Project:

Particle Tracking Analysis

Measuring the Size and Concentration of Nanoparticles using Particle Tracking Analysis (PTA)

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1 Introduction

The sizing and concentration measurement of particles in the nanometre range can be performed directly in aqueous solutions using particle tracking analysis (PTA). This protocol provides procedures for sample preparation and the calculation of the "real" size-distribution for diluted particles in the sub-micrometre range, along with the determination of the particle concentration in particles per millilitre (NPs/mL).

PTA is a companion technique to Dynamic Light Scattering (DLS) (EUNCL-PCC-001) as it measures the hydrodynamic diameter (or radius) of the particles in solution. It is an absolute measurement and as such is not weighted towards particle averages, and thus allows for the measurement of monodisperse and complex sample mixtures.

This SOP deals with the measurement of the hydrodynamic diameter (and its distribution) of NPs dispersed in aqueous solutions, through the determination of their diffusion coefficient. Guidelines for making successful PTA measurements are provided, as well as a discussion of relevant standards for quality control and criteria for data analysis. This document describes the SOPs to be used with the PTA system produced by Malvern (Nanoparticle Tracking Analysis (NTA), Nanosight series of products). The protocol should be modified to be applicable to other systems

2 Principle of the Method

Submicron NPs dispersed in a liquid are in constant random Brownian motion due to the interaction with the liquid molecules. At very low concentration the movement of particles depends by the liquid viscosity, temperature, and the size of particles. This relationship is expressed by the Stokes-Einstein equation used to calculate the hydrodynamic diameter, d_H, from the measured diffusion coefficient.

$$d_H = kT/3\pi\eta D$$

where k in the Boltzmann constant, T is the temperature, η is the viscosity of the liquid and D is the diffusion coefficient.

By definition, the hydrodynamic diameter is the diameter of a hypothetical hard sphere that diffuses with the same speed as the particle being measured. In practice, particles are solvated and can be spherical, spherical-like, or non-spherical, while moving dynamically in solution. The determined diameter is therefore an indicator of the apparent size of the solvated particle that is approximated as being spherical. As the particle surface becomes modified, either through functionalisation, or agglomeration and aggregation, the approximation of the hydrodynamic diameter changes which can lead to uncertainties in the subsequent calculated dimensions. This can be critical in high resolution analysis or using high resolution instruments, where the obtained results can be of questionable validity.

PTA is becoming a standard technique for the characterisation of the nanoparticles (NPs) in suspension, with both being developed into ASTM [1] and ISO standard [2] for the measurement of particle size distributions. Both DLS and PTA operate around the principle of light scattering caused by the NPs moving under Brownian motion, but differ in the manner in which the data is acquired. DLS measures the fluctuations in scattered light intensity with this being correlated to the particle

hydrodynamic diameter *via* the correlation function and the Stokes-Einstein equation [3]. However, due to Rayleigh theory, the intensity of scattered light is proportional to the sixth power of the diameter, and thus the analysis is heavily weighted towards larger particle size. As a result, the presence of NP aggregates will result in a biasing of particle size distribution, resulting in a loss in accurate size determination [4]. More concisely, DLS determines the overall (average) particle behaviour, with a 10^6 bias towards larger particles.

In contrast, PTA is a single particle technique, where the quality of the results is determined by the limits of detection of the equipment, and by the number of particles analysed. However, given enough particles tracked and analysed, PTA can enable the high-resolution true particle size distribution in the sample. Here, the software tracks individual particle movements to calculate the diffusion coefficient for each individual particle. It is a high-resolution analysis technique that is able to distinguish small differences between two particles or populations, either based on diffusion and Brownian motion, or light scattering intensity. The proprietary NTA software for Malvern's system records a series of video files (of typically 30-60 second duration) of the particles viewed and then simultaneously identifies and tracks the centre of each particle on a frame-by-frame basis. The image analysis software then determines the average distance moved by each particle in the x and y directions. This value allows the particle diffusion coefficient (D_t) to be determined and using the Stokes-Einstein equation, the hydrodynamic diameter can be calculated.

3 Applicability and Limitations (Scope)

This SOP deals primarily with the NanoSight series of PTA instruments. There may be difference in the manner in which other PTA systems operate, and as such users should ensure that that they follow the instructions for use provided by the manufacturer in the first instance to ensure correct operation of their instrument.

3.1 Nanoparticle concentration and size limits

NP concentration should be maintained in a range that allows both to obtain a good signal to noise ratio, while at the same time avoiding multiple scattering interferences. NTA has been shown to have good linearity in the measurement of between 8.6E6 and 5.7E9 particles per mL [5]. This covers the optimal NTA concentration range, allowing accurate, reproducible concentration results at the extremes of concentration for accurate NTA particle sizing. In some cases, it may be necessary to dilute the samples (see later for procedure). A good parameter to keep track of the concentration of the samples is the particles per frame indicator in the NTA software.

The size limit for detection is strongly dependent on the refractive index and light scattering potential of the nanoparticle under assessment. NTA has been validated by means of an interlaboratory comparisons to have a limit of detection between 30-600 nm [5]. Particle outside of this window may still be detected, based on material type, and system configuration.

Table 1: Approximate lower limits of detection for monodisperse suspensions of nanoparticles from ISO 19430:2016 [2]

Particle Material	Lower detection limit (hydrodynamic diameter in nm)
Gold	15
Polystyrene	45
Silica	75
Biological Material	60
Other metals	25

3.2 Camera dynamic range and laser setup

While the visualisation of particle light scattering is a distinct advantage for PTA over other techniques, the dynamic range of the camera and the laser setup can also introduce the errors and uncertainties. Under Rayleigh and Mie Scattering, a shorter wavelength light source is required to visualise smaller nanoparticles due to their light scattering potential. The use of a short wavelength laser in NTA setups, such as the 405 nm laser, can allow for the detection of the smaller components of a polydispersed sample. However, this can cause the larger particles to blind the detector, leading to the small particles becoming obscured.

3.3 Temperature control

In the Stokes-Einstein equation the hydrodynamic diameter is a function of diffusion coefficient, solvent viscosity and temperature. Therefore, during the measurement the temperature of the cuvette has to be stable. For this reason, an accurate and robust measurement of the cell temperature is required.

As the mean step size of a particle is portions to its diffusion. As such, an increase of the temperature increases the squared mean travelled distance. In addition, slight differences in the temperature field can lead to convection currents, altering the movement and diffusion of the particles.

3.4 Effects of Salt Concentration, Stabilizers, and pH

Like DLS (EUNCL-PCC-001), the hydrodynamic size derived from NTA can depend on the salt concentration of the suspending medium, pH and concentration of dispersant. In deionized water the additional drag induced by the extension of the double-layer into adjacent bulk solution causes an apparent increase in size. This effect can be particularly relevant for small particles. Therefore, a preliminary study of the effects of salt concentration could be useful in some situations. In some cases, NP are stabilized by the presence of dispersants (such as citrate for gold and silver NP, or surfactants) and their dispersions are stable only in a limited range of pH values. When performing sample dilutions to obtain the optimal concentration for NTA measurement it is important to ensure that the pH and dispersant concentration are kept constant. The reduction of dispersant concentration and changes in pH can lead to NP aggregation and increase in polydispersity of the sample.

The viscosity of the solvent has a direct effect on the Brownian motion of the particles, and therefore on their sizes. Increased viscosity solvents, relative to that of water, can impede the diffusion of the particles, making them appear slower, and ultimately resulting in a larger calculated size. In general, the viscosity of the solvent directly depends on the type of solvent being used, its purity and its temperature. Similarly, different solvents or solvent mixtures can affect the hydrodynamic diameter of the particles though van der Waals interactions and binding of solvent molecules to the particle.

4 Equipment and Reagents

4.1 Equipment

- PTA system such as a Nanosight LM10, NS300 or NS500 (Malvern Panalytical) or equivalent
- PTA software such as NTA version 3.1, or above (Malvern Panalytical) or equivalent
    - NTA concentration upgrade is recommended for concentration measurements
- Disposable syringes (LM10, NS300 systems with/without syringe pump)
- Sample container/holder (NS500)

4.2 Standards for QC

It is advisable to select a standard in the same size ranges of the NPs to be analysed. For example, two different polystyrene nanosphere size standards (NIST Traceable, distributed by Thermoscientific), with diameters of 60 and 203 nm can be used, accordingly to the size range of the NPs you are planning to image (60 nm standard for NPs with average diameter<100-150 nm, 200 nm standard for NPs with a diameter >150 nm). Details of reference samples, along with the expected mode sizes and coefficients of variation (% CV) are available in [5, 6]. Samples should be prepared in high quality, particle free (less than 3 particles per frame) water. If a solvent other than water is to be used, the viscosity of the solvent should be entered into the software.

In the case of concentration measurements, it is recommended that the concentration upgrade is applied (optional instrument specific calibration available for purchase from Malvern Panalytical). This reduced the error in a concentration measurement from 170 % to 10 % [5]. If this is not available, calibration standards should be obtained, with similar light scattering properties to the test material, in order to develop a calibration curve of measured versus theoretical particles concentrations.

4.3 Measurement procedure

4.3.1 System setup

4.3.1.1 Turn on instrument and PC, and launch NTA software to ensure the NTA instrument and hardware is detected

4.3.1.2 Clean optical surface of the laser using lens cleaning tissue and compressed dry air

4.3.1.3 Install the flow cell by lining it up with guide-pins, and securing with spring loaded screws

4.3.1.4 Secure screws sufficiently by hand to prevent leaks while ensuring not to strain the flow cell or laser module

4.3.1.5 LM10/NS300 models:

a. Rinse flow cell with ethanol, followed by water using clean syringes

4.3.1.6 NS500 models:

a. Ensure clean deionized water is in diluent bottle

b. Place sample line into waste connector

c. Select “prime fluidics” in software “Hardware” tab

4.3.2 Preparation of the sample

The optimal determined by the nature (light scattering properties) of the analyte NPs, and it has to be defined case by case. As a starting indication the optimal sample concentration is reached when the particles per frame is between 10-100 distinct particles, as shown in Figure 1. For more robust size and concentration measurements this should be lower, in the region of 10-50 particles per frame (see Figure 1c). It may be necessary to dilute the sample to reach the ideal concentration, ideally diluting them by serial 1:10 dilutions. In this case, care should be taken to control the ionic strength, stabilizer concentration and pH of the dispersants. All these parameters should be documented and reported.

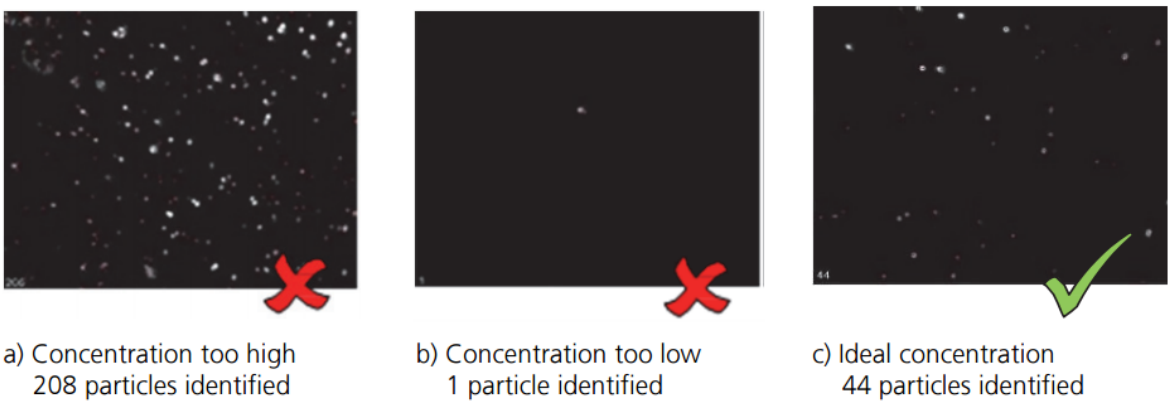


Figure 1: Representative images of particle concentrations for size and concentration measurements of a sample by NTA.

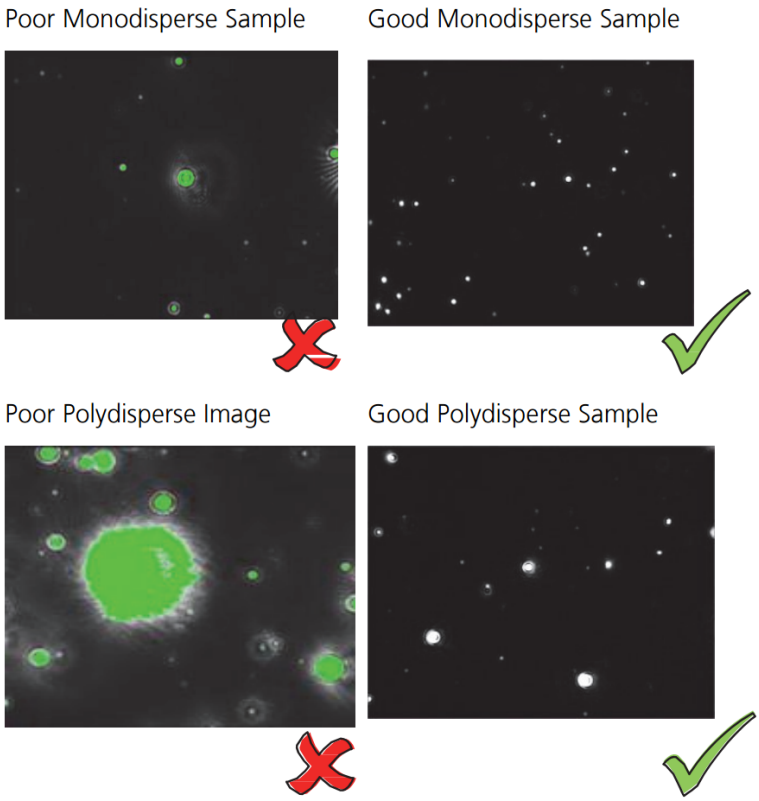


Figure 2: Examples of good and bad camera and focus levels for monodisperse and polydisperse samples

4.3.3 Loading the sample

Correct image camera focus sample should be clearly visible as in shown in Figure 2 (right hand side). Procedure to achieve this is below reported.

4.3.3.1 LM10/NS300 models:

a. Using a 1 mL syringe without needle, introduce the sample into the cell slowly while holding the cell such that the exit port of the flow cell is orientated upwards
b. If during loading, air bubbles are introduced, remove the sample, and reload into the cell
c. Connect the laser to the power cable and switch on laser

4.3.3.2 NS500 models:

a. Place sample in the sample holder and place the sample line into the sample
b. Select load option in software "Hardware" tab

4.3.3.3 All models

a. Select "Start Camera" in NTA
b. Ensure temperature of system is being read appropriately
c. Adjust camera level and focus to ensure that clear distinct particles are visible as shown below (Figure 2)
d. These settings must not be changed between videos captures on the sample the sample

4.3.4 Measuring a sample

Procedure for creating the NTA SOP and entering dilution and viscosity data is below detailed, as shown in Figure 3.

4.3.4.1 Select the SOP tab in the NTA software

4.3.4.2 Select "Standard measurement" from drop-down list

4.3.4.3 Enter the number of captures required, and their duration

a. Typically, 6 captures of 60 seconds in duration

4.3.4.4 Select the name and location for the captured video files by pressing "…" button

a. Store videos in an appropriate folder using a naming scheme that facilitates easier retrieval of the raw data
b. Press "advanced" button to enter dilution factors or solvent viscosities
Click "OK" to store data

4.3.4.5 Click "Create and run script" button

4.3.4.6 A report details prompt will appear

a. Complete form by entering operator name, sample description and any other details required
b. Click "OK" to save data

4.3.4.7 A prompt asking to confirm camera levels will appear

a. Confirm particles are still in focus and camera level is acceptable
b. Click "OK"

4.3.4.8 Follow any additional prompts that may appear

a. LM10/NS300: push through ~0.05 mL using the syringe to load (advance) new sample

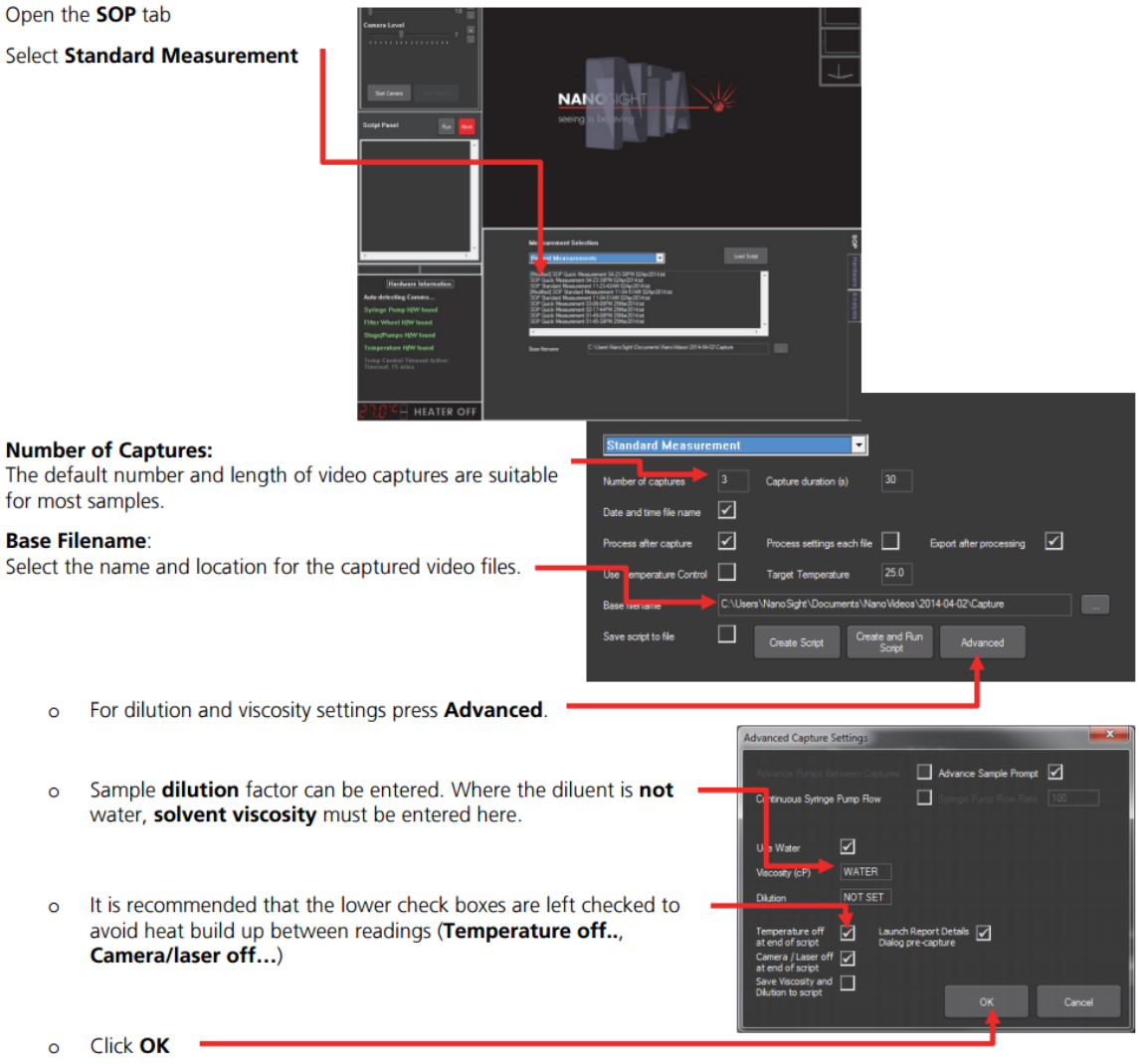


Figure 3: Procedure for creating the NTA SOP and entering dilution and viscosity data

Once the videos have been recorded, analyse the particles as shown in the examples provided in Figure 4.

4.3.4.9 User message asking to adjust detection threshold will appear (see Figure 4).

a. Adjust “detection threshold” slider with + and – buttons to identify the centre of each particle
b. If threshold is too low, “noise” can be tracked
c. If threshold is too high, particles can be excluded
d. Aim to have between 10-40 particle centres per field of view (as shown in Figure 1)
    i. This can be checked by looking at the number in bottom right corner of the video
e. When the user is satisfied with the settings, press “OK” in the user message window

4.3.4.10 After processing is complete and new “export settings” window will open

a. Ensure “include PDF” and “Experiment Summary” are ticked

4.3.4.11 Open “Experiment Summary” file (.csv) in the location where the videos were saved

a. Check “information” section for errors or warning such as high noise, and check for validity of concentration measurements
b. Repeat experiment if necessary/possible if high noise detected, or concertation measurements are invalid

4.3.4.12 LM10/NS300 models

a. Withdraw sample from the flow cell and discard to waste
b. Wash flow cell with deionised water

4.3.4.13 NS500 models

a. Once analysis is complete, remove sample inlet tubing from sample tube and place back in waste connector
b. Press “flush” in Pump/stage settings window in “hardware” tab

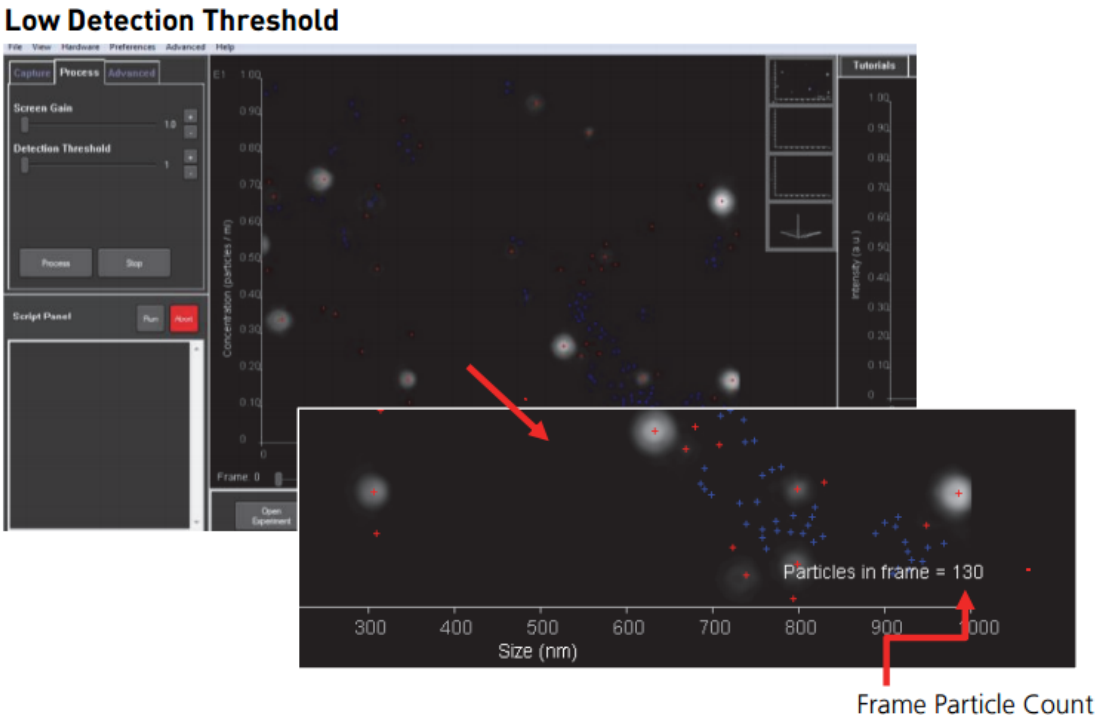


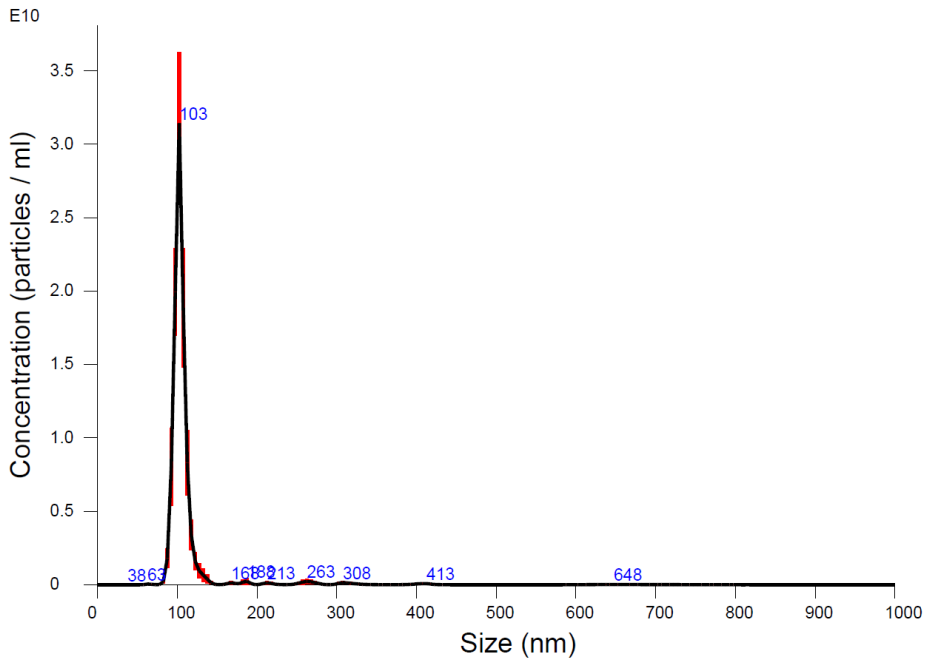
Figure 4: Representative images of high and low detection thresholds. Low thresholds result in noise in the sample being counted and tracked as "true particles" (blue crosses: these should be less than 5 per frame). High thresholds can result in particles being excluded.

4.4 Reporting Data

- Critical information that should be reported includes: particle concentration, sample preparation (dilutions, pre-treatments, etc.), dispersion media composition including NTA size distribution plot, measurement temperature, and any filtration process or procedure used to remove aggregates
- The size of the particles in the sample are generally referred to by the mode diameter ± the standard error of the mean. If the sample contains multiple components, the peak for these should be included
- The distribution percentages (D10, D50, D90 values) can also be included as they give information about the broadness of the sample distribution, as per ISO 19430
- It is good practice to include the measurement results for any calibration or QC standard used
- Full details of reporting requirements are laid down in ISO 19430 and ISO 17025., with additional guidelines available in PD 6699-1:2007 [7] and Thomas *et al.* (2011) [8].

Table 2 reports the details of the above and provide a snapshot of the essential recorded data.

Table 2: Minimum details to report for PTA/NTA measurements as per ISO 19430 and ISO 17025

Sample	Dispersing Media	Number of videos and duration	Concentration (NPs/mL)	Mean size ± SEM (nm)	Mode size ± SEM (nm)	D10 size ± SEM (nm)	D50 size ± SEM (nm)	D90 size ± SEM (nm)
100 nm PSL	DI water	6 videos @ 60 seconds	(9.93 ± 0.43) x 10^{10}	109.6 ± 3.2	101.8 ± 1.1	90.1 ± 1.2	98.1 ± 1.0	110.9 ± 3.8
								

4.5 Flow chart

Flow chart for creating the NTA setup and measurement workflow is shown in Figure 5.

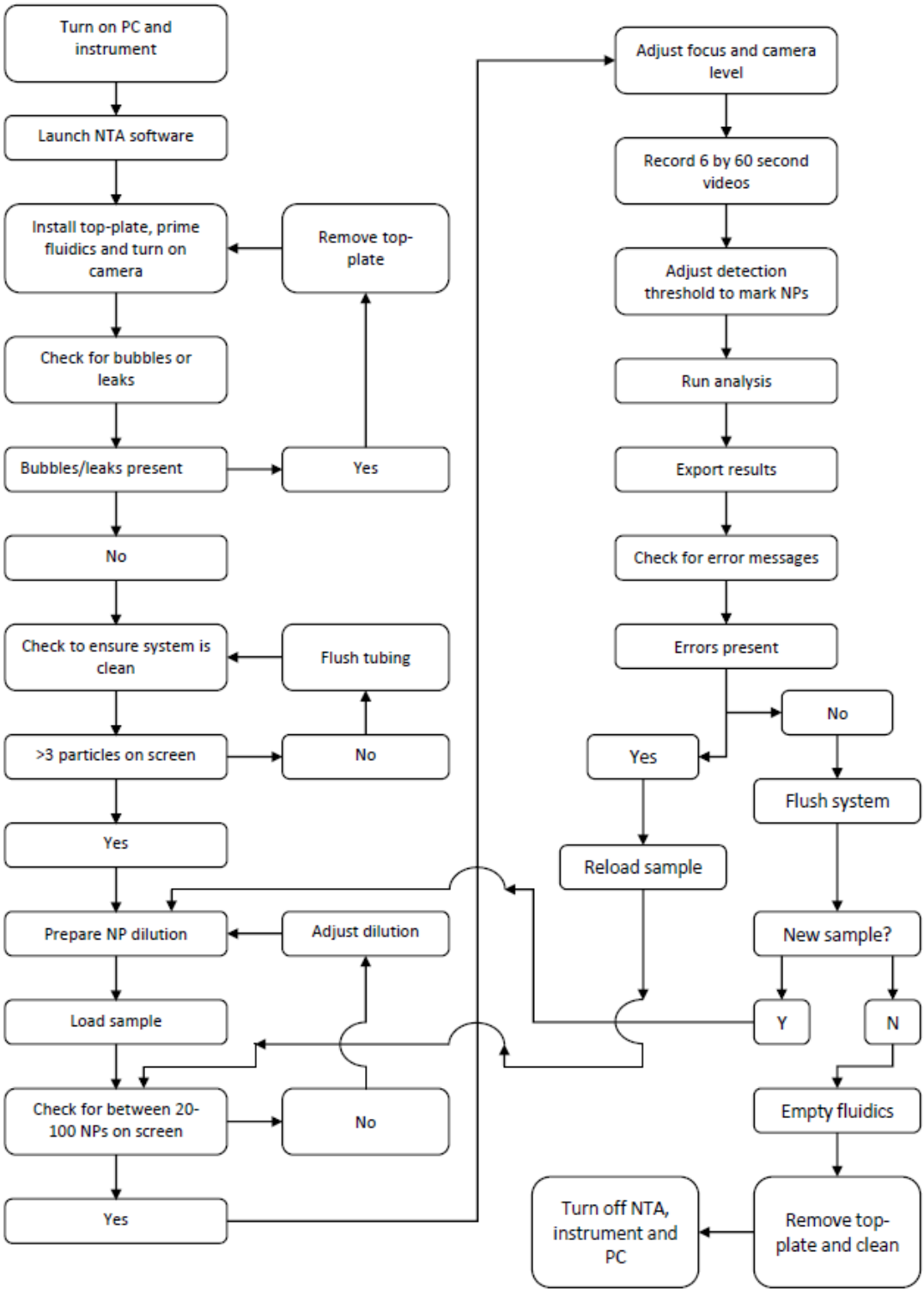


Figure 5: Simplified flow chart for NTA setup, sample loading and analysis of said sample by NTA. Flow chart is based on setup and loading procedure for NS500 running NTA version 3.2. Setup for other systems (LM10, NS300, non-Malvern Panalytical systems) may vary slightly.

5 Quality Control, Quality Assurance, Acceptance Criteria

5.1 Instrument verification

PTA is an absolute method and therefore requires no user calibration. Note that the image size calibration (nm/pixel) is usually performed by the manufacturer and should not require the attention of the operator. However, if the instrument has been modified or new optics have been installed then such a calibration should be performed according to the manufacturer's instructions.

As with other methods (particularly DLS: EUNCL-PCC-001), the instrument should be verified by using an appropriate quality control standard. As the technique is complementary to DLS, similar approaches to instrument verification apply. PTA results are method-defined; hence comparison of measurement results with certified values only makes sense if the certified values were also derived by PTA. If such certified reference materials are not available, values derived by DLS for highly monodisperse suspensions of spherical particles can be an alternative. Alternatively, results from other methods obtained on spherical particles can be used.

For this reason, it is recommended to run a NIST traceable standard before starting each measurement session. Below is a table of accuracy requirements for 100 nm and 150 nm sized particles, as recommended by ISO 19430 (Table 3). Deviations beyond the above stated limits indicate that a problem may exist with the instrument performance, the measurement cell, or the water used to dilute the standard prior to measurement.

Expected values for other materials are also available in the literature [5, 6].

Table 3: Accuracy requirements summary for NTA as per ISO 19430:2016 [2]

	Deviation from the certified values	CV of measured modal values	Recommended number of analysed tracks
100 nm sized particles	± 6 nm	± 6 %	1000
150 nm sized particles	± 9 nm	± 6 %	1000

5.2 Concentration measurement uncertainties

Most PTA systems give an indication of the number of particles in the field of view and this can be used to estimate the total number concentration in the sampling volume, which is instrument specific, but typically between 0.1-1 nL. The uncertainties involved in this calculation are related to the optical properties of the instrument and the polydispersity of the sample. Due to a finite depth of focus (typically ~10 µm), the particles are tracked and counted in that volume only.

Measurement of particles critically depends on the ability of the PTA system to detect them. Larger particles can be detected with more ease than smaller ones. Samples that contain particles of very different sizes may therefore oversample (or overcount) larger particles. Variations in laser setup and camera sensitivity can therefore result in differences in results across different systems. Due to the statistical nature of this measurement the particle track lengths vary. Tracks that are too short or intersecting tracks are rejected by the processing software. Some instrument manufacturers employ

an optimization procedure that optimises the threshold automatically whereas some allow users to set the minimum threshold of tracks manually.

Another effect of large particles is related to their dynamics, smaller particles on average move greater distances than larger particles between frames. In some cases, these particles exit the field of view and in that case, may be disregarded from the calculation. Larger particles at the same edge of the field of view are more likely to be tracked for longer thus contributing more significantly to the overall count. Conversely, a small particle outside the field of view has a greater chance of more rapidly entering the field of view than a large particle at that same position. Therefore, providing the number of particles of a given size *per frame* are being reported on, this in itself does not lead to a bias in the measurement.

The use of the "Concentration upgrade" for the Malvern Panalytical NTA systems can help reduce some of these uncertainties, particularly relation to laser and camera settings. Other manufactures may offer similar options.

5.3 Acceptance criteria for comparison of size and concentration measurements

As detailed in the ASTM and to the ISO standard guidelines [1, 2], a maximum variation of 10% relative standard deviation in the reported modal value which is acceptable when comparing data measured for a sample in the same conditions, if at least 3 measurements are compared.

Similarly, the experimental data files for the measurements detail warnings and cautionary warning relating to vibration and noise levels. If vibration is above 10%, videos should be re-recorded. The reliability of the concentration measurements can also be confirmed (unreliable, use with caution, or ok).

6 Health and Safety Warnings, Cautions and Waste Treatment

To minimize exposure; appropriate safety precautions and protective gear such as gloves, lab coat and goggles must be worn. After the measurement the samples should be discharged as appropriate for nanomaterials.

7 Abbreviations

DLS: dynamic light scattering

NTA: Nanoparticle Tracking Analysis

PTA: Particle Tracking Analysis

NIST: National Institute of Standards and Technology

NP: Nanoparticle

8 Related Documents

Document ID	Document Title	Source
EUNCL-PCC-001	Measuring Batch Mode DLS	http://www.euncl.eu/about-us/assay-cascade/PDFs/Prescreening/EUNCL-PCC-001.pdf?m=1468937875&
EUNCL-PCC-013	Measurement of pH	http://www.euncl.eu/about-us/assay-cascade/PDFs/Prescreening/EUNCL-PCC-013.pdf?m=1468937878&
EUNCL-PCC-021	Measuring NP Aggregation Propensities	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-021.pdf?m=1468937870&

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