Supporting Information
to
Versailles Project on Advanced Materials and Standards (VAMAS) Interlaboratory Study on Measuring the Number Concentration of colloidal gold nanoparticles

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

S1. SAMPLE

S1.1. Liquid analysis:

The 30 nm colloidal gold test sample was measured in liquid to assess the particle size distribution. Figure S1 shown a representative number-based particle size distribution as measured by CLS. The particle size appears narrowly distributed and the sample free from agglomerations.

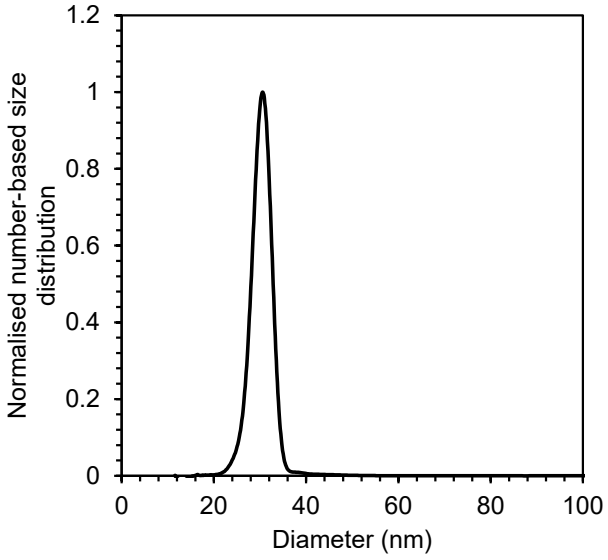

Figure S1. Representative normalised number-based size distribution of the 30 nm gold nanoparticle test sample as measured by CLS, assuming an average effective density of the particle equivalent Stokes' volume of 15 g/cm^3.

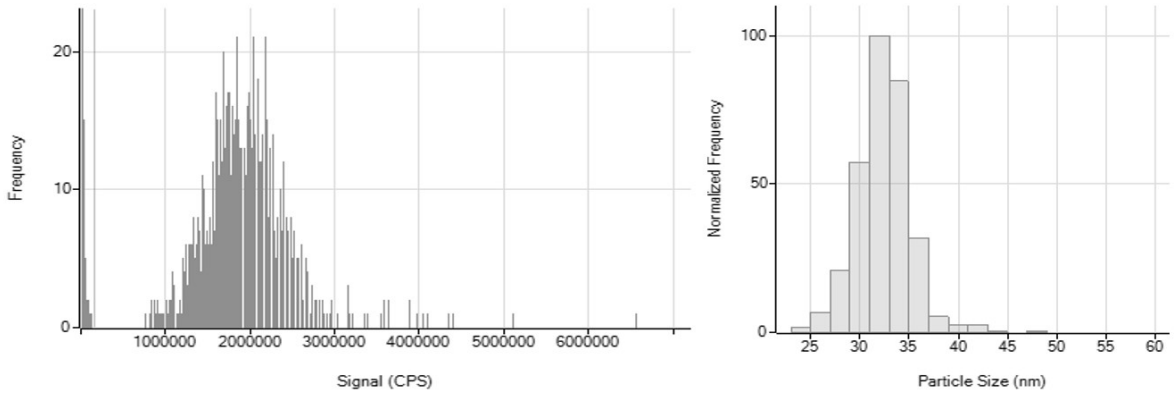

Figure S2. Representative signal distribution (left) and particle size distribution histogram (right) of the 30 nm gold nanoparticle test sample as measured by spICP-MS. The latter was obtained following instrument response factor determination using external calibration strategy with elemental standards, and assuming spherical particle geometry, bulk density of gold and solid single element (gold) composition.

S1.2. Electron microscopy analysis:

The 30 nm colloidal gold test sample was deposited on a Formvar/carbon-coated 200-mesh copper TEM grid for scanning electron microscopy (SEM) imaging. Prior to deposition, the test sample was diluted in ultrapure water to a number concentration of ~7.5×10^{13} kg^{-1}. The TEM grid was glow-discharged and coated with Alcian blue dye before exposure to the nanoparticle suspension. This produced a hydrophilic and positively charged surface which promoted the adsorption of the gold nanoparticles onto the grid.

SEM imaging was performed in an Auriga-60 SEM (Zeiss, Oberkochen, Germany) using 2 kV electrons, 15 µm aperture diameter, and a sample working distance of 2 mm. Figures S2A and B show representative mages of the particles at different magnification. The images were acquired by the in-lens detector, which detects secondary electrons generated near the top surface of the particles and back scattered towards the pole piece. The particles appeared free from agglomeration and of uniform size. The shape of the particles varied across the sample, with most particles appearing close-to-spherical.

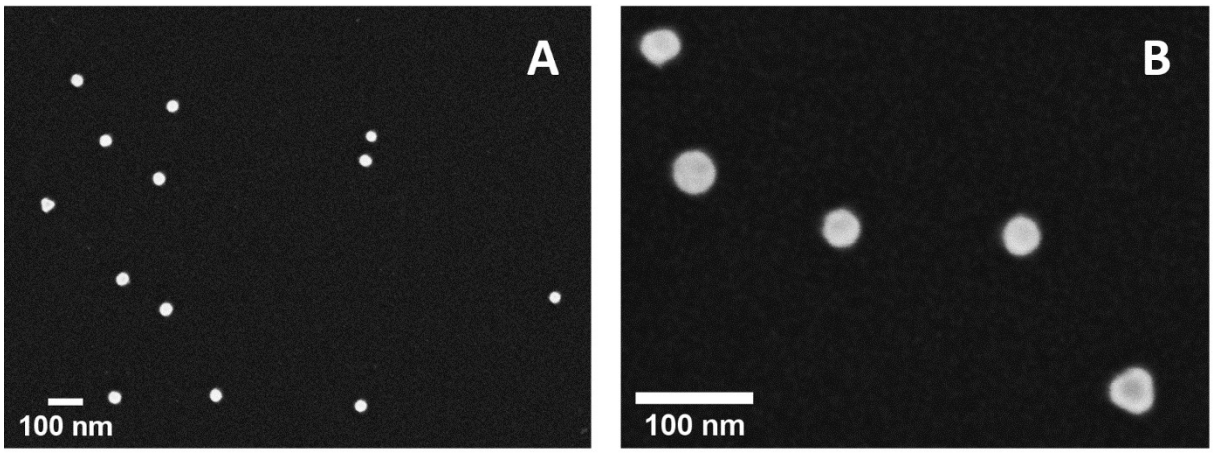

Figure S3: Representative SEM images of the test gold nanoparticles.

S2. MEASUREMENT PROTOCOL

The measurement protocol was distributed to the participant to the study together with the samples. The protocol was published as the "NPL Report AS 98" and is shown in the Appendix to this document.

S3. DEFINITIONS

The definitions below are based on the documentary standard ISO 3534-1 and were adopted in the manuscript:

test result
The value of a characteristic obtained by carrying out a specified test method.
Note 1 to entry: The test method should specify that one or a number of individual observations be made, and their average or another appropriate function (such as the median or the standard deviation) be reported as the test result. It may also require standard corrections to be applied, such as correction of gas volumes to standard temperature and pressure. Thus a test result can be a result calculated from several observed values. In the simple case, the test result is the observed value itself.

accepted reference value
A value that serves as an agreed-upon reference for comparison, and which is derived as:

- a) a theoretical or established value, based on scientific principles;
- b) an assigned or certified value, based on experimental work of some national or international organization;
- c) a consensus or certified value, based on collaborative experimental work under the auspices of a scientific or engineering group;
- d) when a), b) and c) are not available, the expectation of the (measurable) quantity, i.e. the mean of a specified population of measurements.

accuracy
The closeness of agreement between a test result and the accepted reference value.

trueness
The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.

bias
The difference between the expectation of the test results and an accepted reference value.
Note 1 to entry: Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the accepted reference value is reflected by a larger bias value.

In this work, the bias between a test result value x and an accepted reference value x_R was calculated as $|x-x_R|/x_R$.

laboratory bias
The difference between the expectation of the test results from a particular laboratory and an accepted reference value.

bias of the measurement method
The difference between the expectation of test results obtained from all laboratories using that method and an accepted reference value.

precision
The closeness of agreement between independent test results obtained under stipulated conditions.
Note 1 to entry: Precision depends only on the distribution of random errors and does not relate to the true value or the specified value.
Note 2 to entry: The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is reflected by a larger standard deviation.
Note 3 to entry: "Independent test results" means results obtained in a manner not influenced by any previous result on the same or similar test object. Quantitative measures of precision depend critically on the stipulated conditions. Repeatability and reproducibility conditions are particular sets of extreme conditions.

repeatability
Precision under repeatability conditions, i.e. conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.

*In this work the repeatability is expressed by the "**in-lab variability**", or "**repeatability standard deviation**", which was calculated as the relative standard deviation of a set of results obtained with the same method on the provided test material in the same laboratory.*

reproducibility
Precision under reproducibility conditions, i.e. conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

*In this work the reproducibility is expressed by the "**between-labs variability**", or "**reproducibility standard deviation**", which was calculated as the standard deviation of a set of results obtained with the same method on the provided test material in different laboratories with different operators using different equipment.*

Appendix

VAMAS TWA 34 - Project 10:

Inter-laboratory Study: Measurement of number concentration of colloidal nanoparticles - Protocol for sample handling, preparation and measurements.

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LGC

ABSTRACT

This document describes the protocol for sample handling, preparation and measurements in the VAMAS TWA 34 project 10, interlaboratory comparison (ILC) on the measurement of number concentration of colloidal gold nanoparticles (NPs). The techniques selected for this study are UV-Visible spectroscopy, particle tracking analysis (PTA), centrifugal liquid sedimentation (CLS) and single particle inductively coupled plasma mass spectrometry (spICPMS). For the study, five samples labelled as LGCQC5050 are supplied, the concentration of which is known with a standard uncertainty of 20%. Specific guidance is given on sample storage, handling, preparation, measurement and data reporting.

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Alex Shard, Knowledge Leader.

GLOSSARY/ABBREVIATIONS

CLS: centrifugal liquid sedimentation

DI: deionised

DLS: dynamic light scattering

ILC: inter-laboratory comparison

NP: nanoparticle

PTA: particle tracking analysis

SAXS: small angle X-ray scattering

SI: international system of units

spICPMS: single particle-inductively coupled plasma mass spectroscopy

TEM: transmission electron microscopy

TWA: technical working areas

UV-Vis: ultraviolet-visible

VAMAS: Versailles project on advanced materials and standards

EXECUTIVE SUMMARY

This document describes the protocol for sample handling, preparation and measurements in the VAMAS TWA 34 project 10, interlaboratory comparison (ILC) on the measurement of number concentration of colloidal gold nanoparticles (NPs). The techniques selected for this study are UV-Visible spectroscopy, particle tracking analysis (PTA), centrifugal liquid sedimentation (CLS) and single particle inductively coupled plasma mass spectrometry (spICPMS). For the study, five samples labelled as LGCQC5050 are supplied, the concentration of which is known with a standard uncertainty of 20%. Specific guidance is given on sample storage, handling, preparation, measurement and data reporting.

INTRODUCTION

Nanoparticles (NPs) are increasingly used in innovative products manufactured by advanced industries and provide enhanced, unique properties of great commercial and societal value. The demand for high performance materials places increasingly stringent tolerances on the properties of NPs to ensure reliable performance and to meet regulation. Measuring the number concentration of particles in colloidal suspension is a major commercial interest for a large range of industries, such as personal care, fine chemicals, pharmaceutical and biomedical industries. This measurement will enable the optimisation of materials, their specification, design and quality control. Accurate knowledge of NP number concentration will also impact the ability to perform useful risk assessment of the materials, with important implications for the commercialisation and safe use of the products. It will help enable compliance with potential EU legislation linked to the EC definition of a nanomaterial (2011/696/EU) and will help underpin claim related to reliability, performance and lifetime in the formulation of products containing NPs.

Guidance and international documentary standards are essential to those industries which rely upon high performance NPs.

In recent years there have been substantial advances in the ability to directly measure particle number concentration of colloidal suspensions and a number of bench-top techniques have become popular. However, no formal evaluation and validation of these techniques have taken place. The situation is exacerbated by the lack of NP reference materials that are certified for number concentration. Such materials are required to enable the calibration of many of the techniques capable of performing this measurement.

The accuracy of measurements of NP number concentration critically depends on the way the samples are handled. Precipitation, particle agglomeration and inaccurate dilution procedures are only some of the potential sources of inaccuracy of the measurements. In this respect, there is a real need for disseminating best practice among the measurement community and providing users with confidence in the way they operate the tools available to them.

The European project 14IND12 Innanopart of the European Metrology Programme for Innovation and Research (EMPIR) (http://empir.npl.co.uk/innanopart/) is currently trying to address these needs. Project's objectives include the development of reliable methods for NP number concentration measurements with an expanded uncertainty of better than 10 % and the establishment of metrological traceability to the SI unit. The traceable methods selected in Innanopart are Small Angle X-ray Scattering (SAXS) and single particle Inductively Coupled Plasma Mass Spectrometry (spICPMS). The project is also investigating a number of laboratory methods for this measurement, some of which are included in this VAMAS study (i.e. UV-Vis spectrometry, Centrifugal Liquid Sedimentation (CLS) and Particle Tracking Analysis (PTA)).

This VAMAS study is organised within the project Innanopart. Its outcomes will set the basis for the development of relevant standard documentation which is essential to those industries which rely upon high performance NPs.

TIMETABLE

You should complete this work and send the results to Caterina Minelli (caterina.minelli@npl.co.uk) by using the provided spreadsheet by **30th July 2018**. If you cannot do so and you need extra time please inform Caterina Minelli.

THIS PACKAGE

This package contains:

- this protocol,
- 5 x 5 mL vials of 30 nm spherical gold colloids labelled as LGCQC5050 (referred to as NPL1) per technique.
- 3 empty vials, pre-cleaned (**27348 Supelco) per technique**.
- 2 x 0.22 μm filters per technique,
- 2 test weights of different size (NPL SI bots) per technique,
- a Freeze Watch™ Indicator per technique,
- an MSDS and data sheet of sample LGCQC5050,
- 300 mg of citrate buffer (Sodium Citrate Tribasic Dihydrate from Sigma 71402, Lot BCBT5426) labelled "NPL citrate buffer" (for NTA and spICPMS only).
- MSDS of trisodium citrate dehydrate (for NTA and spICPMS only).

Please inspect the packaging to check if it has been opened by customs and if the integrity of the samples has been compromised. The Freeze Watch™ Indicator paper should remain white. If the paper has been stained black, or you are in doubt, please contact Caterina Minelli **(caterina.minelli@npl.co.uk).**

Upon receipt of the samples, please notify us that everything is received in good order.

☐

I have emailed NPL that all is OK with the samples on / / 2018

Figure 1. The package.

LABORATORY EQUIPMENT REQUIRED FOR SAMPLE PREPARATION

We recommend the use of the items below for the preparation of the samples:

- Liquid handling tools such as pipettes or syringes,
- A balance with accuracy of 0.1 mg or less,
- A vortex mixer,
- Ultrapure water with resistivity of 18.2 MΩ cm (at 25 °C). If this type of water is not available in your laboratory, deionised (DI) water can also be used, but should be filtered by using the 0.22 μm filters provided in the package.
- Lab coat, gloves, safety goggles and other protection equipment in accordance to the Health and Safety directives of your laboratory. Vinyl gloves, often used in clean rooms, are coated with a release agent from the moulding process, and should not be used.
- Temperature and humidity monitors.

Depending on the number of dilutions required, you may need some additional vials.

SAMPLES, HANDLING AND STORAGE

NPL1 was produced from BBI solutions EM. GC30 citrate stabilised gold NPs, with average size (equivalent spherical diameter as measured by the manufacturer by TEM) of 30.7 nm. The samples have been bottled in 5 mL vials and gamma irradiated by LGC Limited. The materials is labelled as LGCQC5050.

Upon reception, the samples should immediately be placed into a refrigerator (2-5 °C) for storage. The samples are stable for at least 3 months under such conditions, but should be analysed as soon as it is convenient within this timeframe.

When the samples are removed from the refrigerator for sample preparation and characterisation, allow at least 30 min for them to return to room temperature before opening the container. Use clean, possibly sterile pipettes /pipette tips / syringes for handling the liquids.

MEASUREMENT SCHEME

1.1 UV-VIS AND CLS ILCS

The participants of the UV-Vis and CLS ILCs are invited to measure the number particle concentration of three samples:

1) Sample NPL1, whose concentration between $5 \cdot 10^{10}$ and $9 \cdot 10^{11}$ particles/mL.
2) Sample NPL2, which will be prepared by the participant by diluting sample NPL1 by a known dilution factor, which is suggested to be between 4 and 6.
3) Sample NPL3, which will be prepared by the participant by diluting sample NPL1 by a known dilution factor, which is suggested to be between 9 and 11.

Where possible, sample NPL1 will be measured undiluted.

1.2 PTA AND SPICPMS ILCS

The participant of the PTA and spICPMS ILCs are invited to measure the concentration of only sample NPL1, whose concentration is between $5 \cdot 10^{10}$ and $9 \cdot 10^{11}$ particles/mL. The sample will require dilution, according to the protocol described in this document.

SAMPLE PREPARATION

1.3 SAMPLE INSPECTION

Before the experiment, inspect the samples and ensure that no precipitation is visible at the bottom of the sample container. Please contact Caterina Minelli (caterina.minelli@npl.co.uk) if there is any issue.

Before any sampling from a sample container, vortex the sample for about 30 seconds. After vortexing, please observe the sample and ensure that the content appears homogeneous and no precipitation is present. 1 min bath sonication is also permitted if sedimentation persists.

Invert the ampule several times before opening. Once open, the content should be used within the day. Do not transfer the content of the ampule to another container for storage. Seal the ampule with paraffin film when not in use and until all the dilutions and measurements have been performed.

The following method is recommended to perform the dilutions of the samples NPL1. If you have another method you prefer to use, please feel free to do so and describe your method in full within the data reporting spreadsheet.

1.4 GRAVIMETRIC DILUTION

We recommend to perform the dilutions gravimetrically using a balance with accuracy of 1 mg or less. It is recommended to have the balance calibrated on a regular basis. The balance scale must be tared before use. If there is an internal balance calibration feature, this should be used prior to making measurements on the balance. During the weighing procedures, the vials should be handled with gloves.

To reduce problems with temporal balance drift and water evaporation the following method is recommended for preparing sample dilutions:

- A reference weight is placed on the balance and the reading noted (ref_1).
- An empty container (to be loaded with the test substance) is placed on the balance ($test_1$).
- The container is filled with the test substance and the balance reading taken ($test_2$).
- The reference weight is placed on the balance again and the reading taken (ref_2).

The weight of substance added to the container (w_t) may then be calculated as follows:

$$w_t = \left(test_2 - test_1\right) - \frac{\left(ref_2 - ref_1\right)}{n} \quad (1)$$

where n is the number of steps between ref_2 and ref_1, which in this procedure is 3.

Here, we are assuming a linear drift of the balance with time and that every weighing procedure takes approximately the same amount of time.

In 7.4 we will describe how to adapt this weighing technique to perform the sample dilutions.

Further support for these type of measurements, including calibration, weighing techniques and estimation of the uncertainty, can be found, for example, in the EURAMET Calibration Guide No. 18 or the UK Accreditation Service publication on In-house Calibration and Use of Weighing Machines.

1.5 WEIGHING REPETITION UNCERTAINTY

The repeatability uncertainty of the balance should be measured using the two test weights (one large, one small) provided in the package:

1. The balance is tared and the reading taken ($zero_1$).
2. The larger test weight is placed on the balance ($large_1$)
3. The smaller test weight is placed on top of the larger one and the balance reading taken ($small_1$)
4. The smaller test weight is removed and the reading is taken ($large_2$).
5. Steps (3) and (4) are repeated 4 times to acquire $small_2$, $large_3$, $small_3$, $large_4$, $small_4$, $large_5$ and $small_5$.
6. All the test weights are removed from the balance and the reading is taken ($zero_2$).

Each measurement of the weight of the smaller test weight is calculated as:

$$w_i = small_i - large_i - \frac{zero_2 - zero_1}{11} \tag{2}$$

The repetition uncertainty is calculated as the standard deviation of the w_i values.

Filling in the tab "Weighing repetition uncertainty" in the electronic reporting excel data sheet provided with this protocol will assist you in this task.

1.6 PERFORMING DILUTIONS

Dilutions to prepare NPL2 and NPL3 should be performed preferably by using ultrapure water with resistivity of 18.2 MΩ cm. If this type of water is not available in your laboratory, use DI water filtered by using the 0.22 µm filters provided in the package. Please DO NOT filter samples NPL1, NPL2 or NPL3. Any filtration of diluent must occur before dilutions are performed.

The volume V_i of undiluted particle sample (NPL1) needed for a diluted sample can be calculated according to:

$$C_i \cdot V_i = C_f \cdot V_f \tag{3}$$

Where C_i and C_f are the concentration of the undiluted and diluted samples respectively and V_f is the final volume of the diluted solution. The total mass of the particles contained in the undiluted sample is such that the difference in density between the undiluted and diluted solution can be neglected. For this reason, the following formula is used:

$$C_i \cdot m_i = C_f \cdot m_f \tag{4}$$

where m_i and m_f are the masses of the undiluted and diluted samples respectively.

The schematic below shows the protocol for measuring m_i and m_f.

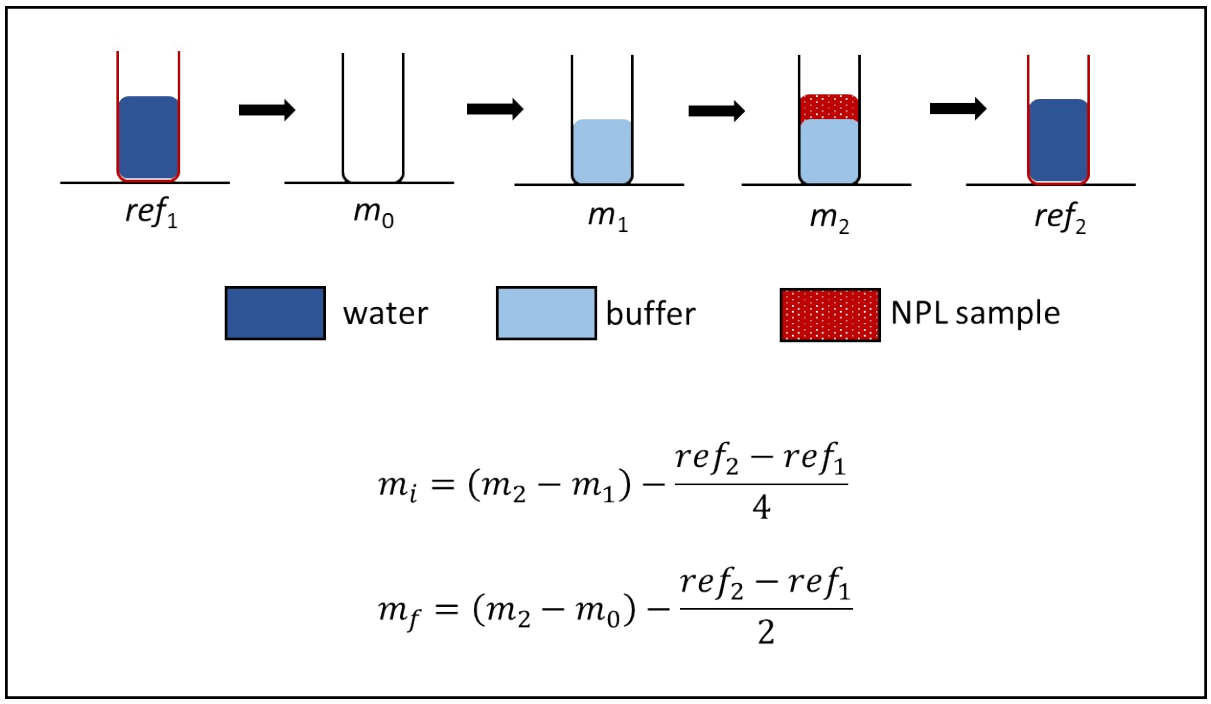

Figure 2. Recommended protocol for measuring m_i and m_f.

Each dilution should be completed within 10 min to limit the uncertainty contribution due to evaporation. Please record the temperature and humidity of your laboratory in the data report sheet if these data are available to you.

To limit the uncertainty due to evaporation, ref_1 and ref_2 should be measured by using one of the empty vials provided in the package filled with about 14 mL of water having a temperature similar to that of the samples. It is important that the vial is left open during the whole weighing procedure to allow evaporation to happen. Here, we are assuming that the evaporation rate is linear in time and that the sample container and the reference container experience similar evaporation rates. Please avoid placing the sample container and reference container close to heat sources or in the sun.

The uncertainty on m_i and m_f can be calculated with various approaches. A full uncertainty budget is outside the scope of this inter-laboratory study. We suggest using the repeatability of the balance as the uncertainty on m_i and m_f, assuming that the drift and the evaporation are treated as shown in the scheme in Figure 2 and any necessary calibration correction is taken into account. If the uncertainty on the repeatability of the balance is null, we suggest using the resolution of the balance divided by $\sqrt{3}$.

The dilution factor d is given by:

$$d = \frac{m_f}{m_i} \tag{5}$$

and its relative standard uncertainty is:

$$\frac{\Delta d}{d} = \sqrt{\left(\frac{\Delta m_f}{m_f}\right)^2 + \left(\frac{\Delta m_i}{m_i}\right)^2} \quad (6)$$

Knowing the dilution factor will be useful to evaluate the results of your measurements and we recommend to follow the protocol in order to produce an accurate value of it.

1.7 DILUTIONS FOR UV-VIS AND CLS TECHNIQUES

The package contains 5 vials of sample NPL1. Please use 3 for performing three independent measurements of NPL1 and the other 2 to prepare samples NPL2 and NPL3 respectively.

Prepare about 14 mL of:

- sample NPL2 by diluting sample NPL1 by a known dilution factor between 4 and 6 and
- sample NPL3 by diluting sample NPL1 by a known dilution factor between 9 and 11.

Aim to perform the measurements on three different aliquots of NPL1, NPL2 and NPL3.

The tab "Sample Handling" of the electronic reporting data sheet provided with this protocol will assist you in this task.

The samples NPL2 and NPL3 prepared in this way are stable for at least 1 week. We recommend that the measurements are performed within a week from the preparation.

1.8 DILUTIONS FOR PTA AND SPICPMS

For these techniques, sample NPL1 will need to be diluted multiple times. For an accurate estimation of the undiluted as received concentration of the sample, it is critical that these dilutions are performed with accuracy and for this reason we recommend the use of the gravimetric method described in 7.4.

For these dilutions, we recommend the use of 1 mM trisodium citrate buffer. To produce the buffer you can use the NPL citrate buffer provided in the package. However, different qualities of citrate buffers can be used at the discretion of the operator. For example, the use of spICPMS may have more stringent buffer requirements with respect to other techniques.

To prepare 0.1 L of 1 mM trisodium citrate buffer, add ultrapure water with resistivity of 18.2 MΩ cm (at 25 °C) to 29.4 mg of the provided salt buffer to reach 0.1 L volume. If this type of water is not available in your laboratory, deionised (DI) water can also be used, but should be filtered by using the 0.22 µm filters provided in the package. The buffer should be filtered when prepared (max pore size of 0.22 µm) and used within 2 weeks.

NEXT STEP

Samples NPL1 and eventually NPL2 and NPL3 are now ready for measurement.

You should have rough estimate of their concentration by knowing that the concentration of NPL1 is ~$2 \cdot 10^{11}$ particles/mL and from the dilution factors that were calculated in the previous section.

The electronic reporting data sheet provided with the study will support you in recording the required information and performing the calculations.

Please go to:

- Section 9 if you are performing UV-Vis measurements.
- Section 10 if you are performing CLS measurements.
- Section 11 if you are performing PTA measurements.
- Section 12 if you are performing spICPMS measurements.

UV-VIS MEASUREMENTS

The instrument should be operated under conditions that give the most stable performance. For the optimum inter-comparison, a common set of operating conditions is needed, however due to the variety of instruments available, no single set of conditions can do this. Instead we give general conditions for guidance to improve comparability.

1.9 TEMPERATURE.

Analysis at room temperature is preferred. Under no circumstances should the samples be exposed to temperatures above 45 °C or below freezing. If the temperature at which the sample is analysed differs significantly from 20 °C, please give details in the 'conditions' section of the data reporting spreadsheet.

If the samples and dispersion media have been stored in the fridge, it is recommended that they reach room temperature before the measurements are performed.

1.10 MEASUREMNT PROTOCOL

It is recommended that the spectrometer is calibrated and regularly tested as recommended by the manufacturer or your usual procedure, for example in terms of wavelength and absorbance accuracy, stray light and linear dynamic range. A range of standards and methods are available to aid you and we recommend following the guidelines provided by the instrument manufacturers (e.g. PerkinElmer technical note on Validating UV/Vis Spectrometers). Other sources of information are generally found in the instrument manual or inspection service reports.

It is generally recommended to switch on the spectrometers, inclusive of their lamps, at least 30 minutes before measurements to ensure equilibrium conditions are reached. Please follow the instrument manufacturer's guidelines in this regard and note in the report.

All measurements if possible should be performed in the same measurement session.

The blank should be filled with water treated in the same way as that used to dilute the samples (see 4 for more details). As well as the samples, a measurement of the water used for the blank and, if used, of the 1 mM buffer in the same wavelength range should also be performed.

The samples and blank can be contained in either glass, plastic or quartz vials with known optical path. Please use, whereever possible, large volume cuvettes. If you are using small volume cuvettes, please ensure that the light beams of the spectrometer fully pass through the blank and the sample volume. This is most important for concentration measurements.

- If your spectrometer is a single beam instrument and you are using a small volume cuvette, please ensure that the cuvette is sitting at the same location in the sample holder for each measurement. If unsure, please try to perform three measurements of the same sample by removing and repositioning the sample between each measurement. The measured spectra should be identical. If this is not the case, please revise the positioning of your cuvette to improve repeatability.

- If you spectrometer is a dual beam instrument, you can verify that the light beams of the spectrometer fully pass through the blank and the sample volume by swapping the sample and control position and comparing measurement results before and after swapping. The two spectra should be identical. If this is not the case, the path of the beams need to be adjusted until the spectra coincide.

Set the instrumental parameters (e.g. slit width) to what you consider is best for the optimum performance.

For each sample, acquire the absorption spectrum of at least three aliquots at wavelengths between 390 nm and 700 nm with increments of 1 nm. For NPL1, measurements should be repeated on at least three independent aliquots, each one from one of the three vials provided. For NPL2 and NPL3 measurements should be performed on at least three different aliquots for each sample. For each aliquot, the absorption spectra should be acquired three times and the resulting spectra should be averaged.

An aliquot of water of the same quality used for the blank should also be measured in triplicate in the same wavelength range. If a buffer was used, this should also be measured in the same way.

Final results should be reported as the arithmetic average of these three measurement results, along with the repeatability uncertainty calculated as their standard deviation. Please feel free to provide any other information on the estimated uncertainty of the measurement.

1.11 DATA ANALYSIS

Use your own in-house procedures to calculate the number concentration of the Au particles in the samples. Please report your results and the method you used. If you do not have a procedure in place, we recommend the use of the method below. This method is based on publications [1] and [2]. Method in [1] is used to calculate the molar extinction coefficient at 450 nm of gold nanoparticles, whose size is measured according to [2] from the position of the local surface plasmon resonance (LSPR) peak.

Calculate the concentration *C* of the particles using the formula below:

$$\left[C(NPs\ mL^{-1})\right] = \frac{0.001 \cdot N_A \cdot A_{450}}{\varepsilon_{450}(M^{-1}cm^{-1}) \cdot L(cm)} \quad (7)$$

Where N_A is the Avogadro constant, A_{450} is the absorption measured at 450 nm, ε_{450} is the molar extinction coefficient of the particles at 450 nm and is equal to $(2.16 \pm 0.12)\cdot 10^9$ $M^{-1}cm^{-1}$ and *L* (in cm) is the optical path length of the cuvette used.

The relative standard uncertainty of a single measurement result can be calculated as:

$$\frac{\Delta C}{C} = \sqrt{\left(\frac{\Delta A_{450}}{A_{450}}\right)^2 + \left(\frac{\Delta \varepsilon_{450}}{\varepsilon_{450}}\right)^2 + \left(\frac{\Delta L}{L}\right)^2} \quad (8)$$

Where the relative standard uncertainty for light absorption at 450 nm is calculated as the standard deviation of the results of three repeated measurements on three different aliquots of the same sample. The relative uncertainty on *L* can typically be neglected.

The major contribution to the uncertainty is expected to come from the model used to measure the concentration, which is in part reflected into the uncertainty on ε_{450}. An accurate estimation of this uncertainty has not been performed and this ILC will contribute to determine it. It is expected that this uncertainty is below 20% [1].

1.12 EXAMPLE OF MEASUREMENT

An example of UV-Vis spectrum of 30 nm Au NPs similar to sample NPL1 is shown in Figure 3. The sample is undiluted.

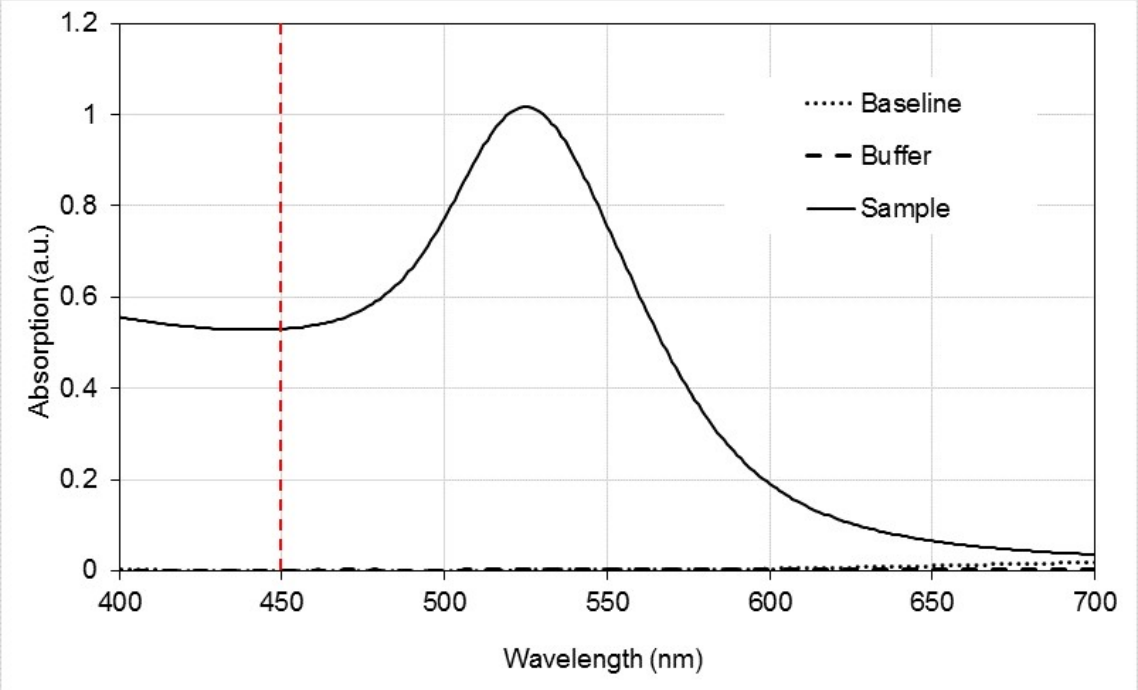

Figure 3: Example of UV-Visible spectrum of undiluted 30 nm Au NPs similar to NPL1.

Here, the measurements were taken with a Perkin Elmer LAMBDA 850 using a quartz cuvette with optical path length of 1 cm. The optical absorption measured at 450 nm after blank correction is 0.556 ± 0.036, where the uncertainty corresponds to the standard deviation calculated from three repeated measurement results. The molar extinction coefficient of these particles is equal to $2.00 \cdot 10^{9}$ $M^{-1}cm^{-1}$ with a 30% relative uncertainty. The resulting NP concentration is $2.78 \cdot 10^{-10}$ M, which equates to $(1.85 \pm 0.67) \cdot 10^{11}$ NPs/mL.

CLS MEASUREMENTS

The instrument should be operated under conditions that give the most stable performance. For the optimum inter-comparison, a common set of operating conditions is needed, however due to the variety of instruments available, no single set of conditions can do this. Instead we give general conditions for guidance to improve comparability.

1.13 TEMPERATURE

Analysis at room temperature is preferred. Under no circumstances should the samples be exposed to temperatures above 45 °C or below freezing. It is known that for some instruments the temperature of the medium containing the sample will raise with respect to room temperature. Please record this temperature at the end of the measurement on the data reporting spreadsheet.

If the samples and dispersion media have been stored in the fridge, they have to be brought to room temperature before the measurements are performed.

1.14 DETECTOR POSITION FOR LINE-START CLS

In order to know the density and viscosity of the gradient at the detector, it is recommended that the detector position is measured. One way to measure it is to inject subsequent volumes of water in the spinning disc and record the detector readings. This is the procedure:

- Set the disc spinning at about 5,000 rpm.
- Start a calibration, but without actually injecting the calibrant.
- Record the initial detector reading.
- Inject 200 µL of clean water and record the detector reading 10 s after injection.
- Continue to inject 200 µL of clean water and record the detector reading 10 s after injection. After a number of injections, you will see the readings decreasing and then rising again to then reach a plateaux (see Figure 4).
- The total volume of liquid injected at the minimum of this curve provides indication of the position of the detector.
- For example, in Figure 4 the volume of liquid between the edge of the disc and the position of the detector is 4 mL. This means that if, for example, the sucrose gradient is produced by injecting a total volume of sucrose of 14.4 mL, the portion of gradient that the particle travel through is only 10.4 mL. The maximum density experienced by the particles is about 72% of the maximum density of the gradient.
- Please calculate the average density and viscosity of the gradient between the gradient's meniscus and the position of the detector and calculate the refractive index of the gradient at position of the detector. Use these values in the instrument software.

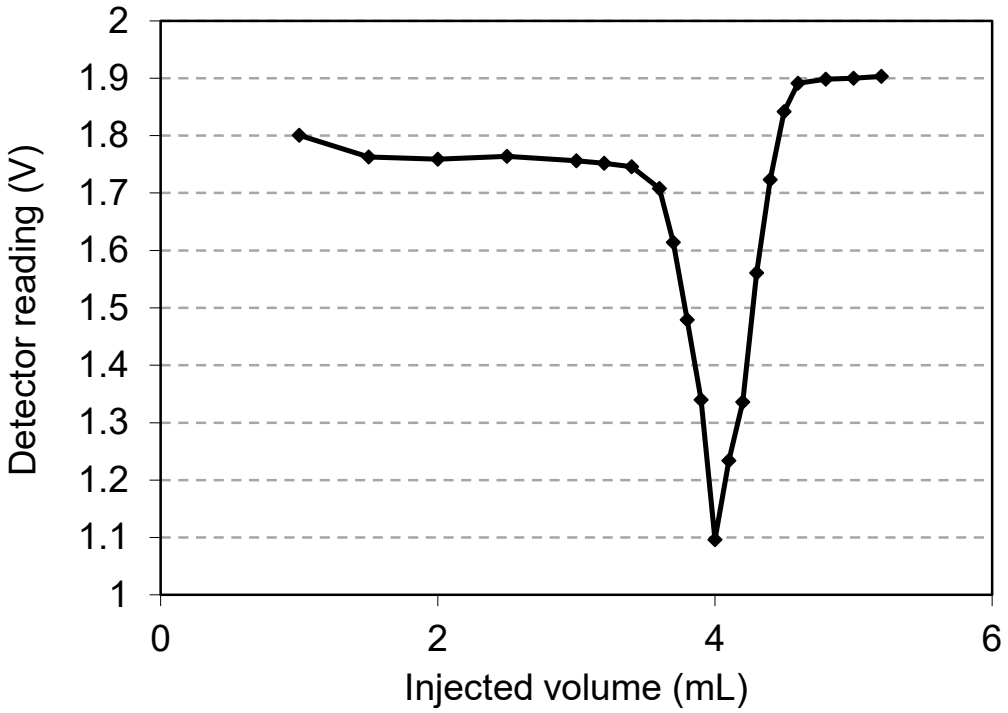

Figure 4: Detector reading as a function of injected volume of water to determine the detector position.

1.15 GRADIENT FOR LINE START DISC CENTRIFUGES

Prepare a sucrose (or equivalent) gradient to perform the measurements as recommended by the manufacturer. We suggest to use minimum and maximum sucrose concentrations of 8 % (w/w) and 24 % (w/w) respectively. A typical total sucrose volume is 14.4 mL (made of 12 injections of 1.2 mL each). Use 0.5 mL of a capping liquid such as dodecane as a final injection to prevent gradient evaporation.

1.16 MEASUREMENT PROTOCOL

It is recommended that the instrument is regularly tested as indicated by the manufacturer. It is generally recommended to switch on the instrument at least 1 day before any measurement is taken to ensure equilibrium conditions are reached. Please follow the instrument manufacturer's guidelines in regard.

All the measurements should be performed in the same measurement session where possible.

For each sample, measure the size distribution of at least three aliquots. For NPL1, measurements should be repeated on at least three independent aliquots, each one from one of the three vials provided. Also measure 2 additional independent aliquots of one of the vials of NPL1. For NPL2 and NPL3 measurements should be performed on at least three different aliquots for each sample.

Set the instrumental parameters, including the rotational speed, as you consider is best for the instrument performance and record them in the electronic data sheet.

If the instrument requires calibration, perform the calibration before each sample injection using a particle calibrant of which the particle diameter and average effective particle density are accurately known.

Input the necessary data about the material and the experimental set up.

Use 15.0 g/cm^3 for the density of the gold particles and 1 for their non-sphericity factor. This density value takes into account the average density of the particle Stokes' volume (which also include organic and solvent molecules at the surface) as well as the overall effect of any deviation from spherical shape. Using this density value, the modal size of the particle should result close to its average TEM diameter (~31 nm).

Use the appropriate refractive index (*n*) and absorption (*k*) of gold depending on the wavelength used by your instrument. This data is available, for example at the link below:

https://refractiveindex.info/?shelf=main&book=Au&page=Johnson

For example, if your instrument uses a diode laser with emission around 405 nm in wavelength, then the RI for gold is 1.465 and the particle absorption (K) 1.95. We are neglecting here the effect of the particle coating on the RI.

If you need to input data about the gradient, please use the values of its average density, viscosity and that of the refractive index calculated on the portion of gradient between the injection and the detector position. If this is not possible, please use the values that you would normally use for the analysis. In any case, please record the used values in the electronic datasheet.

Vortex both the calibrant and samples before use. Bath sonication of the samples is also allowed, but for no longer than 1 minute.

If you are injecting the sample in the instrument, then weigh the syringe before each injection (loaded with the sample) and after each injection. The difference will provide you with the mass (and therefore the volume assuming this is mainly water) of the sample that was effectively injected. This is needed to compute the mass of the NPs contained in 1 mL of the same solution (see below).

1.17 DATA ANALYSIS

Use your own in-house procedures to calculate the concentration of the samples.

Alternatively, in case of a disc centrifuge, we recommend computing the absolute area under the mass-based size distribution measured by CLS. This area represents the total mass of the NPs contained in the volume of sample that was injected (typically 100 – 200 µL). To estimate the concentration of the particles we recommend to:

- Measure the area under the weight-based size distribution. This is typically measured in µg and is the mass (assuming no losses) of the particles contained in the sample volume injected in the instrument. This volume is given by the difference of the syringe weights before and after sample injection.

- Compute the mass of the NPs contained in 1 mL of the same solution.
- Compute the mass of 1 single NP by modelling this as a sphere having the diameter equal to the mode of the size distribution measured by CLS and the density equal to the density value of the sample particles used for the measurement (i.e. 15.0 g/cm^3)
- Divide the mass of the NPs contained in 1 mL of sample by the mass of one single particle to obtain a measurement of the concentration of the sample in NPs/mL.

Measure in this way the concentration of at least three aliquots of each sample. The concentration of NPL1, NPL2 and NPL3 is then found by computing the average among aliquot results. Report also the standard deviation among the aliquots.

This method has a number of sources of uncertainty associated with it and this ILC will contribute to elucidate them. These uncertainties mainly affect the absolute measurement of particle concentration. However, the measurement of relative concentration is more robust.

1.18 EXAMPLE OF MEASUREMENT

An example of high resolution weight-based size distribution of 30 nm Au NPs similar to sample NPL1 is shown in Figure 5. This was measured by using a line-start disc centrifuge.

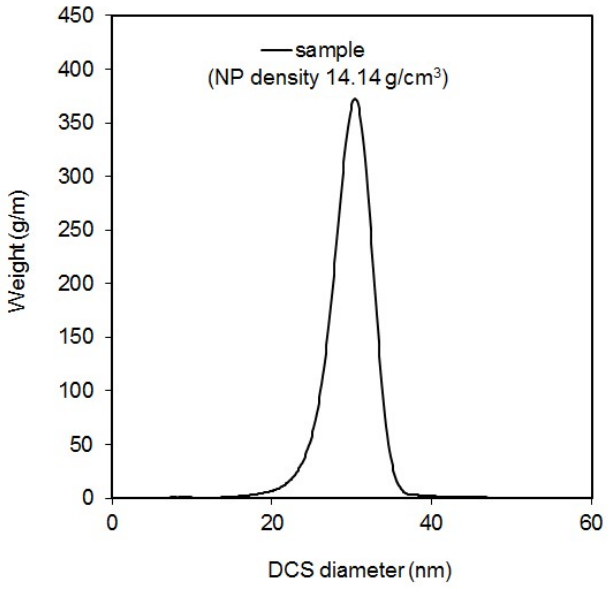

Figure 5: Example of CLS high resolution mass-based size distribution of undiluted 30 nm Au NPs similar to NPL1.

Here, the measurements were taken with a 24,000 CPS Disc centrifuge. The injected volume was measured to be 101.8 µL (instead of 100 µL set in the instrument software). The modal diameter of the particle size distribution is 30.7 nm and the (set) particle density is 14.14 g/cm^3. The area measured under the distribution was 2.30 µg and the injected volume was measured to be 101.8 µL. This is equivalent to a concentration of the sample of $1.7 \cdot 10^{11}$ NPs/mL.

It is interesting to observe that if we were to use a density of the gold particles of 19.3 g/cm^3, we would have measured a sample concentration of $6.5 \cdot 10^{10}$ NPs/mL, which is ~62% that estimated previously.

PTA MEASUREMENTS

This section does not intend to provide a standard operation procedure but guidance to participants on key aspects of PTA procedures that may impact the comparability of results if not properly considered.

1.19 SAMPLE HANDLING

Analysis at room temperature is preferred. Under no circumstances should the samples be exposed to temperatures above 45 °C or below freezing. If the temperature at which the sample is analysed differs significantly from 20 °C, please give details in the 'comments' section of the data reporting spreadsheet. It is recommended that the temperature is controlled and monitored throughout the measurements. Therefore, instruments equipped with a temperature controller are preferred. Samples and buffer should be allowed to reach room temperature before the dilutions and the measurements are performed.

Only sample NPL1 is relevant for PTA. This sample will require dilution before measurement. For dilutions, it is recommended to use trisodium citrate buffer which was provided in the package. If own source of trisodium citrate was used, details should be recorded in the electronic reporting form. The recommended buffer concentration is 1 mM in ultrapure water. The buffer should be filtered after preparation (max pore size of 0.22 µm) and used within 2 weeks. It is recommended to dilute NPL1 sample in 1 mM citrate buffer according to a protocol stated in paragraph 7 (i.e. gravimetrically), so that the final dilution aids in 50-70 particles per field of view. Reference Material (RM) if used should also be diluted in 1 mM citrate buffer. Please note that no treatment should be applied to NPL1 sample, RM or their dilutions other than vortexing or bath sonication for up to 1 min.

1.20 MEASUREMENT PROTOCOL

It is recommended that PTA is calibrated by the manufacturer and its performance is regularly verified by a qualified engineer. Instruments operating either in static or flow modes can be used for the measurements. Please ensure that the mode is recorded in the electronic reporting form.

It is generally recommended to switch on the instrument, at least 30 min. before measurements, to ensure equilibrium conditions are reached. It is recommended to set the instrumental parameters (e.g. focus, camera levels, syringe pump flow, etc.) as considered best for the instrument's performance. It is important to clean the instrument's flow cell and tubing with ultrapure water and dry prior to measurements to ensure minimum background particle counts.

Perform a blank measurement using 1 mM citrate buffer before preparing NPL1 dilutions. If an unusual level of particles is present in the buffer (typically not more than 5 particles per field of view are expected) it is recommended that a fresh buffer is prepared. The concentration of particles measured in the buffer can be taken into account when analysing sample data. Please note that for a fair assessment of the blank contribution, the buffer should be measured and analysed using the same instrument settings as used for the measurements of NPL1 sample. It is also recommended that measurements of the blanks are repeated several times during each measurement session (batch, day etc.).

Measure the 5 vials of the NPL1 sample and record the obtained 5 independent particle number concentration values in the electronic reporting form, together with the dilution factor used and other information. To ensure representative sampling, it is recommended to acquire at least 5 independent videos of 60 s each per aliquot under the repeatability conditions (measurement repeats). The participants should use their own *in house* practise to choose the number of aliquots (i.e. independently prepared dilutions) measured per vial.

It is recommended to also measure RM or Representative Test Material (RTM) at the beginning, in the middle and at the end of each measurement session (batch, day etc.) to monitor the instrument's performance and to estimate the method recovery. Since no such materials are being provided for this study, the participants who wish to perform such measurements are asked to identify suitable materials and record relevant information in the electronic reporting form.

It is recommended to use 1mM citrate buffer to rinse between samples, blanks and RMs if used.

1.21 DATA ANALYSIS

The participants should use their own *in house* practice to calculate the number-based concentration of particles in NPL1 sample and the associated measurement uncertainty. If measurement uncertainty cannot be calculated, standard deviation of the measurement can also be reported. The raw number concentration of the particles *C* (NP/ml) in each aliquot can be calculated as follows:

$$C_i = d_{i,\,NTA} \cdot C_{i,\,meas} \tag{9}$$

where $C_{i,\ \text{meas}}$ is the average particle number concentration of aliquot *i* measured across different repeats and d_{NTA} is the dilution factor.

The associated measurement uncertainty (ΔC) can be calculated as:

$$\Delta C_i = C_i \cdot \sqrt{\left(\frac{\Delta d_{NTA}}{d_{NTA}}\right)^2 + \left(\frac{\Delta C_{i,meas}}{C_{i,meas}}\right)^2} \tag{10}$$

Where Δd_{NTA} is the uncertainty on the dilution factor and $\Delta C_{i,meas}$ is the standard deviation from measurement repeats performed on the same aliquot.

We expect that the measured C_i are consistent and ΔC_i are of the same order for all measurements. If this is not the case, you can reject outlier measurements and provide details of this. In general, the average raw concentration of each sample measured across different aliquots can be expressed as the weighted average of the concentration measured for each aliquot as:

$$C = \frac{\sum_i w_i C_i}{\sum_i w_i} \tag{11}$$

where

$$w_i = \frac{1}{\Delta C_i^2} \tag{12}$$

$$\Delta C = \frac{1}{\sqrt{\sum_i w_i}}$$

and: (13)

It is important to note that the uncertainty estimated in this way only takes into account the repeatability of the method and not the method trueness. This may lead to underestimation of the measurement error and affect the results comparability between the participants and different techniques. Participants can attempt an estimation of the uncertainty associated to the trueness of the method following their own practice. Please give details of this approach. For example, two important parameters to take into account are the Detection Threshold and the Recovery parameters.

The Detection Threshold is set by the user and the related uncertainty can be estimated experimentally by varying it over a reasonable range (input parameter, X axis) and looking at the effect this has on the measured particle number (measurement result, Y axis).

The recovery relates to the fact that there is a bias between the number of particles present in a samples and the number of particles that are actually detected. This bias can be measured by using a RM which is certified for the number concentration of particles (if no certified value for the particle-number concentration is available, this can be calculated from the TEM size and the total element content for non-dissolving particles). The method recovery R_m and its standard uncertainty ΔR_m can be calculated as:

$$R_m = \frac{C_{meas}^{RM}}{C_{RM}} \quad (14)$$

and

$$\frac{\Delta R_m}{R_m} = \sqrt{\left(\frac{\Delta C_{meas}^{RM}}{C_{meas}^{RM}}\right)^2 + \left(\frac{\Delta C_{RM}}{C_{RM}}\right)^2} \quad (15)$$

where C_{RM} is the nominal concentration of the RM and C_{meas}^{RM} is the RM concentration measured by PTA. An improved estimation of the concentration of the particles as measured by NTA C^* is then given by $C^*=C/R_m$.

Since no RM are being provided for this study, the participants who wish to perform such measurements are asked to identify suitable materials and record relevant information in the electronic reporting form.

1.22 EXAMPLE OF MEASUREMENT

An example of 500-fold diluted sample of 30 nm Au NPs similar to sample NPL1 is shown in Figure 6.

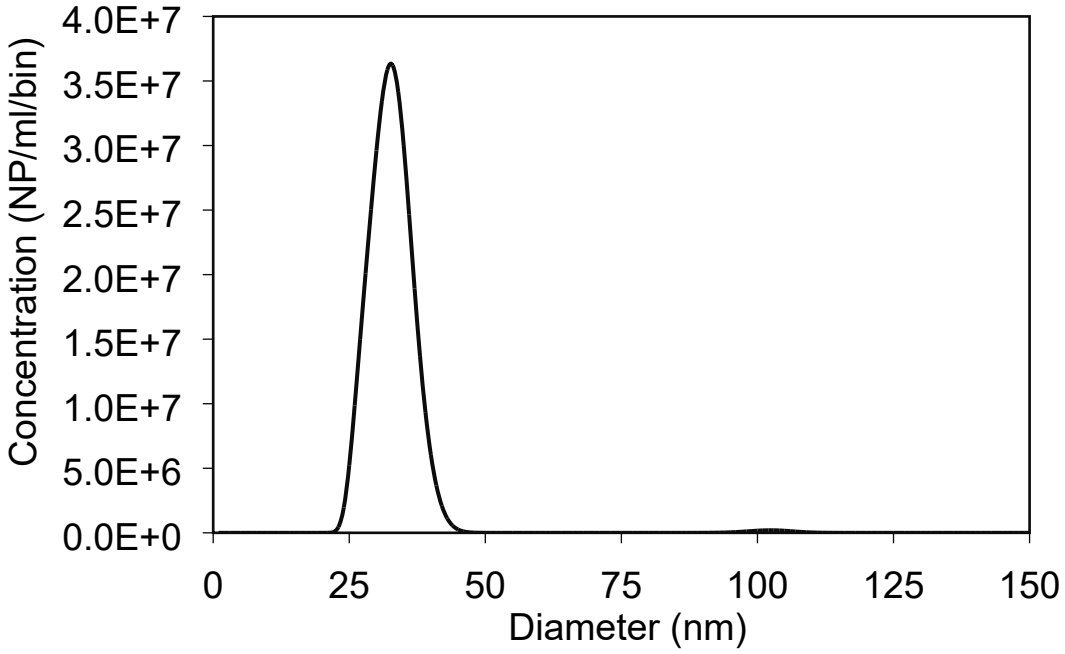

Figure 6: Example of PTA measurements of 500-fold diluted 30 nm Au NPs similar to NPL1.

Here, ~55 particles per field of view were measured. The resulting concentration was ~$3.61 \cdot 10^{8}$ NPs/mL, whilst the calculated particle concentration in the sample was $(1.78 \pm 0.03) \cdot 10^{11}$ NPs/mL (mean ± stdev, n=3).

1.23 FURTHER READING

For detailed information on measurement protocols for PTA method we recommend the ISO 19430 standard entitled "Particle size analysis – Particle tracking analysis (PTA) method". Although the standard is dedicated to size measurements, it is very informative on the use of the PTA method and related analysis.

SPICPMS MEASUREMENTS

This section does not intend to provide a standard operation procedure but guidance to participants on key aspects of spICPMS procedures that may impact the comparability of results if not properly considered.

1.24 SAMPLE HANDLING

Under no circumstances should the samples be exposed to temperatures above 45 °C or below freezing. Samples and buffer should be allowed to reach room temperature before the measurements are performed.

Only sample NPL1 is relevant for spICPMS. This sample will require dilution. For dilutions, it is recommended to use trisodium citrate buffer which was provided in the package. If you use your own source of trisodium citrate details should be recorded in the electronic reporting form. The recommended buffer concentration is 1 mM in ultrapure water. The buffer should be filtered after preparation (max pore size of 0.22 µm) and used within 2 weeks. It is recommended to dilute NPL1 sample in 1 mM citrate buffer according to the protocol stated in section 7 (i.e. gravimetrically), so that the final dilution provides enough particle events to ensure sufficient statistics, but at the same time ensures that only one particle arrives at the detector at a time. Ionic gold standard and RM if used should also be diluted in 1mM citrate buffer. Please note that no other treatment should be applied to NPL1 sample, RM, ionic gold or their dilutions other than vortexing or bath sonication for up to 1 min.

1.25 MEASUREMENT PROTOCOL

It is recommended that the ICPMS performance is verified on day-to-day basis to achieve maximum signal to noise ratio, in particular for the medium *m/z* range or specifically for *m/z* 197. It is important to clean the instrument's glassware, cones and tubing prior to measurements to ensure minimum background signal.

The participants should use their own *in house* capabilities and practice experience of to select the most appropriate dwell time. Overall, dwell time resulting in baseline separation between background signal and particles population are recommended. Guidance for choosing dwell time and analysis time can be found in e.g. ISO/TS 19590:2017 or NIST publication [3]. Information regarding the dwell time used should be recorded in the electronic reporting form.

Transport efficiency should be determined following the *in house* capabilities and practice experience of each participant, but it is recommended to assess transport efficiency at the start, middle and at the end of the measurement session (batch, day etc.) to be able to assess the variability of this parameter. Guidance on transport efficiency determination can be found in e.g. ISO/TS 19590:2017 or NIST publication [3]. Information regarding the transport efficiency determination approach followed should be recorded in the electronic reporting form.

It is recommended to perform a blank measurement using 1 mM citrate buffer. The concentration of particles measured in the buffer can be taken into account when analysing sample data. Please note that for a fair assessment of the blank contribution, the buffer should be measured and analysed using the same instrument settings as used for the measurements of NPL1 sample. It is also recommended that the measurement of the blank is repeated several times during each measurement session.

Measure the 5 vials of the NPL1 sample and record the obtained 5 independent particle number concentration values in the electronic reporting form, together with the dilution factor used and other information. To ensure representative sampling, it is recommended to acquire at least 5 replicate measurements of 1 min long time scans per aliquot under repeatable conditions. The participants should use their own *in house* practice to choose the number of aliquots (i.e. independently diluted) measured per vial.

It is recommended to also measure RM or RTM at the beginning, in the middle and at the end of each measurement session (batch, day etc.) to monitor the instrument's performance. Since no such materials are being provided for this study, the participants who wish to perform such measurements are asked to identify suitable materials and record relevant information in the electronic reporting form.

It is recommended to use Milli-Q water, followed by 3 % Aqua Regia followed by Milli-Q water to rinse between samples, blanks and standards (such as ionic gold or RM) if used.

1.26 DATA ANALYSIS

The participants should use their own *in house* practice to calculate the number-based concentration of particles in NPL1 sample and the associated measurement uncertainty. If measurement uncertainty cannot be calculated, standard deviation of the measurement can also be reported. However, it is important to note that this may lead to underestimation of the measurement error and influence the results comparability between the participants. In case, participants do not have data analysis procedure in place, the number concentration of the particles *C* (NP/g or NP/mL) can be calculated as follows:

The number concentration of the particles C_i (NP/mL) of each aliquot *i* can be calculated as follows:

$$C_i = \frac{N \cdot d_{i,MS}}{\eta \cdot V} \tag{16}$$

Where *N* is the average number of particles detected per 1 min time scan (NP/min), $d_{i,\mathrm{MS}}$ is the dilution factor, η is the average transport efficiency and *V* is the average sample uptake flow (g/min or ml/min).

The uncertainty of the measurement (ΔC_i) can be calculated as as:

$$\Delta C_i = C \cdot \sqrt{\left(\frac{\Delta N}{N}\right)^2 + \left(\frac{\Delta d_{MS}}{d_{MS}}\right)^2 + \left(\frac{\Delta \eta}{\eta}\right)^2 + \left(\frac{\Delta V}{V}\right)^2} \tag{17}$$

Where Δd_{MS} is the uncertainty on the dilution factor and ΔN is the standard deviation from measurement repeats performed on the same aliquot.

We expect that the measured C_i are consistent and ΔC_i are of the same order for all measurements. If this is not the case, you can reject outlier measurements and provide details of this. In general, the average raw concentration of each sample measured across different aliquots can be expressed as the weighted average of the concentration measured for each aliquot as:

$$C = \frac{\sum_i w_i C_i}{\sum_i w_i} \tag{18}$$

where

$$w_i = \frac{1}{\Delta C_i^2} \tag{19}$$

and:

$$\Delta C = \frac{1}{\sqrt{\sum_i w_i}} \tag{20}$$

1.27 EXAMPLE OF ACQUIRED DATA

An example of 4 500 000 - fold diluted sample of 30 nm Au NPs similar to sample NPL1 is shown in Figure 7.

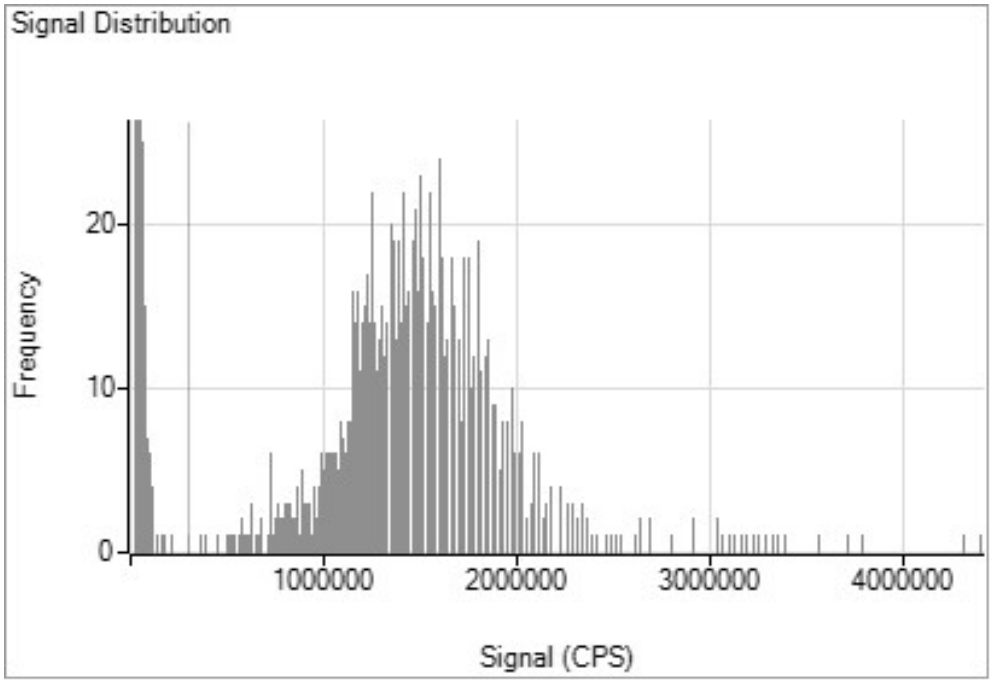

Figure 7: Example of spICPMS measurements of 4,500,000 - fold diluted 30 nm Au NPs similar to NPL1.

Here, ~1000 particles per 1 min time scan were measured. The calculated particle concentration in the sample was $(1.85 \pm 0.04) \cdot 10^{11}$ NPs/g (mean ± stdev, n=3).

DATA REPORTING

An electronic reporting data form is supplied as part of this study. Please report the details of your analysis conditions and methods, alongside your results, in the appropriate section of the spreadsheet. Please also return raw data in the spreadsheet or in .txt format, with the filename for each dataset clearly noted in the 'data summary' part of the data reporting spreadsheet.

CONFIDENTIALITY

The samples supplied in this ILC are not certified reference materials, but have been produced to the best of our abilities. They are sent to you in confidence, and if there are any problems with them we ask that you contact us immediately so that we can determine whether the problem is generic or restricted to a single batch or sample.

Please do not publish your individual results or any further or different analyses of these materials without consulting or informing NPL.

A coded name for the participating laboratories will be utilised in any report or publication from this study so that laboratory anonymity is maintained. If, for commercial reasons or others, you do not wish to be identified in our final report, please note this in your report.

QUESTIONS

If you have any questions or are unsure of any then do contact Caterina Minelli (caterina.minelli@npl.co.uk).

16 ACKNOWLEDGEMENTS

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