# Journal of Visualized Experiments

# UV-Vis Spectroscopy Characterization of Nanomaterials in Aqueous Media

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE61764R1 |
| **Full Title:** | UV-Vis Spectroscopy Characterization of Nanomaterials in Aqueous Media |
| **Corresponding Author:** | Eugenia Valsami-Jones, PhD<br>University of Birmingham<br>Birmingham, West Midlands UNITED KINGDOM |
| **Corresponding Author's Institution:** | University of Birmingham |
| **Corresponding Author E-Mail:** | E.ValsamiJones@bham.ac.uk |
| **Order of Authors:** | Eugenia Valsami-Jones, PhD |
| | Ana C. Quevedo |
| | Emily Guggenheim |
| | Sophie M. Briffa |
| | Jessica Adams |
| | Stephen Lofts |
| | Minjeong Kwak |
| | Tae Geol Lee |
| | Colin Johnston |
| | Stephan Wagner |
| | Timothy R. Holbrook |
| | Yves U. Hachenberger |
| | Jutta Tentschert |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Open Access (US$4,200) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Birmingham, West midlands, United Kingdom |
| Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below: | I agree to the UK Author License Agreement (for UK authors only) |
| Please specify the section of the submitted manuscript. | Engineering |
| Please provide any comments to the journal here. | |

# UV-Vis Spectroscopic Characterization of Nanomaterials in Aqueous Media

Ana C. Quevedo[1], Emily Guggenheim[1], Sophie M. Briffa[1], Jessica Adams[2,3], Stephen Lofts[2,] Minjeong Kwak[4], Tae Geol Lee[4], Colin Johnston[5], Stephan Wagner[6], Timothy R. Holbrook[6], Yves U. Hachenberger[7], Jutta Tentschert[7], and Eugenia Valsami-Jones[1]

[1] School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, UK
[2] UK Centre for Ecology and Hydrology, Lancaster Environment Centre, Lancaster, UK
[3] Natural England, Foss House, Kings Pool, 1-2 Peasholme Green, York, UK
[4] Center for Nanosafety Metrology, Korea Research Institute of Standards and Science (KRISS), Daejeon, Republic of Korea
[5] Department of Materials, University of Oxford, Begbroke Science Park, Oxford, UK
[6] Helmholtz-Centre for Environmental Research, Department of Analytical Chemistry, Permoserstraße 15, Leipzig, Germany
[7] Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, Berlin, Germany

Ana C. Quevedo (AIC585@bham.ac.uk)
Emily Guggenheim (ejg843@gmail.com)
Sophie M. Briffa (S.M.Briffa@bham.ac.uk)
Jessica Adams (adamsjl2@hotmail.co.uk)
Stephen Lofts (stlo@ceh.ac.uk)
Minjeong Kwak (kwakmj@kriss.re.kr)
Tae Geol Lee (tglee@kriss.re.kr)
Colin Johnston (colin.johnston@materials.ox.ac.uk)
Stephan Wagner (stephan.wagner@ufz.de)
Timothy R. Holbrook (timothy-ronald.holbrook@ufz.de)
Yves U. Hachenberger (Yves.Hachenberger@bfr.bund.de)
Jutta Tentschert (Jutta.Tentschert@bfr.bund.de)

*Corresponding author:
Eugenia Valsami-Jones (E.ValsamiJones@bham.ac.uk)

**KEYWORDS:**

Ultraviolet-Visible Spectroscopy (UV-Vis), Gold nanoparticles (AuNP), Characterization, Interlaboratory Comparison (ILC), Standard Operating Procedure (SOP), Nanomaterials.

## SUMMARY:

This study presents the benchmarking results for an interlaboratory comparison (ILC) designed to test the standard operating procedure (SOP) developed for gold (Au) colloid dispersions characterized by ultraviolet-visible Spectroscopy (UV-Vis), amongst six partners from the H2020 ACEnano project for sample preparation, measurement, and analysis of the results.

## ABSTRACT:

The physicochemical characterization of nanomaterials (NMs) is often an analytical challenge, due to their small size (at least one dimension in the nanoscale, i.e. 1–100 nm), dynamic nature, and diverse properties. At the same time, reliable and repeatable characterization is paramount to ensure safety and quality in the manufacturing of NM-bearing products. There are several methods available to monitor and achieve reliable measurement of nanoscale-related properties, one example of which is Ultraviolet-Visible Spectroscopy (UV-Vis). This is a well-established, simple, and inexpensive technique that provides non-invasive and fast real-time screening evaluation of NM size, concentration, and aggregation state. Such features make UV-Vis an ideal methodology to assess the proficiency testing schemes (PTS) of a validated standard operating procedure (SOP) intended to evaluate the performance and reproducibility of a characterization method. In this paper, the PTS of six partner laboratories from the H2020 project ACEnano were assessed through an interlaboratory comparison (ILC). Standard gold (Au) colloid suspensions of different sizes (ranging 5–100 nm) were characterized by UV-Vis at the different institutions to develop an implementable and robust protocol for NM size characterization.

## INTRODUCTION:

Nanomaterials (NMs) have become popular due to their unique properties in the nanoscale (1 to 100 nm), which differ from the properties of their bulk counterparts, either due to size-related or quantum effects (e.g., increased specific surface area by volume) along with distinct reactivity, optical, thermal, electrical, and magnetic properties[1,2]. The potential applications of NMs in society are diverse and widely related to fields such as health care, food industry, cosmetics, paints, coatings, and electronics[3-5]. Gold nanoparticles (AuNPs) are widely applied in nanotechnology (e.g., in health care, cosmetics, and electronic applications), mainly due to their simple fabrication, size-dependent optical features, surface functionalization potential, and physicochemical properties, which can be suitable for many key applications[6,7].

Quality and reproducibility in the synthesis and characterization of NMs are extremely important for quality assurance, but also for the safe manufacture of nano-based products, especially due to the reactivity of NMs, notably in complex environments, where NM properties, such as size distribution and morphology, may undergo rapid changes[8,9]. Numerous methods are available to monitor nanoscale-related properties. For example, scanning/transmission electron microscopy (SEM/TEM) are techniques used to obtain high-resolution (down to sub-nanometer) optical and compositional information of NMs; atomic force microscopy (AFM) provides nanoscale resolution in the vertical (z axis) dimension; and X-ray diffraction (XRD) provides information on the atomic structure of NMs; all these methods can only be used on dry samples (powders)[10,11]. Techniques suitable for the characterization of NMs in liquid media include field flow fractionation (FFF), which allows the separation of large molecules, aggregates, and particles based on their size; dynamic light scattering (DLS); and nanoparticle tracking analysis (NTA)—two methods widely used to determine the size distribution profile of particles using Brownian motion—and ultraviolet-visible spectrophotometry (UV-Vis), which allows the assessment of NM characteristics such as size, aggregation state, and refractive index by a simple absorption measurement[11-13]. Although all these techniques allow NM characterization, their performance is dependent on instrument setup, instrument-related differences, complex methodology for sample preparation, and the user's level of expertise. Moreover, most of the techniques do not allow real-time monitoring of NM size, sample integrity, or differentiation between dispersed or aggregated particles[6]. UV-Vis spectroscopy is a widely used technique that provides non-invasive and fast real-time evaluation of NM size, concentration, and aggregation state. Additionally, it is a simple and inexpensive process with minimal sample preparation, which makes this technique an essential tool that is extensively used in numerous laboratories within many disciplines and markets[6,12,14]. UV-Vis works by measuring the transmittance of electromagnetic radiation of a wavelength between 180 and 1100 nm through a liquid sample. The UV and VIS spectral ranges cover the wavelength range for the ultraviolet (170 nm to 380 nm), visible (380 nm to 780 nm), and near-infrared (780 nm to 3300 nm)[4,14]. The wavelength of light passing through the sample cell is measured; the intensity of light entering the sample is referred to as $I_0$, and the intensity of the light emerging on the other side is designated as $I_1$[14]. The Beer-Lambert law reflects the relationship between A (absorbance) as a function of sample concentration C, the sample extinction coefficient ϵ, and the two intensities[14]. Absorption measurements can be collected at a single wavelength or over an extended spectral range; the measured light transmittance is transformed into an absorbance measurement by following the Beer-Lambert law equation. The standard equation for absorbance is A = εlc, where (A) is the amount of light absorbed by the sample for a given wavelength (ε) is the molar attenuation coefficient (absorbance/(g/dm$^3$) (l) is the distance the light travels through the solution (cm), and (c) is the concentration per unit volume (g/dm$^3$). The absorbance is calculated as the ratio between the intensity of a reference sample ($I_0$) and the unknown sample (I), as described in the following equation[14]:

$$A = log10\left(\frac{I_0}{I}\right) = \varepsilon lC$$

The simplicity of UV-Vis makes it an ideal technique to compare PTS of an established measurement protocol[6,12,15]. The objective of an ILC or PTS is to verify the performance and reproducibility of a method using an SOP[15]. This, in turn, provides a standardized approach for quick characterization of nanoparticle suspensions for other users.

To assess the proficiency, consistency, and reliability of the method presented here, six laboratories participated in an ILC as members of the Horizon 2020 ACEnano project (https://cordis.europa.eu/project/id/720952). The ILC involved UV-Vis characterization of standard Au colloid dispersions of different particle sizes (5–100 nm). An SOP was provided to all the involved laboratories to ensure the identical preparation of AuNP suspensions, evaluation, and reporting of results to contribute towards the development of an implementable and robust tiered approach in NM physicochemical characterization, data interpretation, and improvement of best practice protocols for industrial and regulatory needs[8].

## PROTOCOL:

### 1. Delivery of the AuNP samples:

1.1. Prepare aliquots of 5 mL of Au colloid dispersions with sizes of 5, 20, 40, 60, and 100 nm including a 50 µg/mL sample of 'unknown size' (See **Table of Materials** for more specific details about the nanomaterials used).

1.2 Send the samples in 7 mL polystyrene containers with gel packs to each participating laboratory to maintain a suitable temperature during the shipping. Store the samples at 4 °C immediately.

NOTE: The ‘unknown size’ sample must present a size of 80 nm; this information should be known by the partner distributing the material, but not disclosed to the other partners.

## 2. Calibration of the spectrophotometer:

2.1. Turn on the UV-Vis spectrometer for at least 20 min to allow the lamp to heat up.

NOTE: Refer to the **Table of Materials** for the model and brand of the spectrophotometer used.

2.2. In the software, select the option**Spectrum scan** from the mode window, which displays the operating modes.

2.3. Adjust the parameter settings in **Instrument | Settings and parameters** in the software before proceeding with measurements: **Measurement Mode | Spectrum scan**, **Data Mode | ABS**, **Start wavelength** of 680 nm, **End Wavelength** of 380 nm, **Scan Speed** of 400 nm/min, **Sampling interval** of 0.5, **Slit Width** of 1.5, and **Path Length** of 10.

2.4. After the parameters have been set, fill two cuvettes (3 mL; polystyrene) with 1 mL of ultrapure water (UPW) (18.2 M·Ω·cm). Place the cuvettes in the reference cell holder (rear) and the sample cell holder (front) to cover the light path (See **Table of Materials** for the specific brand and model of the cuvettes used).

NOTE: Make sure the cuvettes are positioned and aligned correctly to cancel the noise effect and other environmental effects that are not sample-related.

2.5. Close the UV-Vis instrument cover and continue with the blank calibration by selecting **Blank** from the command bar. The baseline correction is performed by running a reference with the two cuvettes filled with 1 mL of UPW placed in the sample holders. For alternative protocols used by other partners, please see **Supplementary Information (SI).**

## 3. Preparation of the samples

3.1. Take a subsample of 500 µL for each AuNP of 5, 20, 40, 60, 100 nm, and the unknown size, and prepare a dilution with 500 µL of UPW.

3.2. Place the diluted suspensions in 1 mL cuvettes; the total dilution ratio should be 1:1 and final concentration 25 µg/mL.

NOTE: The diluted sample must be prepared immediately before the UV-Vis measurement.

## 4. Measurement of the nanoparticle dispersions

4.1. After the blank calibration has been performed, and a fresh sample has been prepared, replace one of the blank cuvettes in the sample cell holder (front) with the AuNP dispersion sample; the other reference cuvette filled with 1 mL of UPW must be left untouched.

NOTE: Use a new disposable cuvette for different samples to avoid cross-contamination between samples. When using quartz cuvettes, rinse the sample cuvette with UPW between samples.

4.2. Select the option **Measure/Start** from the command bar to run the spectrum scans for each diluted AuNP dispersion. Three spectrum scan runs should be obtained for each AuNP sample, including the unknown size sample.

NOTE: Ensure that the blank cuvette remains in the reference cell holder when running a measurement.

## 5. Reporting results

5.1. Extract the raw experimental data for each measurement in a spreadsheet-compatible file by selecting **File menu** and clicking **Export report (*.csv) file**.

5.2. Note the maximum absorption wavelength ($Abs_{max}$) and lambda ($\lambda_{max}$) for each of the UV-Vis readings and record them in the provided template.

NOTE: The predesigned template was provided to the ACEnano partners to automatically calculate the wavelengths' average standard deviations by setting the appropriate calculation formula in the workbook. For further details and access to the template, see **Supplementary Information (SI)**.

5.3. In the workbook, plot a calibration curve with the average of the $\lambda_{max}$ (y-axis) against the known nanoparticle size (nm) (5, 20, 40, 60, and 100 nm). For example, in the spreadsheet, create the calibration curve by selecting in the command bar **Data | Insert Graph | Scatter Plot | Add Trendline | Polynomial Curve (Power 2)**.

5.4. Include the polynomial equation for the calibration curve: select **Trendline options | Display Equation On Chart** from the command bar (**Figure 1**).

5.5. Finally, to calculate the unknown size of the AuNP sample, isolate the polynomial equation from the calibration curve to fit the mean value for the unknown $\lambda_{max}$, using a derivation of the quadratic formula (**Figure 1**). The calculated size can be included in the template to complete a full summary of the data for consistency, faster interpretation, and evaluation of the results (see SI).

**Insert Figure 1 here**

## REPRESENTATIVE RESULTS:

UV-Vis is one of the most popular techniques for nanoparticle characterization as it allows the user to obtain precise analysis of properties of NMs such as $\text{Abs}_{\text{max}}$ and $\lambda_{\text{max}}$[6,12]. Results of the present study represent the UV-Vis characterization of AuNP dispersions through an ILC between six participating labs.

**Insert Figure 2 here**

Results for the $\lambda_{\text{max}}$ wavelengths showed close repeatability among the partners (**Figure 2A**). This was also the case for the calculated range, which was used to assess the difference between values, and which showed small differences ranging between 1.00 and 2.40 ($\lambda_{\text{max}}$) for most of the AuNP sizes (**Table 1**). The overall $\lambda_{\text{max}}$ mean, calculated using the recorded mean for each laboratory for each AuNP size, similarly displayed low standard deviations for most of the sizes. The 100 nm size was the only exemption, as it displayed a high variation range (4.66 $\lambda_{\text{max}}$) between partners, leading to a greater standard deviation (572 ± 2.00 nm) compared to other AuNP sizes (**Table 1**). It is important to mention that laboratory 5 was not able to perform any measurements for the 100 nm size particles, due to contamination issues that might have compromised the repeatability of the results.

In contrast, absorbance results ($\text{Abs}_{\text{max}}$) exhibited a more scattered range of data values (**Figure 2B**) compared to $\lambda_{\text{max}}$ results. Despite the apparently higher variability of these results between laboratories, the analysis displayed overall means with lower standard deviations and unexpected inferior variation ranges (0.11–0.21 $\text{Abs}_{\text{max}}$) between laboratories compared to the $\lambda_{\text{max}}$ results (**Table 1**).

**Insert Table 1 here**

The Z-score values were also calculated to note the distance of individual values from the overall mean. The analysis of Z-scores provided information about the confidence of the ILC results, as the scores are directly related to the population distribution by displaying, in a number of standard deviations, how far a data point is from the mean[16]. In the results, most of the laboratories showed positive Z-score values of 0.01–1.93 for $\lambda_{\text{max}}$, indicating that most of the results were close to the mean and presented a normal distribution curve, as Z-scores greater than the absolute value of 2 and -2 are considered values that are distant from the mean and do not have a normal distribution[16]. The highest Z-score for $\text{Abs}_{\text{max}}$ was recorded for the 40 nm size reported by laboratory 1, with a value of 1.93 and an $\text{Abs}_{\text{max}}$ average of 530 ± 0, compared to the overall mean of 527.82 ± 1.13 (**Figure 3A**). The maximum Z-score value of 1.23 for $\lambda_{\text{max}}$ was reported by laboratory 3 along with a reported $\lambda_{\text{max}}$ of 0.454 ± 0 for 5 nm AuNP size compared to the overall mean of 0.395 ± 0.04. This was followed by the 60 nm AuNP with a Z-score of 1.18

and an $\lambda_{max}$ mean of 0.754 ± 0 compared to the overall average of 0.689 ± 0.05. The remaining sizes displayed Z-score values from -0.04 to-1.23 (**Figure 3B**).

**Insert Figure 3 here**

Results for the unknown sample showed that most of the partners calculated the size to be 76–80 nm. The mean of laboratories 1-4 and 6 was recorded as 78.02 ± 1.36 nm. Laboratory 5 reported a larger size of 109 nm, broadening the overall average and standard deviation up to 83.18 ± 12.70 nm, suggesting that this value was an outlier (**Figure 4A**). The Z-scores were calculated to be between -0.25 to -0.56 for all the laboratories; the only exception was for the unknown size reported by laboratory 6, which displayed the highest positive Z-score (2.03) compared to all the measurements, which can be considered as a value that is distant from the mean (**Figure 4B**).

**Insert Figure 4 here**

## FIGURE AND TABLE LEGENDS:

**Figure 1.** Calibration curve to calculate the size of the unknown sample. The plot represents the wavelengths ($\lambda_{max}$) and the size of the AuNPs used to plot the calibration. The plot shows only one calibration curve from one partner.

**Figure 2. Lambda and absorbance results.** The figures show the plots for the results reported by each laboratory for different AuNP sizes. **A)** Lambda max results. **B)** Absorbance max results. Laboratory 5 was not able to report data for 100 nm due to sample contamination.

**Figure 3. Lambda and Absorbance Z-scores.** Z-scores were calculated using the results reportd by each laboratory against the overall mean. **A)** Calculated Lambda max Z-scores. **B)** Calculated Absorbance max Z-scores.

**Figure 4. Unknown sample size and Z-scores. A)** Reported size for each laboratory for the provided unknown sample. **B)** Calculated Z-scores for each individual result against the overall mean of 83.18 ± 12.70 nm.

**Table 1. Lambda and Absorbance calculated range and means**. The range and overall mean and standard deviation for each AuNP size are shown. Results were calculated using the reported mean for lambda and absorbance for each laboratory (six measurements), except for the 100 nm size for which only 5 measurements were used to calculate the values due to a sample contamination reported by laboratory 5.

## DISCUSSION

Several methods are available for the characterization of nanoscale-related properties (e.g., analytical ultracentrifugation (AUC), SEM/TEM, and DLS[10,11]). However, these techniques lack the simplicity of UV-Vis to obtain primary results in the characterization of NMs[12,13]. UV-Vis is a common instrument even in not-so-well equipped laboratories, making it an unbeatable tool for the characterization of NMs[6]. When characterizing NMs, it is important to consider the limitations, strengths, and weaknesses of the techniques to be applied. In the UV-Vis spectrometer, the light beam passes through the sample compartment resulting in absorption values; as a result, external vibrations, outside light, contaminants, and the user's performance may interfere with the measurement and results[4,12]. Similarly, when plotting a calibration curve to determine the size of an unknown sample, it is important to register all the measurements needed to construct the calibration, as missing factors may contribute to variations among measurements and users.

For example, the high variation in the overall $\mathrm{Abs}_{\mathrm{max}}$ mean of the unknown sample might be linked to differences between the laboratories due to the dependence between the beam intensity, position, and the instrument itself[17,18]. Furthermore, the missing data for the 100 nm size from laboratory 5, due to a contamination problem, may also contribute to the high differences between the results, as the missing data may have affected the calibration curve and the plotted polynomial equation used to calculate the size of the unknown AuNP suspension. Certainly, reproducibility between protocols and laboratories can be complicated, as many factors might contribute to the lack of consistency in laboratory activities, resulting in researchers being occasionally unable to reproduce findings from other labs, which may lead to slower scientific progress, wasted time, money, and resources[19]. The successful characterization of physicochemical properties of NMs, particularly size, requires an easy-to-execute method by all participating laboratories, which can mostly be addressed by following a systematic and conceptual replication, such as the creation of an SOP, instrument training, and avoiding the use of misidentified or cross-contaminated samples[15,19].

Similarly, the quality and stability of the colloid suspension are also important factors to consider, as changes in their physicochemical properties may lead to different outcomes. Therefore, to ensure their stability for longer periods, nanoparticle suspensions should be stored in the dark at 4 °C. Likewise, during the shipping process, the aliquoted samples should be kept cold, as long periods at room temperature may lead to significant aggregation[20]. Additionally, to overcome failures in NM characterization, it is necessary to provide access to the original data, protocols, and key research materials between collaborating labs, especially, when assessing the proficiency, consistency, and reliability through an ILC[15]. Making these factors clear and accessible is key to achieving a successful NM characterization by any laboratory or equipment. Disregarding these aspects might result in a lack of reproducibility, accuracy, and misleading or erroneous results[15]. Although UV-Vis spectroscopy has been demonstrated to be the gold standard in NM characterization, it can be exploited in many other fields as it allows quantitative determination of an extended dynamic range of solutions in both inorganic and organic compounds[6,21].

Besides, UV-Vis can be easily combined with other tools to measure a large variety of attributes, thereby improving the quality of any analysis[22]. Based on these features, UV-Vis is widely used in many areas such as in the biopharmaceutical field by measuring UV-Vis spectra in high concentration protein solutions, in environmental control when comparing similarities between contaminants and their product-related impurities in real time, in industrial wastewater treatments plants as part of regulations for wastewater color determination and acceptability level[22,23]. Certainly, as technology progresses and more advanced features and experience become available in spectrophotometry, further broadening of the applications and parameters that can be measured using this technique will occur[22]. For example, in field applications, on-line UV-Vis spectrometry is a valuable tool for monitoring numerous parameters in real time and in various types of liquids, which is an exceptional feature among online sensor systems[22].

The ILC described here was designed as a test of the SOP developed for UV-Vis amongst six participating labs involved in the H2020 ACEnano project. The analysis of the results demonstrated that an ILC provides valuable information to allow technical confidence in an internal method for NM characterization by each participant laboratory. Data collection in an established template confirmed consistency and faster interpretation of the results and provided a model for the estimation of the size of an unknown AuNP sample, which also displayed repeatability between results when sufficient points in the calibration curve were included. Furthermore, the results validated the effectiveness of UV-Vis for NM characterization as well as the importance of the creation of best practice protocols. Such an approach further provides an opportunity for the implemented procedure to contribute towards the development of a legislative framework through reproducible NM characterization protocols based on method selection and data interpretation that are relevant for accreditation regulators and research management bodies.

## ACKNOWLEDGMENTS:

ACQ would like to thank The National Council for Science and Technology (CONACyT) in Mexico for funding her PhD studies. All authors acknowledge support from the European Union Horizon 2020 Programme (H2020) under grant agreement nº 720952, project ACEnano (call NMBP-26-2016).

## DISCLOSURES:

The authors declare that they have no competing interests.

## REFERENCES

1 Rauscher, H., Rasmussen, K., Sokull-Klüttgen, B. Regulatory aspects of nanomaterials in the EU. *Chemie Ingenieur Technik.* **89** (3), 224-231 (2017).

2 Hassellöv, M., Kaegi, R. in *Environmental and Human Health Impacts of Nanotechnology* eds J. R. Lead, E. Smith, 211-266 Blackwell Publishing Ltd (2009).

3 Shafiq, M., Anjum, S., Hano, C., Anjum, I., Abbasi, B. H. An overview of the applications of nanomaterials and nanodevices in the food industry. *Foods.* **9** (2) (2020).

4 Venkatachalam, S. in *Spectroscopy of Polymer Nanocomposites* eds S. Thomas, D. Rouxel, D. Ponnamma, 130-157 William Andrew Publishing (2016).

5 Bharmoria, P., Ventura, S. in *Nanomaterials for healthcare, energy and environment*. eds A. H. Bhat et al. 1-29, doi:10.1007/978-981-13-9833-9_1, Springer, Singapore (2019).

6 Amendola, V., Meneghetti, M. Size evaluation of gold nanoparticles by UV−vis spectroscopy. *The Journal of Physical Chemistry C.* **113** (11), 4277-4285 (2009).

7 Yeh, Y.-C., Creran, B., Rotello, V. M. Gold nanoparticles: preparation, properties, and applications in bionanotechnology. *Nanoscale.* **4** (6), 1871-1880 (2012).

8 European Commission. *Analytical and Characterisation Excellence in nanomaterial risk assessment: A tiered approach*, <https://cordis.europa.eu/project/id/720952> (2019).

9 Ikhmayies, S. J. Characterization of nanomaterials. *The Journal of Operations Management.* **66** (1), 28-29 (2014).

10 Mourdikoudis, S., Pallares, R. M., Thanh, N. T. K. Characterization techniques for nanoparticles: comparison and complementarity upon studying nanoparticle properties. *Nanoscale.* **10** (27), 12871-12934 (2018).

11 Mayeen, A., Shaji, L. K., Nair, A. K., Kalarikkal, N. in *Characterization of Nanomaterials* eds S. M. Bhagyaraj, O. S. Oluwafemi, N. Kalarikkal, S. Thomas, 335-364 ,Woodhead Publishing (2018).

12 Tomaszewska, E. et al. Detection limits of DLS and UV-Vis spectroscopy in characterization of polydisperse nanoparticles colloids. *Journal of Nanomaterials.* **2013**, 10 (2013).

13 Singer, A., Barakat, Z., Mohapatra, S., Mohapatra, S. S. in *Nanocarriers for Drug Delivery* eds S. S. Mohapatra et al. 395-419, Elsevier (2019).

14 Perkampus, H. H. *UV-VIS spectroscopy and its applications*. Springer-Verlag (1992).

15 Delčev, S., Zaimović-Uzunović, N., Basić, H. Participation of accredited laboratories in proficiency testing schemes and interlaboratory comparisons. *Key Engineering Materials.* **637** (2015).

16 Hayes, A. *Financial ratios: Z-Scores values*, <https://www.investopedia.com/terms/z/zscore.asp> (2020).

17 Oliveira, O. N., Li, L., Kumar, J., Tripathy, S. K. in *Photoreactive Organic Thin Films* eds Zouheir Sekkat & Wolfgang Knoll, 429-I, Academic Press (2002).

18 Sakhno, O., Goldenberg, L. M., Wegener, M., Stumpe, J. Deep surface relief grating in azobenzene-containing materials using a low-intensity 532 nm laser. *Optical Materials: X.* **1** 100006 (2019).

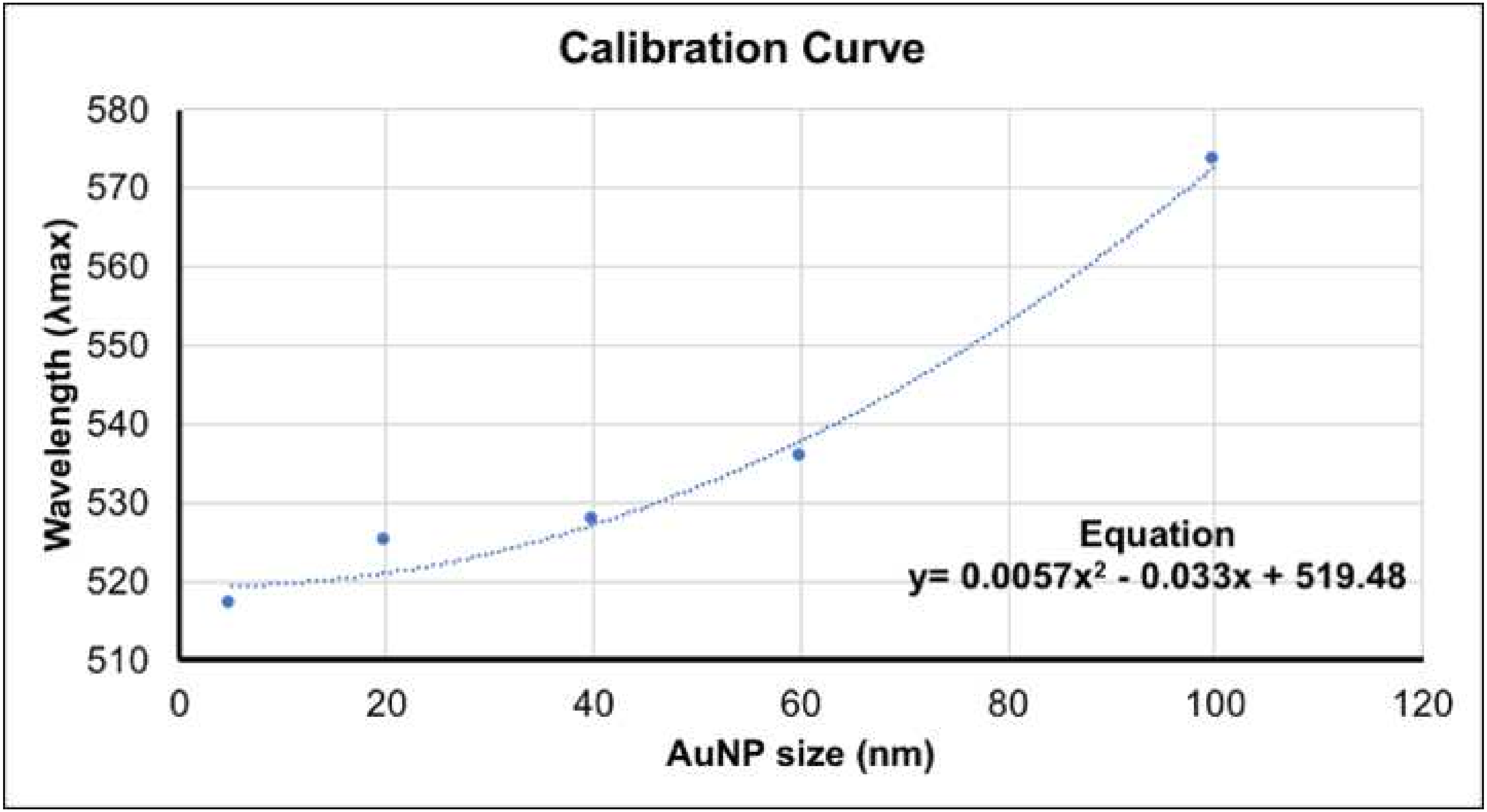
19 ATCC. *Six factors affecting reproducibility in life science research and how to handle them.*, <https://www.nature.com/articles/d42473-019-00004-y#ref-CR16> (2020).

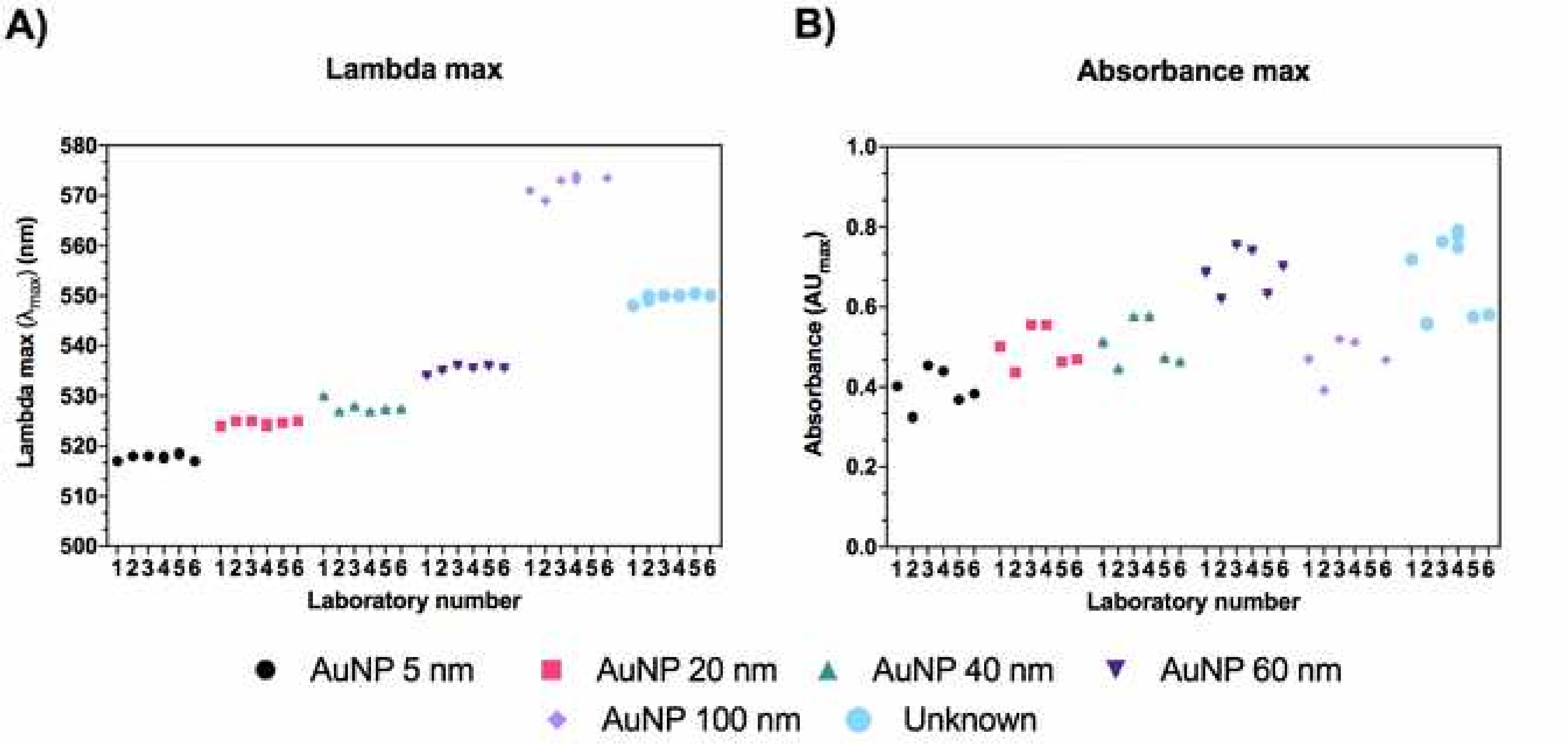
20 Balasubramanian, S. K. et al. Characterization, purification, and stability of gold nanoparticles. *Biomaterials.* **31** (34), 9023-9030 (2010).

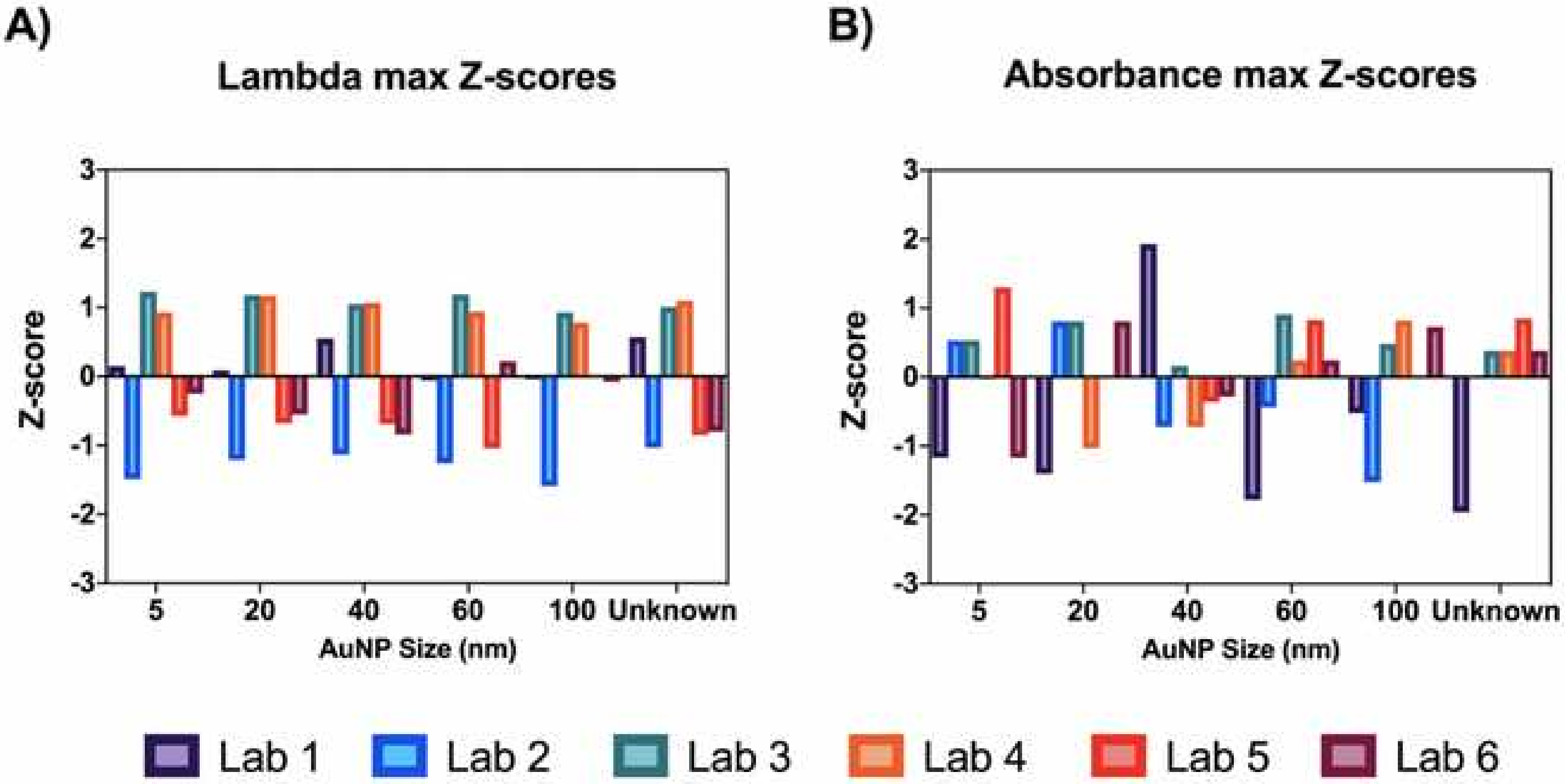
21 Łobiński, R. , Marczenko, Z. Recent advances in ultraviolet-visible spectrophotometry. *Critical Reviews in Analytical Chemistry.* **23** (1-2), 55-111 (1992).

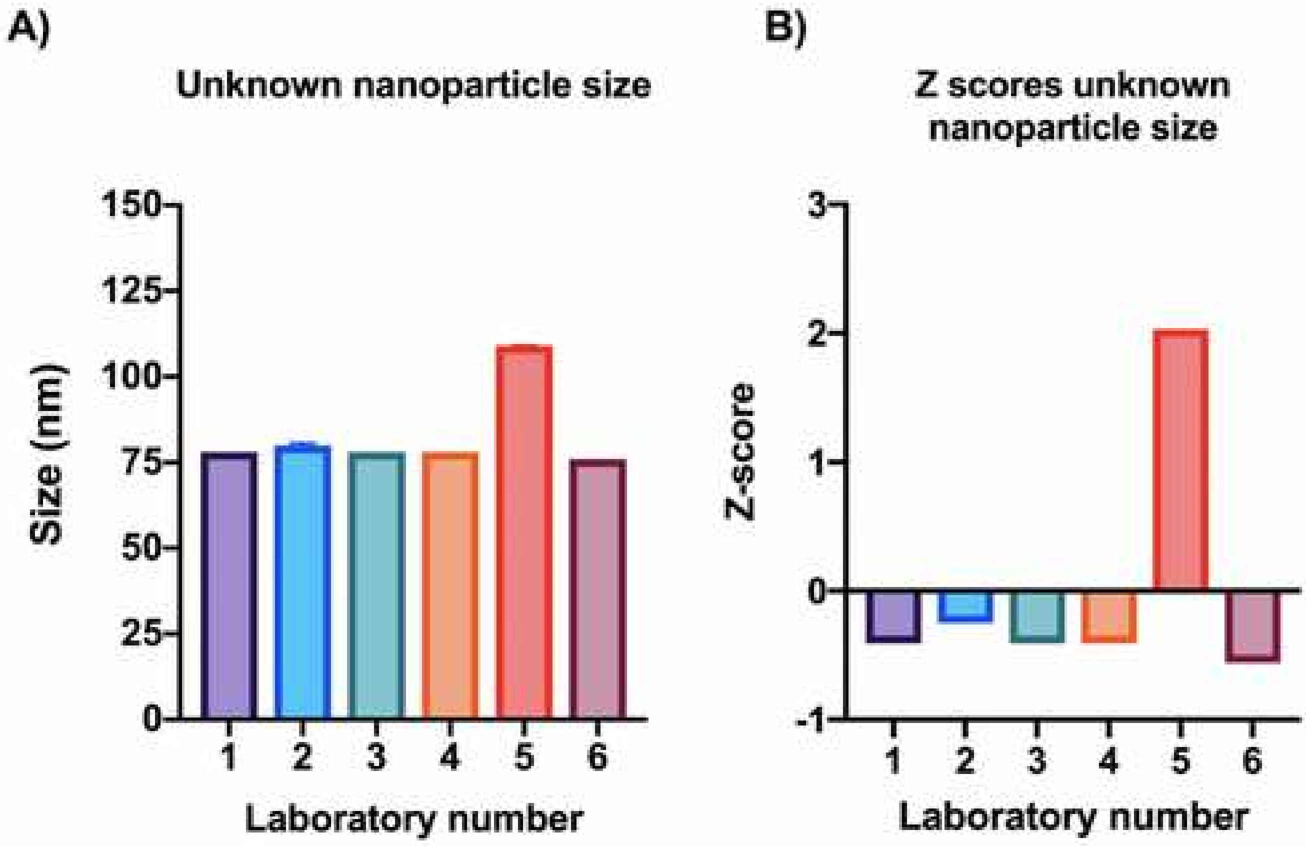
22 Ojeda, C. B., Rojas, F. S. Process analytical chemistry: applications of ultraviolet/visible spectrometry in environmental analysis: an overview. *Applied Spectroscopy Reviews.* **44** (3), 245-265(2009).

23 Rolinger, L., Rüdt, M., Hubbuch, J. A critical review of recent trends, and a future perspective of optical spectroscopy as PAT in biopharmaceutical downstream processing. *Analytical and Bioanalytical Chemistry.* **412** (9), 2047-2064 (2020).


Calibration Curve
Wavelength (λmax)
580
570
560
550
540
530
520
510
0
20
40
60
80
100
120
AuNP size (nm)
Equation
y= 0.0057x² - 0.033x + 519.48


A)
Lambda max
Lambda max ($\lambda_{max}$) (nm)
580
570
560
550
540
530
520
510
500
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Laboratory number
B)
Absorbance max
Absorbance ($AU_{max}$)
1.0
0.8
0.6
0.4
0.2
0.0
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6
Laboratory number
● AuNP 5 nm
■ AuNP 20 nm
▲ AuNP 40 nm
▼ AuNP 60 nm
◆ AuNP 100 nm
● Unknown


A)
Lambda max Z-scores
B)
Absorbance max Z-scores
Z-score
3
2
1
0
-1
-2
-3
5
20
40
60
100
Unknown
AuNP Size (nm)
Lab 1
Lab 2
Lab 3
Lab 4
Lab 5
Lab 6


A)
Unknown nanoparticle size
Size (nm)
150
125
100
75
50
25
0
1
2
3
4
5
6
Laboratory number
B)
Z scores unknown nanoparticle size
Z-score
3
2
1
0
-1
1
2
3
4
5
6
Laboratory number

| Value | AuNP (nm) | | | | | |
|---|---|---|---|---|---|---|
| | **5** | **20** | **40** | **60** | **100** | **Unknown** |
| Range $\lambda_{max}$ | 1.45 | 1.00 | 3.00 | 2.00 | 4.66 | 2.40 |
| Range $Au_{max}$ | 0.12 | 0.11 | 0.13 | 0.13 | 0.12 | 0.21 |
| Mean $\lambda_{max}$ | 517.7 ± 0.59 | 524.6 ± 0.45 | 527.8 ± 1.13 | 535.3 ± 0.74 | 572 ± 2.00 | 549.7 ± 0.85 |
| Mean $Au_{max}$ | .395 ± 0.048 | .497 ± 0.050 | .509 ± 0.057 | .689 ± 0.055 | .472 ± 0.051 | .661 ± 0.101 |

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| Absorption Ultra-Micro-cuvette, 200 µL | Hellma | 105.201-QS |
| Cary 5000 spectrophotometer ( Spectrophotometer C) | Agilent | Cary 5000 |
| Gold nanoparticles 5 nm | BBI solutions | EM.GC5 |
| Gold nanoparticles 20 nm | BBI solutions | EM.GC20 |
| Gold nanoparticles 40 nm | BBI solutions | EM.GC40 |
| Gold nanoparticles 60 nm | BBI solutions | EM.GC60 |
| Gold nanoparticles 80 nm | BBI solutions | EM.GC80 |
| Gold nanoparticles 100 nm | BBI solutions | EM.GC100 |
| Agilent / HP 8453 ( Spectrophotometer E) | | |
| Jenway 6800 spectrophotometer ( Spectrophotometer A) | Jenway | UV6800 |
| Polystyrene cuvette, 1.5 mL, micro 10 mm pathlength | Sigma | 759015 |
| Polystyrene cuvette, 3 mL (10 mm x 10 mm x 45 mm) | Sarstedt Inc | 67.742 |
| Semi-micro quartz cuvette, 1mL (1 mm x 10 mm x 45 mm) | Agilent | 6610001 |
| Ultrapure water (UPW) (18.2 MΩcm). | / | / |
| UV-1800 spectrophotometer ( Spectrophotometer B) | Shimadzu | UV1800 |
| Varian Cary 50 spectrophotometer ( Spectrophotometer D) | Agilent | Cary 50 |

## Comments/Description

# Response to Reviewers Comments

Thank you for your positive response to our journal submission. We appreciate the constructive feedback. Changes in the manuscript are shown in red. The responses for the different comments are the following:

**Editorial Comments:**

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
- Avoid punctuating the title.
- Please list a minimum of 6 keywords/phrases

1) Thank you for your comments, the manuscript has been thoroughly reviewed to make sure it is correctly written and does not contain any grammatical errors.
2) Title was revised accordingly.
3) A list of 6 keywords has been updated in the manuscript.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Some examples NOT in the imperative: 1.1, 2.1.1, etc.

The protocol has been thoroughly reviewed and changed to imperative tense.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The protocol has been reviewed and modified to ensure that all specific details are clear, so the viewers can easily understand and replicate the protocol by following the guidelines.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Key parts of the protocol have been highlighted to include all the relevant details and following a cohesive story to make the protocol easy to follow and replicate for all the viewers.

• **Results:** Split the representative results into a separate section.

Results section has been split from discussion.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Discussion has been rewritten to cover all previously suggested aspects as followed:

There are a large number of methods available to characterise nanoscale related properties, such as Analytical Ultracentrifugation (AUC), Scanning/Transmission Electron Microscopy (SEM/TEM), and Dynamic Light Scattering (DLS)[10,11]. However, these techniques differ from the simplicity of the Ultraviolet-Visible Spectroscopy (UV-Vis) to obtain primary results in the characterization of NMs[12,13]. UV-Vis is a common instrument existing even in not-so-well equipped laboratories, making it an unbeatable tool for the characterizations of nanomaterials (NMs)[6].
When characterising NMs, it is important to consider the limitations, strengths, and weaknesses of applied techniques. In the UV-Vis spectrometer, the light beam passes through the sample compartment resulting in absorption values; as a result, external vibrations, outside light, contaminants, and the user's performance may interfere with the measurement and results[4,12]. Similarly, when building a calibration curve to determine the size of an unknown sample, it is important to register all the

measurements needed to build the calibration, as missing factors may contribute to variations among measurements and users. For example, the high variation for the overall $Abs_{max}$ mean of the unknown sample might be linked to differences between the laboratories due to the dependence between the beam intensity, position, and the instrument itself [17,18]. Furthermore, the missing data for 100 nm size for laboratory 5, due to a contamination problem, may also contribute to higher differences between the results, as the missing data may affected the calibration curve and the plotted polynomial equation used to calculate the size of the unknown AuNP suspension.

Certainly, reproducibility between protocols and laboratories can be complicated, as many factors might contribute to the lack of consistency in laboratory activities, and resulting in researchers being occasionally unable to reproduce findings from other labs; leading to slower scientific progress, wasted time, money, and resources[19]. The successful characterization of nanomaterial physicochemical properties, particularly size, requires an easy to execute method by all participating laboratories, which can mostly be addressed by following a systematic and conceptual replication, such as the creation of a Standard Operating Procedure (SOP), instrument training, and avoiding the use of misidentified or cross-contaminated samples[15,19]. Similarly, the quality and stability of the colloid suspension is also an important factor to consider, as changes in their physicochemical properties may lead to different outcomes, therefore to ensure their stability for longer periods, nanoparticle suspensions should be stored in the dark at 4 °C. Likewise, during the shipping process, the aliquoted samples should be kept cold, as long periods at room temperature may lead to significant aggregation[20]. Additionally, to overcome failures in NM characterisation, it is necessary to provide access to the original data, protocols, and key research materials between collaborating labs; especially, when assessing the proficiency, consistency, and reliability through an Interlaboratory comparison (ILC)[15]. Making these factors clear and accessible is key to achieve a successful NM characterisation by any laboratory or equipment. Disregarding these aspects might result in a lack of reproducibility, accuracy, and misleading or erroneous results[15].

UV-Vis spectroscopy has demonstrated to be the gold standard in the NMs characterisation, however, UV-Vis can be exploited in many other fields, as it allows quantitative determination of an extended dynamic range of solutions in both inorganic and organic compounds[6,21]. Besides, UV-Vis methods can be easily combined with other tools to measure a large variety of attributes, increasing the quality of any analysis [22]. Based on these features, UV-Vis is widely used in many areas, such as in the biopharmaceutical field by measuring UV-Vis spectra in high concentration protein solutions, in environmental control, when comparing similarities between contaminants and their product-related impurities in real-time, industrial wastewater treatments plants, as part of regulations for wastewater colour determination and acceptability level and many more[22,23]. Certainly, as technology progresses and more advanced features and experience is gathered in the spectrophotometry field, further broadening of the applications and parameters that can be measured using this technique are likely to increase[22]. For example, in field applications, on-line UV-Vis spectrometry, is a valuable tool for monitoring numerous parameters in real time and in various types of liquids, which is an exceptional feature among online sensor systems[22].

• **Figures:** 1) Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.
2) Remove the legends text from the figure files.

1) The figures have been removed from the manuscript, keeping only the captions.
2) The text from the figure files has been removed.

• **Tables:** 1) Please remove the embedded Tables from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

The tables have been updated to Excel files and keeping the legend at the manuscript.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are BBI Solutions OEM Ltd., Bijou Containers, (Thermofisher, 129A, Jenway 6800 spectrophotometer, UV-1800 Shimadzu, Hellma Ultra-Micro-cuvette 105.201-QS, Agilent Cary 5000, Agilent, Varian Cary 50,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove product names from Table 1.

The commercial language has been rewritten to a more generic language in the manuscript, followed by a reference to check the list of materials.

- **Table of Materials:** Please sort in alphabetical order.

The table of materials has been sorted in alphabetical order.

**Comments from Peer-Reviewers:**

**Reviewer #1:**

This manuscript describes a simple and inexpensive method to characterize the size of gold nanoparticles. Standard gold (Au) colloid suspensions of different sizes (ranging 5 -100 nm) were characterised by UV-Vis firstly. A calibration curve was created with the average of the λmax (nm) (y-axis) against their known nanoparticle size (nm) (5, 20, 40, 60 and 100 nm). The unknown AuNP sample can be calculated based on the fitted quadratic formula. The manuscript is suitable for publication in Journal of Visualized Experiments.

Thank you for your comment, we appreciate that the paper is suitable for JoVE publications.

**Reviewer #2:**
Manuscript Summary:
Journal of Visualized Experiments

Article Type: Methods Article - JoVE Produced Video
Manuscript Number: JoVE61764
Full Title: Characterisation of Nanomaterials in Aqueous Media: protocol optimisation for UV-Vis spectroscopy
Authors:
Ana C. Quevedo, Emily Guggenheim, Sophie M. Briffa, Jessica Adams, Stephen Lofts, Minjeong Kwak, Tae Geol Lee, Colin Johnston, Stephan Wagner, Timothy R. Holbrook, Yves U. Hachenberger, Jutta Tentschert6, and Eugenia Valsami-Jones

I begin with congratulating all authors for this much awaited piece of work.

They have rightly pointed out that, in the manufacturing of NM bearing products the time, reliable and repeatable characterisation are indispensable parameters to ensure safety and quality. They have chosen Ultraviolet-Visible Spectroscopy (UV-Vis) which is a well-established, simple to use and inexpensive technique that provides non-invasive and fast real-time screening evaluation of NM size, concentration, and aggregation state. I do agree with the claim of authors as stated, 'Such features make UV-Vis an ideal methodology to assess the Proficiency Testing Schemes (PTS) of a validated Standard Operating Procedure (SOP), intending to evaluate the performance, and reproducibility of a characterisation method'. Fortunately, this technique can be exploited in the any simplest laboratories without

using high end sophisticated instrument(s).
Though, one has to use high end instruments techniques to reveal morphology features such as TEM, HR-TEM, AFM, STM, the significance of UV-vis spectroscopy to get primary result has been unbeatable especially in the characterizations of nanomaterials. Moreover, we can identify type of nanoparticles along with completion of reaction or else other way around.

In Conclusion: This article would be very useful in characterization of nanomaterials as a basic tool.

Major Concerns:
None

Minor Concerns:
1. Page 4, Line 141; '……………and stored at 4 ºC upon arrival'.
Few samples are temperature sensitive did you check for any changes in the quality of result on storage of sample at 4 ºC and immediately upon arrival?

Thank you for your feedback, we have included a couple of sentences to specify the importance of temperature control in the stability of the nanoparticles. Please see page 5, line 147: 'Send the samples to each participating lab in 7 mL Polystyrene containers with gel packs to maintain a suitable temperature during the shipping. Once the samples are received, store them at 4 ºC immediately'. 'Page 10, line 327: Similarly, the quality and stability of the colloid suspension is also an important factor to consider, as changes in their physicochemical properties may lead to different outcomes, therefore to ensure their stability for longer periods, nanoparticle suspensions should be stored in the dark at 4 °C. Likewise, during the shipping process, the aliquoted samples should be kept cold, as long periods at room temperature may lead to significant aggregation[20]'.

2. Page 6, Line 216; 1.5 mL; polystyrene, micro 10 mm pathlength cuvettes were used, cost wise it is absolutely fine, nonetheless if Quartz cuvettes would have been used that could be a better choice for good quality report.

Quartz cells may improve quality report, also they are typically used for the analysis and detection of low sample concentrations with a larger range of wavelengths (340–2500 nm). Though, polystyrene cuvettes were used in the study due to cost wise and for their common use in the analysis of wavelength range of visible light 380–780 nm, which involved the expected range of the AuNPs. However, we appreciate your comment and we will consider the use quartz cuvettes for our next study to improve good quality report.

3. Page 8 Line 264; I couldn't get why 'AUmax' abbreviation is used; this may be Amax or else Absmax

The term ‘$AU_{max}$’ has been changed to ‘$Abs_{max}$’ in the manuscript.

4. In figure in addition to presented figures the authors could have added 'Absorbance Vs Wavelength (nm)' plots, I am wondering why these plots are not added in the text?

I think, the stated plots would have been quick catchy rather than the presented data.

An 'Absorbance Vs Wavelength (nm)' plot has been added in the protocol section ‘reporting results-5.3’ as figure 1 to complement the instructions to calculate the unknow size of the sample.

5. I think Ref No. 3 is not complete.

6. In Ref 7 Name of the journal JOM need to be expanded.

7. I think references are not uniform, if feasible do add few more relevant references too.

The references have been updated. The manuscript has also been reviewed and more relevant references have been added, mainly as part of the introduction and discussion.

JoVE JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT - UK

| | |
|---|---|
| Title of Article: | Characterisation of Nanomaterials in Aqueous Media: protocol optimisation for UV-Vis spectroscopy |
| Author(s): | Ana C. Quevedo, Emily Guggenheim, Sophie M. Briffa, Jessica Adams, Stephen Lofts, Minjeong Kwak, Tae Geol Lee, Colin Johnston, Stephan Wagner, Timothy R. Holbrook, Yves U. Hachenberger, Jutta Tentschert, and Eugenia Valsami-Jones |

Item 1: The
Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☒ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

## ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode ; "**CRC NonCommercial License**" means the Creative Commons Attribution-NonCommercial 3.0 Agreement (also known as CC-BY-NC), the terms and conditions of which can be found at: http://creativecommons.org/licenses/bync/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other preexisting works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative

Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License. If the "Standard Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC NonCommercial License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties**. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or

misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion andwithout giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contaminationdue to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors. 13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received. 14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

# UV-Vis Spectroscopic Characterization of Nanomaterials in Aqueous Media

Ana C. Quevedo[1], Emily Guggenheim[1], Sophie M. Briffa[1], Jessica Adams[2,3], Stephen Lofts[2,] Minjeong Kwak[4], Tae Geol Lee[4], Colin Johnston[5], Stephan Wagner[6], Timothy R. Holbrook[6], Yves U. Hachenberger[7], Jutta Tentschert[7], and Eugenia Valsami-Jones[1]

[1] School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK
[2] UK Centre for Ecology and Hydrology, Lancaster Environment Centre, Lancaster, LA1 4AP, UK
[3] Natural England, Foss House, Kings Pool, 1-2 Peasholme Green, York YO1 7PX
[4] Center for Nanosafety Metrology, Korea Research Institute of Standards and Science (KRISS), Daejeon 34113, Republic of Korea
[5] Department of Materials, University of Oxford, Begbroke Science Park, Oxford OX5 1PF, UK
[6] Helmholtz-Centre for Environmental Research, Department of Analytical Chemistry, Permoserstraße 15, 04318 Leipzig, Germany
[7] Department of Chemical and Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Ana C. Quevedo (AIC585@bham.ac.uk), Emily Guggenheim (ejg843@gmail.com) Sophie M. Briffa (S.M.Briffa@bham.ac.uk), Jessica Adams (adamsjl2@hotmail.co.uk), Stephen Lofts (stlo@ceh.ac.uk), Minjeong Kwak (kwakmj@kriss.re.kr), Tae Geol Lee (tglee@kriss.re.kr), Colin Johnston (colin.johnston@materials.ox.ac.uk), , Stephan Wagner (stephan.wagner@ufz.de), Timothy R. Holbrook (timothy-ronald.holbrook@ufz.de), Yves U. Hachenberger (Yves.Hachenberger@bfr.bund.de), Jutta Tentschert (Jutta.Tentschert@bfr.bund.de)

*Corresponding author:
Eugenia Valsami-Jones (E.ValsamiJones@bham.ac.uk)

## Supplementary information

Protocols used by other participants in the present study for the calibration of their spectrophotometers.

## 1. Spectrophotometer B

1.1 Turn on the UV-Vis spectrometer for at least 20 min to allow the lamp to heat up. **NOTE:** See Table of materials to refer to the model and brand of the spectrophotometer used.

1.1.1 Open the software and select the option ‘Spectrum scan’ from the mode window that displays the operating modes.

1.1.2 Adjust the parameter settings in ‘Instrument→Settings and parameters’ in the software before proceeding with the measurements. Details of the parameters used for AuNP suspensions are shown in Table 1.

1.1.3 Fill two quartz cuvettes (200 µL, Micro-cuvette) with 200 µL of ultrapure water (UPW) (18.2 MΩ.cm). Place the reference cuvettes inside the cell holder (rear) and the sample cell holder (front) to cover the light path (see Table of materials for the specific cuvette model and brand). **NOTE:** Ensure that the cuvettes are positioned and aligned correctly to cancel the noise effect and other environmental effects that are not sample-related.

1.1.4 Finally, close the UV-Vis instrument cover, and continue with the blank calibration by selecting 'Blank' from the command bar. The baseline correction is obtained by running a reference with the two cuvettes filled with 1 mL of UPW placed in the sample holders.

## 2. Spectrophotometer C

2.1. Turn on the UV-Vis spectrometer for at least 20 min to allow the lamp to heat up. **NOTE:** See Table of materials to refer to the model and brand of the spectrophotometer used.

2.2 Open the software and select the option 'scan' from the mode window that displays the operating modes.

2.3 Adjust the parameter settings in 'Settings→parameters' in the software before proceeding with the measurements. Details of the parameters used for the AuNP suspensions are shown in Table 1.

2.4 Fill two cuvettes (1 mL; Semi-micro quartz) with 1 mL of UPW (18.2 MΩcm). Place the reference cuvettes inside the cell holder (rear) and the sample cell holder (front) to cover the light path (see Table of materials for the specific cuvette model and brand).
**NOTE:** Ensure that the cuvettes are positioned and aligned correctly to cancel the noise effect and any other environmental effects that are not sample-related.

2.5 Finally, close the UV-Vis instrument cover, and continue with the blank calibration by selecting 'Zero' and 'Baseline' from the command bar. The baseline correction is obtained by running a reference with the two cuvettes filled with 1 mL of UPW placed in the sample holders.

## 3. Spectrophotometer D

3.1 Turn on the UV-Vis spectrometer for at least 20 min to allow the lamp to heat up. It is recommended to measure 3 different reference standards as a performance check.
**NOTE:** See Table of materials to refer to the model and brand of the spectrophotometer used.

3.2 Open the software and select the option 'Spectrum scan' from the mode window that displays the operating modes available.

3.3 Adjust the parameter settings in 'Settings→Cary' in the software before proceeding with the measurements. Details of the parameters used for AuNP suspensions are shown in Table 1.

3.4 Fill two cuvettes (1.5 mL; polystyrene cuvette) with 1 mL of UPW (18.2 MΩcm). Place the reference cuvettes in cell holder (rear) and the sample cell holder (front) to cover the light path (See table of materials for the specific cuvette model and brand).
**NOTE:** Ensure that the cuvettes are positioned and aligned correctly to cancel the noise effect and any other environmental effects that are not sample-related.

3.5 Finally, close the UV-Vis instrument cover, and continue with the blank calibration by selecting 'Baseline→start' from the command bar. The baseline correction is obtained by running a reference with the two cuvettes filled with 1 mL of UPW placed in the sample holders.

**Table 1. Parameter settings used for measurements.** The table lists the parameters used to set the UV-Vis spectrometers before the measurements.

| **Settings** | **Equipment model** | | | | **Unit** |
|---|---|---|---|---|---|
| | **Jenway 600** | **UV-1800 Shimadzu** | **Agilent Cary 5000** | **Varian Cary 50** | |
| **Measurement Mode** | Spectrum scan | Spectrum scan | Scan | Spectrum Scan | N/A |
| **Data Mode** | ABS | ABS | ABS | ABS | N/A |
| **Start wavelength** | 680 | 680 | 680 | 680 | nm |
| **End Wavelength** | 380 | 380 | 380 | 380 | nm |
| **Scan Speed** | 400 | 400 | 600 | 400 | nm/min |
| **Sampling interval** | 0.5 | 0.5 | 1 | 0.17 | N/A |
| **Slit Width** | 1.5 | 1.5 | 2 | 1.5 | N/A |
| **Path Length** | 10 | 10 | 10 | 10 | N/A |

*Abbreviations: No Applicable (N/A),* Absorbance (ABS), *Nanometer (nm), and Minute (min).*

## 4. Measurement of the nanoparticle dispersions

For Spectrophotometers A, B, C, and D, refer to section 4 in the main manuscript for a detailed protocol of the nanoparticle's dispersions for the equipment.

### 4.1 Measurement of the nanoparticle dispersions:

Once the blank calibration has been performed and a fresh sample has been prepared:

4.1.1. Place a cuvette holding diluted suspension in the sample cell holder. It is recommended to use a new disposable cuvette for each different sample analyzed to avoid cross-contamination among samples. If using a non-disposable quartz cuvette, the sample cuvette must be rinsed with UPW and dried between samples.

4.1.2. Perform spectral acquisition either by pressing the Sample button on the front of the machine, or the Sample button at the bottom left of the ChemStation window. When measurement is complete the spectrum will be displayed in the Overlaid Sample Spectra window in ChemStation.

4.1.3. Save the spectrum results (see next section) and repeat with the next sample until complete.

### 4.2. Reporting results

4.2.1. After each measurement, select the most recently acquired spectrum in the Overlaid Sample Spectra window and export it to a .csv file using File > Export Selected Spectrum. **NOTE:** Filenames can have a maximum of eight characters only. These files provide an absorbance value and its standard deviation for each measured wavelength.

4.2.2. After acquiring all the spectra, save them into a single native format file by firstly selecting them all on the screen (hold down the Ctrl key to select multiple spectra) and using File > Save > Selected Spectra.
**NOTE:** This file cannot be read by Excel but is a backup that can be loaded into ChemStation to retrieve and export the spectral data.

4.2.3. Analyze the results as described in section 5 in the main manuscript.

## 5. Reporting results

For Spectrophotometers A, B, C, and D, refer to section 4 in the main manuscript for a detailed protocol reporting the extracted results.

## 6. Spectrophotometer E

6.1. Switch on the controlling computer.

6.2. Turn on the HP8453 spectrometer using the power button, and wait for the light on the front of the machine to turn green. Ensure that the sample holding area contains a sample holder suitable for a 1 cm path length cell.

6.3. Start the HP ChemStation software using the ‘Instrument 1 online’ icon on the PC desktop.

6.4. When the software is fully booted, check that the tungsten lamp of the spectrophotometer is on by viewing the image of the lamps at the bottom left of the ChemStation window. If on, the tungsten lamp image (the smaller of the two lamps) should be glowing. If not, right click on the image of the lamp and choose ‘LAMP ON’ from the menu.

6.5. Allow 30 min for the lamp and machine to warm up before processing any samples.

6.6. In the ChemStation window, click Instrument → Setup Spectrophotometer and enter the selected wavelength range (Low wavelength 380 nm, High wavelength 680 nm). Change integration time to 2 s and Interval to 1 s, if not at these values. Click OK when done.

6.7. In the task box at the top left of the ChemStation window, choose ‘Fixed Wavelengths’.

6.8. When the spectrophotometer is warmed up, run a sample blank: fill a 3 mL, 1 cm path length polystyrene or quartz cuvette with 2 mL of UPW (18.2 MΩ/cm). Place in the sample holder with the clear faces of the cuvette in the path of the light beam (i.e. front to back of the machine). Obtain a blank reading by either pressing the Blank button on the front of the machine, or the Blank button in the ChemStation window (bottom left). When measurement is complete a blank spectrum window will appear in ChemStation. Check the blank absorbance for acceptability. If acceptable, minimize the window; otherwise, repeat the blank measurement until it is acceptable.