distribution
q	Scattering vector
SAXS	Small-Angle X-Ray Scattering
SEM	Scanning Electron Microscopy
$2\sigma_{Bt}$	Uncertainty related to using Bootstrapping test
σ_C	Combined measurement uncertainty
σ_g	Geometric Standard Deviation
$2\sigma_P$	Uncertainty related to the method
$2\sigma_{SF}$	Uncertainty related to instrument operator
SOP	Standard Operation Procedure
sp-ICP-MS	Single Particle Inductively Coupled Plasma-Mass Spectrometry
T	Temperature

TEM	Transmission Electron Microscopy
TG	OECD Test Guideline
WNT	Working Group of National Co-ordinators of the TGs programme
WPMN	Working Party on Manufactured Nanomaterials