Toward Achieving Harmonization in a Nanocytotoxicity Assay Measurement Through an Interlaboratory Comparison Study

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Summary

Development of reliable cell-based nanotoxicology assays is important for evaluation of potentially hazardous engineered nanomaterials. Challenges to producing a reliable assay protocol include working with nanoparticle dispersions and living cell lines, and the potential for nano-related interference effects. Here we demonstrate the use of a 96-well plate design with several measurement controls and an interlaboratory comparison study involving five laboratories to characterize the robustness of a nanocytotoxicity MTS cell viability assay based on the A549 cell line. The consensus EC50 values were 22.1 mg/L (95% confidence intervals 16.9 mg/L to 27.2 mg/L) and 52.6 mg/L (44.1 mg/L to 62.6 mg/L) for positively charged polystyrene nanoparticles for the serum-free and serum conditions, respectively, and 49.7 µmol/L (47.5 µmol/L to 51.5 µmol/L) and 77.0 µmol/L (54.3 µmol/L to 99.4 µmol/L) for positive chemical control cadmium sulfate for the serum-free and serum conditions, respectively. Results from the measurement controls can be used to evaluate the sources of variability and their relative magnitudes within and between laboratories. This information revealed steps of the protocol that may need to be modified to improve the overall robustness and precision. The results suggest that protocol details such as cell line ID, media exchange, cell handling, and nanoparticle dispersion are critical to ensure protocol robustness and comparability of nanocytotoxicity assay results. The combination of system control measurements and interlaboratory comparison data yielded insights that would not have been available by either approach by itself.

Keywords: nanotoxicology, MTS assay, interlaboratory comparison, polystyrene nanoparticles, alternatives to animal testing

# authors contributed equally

Abbreviations

DLS, dynamic light scattering; ENM, engineered nanomaterial; ID, identification; MCMC, Markov Chain Monte Carlo; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NH2-PS, positively charged-polystyrene nanoparticles; NP, nanoparticles; OD, optical density; SEM, scanning electron microscopy; STR, short tandem repeat

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1 Introduction

Engineered nanomaterials (ENMs) have unique physicochemical properties due to their small size, high surface to volume ratio and spatially controlled composition. It is expected that sophisticated control of ENM manufacturing will allow the development of advanced materials which have impact in a wide range of fields including energy, textiles, and medicine (De Volder et al., 2013; Wagner et al., 2006; Graetzel et al., 2012). The increasing quantities of manufactured ENM will increase the likelihood of human, animal and organism exposure to these materials (Nel et al., 2006; Schrürs and Lison, 2012; Auffan et al., 2009). Nanotechnology Environmental and Health Safety (nanoEHS) efforts are based on the idea that physicochemical characteristics of ENM may adversely impact components of biological systems. Tests that evaluate the nature of these interactions are important for understanding hazards that may be associated with these materials.

Cell-based toxicity assays can be used as a first tier approach to identify potentially hazardous ENMs (Nel et al., 2013). Advantages of these assays are that they can be rapid, cost effective, mechanistic, used for high-throughput screening, and serve as a pathway for reducing animal testing (Nel et al., 2013; NRC, 2007; Sauer et al., 2013; Horev-Azaria et al., 2013; Clippinger et al., 2016; Landsiedel et al., 2014). However, the use of nanocytotoxicity assays has led to conflicting results from similar ENMs tested in different laboratories (Schrürs and Lison, 2012; Krug and Wick, 2011; Krug, 2014; Kaiser et al., 2011). Undocumented differences in assay protocols, differences in NP dispersions, and inadequate controls for monitoring assay performance are likely responsible for these results (Schrürs and Lison, 2012; Krug and Wick, 2011; Poland et al., 2014; Geys et al., 2010; Monteiro-Riviere et al., 2009). Recognition of these issues has resulted in the request for “standardized” nanocytotoxicity assays (Krug and Wick, 2011; Nel et al., 2013; Landsiedel et al., 2009, 2014). Standardization would indicate that assay results from different operators or laboratories are comparable and that the protocol is robust to small changes in operating conditions.

The challenges associated with developing reliable nanocytotoxicity assays are similar to those associated with the reproducibility of biological measurements. In addition to improved statistical analysis, reproduction of the whole biological measurement within a laboratory, and better reporting of method sections (Poland et al., 2014; Miller, 2014), assessing the reproducibility of biological methods across different laboratories is also critical (Plant et al., 2014). In fact, it is impossible to assess all of the sources of variability in an assay (i.e., dark uncertainty (Thompson and Ellison, 2011)) without the aid of a comprehensive interlaboratory study. Measurements from several different laboratories are more likely to produce results that incorporate additional sources of variability including the unknown factors affecting the results of the assay. Thus, results or protocols obtained from a single laboratory, even if they are shown to be reproducible within that laboratory and utilize good quality assurance/quality control practices, are not sufficient for ensuring similar results among multiple laboratories (Warheit and Donner, 2015). Additional experimental design components such as appropriate control experiments, specifications for ensuring valid method performance, and confirmation of the robustness of the method to unintended variation in the experimental protocol are some of the features of interlaboratory studies which enable confidence in comparing test results among the laboratories (Plant et al., 2014). A measurement science approach that includes systematic understanding of the sources of variability in the assay protocol and provides a comprehensive set of system controls to ensure acceptable assay performance is proposed to improve reproducibility in nanotoxicity and other biological assays.

Here, we show the results of a 5 laboratory (NIST, EMPA, KRISS, JRC, NANOtech) comparison of a nanocytotoxicity assay and the evaluation of the transferability and reproducibility of the assay procedure. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium (MTS) assay is a colorimetric assay for testing cell viability by measur-
ing the reduction of the tetrazolium dye to insoluble formazan, a process that only occurs in live cells, using a plate reader. The MTS assay was chosen because it is widely used in cytotoxicity studies and the protocol has only a few basic steps. We also evaluated the effect of using serum-free in comparison to serum-containing medium during the incubation of ENM as serum may affect the response of cells to ENM. Figure 1 illustrates the serum and serum-free protocols (Protocols S11, S21) that were derived from the manufacturer’s instructions and from the results of a cause-and-effect analysis of the MTS assay (Rösslein et al., 2014). The protocols include 8 system controls to quantify critical sources of variability in the assay (Fig. 2, Tab. 1).

Our study used a single ENM and the human A549 cell line in two variants to demonstrate the value of the measurement science approach in understanding sources of variability in an assay. By not controlling all of the aspects of cell culture, the experimental design of the interlaboratory study mimicked possible sources of variability including differences in cell treatment procedures, sources of serum, culture medium, and cell culture plates (Tab. S12, S22). Some factors were controlled, specifically that all participants had the same stock of NH2-PS nanoparticles (NP) (i.e., positively charged-polystyrene NP), positive chemical control reagent (CdSO4) and the two human A549 cell lines. The NH2-PS NP was chosen as our model system since positively charged NPs have been shown to be toxic to many different cell types (Nemmar et al., 2003; Shen et al., 2009; Hong et al., 2006) and because the NH2-PS NP will not release dissolved ions which may cause toxicity unlike, for example, copper oxide NPs. Cadmium sulfate was chosen as a chemical positive control because it is stable in solution, known to be toxic, is highly soluble in aqueous media, and can be readily quantified, which can help ensure that the same concentration is being used across time and among laboratories during the interlaboratory comparison.

This study differs from a recently published interlaboratory comparison (Xia et al., 2013) in that we systematically evaluate the contributions to the total variability from the various steps of the assay and provide numerous specifications that ensure comparability of the assay measurement process. This study was not designed as a comparison of the effect of different cell lines or various ENMs; the objective was to show the power of using an assay design with system control measurements in combination with an interlaboratory comparison experiment to systematically understand the sources of variability in the assay.

2 Methods

Coordination

Tasks were divided between each of the laboratories. EMPA served as sample and data coordinator. EMPA and NIST were responsible for experimental design, statistical analysis, and preliminary data collection. KRISS was responsible for NP

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1 https://doi.org/10.14573/altex.1605021p
2 https://doi.org/10.14573/altex.1605021s
Characterization of the NH$_2$-PS NP

The original suspension of NH$_2$-PS NP received from EMPA was diluted by a factor of 1000 in 18 MΩ water, cell culture medium (RPMI-1640) without serum, cell culture medium (RPMI-1640) containing 0.1% fetal bovine serum (FBS), or cell culture medium (RPMI-1640) containing 10% FBS. The sample suspensions were measured for size and zeta potential immediately after the dilution and after incubation for 24 and 48 h (37°C, 5% atmospheric CO$_2$).

The hydrodynamic diameter was measured by dynamic light scattering (DLS) with a particle size analyzer (ELS-Z, Otsuka electronics Co. Ltd.). Twelve repetitive measurements were performed with 5 sub-runs for each measurement using a 2-sec detection time. To remove potential interference from dust scattering, the six measurement results with the lowest DLS values were selected to calculate the average ENM size and its uncertainty. The scattering intensity of the nanoparticle suspension (200 µl) and CdSO$_4$ solution (4 ml) were shipped overnight to each of the laboratories under ambient conditions. MTS reagents (Promega, Madison WI) were purchased by each laboratory. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the NIST nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

The precision of the plate readers was tested by measuring a single plate with cells treated with the MTS reagent multiple times.

### Reagents and equipment

NH$_2$-PS NP suspended in H$_2$O were obtained from Bangs Laboratories Inc. (Fishers, Indiana, US), lot number 10351 and inventory number L120117F, 10% (w/v). CdSO$_4$-7H$_2$O (Sigma-Aldrich) was dissolved in 18 MΩ water to make a final concentration of 10 mmol/L. Aliquots of both the nanoparticle suspension (200 µl) and CdSO$_4$ solution (4 ml) were shipped overnight to each of the laboratories under ambient conditions. MTS reagents (Promega, Madison WI) were purchased by each laboratory. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the NIST nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

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### Tab. 1: Control settings deduced from the cause-and-effect analysis and implemented into a 96-well plate layout

This table is modified and reprinted with permission from Rösslein et al. (2014).

| Control Number | Brief Control Description |
|----------------|---------------------------|
| 1              | Background correction for the chemical dosing. Allows detecting if increasing concentrations of the reference chemical change the final MTS measurement result. |
| 2              | Triplicate reference chemical control. Shows that the assay worked as expected. |
| 3              | Assesses within multichannel pipetting variance. Non-treated cells seeded with a single multichannel pipette ejection step. Absolute absorbance measurement provides insight on nominal cell growth. Indicates technical problems with the pipette. |
| 4              | These wells contain no cells but MTS reagent (last step of assay procedure). Allows for background absorbance correction and variance correction. Together with outermost wells (control No. 4) assesses possible internal measurement gradients due to instrument malfunction or culture plate variability. Large variations may further indicate issues with the MTS reagent. |
| 5              | Background correction for the ENM dosing. Allows detecting if increasing concentrations of the ENM change the final MTS measurement result, i.e., interfere with the final readout. |
| 6              | Additional considerations that exceed those described in No. 3 "no cells no treatment": these wells contain medium from the time of cell seeding on. This helps to circumvent so-called edge effects that might occur during longer incubation times of cells seeded in small volumes in the outermost wells (i.e., evaporation). |
| 7+8            | Assesses between multichannel pipetting variance. Solvent treated cells (compare B3-B5 for chemical control, B8-B10 for ENMs) seeded in different ejection steps. This control indicates handling problems of the operator during the seeding procedure and possible effects of the solvent if compared to "no treatment" wells (B6-G6). |
10% serum but without NPs, which suggests that the serum had a negligible impact on the DLS results. The zeta potential of the NH2-PS NP in the cell culture media suspensions (without serum, with 1% serum, and with 10% serum) was measured using an electrophoretic mobility analyzer (Zetasizer Nano Z, Malvern Instrument). Values from five independent measurements were used to calculate the average zeta potential and its uncertainty.

Primary particle size was measured via scanning electron microscopy (SEM) using a Zeiss NVision 40 focused ion beam/ scanning electron microscope operating at 15 kV.

Cell lines
Two A549 human lung cancer cell lines were used in this study. One cell line (A549-B) was purchased from ATCC (Manassas, VA) immediately before the study began. Seed and working stock distribution vials were prepared at NIST and working stock vials were sent to EMPA who prepared a working stock and distributed it to the other laboratories. A second A549 cell line (A549-A) purchased from I.A.Z. Institute (Munich, Germany) in 2005 was also distributed to each of the laboratories. The passage number after acquisition of the cell lines from the source was less than 25 for both cell lines for all experiments. The two cell lines were sent to each laboratory at passage number 5 and were cultured for at least 3 passages before being used in the interlaboratory comparison. Information was not given by the providers of the cells about the passage number prior to shipping the cells. Each laboratory then prepared seed and working stocks of the cell lines.

Cells were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum, L-glutamine and an antibiotic mixture (penicillin and streptomycin at concentrations of 100 IU/mL and 10 µg/mL, respectively). Sources of media, serum, antibiotics and other cell culture items such as plastic ware are minimally specified in the protocols (Protocols S1, S2), and each laboratory obtained the supplies from different vendors to mimic typical laboratory conditions; information about the sources are provided in Table S12.

Mycoplasma was tested after the cells were received and periodically by several of the laboratories that were not considered to be outliers. Control experiments were generated from data collected in all rounds and laboratories that were not considered to be outliers.

Conventional statistical analysis such as calculation of median and mean values, 95% confidence intervals and median absolute deviation values for the control experiments grouped by laboratory were performed using the R software package and ggplot2 to produce related figures. Both within-plate for a single laboratory and between-laboratory round statistics were calculated and plotted to identify potential systematic trends and widely dispersed data. Performance specifications for the assay control experiments were generated from data collected in all rounds and laboratories that were not considered to be outliers.

24 h in serum-free medium or 48 h in medium containing 10% serum but without NPs, which suggests that the serum had a negligible impact on the DLS results. The zeta potential of the NH2-PS NP in the cell culture media suspensions (without serum, with 1% serum, and with 10% serum) was measured using an electrophoretic mobility analyzer (Zetasizer Nano Z, Malvern Instrument). Values from five independent measurements were used to calculate the average zeta potential and its uncertainty.

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Cell line characteristics including proliferation rate, average cell volume and DNA short tandem repeats (i.e., cell line ID) were performed on each of the cell stocks used for experiments (Tab. S3) (Yu et al., 2015). Mycoplasma was tested after the cells were received and periodically by several of the laboratories using the ATCC assay and all tests were negative.

Abbreviated protocols
All culturing of A549-A and A549-B cells was performed at 37°C, 5% CO2 in humidified air. Maintenance of cells was performed in 75 cm² cell culture flasks which were passaged and seeded as described in Table S2. Cell harvesting was performed using trypsin and counting the cells using a hemocytometer in the presence of trypan blue using the procedure described in Protocols S1 and S2. The cell suspension was evaluated microscopically to confirm that the cells were single cell suspensions (no clumping) prior to cell counting. Cells were seeded at 1.5 x 10⁴ cells per well; additional wells were also prepared using cell culture medium without cells. The plate was then cultured for 24 h. For the cells exposed in the presence of serum, the medium was removed and fresh complete medium was added in addition to CdSO₄ or NP. For the cells in the serum-free condition, the same procedure was followed except that the cells were first washed three times with PBS to ensure removal of the serum and medium containing no serum was added in addition to CdSO₄ or NP. The exposure period was 24 h in serum-free medium or 48 h in medium containing serum. After the exposure period, the medium was removed from all wells, and the MTS reagent mixed with phenol red-free RPMI-1640 (without L-glutamine or antibiotics) was added to each well. The plates were then incubated for 60 min. Then, absorption measurements were performed with a plate reader at a wavelength of 490 nm.

Statistical analysis
Relative absorbance values were calculated for cells treated with CdSO₄ and NP for each treatment condition by dividing the median of the treatment condition less the median of the no cell background value by the median of the vehicle control condition less the median of the no cell background value.

Conventional statistical analysis such as calculation of median and mean values, 95% confidence intervals and median absolute deviation values for the control experiments grouped by laboratory were performed using the R software package and ggplot2 to produce related figures. Both within-plate for a single laboratory and between-laboratory round statistics were calculated and plotted to identify potential systematic trends and widely dispersed data. Performance specifications for the assay control experiments were generated from data collected in all rounds and laboratories that were not considered to be outliers.

The EC₅₀ values (i.e., concentration that causes a 50% change in the assay readout) and the uncertainty of the EC₅₀ values were calculated using Markov Chain Monte Carlo (MCMC) fitting procedures (Cornfield and Mantel, 1950) on the following statistical model. The data were the responses ri observed at i = 1, ..., 6 increasing dosing concentrations. The function of concentration versus rᵢ was used to form a dose-response curve, specifically, a logistic regression curve of the following form

\[ E(1-rᵢ) = \frac{γᵢ}{1 + e^{-(aᵢ-rᵢ)/bᵢ}} \]  
for i = 1,...,5 and j = 1,...,6 \hspace{1cm} (1)

\[ aᵢ ~ N(a, σᵃ) \]  
(2)

\[ log(βᵢ) ~ N(β, σ β) \]  
(3)

\[ log(γᵢ) ~ N(γ, σ γ) \]  
(4)

Here \( E \) is the expected value of the response, \( N(a, b) \) stands for Gaussian distribution with mean \( a \) and variance \( b \). In this model, \( aᵢ \) is the EC₅₀ for the \( i^{th} \) lab. In this hierarchical statistical model (Gelman et al., 2008) the \( aᵢ, βᵢ, \) and \( γᵢ \) parameters of (1) in the top hierarchy determine the dose-response curves for each lab and in the second hierarchy the \( a, β, \) and \( γ \) of expressions (2),...
Tab. 2: Comparison of within lab uncertainty to total uncertainty for determination of the EC_{50} values

| Condition                        | Total uncertainty (span of 95% confidence interval for consensus values) | Within lab uncertainty (average span of 95% confidence interval for each laboratory a) | Proportion of within lab uncertainty to total uncertainty b |
|----------------------------------|---------------------------------------------------------------------------|------------------------------------------------------------------------------------|---------------------------------------------------------------|
| NP serum-free A cell (mg/l)      | 11.9                                                                      | 2.1                                                                                | 18%                                                           |
| NP serum-free B cell (mg/l)      | 10.3                                                                      | 3.6                                                                                | 35%                                                           |
| NP serum A cell (mg/l)           | 24                                                                         | 14.3                                                                               | 59%                                                           |
| NP serum B cell (mg/l)           | 18.5                                                                      | 6.1                                                                                | 33%                                                           |
| CD serum-free A cell (µmol/l)    | 4.3                                                                       | 2.9                                                                                | 67%                                                           |
| CD serum-free B cell (µmol/l)    | 4.0                                                                       | 3.9                                                                                | 98%                                                           |
| CD serum A cell (µmol/l)         | 32                                                                         | 32                                                                                 | 100%                                                          |
| CD serum B cell (µmol/l)         | 45.1                                                                      | 45.1                                                                               | 100%                                                          |

a Outliers identified elsewhere were excluded in this analysis.

b Outliers identified elsewhere were excluded in this analysis. A value of 100% indicates that all of the total uncertainty in the test result can be explained by the within lab uncertainty. This would indicate a negligible contribution from between lab variability.

(3), and (4) represent the parameters of a common consensus curve. This random effects interlaboratory model (Toman and Possolo, 2009) is generalized to logistic regression. The model takes full advantage of the additional between-laboratory variability information produced by the interlaboratory study and estimates the variability that is due to unknown factors which differ among laboratories (Thompson and Ellison, 2011).

The parameters were estimated using Bayesian MCMC methods (Congdon, 2001) with non-informative prior distributions implemented using the freeware OpenBUGS (Lunn et al., 2009). The Bayesian analysis resulted in point estimates of the parameters as well as asymmetric 95% probability confidence intervals.

In cases where the logistic regression model resulted in a poor fit (i.e., the positive chemical control in the serum containing experiments, Fig. 10) other regression models such as a straight-line model were used to fit the data. The resulting EC_{50} values were not statistically different and thus a logistic regression model was used for all fitting. Correlation plots between control statistics and the EC_{50} values were used to identify potential control measurements whose value directly affects the measured EC_{50} value. Additional details about the MCMC approach used in this study are provided in a recent publication (Toman et al., 2016). In some cases, the 95% confidence interval of the dose-response curve extended below 0 absorbance units. This is an artifact of the fitting procedure and does not have physical significance.

Two different approaches were used to further study the variability in the EC_{50} values. Multivariate analysis of variance (ANOVA) of the EC_{50} values consistently revealed significant differences (p < 0.05) among laboratories under all conditions (both cell types; serum-containing and serum-free medium; and for the CdSO_{4} and PS-NH_{2} NP). However, the ANOVA approach only compared the EC_{50} values and did not include the substantial uncertainty in these values from the logistic curve fitting procedure. The second approach was a comparison of the 95% confidence intervals for the EC_{50} values for each laboratory (excluding outliers) to the 95% confidence interval for the consensus EC_{50} values (Tab. S5\textsuperscript{2}, S9\textsuperscript{2}). These confidence intervals were produced as part of the curve fitting and thus accounted for all uncertainty, including the between-laboratory uncertainty, and as a consequence show much better agreement among the measured EC_{50} values. It is important to note that this approach is capable of calculating these values even though the number of rounds performed varied among laboratories. In summary, Table 2 shows the total uncertainty, average within-labor uncertainty, and the proportion of within-laboratory uncertainty to total uncertainty for all of the conditions tested. When the average within-laboratory uncertainty was comparable to the total uncertainty, the data was considered to be harmonized. When the between-laboratory uncertainty was substantially larger than the within-laboratory uncertainty, control measurements were used to identify protocol steps that contributed to the larger between-laboratory variability.

In vitro sedimentation, diffusion and dosimetry model for nanomaterials

Target cell dose as a function of time and exposure conditions, i.e., the effectively deposited dose at the bottom of the exposure well, was computationally estimated by the In vitro Sedimentation, Diffusion and Dosimetry model known as ISDD. The model numerically solves a partial differential equation for both diffusion and sedimentation. It is available as Matlab code and as a Windows Executable from its developers (Hinderliter et al., 2010). It can be downloaded from http://nanodose.pnl.gov/ModelDownload.aspx. Further details and modeling parameters can be found in the supplementary information\textsuperscript{2}.
3 Results

3.1 Cell line characterization

Two A549 cell lines from different sources were used in the interlaboratory study. While the mean generation times of the two cell lines were statistically identical (22.6 h ± 2.2 h for A549-A and 22.5 h ± 2.5 h for A549-B, Tab. S3), genotyping of the genomic composition of the cell lines using commercial human-specific STR assays revealed that the A549-A cell line exhibited a dropout of the 12th allele at the CSF1PO position of chromosome 5 (Fig. S1). All other 23 markers were identical between the two cell lines and were consistent with the STR profile determined by ATCC. The short tandem repeat (STR) DNA analysis was repeated on the initial parent stock cell lines with the same results, indicating the A549-A cells arrived from the original vendor with the missing allele. No mycoplasma contamination was detected in the stock solutions sent out to the laboratories or in a receiving laboratory.

3.2 The effect of serum on NH2-PS NP

Incubation of the NH2-PS NP in 10% FBS but not in the serum-free medium (Tab. S4) led to substantial agglomeration (56 nm to 1258 nm) and change in zeta potential (48.8 mV to -10 mV). The size distribution of the primary particles determined by scanning electron microscopy was 51 nm ± 9 nm (uncertainty indicates standard deviation value of 200 particles; Fig. S2) (Hanna et al., 2016).

Therefore, it was decided to test the effects of NH2-PS NP in serum-containing and serum-free conditions to assess the effect of agglomeration on the variability in the assay results.

3.3 The effect of NH2-PS NP on A549 cell response

Figure 3 shows the dose-response curves obtained for A549-A and A549-B cells treated with NH2-PS NP in serum-free and serum-containing media in the 5 laboratories using Protocols S11 and S21, respectively. Figure 4 shows the estimated EC50 values for each of the average NP dose-response curves over all the rounds for each laboratory as determined by fitting the data with a MCMC simulated logistic curve.

Under serum-free conditions (Fig. 4A), the consensus EC50 values (excluding the laboratory A outlier) for the NH2-PS NP on the A549-A and A549-B cells were both approximately 22 µg/mL (see Tab. S5 for exact values), indicating a similar effect of the NP on both cell lines. Within-laboratory variabil-
ity for the EC$_{50}$ value for the A549-A and A549-B cell lines is only 18% and 35%, respectively, of the total laboratory variability represented by the confidence intervals on the consensus value (Tab. 2). This suggests that despite the use of a detailed protocol, differences among the laboratories, such as reagents and experimental technique, contribute to greater than 65% of the total variability associated with the consensus value. Our experimental design did not allow the further separation of the variability that results specifically from differences in reagents or experimental techniques among laboratories.

The consensus EC$_{50}$ values for NH$_2$-PS NP incubated in the presence of serum with the A549-A or A549-B cells (Fig. 4B, Tab. S5) were 57 µg/mL and 53 µg/mL, respectively, and exhibited overlapping 95% confidence intervals. These intervals were significantly larger than those observed under serum-free conditions. The within-laboratory variability for the A549-A and A549-B cell lines was 59% and 33%, respectively, of the total variability observed in the consensus value (Tab. 2). Testing NH$_2$-PS NP in the presence of serum thus increased the within-laboratory variability, suggesting that parts of the protocol involved in the addition of NP to serum-containing media increase variability in the test result even when repeated within the same laboratory. The variability between laboratories was at least 41% of the total variability, indicating that differences between reagents and experimental technique among the laboratories were also significant contributors to the total variability in the consensus value.

The EC$_{50}$ values reported in Table S5$^2$ are based on the nominal mass concentration of NP in the cell culture media. Modified dosing metrics that account for the diffusion and sedimentation properties of the nanomaterials in the media were generated using the ISDD model (see Supplemental Materials$^2$ for additional details and discussion). This model estimates the delivered dose of the NP to the surface of the cells based on the measured properties of the NP and the respective medium (see Tab. S6$^2$ for the delivered doses and Tab. S8$^2$ for modeling parameters). The corrected EC$_{50}$ mass dose for the NP in both serum-containing and serum-free medium was approximately 2.3 µg/cm$^2$ (Tab. S7$^2$). Although this value is reported in mass per surface area, the similarity in the value between the two conditions suggests that the increased measured EC$_{50}$ value for NP in serum is due to a reduction in available NP that can interact with cells due to agglomeration. Further studies are required to understand the mechanisms responsible for the difference in EC$_{50}$ values between the serum-containing and serum-free conditions.

### 3.4 System control experiments

Several system control experiments were incorporated in the 96-well plate design and the protocol to further evaluate potential sources of variability in the measurement process.

**Within-column cell density control (control 3)**

Protocols S1$^1$ and S2$^1$ specified that a single multi-channel pipetter is used to seed cells into each designated column of a 96-well plate. The nominal number of cells remaining in a well after rinsing and the variability in the cell density along the wells in the column were assessed by control 3 (Tab. 1, Fig. 2). The median absolute optical density of this control in both serum-containing and serum-free conditions for each round was on average 1.8 with a CV of < 10% (Fig. 5A). Laboratory B exhibited a CV larger than 10% but the highest OD values were only observed for the A549-B cells under serum-containing conditions. Laboratory A showed a cluster of data points from all the serum-free experiments with an average OD value of 1. This
outlier data cluster was lower than in the control experiments for the serum condition in that laboratory and the results from the other laboratories and correlated with the outlier EC<sub>50</sub> value data shown in Figure 3A and 4A, suggesting that the experimental steps that this laboratory used during the execution of the serum-free Protocol S1 resulted in a low value for this control and a low EC<sub>50</sub> value for the NP toxicity experiment.

Sensitivity analysis was used to determine if the estimated EC<sub>50</sub> values for the NH<sub>2</sub>-PS NP in all experiments were correlated with the cell densities measured in the control 3 wells. A correlation plot between the median OD values of control 3 and the EC<sub>50</sub> data from each round from all of the laboratories (Fig. 6A) indicated that significant correlations were only observed under the serum-free conditions when including the outlier data cluster from laboratory A. This result suggests that an OD value below 1 in control 3 wells can decrease the EC<sub>50</sub> value of the test result. There is no correlation with the OD value in control 3 wells and the NH<sub>2</sub>-PS NP EC<sub>50</sub> value if the control wells have an OD value between approximately 1.4 and 2.7. This analysis helps to explain the outlier dose-response curve shown for A549-A cells after NP treatment in serum-free conditions observed for Laboratory A in Figure 3A because the OD values for control 3 wells were below this range.

The wells in control 3 also provide a measure of within-column well-to-well variability in cell density. This control was prepared with a single multi-channel pipette ejection, and thus, large standard deviations between the wells can indicate pipette malfunction, inhomogeneous filling of the pipettes with cells, or aggressive rinsing techniques that dislodge cells from the surface inhomogeneously. The within-column well variability was as high as 20% in some cases but most of the rounds showed variability below 10% (Fig. 5B). Only laboratories B and C exhibited within-column well-to-well variability of less than 10% for all rounds.

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**Fig. 5:** Median values (A) and relative median average deviation (MAD) (B) of control 3 – within pipette cell seeding density of non-treated cells

Values for serum-free conditions included a rinsing step. An outlier set of data (red box) was observed in laboratory A. Each data point is the median or MAD value of a column of a plate for each round from a laboratory. Different levels of variability observed between laboratories suggests that there may be additional day to day variability in the rinsing step.

**Fig. 6:** Correlation of NH<sub>2</sub>-PS NP EC<sub>50</sub> values (A) and CdSO<sub>4</sub> EC<sub>50</sub> values (B) with mean OD no treatment cell control values for both cell lines (control 3)

Outlier data is indicated by red boxes. The solid line is the linear regression fit with outliers, while the dotted line is the fit without outliers. When outliers are included for the serum-free treatments for the CdSO<sub>4</sub> and the NH<sub>2</sub>-PS NP (OD below 1.0), the slopes are statistically different from 0, indicating that the EC<sub>50</sub> value is correlated with control 3 values. However, EC<sub>50</sub> values are not correlated with the non-treatment mean OD when these outliers are removed or for the serum treatment condition.
average deviation, a robust measurement of the variability of a univariate sample, between the wells in control 7 and 8 from all laboratories in both serum-containing and serum-free conditions is approximately 10% (Fig. 7C,D). Using medium containing serum, laboratories A, C and D showed variability of less than 5%, which is less than the average variability of 7% from all the laboratories. This suggests that pipetting or cell resuspension techniques in laboratory B and E should be compared to laboratories A, C, and D to identify possible differences in executing the cell seeding steps. The average within-laboratory variability between the control wells under serum-free conditions is significantly larger (approximately 12%) than under serum-containing conditions. This suggests that the additional rinsing step in the serum-free protocol may introduce additional variability. Correlations between either the median OD or the relative median average deviation values of these controls and the EC50 value for the NH2-PS NP and CdSO4 were not detected, suggesting that the assay can tolerate the level of variability shown in Figure 7 for controls 7 and 8.

**Between-column cell density control**
Controls 7 and 8 are a second set of pipetting controls that evaluate cell density variability between pipetting steps. They also report on the impact of different solvent vehicles for the positive chemical control and NP on a cell density measurement (Fig. 2). The average median OD values for both controls were significantly lower in serum-free conditions compared with serum-containing conditions (Fig. 7A,B). This could be due to a combination of longer incubation and fewer rinsing steps for the serum condition. The variability in these control values is similar between the laboratories (approximately 10%), but there is a systematic shift of the cluster average dependent on the laboratory. This suggests that reagents and protocol techniques specific to each laboratory such as cell resuspension during seeding, rinsing or cell counting before seeding steps introduced a systematic bias. An exception to this result was observed in laboratory B where clear separation of the A549-A and A549-B data occurred under the serum conditions (Fig. 7B). The cause of this systematic difference is unknown. The relative median average deviation, a robust measurement of the variability of a univariate sample, between the wells in control 7 and 8 from all laboratories in both serum-containing and serum-free conditions is approximately 10% (Fig. 7C,D). Using medium containing serum, laboratories A, C and D showed variability of less than 5%, which is less than the average variability of 7% from all the laboratories. This suggests that pipetting or cell resuspension techniques in laboratory B and E should be compared to laboratories A, C, and D to identify possible differences in executing the cell seeding steps. The average within-laboratory variability between the control wells under serum-free conditions is significantly larger (approximately 12%) than under serum-containing conditions. This suggests that the additional rinsing step in the serum-free protocol may introduce additional variability. Correlations between either the median OD or the relative median average deviation values of these controls and the EC50 value for the NH2-PS NP and CdSO4 were not detected, suggesting that the assay can tolerate the level of variability shown in Figure 7 for controls 7 and 8.
respectively, on the assay readout (i.e., absorption) in the absence
of cells. The absorbance values from control 1 were statistically
identical to those from control 4 for all rounds (data not shown),
indicating that the positive control CdSO\textsubscript{4} did not interfere with
the MTS assay readout system. However, comparing control
4 to control 5 results indicated that the NPs can interfere with
the assay readout in the presence of serum but not in serum-free
conditions (Fig. 9). In the presence of serum, three of the labora-
tories (B, C and E) show an absorbance change from 0.05 to up to
0.3 OD units (data average from all rounds) as the NP dosing
concentration increases. Laboratories A and D showed no sig-
nificant changes in absorbance value over these same concentra-
tions. This control indicates that procedural differences between
the laboratories when using NP in serum conditions can result in
a NP interference effect during the measurement of the OD value.

Non-cellular controls
The 96-well plate design has three control columns that do not
contain cells. Control 4 (within pipette volume control), which
is used to assess MTS background absorbance, showed tightly
clustered median OD values of less than 6\% from all laborato-
ries (Fig. 8A). Laboratory C demonstrated a within-laboratory
variability consistently less than 3\%, suggesting that there is a
procedure difference in the other laboratories that introduces up
to 50\% more variability in this control between rounds. Labora-
tory D exhibited a systematic upward bias in the median OD
value. This suggests that the MTS reagent or pipetting volume
setting in laboratory D introduced a systematic bias. The relative
median average distribution of the wells in the control 4 column
for all rounds and all laboratories is less than 5\% (Fig. 8B). Labo-
rary C and D showed variations of less than 2.5\%, indicating
that larger variability between the pipetting tip volumes in the
multichannel pipette occurred in the other laboratories.

Comparison of the values from control 4 to those from control 1
and 5 assesses the impacts of the positive chemical control or NP,
Interestingly, the NP dose-response curves showed no significant sensitivity to the two A549 cell lines used in this study (see Section 3.3, Fig. 4).

We observed a high level of agreement between each of the laboratories for the CdSO₄ EC₅₀ values in the presence of serum for both cell lines, but also a large uncertainty associated with the EC₅₀ estimation (Fig. 10). Similar to the results from the serum-free condition, there appeared to be a difference in the say system is operating as expected. There was a high level of agreement between the laboratories for the CdSO₄ control under serum-free conditions (Fig. 10) but the results significantly (p < 0.001) differed for the two cells lines (Tab. S9² shows exact EC₅₀ values). For each cell line, the within-laboratory variability is comparable to the total variability, suggesting that overall the protocol techniques used in the different laboratories can result in a well harmonized EC₅₀ value for a chemical response (Tab. 2). Interestingly, the NP dose-response curves showed no significant sensitivity to the two A549 cell lines used in this study (see Section 3.3, Fig. 4).

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**Tab. 3: System specifications for the MTS assay as defined by the interlaboratory comparison for A549 cells**

Meeting these control specifications is critical for achieving measurement assurance in the MTS nanocytotoxicity assay.

| Control                                      | Serum-free                  | Serum                          |
|----------------------------------------------|-----------------------------|--------------------------------|
|                                              | target value | range | variability | target value | range | variability |
| Control 1ᵃ (background chemical control)     | 0.06 OD       | 0.05-0.09 OD | < 6%       | see serum-free values |
| Control 2ᵇ (CdSO₄ control (EC₅₀ value))      | 49.9 µmol/l  | 47.5-51.5 µmol/l | 77.2 µmol/l | 54.3-99.4 µmol/l |
| Control 3 (within pipette variability)       | 1.8 OD        | 1.5-2.0 OD | < 10%       | 2.0 OD        | 1.8-2.3 | < 7%       |
| Control 4 (no cell and no treatment control) | 0.06 OD       | 0.05-0.09 OD | < 6%       | see serum-free values |
| Control 5ᶜ (background NP B11-G11)           | see Fig. 9    |        |             |                |        |            |
| Control 6                                    | not tested     |        |             |                |        |            |
| Control 7+8 (between pipette variability)    | 1.5 OD        | 1.3-1.8 OD | < 12%       | 2.2 OD        | 1.8-2.8 | < 7%       |

ᵃ If no additional background from the chemical reaction control is observed.
ᵇ Values of the A549-B cell line are given. They were fresh out of storage from ATCC and were passaged once and then sent to the participants.
ᶜ No values given, because some of the laboratories observed a background signal while others did not.
have shown decreased agglomeration of NPs in media with proteins (Schulze et al., 2008; Sager et al., 2007; Tantra et al., 2010; Kwon et al., 2014), increased agglomeration from serum proteins has been observed in other studies (Murdock et al., 2008; Caracciolo et al., 2014). In the serum-containing media, the lower zeta potential reduced electrostatic stabilization while stabilization by steric repulsion depends on the protein corona which in turn depends on the protein loading on the surface, composition of the proteins in the serum, and the surface chemistry of the particles, which in this case was not sufficient to stabilize the NH2-PS NP. This may be at least partly attributable to the high ionic strength in the serum-containing medium (≈ 1.5 mol/L) causing compression of the protein coating to 1 nm at which point van der Waals forces would become dominant.

An advantage of expressing in vitro exposures based on surface area dose metrics is that they can be compared to in vivo exposure results, but differences in the method of nano-material exposures should be considered. For example, in vivo studies are often carried out with lower doses per lung surface (excluding overload experiments). For various carbonaceous materials, it has been demonstrated that at similar doses, carbon black and graphite have no effect (0.1 or 0.03 µg/cm², respectively; an estimate of the rat lung surface area 0.3 m² was used in this calculation (Brown et al., 2005)), but graphene and multiwall carbon nanotubes do have an effect (0.03 or 0.01 µg/cm², respectively (Ma-Hock et al., 2013)). Moreover, in vivo investigations have shown that both the dosing rate and the delivered dose are responsible for toxicological effects. In a recent study, it could be demonstrated that at the same applied dose of approximately 160 µm TiO2 per rat lung, the time of application was important for the adverse effect. At high dose rates (> 105 µg/minute via instillation), the severe inflammatory effects were observed whereas at more typical dose rates of < 1 µg/min via inhalation, the nano-titanium dioxide had

response between the A549-A and A549-B cells, but it was not statistically significant due to the considerable overlap of the 95% confidence intervals. Evaluation of the sigmoidal curve fitting process for EC50 determination suggests that the large uncertainty for this condition was not entirely due to assay performance, but also due to the EC50 value falling between the last two dosing intervals (50 µmol/L and 100 µmol/L) of the assay. By having few dosing concentrations around the EC50 value, the error due to curve fitting the EC50 value is increased.

Performance specifications
The results from the system control experiments (Tab. 1) for all laboratories and all rounds excluding outliers were consolidated and summarized to form a set of system performance specifications (Tab. 3).

4 Discussion
The major focus of the study was the use of an assay plate design and an interlaboratory comparison study to evaluate the robustness of the steps of an MTS assay protocol. Although the biological effects of the NH2-PS NP on the A549 cell line are interesting, further investigation into the mechanism of these effects was not considered in this study. We also did not consider how the use of different cell lines and nanoparticles would influence the values that were measured in this study. Although the protocols described here may need to be modified for a particular cell line and nanoparticle, the general concepts introduced in this study should be applicable to most nanocytotoxicity tests.

The characterization of the NPs in the absence or presence of serum revealed substantial agglomeration and a drop in the zeta potential in the presence of serum. While many studies have shown decreased agglomeration of NPs in media with proteins (Schulze et al., 2008; Sager et al., 2007; Tantra et al., 2010; Kwon et al., 2014), increased agglomeration from serum proteins has been observed in other studies (Murdock et al., 2008; Caracciolo et al., 2014). In the serum-containing media, the lower zeta potential reduced electrostatic stabilization while stabilization by steric repulsion depends on the protein corona which in turn depends on the protein loading on the surface, composition of the proteins in the serum, and the surface chemistry of the particles, which in this case was not sufficient to stabilize the NH2-PS NP. This may be at least partly attributable to the high ionic strength in the serum-containing medium (≈ 1.5 mol/L) causing compression of the protein coating to 1 nm at which point van der Waals forces would become dominant.

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no effect in the lungs of the rats (Baisch et al., 2014). Further studies may be required to clarify which physiologically relevant parameters of an in vivo system are captured in actual in vitro experiments.

Each laboratory performed three to eight rounds of the experiment which allowed assessment of both the within-laboratory variability and the between-laboratory variability of the MTS assay result. Comparison of the within-laboratory variability to the between-laboratory variability revealed apparent harmonization of the positive chemical control CdSO₄ EC₅₀ results while only moderate harmonization was observed for the NH₂-PS NP treatment. Evaluation of the system controls and comparing the system controls under serum-containing and serum-free conditions provides insights into which steps of the protocol may need further refinement to improve interlaboratory comparability of this assay especially when used with NPs.

When evaluating the within pipette cell seeding density of non-treated cells (control 3; Fig. 5A), the values from laboratory A clearly indicate seeding of a lower cell concentration under serum-free conditions than in the other laboratories and these lower cell concentrations resulted in lower EC₅₀ values (Fig. 6). These results agree with findings which were obtained in a previous study demonstrating that the Min-U-Sil particles, a colloidal crystalline silica particle, caused a greater decrease in viability with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay performed at lower cell densities (Geys et al., 2010). However, the cell concentrations in this previous study were intentionally varied while those in this interlaboratory comparison study occurred unintentionally as a result of unexpected differences in rinsing procedures. Discussion among participating laboratories revealed that laboratory A used a more rigorous cell rinsing aspiration technique than the other laboratories, which appears to have resulted in cell detachment. Confirmatory experiments (Fig. 11) comparing the rigorous cell rinsing technique (vacuum aspiration) with a gentler rinsing technique (pipetting) demonstrated this to be the cause of the lower optical density in Figure 6 and the likely cause of the outlier data for laboratory A shown in Figures 3A, 4A and 5A. These data indicate that the rinsing procedure used to remove media from the cells should be more clearly specified in the protocol. The inclusion of the triple rinsing step in only the serum-free protocol was intended to minimize the number of assay steps and it was not anticipated prior to the interlaboratory study that the rinsing procedure might impact the assay results.

Evaluation of the NP interference control (control 5; Fig. 9) reveals that the rinsing procedures for removing media containing NP also need to be more clearly specified in the protocol. The increase in the optical density for control 5 at the two highest dosing concentrations indicates that laboratory B, C and E did not fully remove the NP before adding the MTS reagents; this could have increased variability in the measured NP EC₅₀ value. Discussion between the laboratories did not fully identify a cause for this effect, but a likely reason could be related to the laboratories incompletely removing the media from the cell culture wells prior to the addition of the MTS reagent. This issue was also observed in previous ENM inter-laboratory comparison using the MTS assay. Xia et al. added a centrifugation step to their protocol to remove NP interference effects (Xia et al., 2013). Testing the impact of a centrifugation step or modified rinsing steps to decrease ENM interference will be important for optimizing the MTS protocol described here for better harmonization between laboratories.

Results from the within pipette volume control (control 4) show harmonization between laboratories with respect to their ability to pipette volumes within a pipetting step (Fig. 8). Figure 8B shows there is less than 5% variability between pipette volumes within a pipette step over all rounds performed.
in all laboratories during this experiment. This suggests that pipetting a volume of liquid is not a major contributor to the variability in the assay results. When this data is compared to pipetting with cells (control 3), the variability between rounds for a single laboratory increases to as high as 20%, and there is more variability among the laboratories. This reveals that the combination of cell counting, cell seeding, and cell rinsing is a significant cause of the variability in the assay results. This suggests that the methods that are used for these steps may need to be more clearly defined in the protocol. Additional experiments may be required to establish the best cell handling techniques.

Variability of EC$_{50}$ values for the CdSO$_4$ positive control (control 2) and the NH$_2$-PS NP material within each of the laboratories increased in the order of serum-free CdSO$_4$ < serum-free NP < serum CdSO$_4$ ≈ serum NP (Fig. 4, 5). This suggests that the use of NP introduces additional variability to the assay for the serum-free condition, possibly due to the more complicated behaviors of the dispersed NP compared to well-dissolved chemicals (Cohen et al., 2015; Teeguarden et al., 2007).

Overall, serum does appear to have a protective effect for both cell lines exposed to both toxicants as the consensus EC$_{50}$ are significantly increased in the presence of serum (Fig. 4, 5). The source of this effect is unclear as the serum could affect both the cell physiology and the NP agglomeration state and surface charge (Tab. S4) and may interact with Cd. Serum is known to induce a protein corona around NP, which can insulate the cell from NP surface effects (Cedervall et al., 2007; Lundqvist et al., 2008). These factors may also explain the increased variability observed for NP under the serum condition. The NP dispersion step of the protocol is not precisely defined and the timing between the dispersion step and the dosing step is not specified, which can influence the extent of agglomeration during the exposure or the structure/composition of the protein corona. If variable precipitation of the NP agglomerates occurred during the separate rounds, the cells may have been effectively exposed to different dosing conditions (Petersen et al., 2014). Improved dispersion procedures or the use of more automation in preparing the dosing plate could reduce the variability observed within the laboratory when using NH$_2$-PS NP and serum. This factor may also contribute to the variability of the EC$_{50}$ value for the NH$_2$-PS NP material between laboratories. The experimental design used here does not provide specific insight on this source of variability as several reagents including the sources of serum (Tab. S1) differ between the laboratories. The different sources of serum could biologically influence the cellular response to both the CdSO$_4$ and NH$_2$-PS NP treatments. Further experiments are needed to evaluate how these factors influence the test response. If serum is shown to induce significant variability in the test results, a recommendation would be to share the serum between the laboratories to improve the comparability of test results. However, legal regulations involving bovine serum products may prevent sharing of serum between laboratories in different countries. Alternatives such as chemically defined cell culture media should be investigated for future studies.

An interesting source of variability that was clearly observed in the CdSO$_4$ positive control (control 2) only under serum-free conditions was the source of the A549 cell line (Fig. 10). Although the stocks for both cell lines were at early passage times (less than passage 25), the EC$_{50}$ value for CdSO$_4$ was 2-fold larger for the A549-B cell line when compared to the A549-A cell line despite the cell lines having statistically identical growth rates (Tab. S3). This difference may be attributable to the genetic modification detected when comparing the STR typing profiles between the A549 cell lines (Fig. S1). The CdSO$_4$ positive control was added to the plate layout as a system control that could verify the nominal operation of the MTS assay. In this study, it revealed how cell lines with the same name but from a different source can influence a toxicity result. These results suggest that it is critical that laboratories share a genetically identical cell line to ensure the comparability of their results. Furthermore, genetic identification should be established routinely to ensure the cell line is not changing with time (Chatterjee, 2007; Almeida et al., 2011).

The dosing intervals can affect the 95% confidence interval predicted for EC$_{50}$ values due to limitations in modeling the cell response between the dose interval values (Robinson et al., 2009). In this study, the calculated 95% confidence intervals in the EC$_{50}$ value for both NH$_2$-PS NP and CdSO$_4$ in the presence of serum for all laboratories were several-fold larger than those calculated for the treatments in serum-free conditions (Tab. S5, S9, Fig. 4, 10). Although the experimental details are responsible for some fraction of the observed variability, it is important to note that both of the EC$_{50}$ values for NH$_2$-PS NP and CdSO$_4$ in the presence of serum occurred in the last dosing interval resulting in additional apparent variability during the fitting process. Improved dosing intervals for NP toxicity experiments will reduce variability due to curve fitting uncertainty. Pilot studies could be used to estimate the EC$_{50}$ value and dosing intervals could be designed around these values (Robinson et al., 2009). Additional studies are needed to determine what fraction of the variability in the EC$_{50}$ value is derived from curve fitting with selected dosing intervals.

An important use of the system control data obtained from this interlaboratory comparison is to set the ranges for system specifications to ensure comparability in the assay protocol and confidence in the assay test result (Plant et al., 2014). Table 3 shows several performance specifications for the MTS assay protocol. Correlation analysis of these control values and the EC$_{50}$ value of the NH$_2$-PS NP test results suggest that these ranges do not directly influence the measured EC$_{50}$ values (Fig. 6, results for other controls not shown). The test result would be considered “valid” or at least comparable to data described here if each of these specifications is met. Each of the specifications provides unique information about the protocol steps, and failure to meet a specification can allow troubleshooting of the assay procedure to identify possible improvements in executing the protocol. Charting the system control measurements over time enables the observation of trends indicative of changes in assay performance. Developing and using assay
protocols that provide performance specifications and using control charts will aid in establishing quality management of a cell-based assay and improve confidence in short- and long-term comparability of assay results within a laboratory and between laboratories.

In conclusion, the combination of system control measurements and interlaboratory comparison data yielded insights that would not have been available by either approach by itself. While an interlaboratory comparison, without the control measurements and common assay design, would provide measures of within- and between-laboratory variability, it would not reveal the specific causes of variability associated with different steps of the assay. Similarly, a single laboratory assessing an assay with system control measurements would provide insight about the relative sources of variability for the assay within that laboratory. However, this approach would not provide information about the unknown sources of variability among different laboratories and how different interpretations of steps of the protocol or differing precision in a single step among laboratories affect the test results. Our study revealed that cell line ID, rinsing procedures, cell handling, NP dispersion, and choice of dosing intervals can significantly influence the assay results. The approach described here can be used in future studies to test a broader array of assays with a larger number of cell lines. It may be necessary to modify the protocol before use with other cytotoxicity readouts (e.g., MTT and XTT assay) to tailor the control measurements for assay-specific sources of variability which may differ from the MTS assay. This approach is designed to produce robust assays which will enable better decision-making during risk analysis of engineered nanomaterials.

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Author contributions
JTE, MR, MLS, HFK, NWS, and PW designed the experimental protocols. JTE, MR, NWS, AKO, RM, FS, JL, SJK, FR, CH, and WS conducted the cytotoxicity experiments. ELR conducted the STR analysis. BT conducted the Markov Chain Monte Carlo fitting simulation and the comparison of the within-laboratory variability to total variability. JTE, MR, and EJP contributed to the data analysis and writing the manuscript. All coauthors reviewed the manuscript.

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Conflict of interest
The authors declare that they have no conflict of interest.