Research Article
Toward Achieving Harmonization in a Nanocytotoxicity Assay Measurement through an Interlaboratory Comparison Study

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Summary
Design and development of reliable cell-based nanotoxicology assays are 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 nano-cytotoxicity MTS cell viability assay. 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 the A549 cell line from ATCC for positively charged polystyrene nanoparticles for the serum free and serum conditions, respectively, and were 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 nano-cytotoxicity 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

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Received May 2, 2016; Accepted September 20, 2016; Epub September 29, 2016; http://dx.doi.org/10.14573/altex.1605021
1 Introduction

Engineered nanomaterials (ENMs) have unique physicochemical properties due to their small size, high surface to volume ratio and spatially controlled compositions. 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; Schrurs 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 such as through the use of in vivo to in vitro comparisons to compare results from toxicological studies with in vitro lung models to those from 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 in different laboratories (Schrurs 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 (Schrurs 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 “validated” nano-cytotoxicity assays (Krug and Wick, 2011; Nel et al., 2013; Landsiedel et al., 2009; Landsiedel et al., 2014). Validation in this context would indicate that there is data to demonstrate that assay results from different operator or laboratories are comparable and that the protocol is robust to small changes in operating conditions.

The challenges associated with developing reliable nano-cytotoxicity 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 which are obtained from results in multiple laboratories, 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, NANOtec) comparison of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) nano-cytotoxicity assay and the evaluation of the transferability and reproducibility of the assay procedure. The MTS assay is a colorimetric assay for testing cell viability by measuring the reduction of the tetrazolium dyes to its 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 has only a few basic steps in the protocol. Figure 1 illustrates the serum and serum-free protocols (Protocols S1 and S2) that were derived from the manufacturer’s instructions and the results from a cause-and-effect analysis of the MTS assay (Risslein et al., 2014). The protocols include 8 system controls to quantify critical sources of variability in the assay (Figure 2 and Table 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 media, and cell culture plates (Tables S1 and S2). Some factors were controlled, specifically that all participants had the same stock of NH2-Ps nanoparticles (NP) (i.e. positively charged-polystyrene NP), the positive chemical control reagent (GdSO4) and the two human A549 cell lines. The NH2-Ps

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1 List of abbreviations

CV, Coefficient of variation; 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; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nanoEHS, Nanotechnology environmental and health safety; NH2-Ps, positively charged-polystyrene nanoparticles; NP, Nanoparticles; OD, Optical density; SEM, Scanning electron microscopy; STR, Short tandem repeat; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
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 characterization in serum and serum-free media. NIST conducted the SEM analysis.

Protocol Development and Experimental Design of the Interlaboratory Comparison

The final measurement protocol is a modified version of the MTS manufacturer’s protocol and is contained in the supplemental material (Protocols S1 and S2). The protocol flowchart is shown in Figure 1 and is summarized below. The 96-well plate design was based on previous work at EMPA and NIST for studies in the International Alliance of NanoEHS Harmonization (IANH). An image of the 96-well plate design and the description of the controls are shown in Figure 2.

All replicate experiments or “round” from each lab was a full implementation of the protocol. Each round included four 96-well plates: 2 plates contained A549-A cells and 2 plates contained A549-B cells. One plate with each cell line was used with NP dispersed in serum or serum-free media. Each plate contained both a positive chemical control with CdSO₄, a test NP, and several control experiments encoded into the 96-well plate design (Figure 2 and Table 1). Laboratories A, B, C, D and E performed 8, 6, 6, 4, and 3 rounds, respectively, before the data was sent to EMPA for statistical analysis.

Reagents

NH₂-PS NP suspended in H₂O were obtained from Bangs Laboratories Inc (Fishers, Indiana, US), lot number 10351 and inventory number L120117F, 10% (w/v). CdSO₄·7H₂O (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 CdSO₄ 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.

Characterization of the NH₂-PS NP

The original suspension of PS-NH₂ NP received from EMPA was diluted by a factor of 1000 in 18 MΩ water, cell culture media without serum, 0.1 % fetal bovine serum (FBS) media (0.1 % FBS added into cell culture media without serum) and 10% FBS in cell culture media, respectively. The sample suspensions were measured for size and zeta potential just after the dilution and after incubation for 24 h and 48 h (37 °C, 5 % atmospheric CO₂).

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 two second detection time. To remove potential interference from dust scattering, the six measurement results with the lowest DLS sizes were selected to calculate the average ENM size and its uncertainty. The scattering intensity of the nanoparticle suspension was ≈ 25 times greater than that of the serum containing media, which suggests that the serum had a negligible impact on the DLS results. The zeta potential of the PS-NH₂ NP in the cell culture media suspension were measured with 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 of the particles was measured via scanning electron microscopy 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. The first 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 to the other laboratories. A second A549 cell line (A549-A) purchased from LAZ, 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 cells 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 provided from the providers of the cells about the passage number prior to shipping the cells. Each laboratory then prepared seed and working stocks of this cell line. Cells
were maintained in Roswell Park Memorial Institute (RPMI) media containing 10% fetal bovine serum containing an antibiotic mixture and were passaged as described in Table S1. 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 (Table S3) (Yu et al., 2015). The STR DNA analysis was identical when repeated on the initial parent stock cell lines suggesting the A549-A cells arrived from the original vendor with the missing allele. Other culture items, serum sources, media and antibiotics (penicillin and streptomycin, L-glutamine and 10% fetal calf serum for the serum condition. All cell culturing of A549-A and A549-B 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 after trypan blue staining 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. To prepare the cells for seeding for the MTS assay, 10 mL of cell culture medium per 96 well plate with a cell concentration of 7.5 x 10^3 cells/mL was prepared. Cells were seeded at 1.5 x 10^5 cells per well using the pipetting procedure described in Protocols S1 and S2; additional wells were also prepared using cell culture medium but without cells. The plate was then cultured for 24 h using the conditions described above. Preparation of the working concentrations of the chemical control and nanoparticles were prepared as described in Protocols S1 and S2. For the cells exposed in the serum condition, the media was removed and then additional media was added in addition to the chemical control or nanoparticles. For the cells in the no serum condition, the same procedure was followed except that the cells were first washed three times with PBS to ensure removal of the serum. Cells were exposed for 24 h for the no serum condition or for 48 h for the serum condition. At the termination of the exposure period, the content was removed from all wells, and the MTS reagent mixed with RPMI-1640 (but not L-glutamine or antibiotics) was added to each well. The plates were then incubated for 60 minutes using the culturing conditions described above. Then, absorption measurements were performed with a plate reader at a wavelength of 490 nm. In a separate experiment, the precision of the plate readers was tested by measuring a single plate with cells treated with the MTS reagent multiple times.

Statistical Analysis
Relative absorbance values were calculated for cells treated with CdSO₄ and the NH₂-PS NP for each treatment condition. For the CdSO₄ treatment conditions, the relative absorbance values were calculated using the ratio of the absorbance values at 490 nm for the treatment condition subtracted by the no cell background values (control 1) to the absorbance value for the CdSO₄ vehicle control cells (control 7) subtracted by the no cell background values (control 1). The following are the equations used for several of the treatment conditions (Figure 2 shows the plate layout).

Relative absorbance (0) = (median (B3-B5) - median (B2-G2)) / (median(B3-B5) - median (B2-G2)) = 1
Relative absorbance (1) = (median (C3-C5) - median (B2-G2)) / (median(B3-B5) - median (B2-G2))
Relative absorbance (10) = (median (D3-D5) - median (B2-G2)) / (median(B3-B5) - median (B2-G2))
Relative absorbance (25) = (median (E3-E5) - median (B2-G2)) / (median(B3-B5) - median (B2-G2))

For the NP treatment conditions, the relative absorbance values were calculated using the ratio of the absorbance values at 490 nm for the treatment conditions subtracted by background (no cells but with NP addition at the treatment concentration) to the absorbance value for the NP vehicle control cells (control 8) subtracted by the no cell background. The following are the equations used for several of the treatment conditions (Figure 2 shows the plate layout).

Relative absorbance (0) = (median (B5-B10) - value (B11)) / (median(B8-B10) - value (B11)) = 1
Relative absorbance (1) = (median (C8-C10) - value (C11)) / (median(B8-B10) - value (B11))
Relative absorbance (10) = (median (D8-D10) - value (D11)) / (median(B8-B10) - value (B11))
Relative absorbance (25) = (median (E8-E10) - value (E11)) / (median(B8-B10) - value (B11))

Conventional statistical analysis such as calculation of median and mean values, 95 % confidence intervals and median absolute deviation values for the control experiments as grouped by laboratory were generated from data collected in all rounds and laboratories that were not considered to be outliers.

The EC₅₀ values (e.g. concentration of either chemical or ENM toxin that causes a 50 % change in the assay readout) and the uncertainty in the EC₅₀ value 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 1- rᵢ was used to form a dose-response curve, specifically, a logistic regression curve of the following form:

\[ rᵢ = \frac{1}{1 + e^{-\beta cᵢ}} \]
\[ E(1 - r^i_j) = \frac{y_i}{1 + e^{(y_i - \gamma^i_j) / \alpha^i_j}} \quad \text{for } i=1,...,5 \text{ and } j=1,...,6 \]  

Here, \( E(\cdot) \) stands for expected value of the response, \( N(a, b) \) stands for Gaussian distribution with mean \( a \) and variance \( b \). In this model, the \( \alpha^i_j \) is the EC\( 50 \) for the \( i^{\text{th}} \) lab. This is a hierarchical statistical model (Gelman et al., 2008) with the \( \alpha^i, \beta^i \), and \( \gamma^i_j \) parameters of (1) in the top hierarchy determining the dose-response curves for each lab and in the second hierarchy the \( \alpha, \beta, \) and \( \gamma \) of expressions (2), (3), and (4) representing the parameters of a common consensus curve. This is a random effects inter-laboratory model (Toman and Possolo, 2009) generalized to logistic regression. This model takes full advantage of the additional between laboratory variability information produced by the inter-laboratory study and better estimates the variability that is due to unknown factors that differ among laboratories (Thompson and Ellison, 2011).

The parameters were estimated using Bayesian Markov Chain Monte Carlo 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, Figure 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 is 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 and no serum; 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 (Tables S5, S9). 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 of the experiment performed varied among laboratories. In summary, Table 2 shows the total uncertainty, average within lab 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 supplemental information.

### 3 Results

#### 3.1 Cell line characterization

Two A549 cell lines from different sources were introduced into the design of 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, Table S3), genotyping of the genomic composition of the cell lines using commercial human-specific short tandem repeat (STR) assays revealed that the A549-A cell line exhibited a dropout of the 12 allele at the CSF1PO position of chromosome 5 (Figure S1). All 23 other markers identified were identical between the two cell lines and were consistent with the STR profile determined by ATCC.

#### 3.2 The effect of NH\(_2\)-PS NP on A549 cell response

Figure 3 shows the dose-response curves obtained for A549-A and A549-B cells treated with NH\(_2\)-PS NP in serum-free and serum media from the 5 laboratories using protocols S1 and S2, respectively. Microscopic visualization of the cells in the 96-well plate was performed periodically to confirm that the decrease in MTS signal corresponded to a decrease in cell number. The decision to test the effects of NH\(_2\)-PS NP in serum and serum-free conditions was based on the substantial agglomeration (56 nm to 1258 nm) and change in zeta potential (48.8 mV to -10 mV) that occurred to the NH\(_2\)-PS NP after
incubation in 10 % FBS but not in the serum-free condition (Table S4). This allowed testing of the effect of agglomerate on the variability in the assay results. 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 surface, and the surface chemistry of the particles which in this case was not sufficient to stabilize the NH$_2$-PS NP. This may be at least partly attributable to the high ionic strength in the serum containing media ($\approx$ 1.5 mol/l) causing compression of the protein coating to 1 nm at which point van der Waals forces would become dominant. The size distribution of the primary particles determined by scanning electron microscopy was 51 nm \pm 9 nm (uncertainty indicates standard deviation value of 200 particles; Figure S2) (Hanna et al., 2016).

Figure 4 shows the estimated EC$_{50}$ 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 Markov Chain Monte Carlo (MCMC) simulated logistic curve. Under serum-free conditions (Figure 4a), the consensus EC$_{50}$ values (excluding the laboratory A outlier) for the NH$_2$-PS NP and the A549-A and A549-B cells were approximately 22 µg/mL (see Table S5 for exact values), indicating a similar effect on both cell lines. Within laboratory variability for the EC$_{50}$ value for A549-A and A549-B cell line is only 18 % and 35 %, respectively, of the total laboratory variability represented by the confidence intervals on the consensus value (Table 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 dispersed in serum conditions with the A549-A or A549-B cells (Figure 4b and Table S5) were 57 µg/mL and 53 µg/mL, respectively, and exhibited overlapping 95 % confidence intervals. These intervals were significantly larger than those observed in serum-free conditions. The within laboratory variability for the A549-A and A549-B cell lines were 59 % and 33 %, respectively, of the total variability observed in the consensus value (Table 2). Testing NH$_2$-PS NP in serum conditions 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 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 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 media (see Table S6 for the delivered doses and Table S8 for modeling parameters). The corrected EC$_{50}$ mass dose for the NP in both serum and serum free media was approximately 2.3 µg/cm$^2$ (Table S7). 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 and serum-free conditions.

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 nanomaterial 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/cm$^2$, respectively; an estimate of the rat lung surface area 0.3 m$^2$ 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/cm$^2$, 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 TiO$_2$ per rat lung, the time of application was important for the adverse effect. At high dose rates ($\approx$10$^4$ 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 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.

3.3 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 and S2 dictated that a single multi-channel pipetter is used to seed cells in a single column of a 96-well plate. The nominal number of cells in a well after rinsing and the variability in the cell density along the wells in the column were assessed by control 3 (Table 1 and Figure 2). The median absolute optical density of this control in both serum and serum-free conditions for each round was on average 1.8 with a CV of <10 % (Figure 5a). Laboratory B exhibited a CV larger than 10 % but the highest OD values were only observed for the A549-B cells under serum conditions. Laboratory A showed a cluster of data points from all the serum-free experiments at an average OD value of 1. This outlier data cluster appeared lower than the control experiments for the serum condition and the results from the other laboratories. This cluster of low OD values for control 3 correlated with the outlier EC$_{50}$ value data shown in Figure 3a and 4a suggesting that the
Laboratories A and D showed no sign of absorbance change from 0.

All chemical control compound and the absorbance values of chemical control or NP, respectively, on the assay readout of all laboratories.

Of the pipetting volume setting laboratories (Figure 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. Laboratory 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 round and all laboratories is less than 5 % (Figure 8b). Laboratory 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, respectively, on the assay readout (i.e. absorption) in the absence of cells. CdSO₄ was used as a chemical control compound and the absorbance values from control 1 were statistically identical to those from control 4 for all rounds (data not shown), indicating that CdSO₄ 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 under serum conditions but not in the serum-free condition (Figure 9). Under serum conditions, three of the laboratories (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 significant changes in absorbance value over these same concentrations. 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.
Positive Chemical Control

The assay plate design also incorporates chemical response measurements (control 2) that serve as a system control to confirm that the complete assay system is operating as expected. There is a high level of agreement between the laboratories when the CdSO_4 control is performed under serum-free conditions (Figure 10) but the results significantly ($p<0.001$) differed for the two cells lines (Table S9 shows exact EC_{50} 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 EC_{50} value for a chemical response (Table 2). Interestingly, the NP dose-response curves showed no significant sensitivity to the two A549 cell lines used in this study (see Figure 4).

We observed a high level of agreement between each of the laboratories for the CdSO_4 EC_{50} values under serum conditions for each cell line, but also a large uncertainty associated with the EC_{50} estimation (Figure 10). Similarly to the results from the serum-free condition, there appeared to be a difference in the response between the A549-B and A549-A 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 EC_{50} determination suggests that the large uncertainty for this condition was not entirely due to assay performance, but also due to the EC_{50} 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 EC_{50} value, the error due to curve fitting the EC_{50} value is increased.

Performance Specifications

The results from the system control experiments (Table 1) for all laboratories and all rounds excluding outliers were consolidated and summarized to form a set of system performance specifications (Table 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 in an MTS assay protocol. Although the biological effects of the NH_4-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. 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 chemical control CdSO_4 EC_{50} results while only moderate harmonization was observed for the NH_4-PS NP treatment. Evaluation of the system controls and comparing the system controls under serum 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 control 3, the within pipette cell seeding density of non-treated cells (Figure 5a), the measured optical density from laboratory A clearly indicates a lower cell concentration under serum free cells conditions that was not observed in the other laboratories. Discussion among participating laboratories revealed that laboratory A used a more rigorous cell rinsing aspiration technique than the other laboratories which resulted in apparent cell detachment under the serum-free conditions. Confirmatory experiments (Figure 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 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 control 5, the NP interference control (Figure 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 indicate that laboratory B, C and D did not fully remove the NP before treating with the MTS reagents: this could increase in variability in the measured NP EC_{50} value. Discussion between the laboratories did not fully identify a cause for this effect, but a likely reason could be related to the laboratory completely removing the media from the cell culture wells prior to the addition of the MTS reagent. This issue was also observed in previous ENM interlaboratory comparison using the MTS assay. These authors added a centrifugation step to their protocol to remove the 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 Control 4, the within pipette volume control, show harmonization between laboratories with respect to their ability to pipette volumes within a pipetting step (Figure 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 control 3 which includes pipetting with cells, 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 are 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 techniques cell handling.
Variability of EC₅₀ values for control 2, the CdSO₄ positive control, and the NH₂-PS NP material within each of the laboratories increased in the order of serum-free CdSO₄ < serum-free NP < serum CdSO₄ < serum NP (Figures 4 and 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., 2017; Rosenberg et al., 2007). Overall, serum does appear to have a protective effect for both cell lines exposed to both toxicants as the consensus EC₅₀ are significantly increased under serum conditions (Figure 4 and 5). The source of this effect is unclear as the serum can affect both the cell physiology and the NP agglomeration state and surface charge (Table S4) and may interact with Cd. Also, serum is known to induce a protein corona around NP, which can insulate the cell from NP surface effects (Cederwall 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 NH₂-PS NP and serum. This factor may also contribute to the variability of the EC₅₀ value for the NH₂-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 (Table S1) differ between the laboratories. The different sources of variability could biologically influence the cellular response of both the CdSO₄ and NH₂-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 control 2, the CdSO₄ positive control, under serum-free conditions was the source of the A549 cell line (Figure 10). Although the stocks for both cell lines were at early passage times (less than passage 25), the EC₅₀ value for CdSO₄ 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 (Table S3). The cell line-dependent response was not statistically significant (overlapping 95% confidence intervals) when the CdSO₄ treatment was performed in serum conditions or with the NH₂-PS NP under either condition. This difference may be attributable to the genetic modification detected when comparing the STR typing profiles between the A549 cell lines (Figure S1). The CdSO₄ 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 EC₅₀ 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 EC₅₀ value for both NH₂-PS NP and CdSO₄ in serum conditions for all laboratories were several-fold larger than those calculated for the treatments in serum-free conditions (Tables S5 and S9 and Figures 4 and 10). Although the experimental details are responsible for some fraction of the observed variability, it is important to note that both of the EC₅₀ values for NH₂-PS NP and CdSO₄ in serum conditions occur in the middle of the last dosing interval (50 units to 100 units) resulting in additional apparent variability during the fitting process. Improved dosing intervals for NP toxicity experiments will reduce the variability due to curve fitting uncertainty. In some cases, pilot studies are used to estimate the EC₅₀ value and dosing intervals are designed around these values (Robinson et al., 2009). Additional studies are needed to determine what fraction of the variability in the EC₅₀ 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 ensuring comparability in the assay process 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 EC₅₀ value of the NH₂-PS NP test results suggest that these ranges do not directly influence the measured EC₅₀ values (Figure 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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http://dx.doi.org/10.1021/tx200645n.


http://dx.doi.org/10.1088/0026-9290/53/1/S40.


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

Acknowledgements
This project was supported by the Nano Material Technology Development Program (NRF-2011-0020504 and NRF-2014M3A7B6020163) of MSIP/NRF, the NanoScreen Materials Challenge co-funded by the Competence Centre for Materials Science and Technology (CCMX), and the Bilateral Korean-Swiss S&T program in 2012. Research performed in part at the NIST Center for Nanoscale Science and Technology.

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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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).

<table>
<thead>
<tr>
<th>Control Number</th>
<th>Brief Control Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Background correction for the chemical dosing. Allows detecting if increasing concentrations of the reference chemical change the final MTS measurement result.</td>
</tr>
<tr>
<td>2</td>
<td>Triplicate reference chemical control. Shows that the assay worked as expected.</td>
</tr>
<tr>
<td>3</td>
<td>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.</td>
</tr>
<tr>
<td>4</td>
<td>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.</td>
</tr>
<tr>
<td>5</td>
<td>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.</td>
</tr>
<tr>
<td>6</td>
<td>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).</td>
</tr>
<tr>
<td>7+8</td>
<td>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 seeding procedure and possible effects of the solvent if compared to “no treatment” wells (B6-G6).</td>
</tr>
</tbody>
</table>
Tab. 2: Comparison of within lab uncertainty to total uncertainty for determination of the EC\textsubscript{50} values

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total uncertainty (span of 95% confidence interval for consensus values)</th>
<th>Within lab uncertainty (average span of 95% confidence interval for each laboratory\textsuperscript{1})</th>
<th>Proportion of within lab uncertainty to total uncertainty\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP serum free A cell (mg/l)</td>
<td>11.9</td>
<td>2.1</td>
<td>18%</td>
</tr>
<tr>
<td>NP serum free B cell (mg/l)</td>
<td>10.3</td>
<td>3.6</td>
<td>35%</td>
</tr>
<tr>
<td>NP serum A cell (mg/l)</td>
<td>24</td>
<td>14.3</td>
<td>59%</td>
</tr>
<tr>
<td>NP serum B cell (mg/l)</td>
<td>18.5</td>
<td>6.1</td>
<td>33%</td>
</tr>
<tr>
<td>CD serum free A cell (µmol/l)</td>
<td>4.3</td>
<td>2.9</td>
<td>67%</td>
</tr>
<tr>
<td>CD serum free B cell (µmol/l)</td>
<td>4.0</td>
<td>3.9</td>
<td>98%</td>
</tr>
<tr>
<td>CD serum A cell (µmol/l)</td>
<td>32</td>
<td>32</td>
<td>100%</td>
</tr>
<tr>
<td>CD serum B cell (µmol/l)</td>
<td>45.1</td>
<td>45.1</td>
<td>100%</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Outliers identified elsewhere were excluded in this analysis.

\textsuperscript{2} 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.
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 nano-cytotoxicity assay.

<table>
<thead>
<tr>
<th>Control</th>
<th>Serum free</th>
<th>Serum free</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>target value</td>
<td>range</td>
</tr>
<tr>
<td>Control 1 1) (background chemical control)</td>
<td>0.06 OD</td>
<td>0.05-0.09 OD</td>
</tr>
<tr>
<td></td>
<td>&lt;6%</td>
<td>see serum-free values</td>
</tr>
<tr>
<td>Control 2 2) (CdSO4 control (EC50 value))</td>
<td>49.9 µmol/l</td>
<td>47.5-51.5 µmol/l</td>
</tr>
<tr>
<td></td>
<td>77.2 µmol/l</td>
<td>54.3-99.4 µmol/l</td>
</tr>
<tr>
<td>Control 3 (within pipette variability)</td>
<td>1.8 OD</td>
<td>1.5-2.0 OD</td>
</tr>
<tr>
<td></td>
<td>&lt;10%</td>
<td>2.0 OD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8-2.3</td>
</tr>
<tr>
<td>Control 4 (no cell and no treatment control)</td>
<td>0.06 OD</td>
<td>0.05-0.09 OD</td>
</tr>
<tr>
<td></td>
<td>&lt;6%</td>
<td>see serum-free values</td>
</tr>
<tr>
<td>Control 5 3) (Background NP B11-G11)</td>
<td>See figure 9</td>
<td></td>
</tr>
<tr>
<td>Control 6</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>Control 7+8 (between pipette variability)</td>
<td>1.5 OD</td>
<td>1.3-1.8 OD</td>
</tr>
<tr>
<td></td>
<td>&lt;12%</td>
<td>2.2 OD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8-2.8</td>
</tr>
</tbody>
</table>

1) If no additional background from the chemical reaction control is observed
2) Values of the A549-B cell line are given. They are fresh out of storage from ATCC and were passaged once and then sent to the participants.
3) No values given, because some of the laboratories observed a background signal while others did not.
Figure 1: Flow chart describing the modified MTS protocol for this study.
Figure 2: 96-well plate layout and 8 control measurements. Modified and reprinted with permission from (Rösslein et al., 2014).
Figure 3: NH$_2$-PS NP dose response curves for A549-A cells (A, C) and A549-B cells (B, D) conducted in serum-free (A, B) and serum (C, D) conditions. Relative absorbance value calculations are described in the Method section. All rounds from each laboratory except those identified as outliers were used in a Bayesian statistical model to generate the dose-response curves. For part A, Laboratory A was identified as an outlier under these conditions. The grey area represents the 95% confidence interval of the consensus curve as determined by Bayesian statistical modeling of all data.
Figure 4: NH$_2$-PS EC$_{50}$ values for A549-A and A549-B cells in serum-free (A) and serum (B) conditions. Whisker error bars represent 95% confidence interval from all rounds from each laboratory. Consensus values were generated by Bayesian modeling of all rounds of data from all of the laboratories that were not considered outliers. Only one set of data (serum-free condition, A549-A cells, Lab A) was considered an outlier. This data set was marked with an asterisk.
Figure 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.
Figure 6: Correlation of NH$_2$-PS NP EC$_{50}$ values (A) and CdSO$_4$ EC$_{50}$ 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$_4$ and the NH$_2$-PS NP (OD below 1.0), the slopes are statistically different from 0, indicating that the EC$_{50}$ value is correlated with control 3 values. However, EC$_{50}$ values are not correlated with the non-treatment mean OD when these outliers are removed or for the serum treatment condition.
Figure 7: Median values (A, B) and relative median average deviation (MAD) (C, D) of controls 7 + 8 – between pipette variability in serum free (A, C) and serum (B, D) conditions. Values for serum free conditions included a rinsing step. Each data point is the median or MAD value of a plate for each round from a laboratory. Higher MAD values are generally observed in the serum free conditions, which includes a rinsing step. The similar values observed here as compared to figure S4 indicate minimal vehicle effect.
Figure 8: Median values (A) and relative median average deviation (MAD) (B) of control 4 – within pipette volume.
Absorption values for all rounds in each laboratory were averaged and plotted for the Nanoparticle Interference Control column. Dose-dependent increases in absorption at 490 nm were observed in 3 laboratories (A, C and E) when the NH$_2$-PS NP were dispersed in serum containing culture media. This effect was not observed for any of the laboratories when the NH$_2$-PS NP were dispersed in serum-free cell culture media. Light scattering experiments indicated that the NP agglomerated in serum containing media (Table S4) suggesting this increase in absorption is related to absorbance from NP remaining in the well. This effect was not observed in all laboratories suggesting that differences in laboratory media removal protocols may play a role in minimizing this effect. Error bars represent two times the standard deviation values.
Figure 10: CdSO₄ EC₅₀ values for A549-A and A549-B cells in serum-free (A) and serum (B, D) conditions. Whisker error bars represent 95 % confidence interval from all rounds from each laboratory. Consensus values were generated by Bayesian modeling of all rounds of data from all of the laboratories that were not considered outliers. Only one set of data (serum-free condition, A549-B cells, Lab A) was considered an outlier. This data set was marked with an asterisk.
Figure 11: CdSO$_4$ EC$_{50}$ values for A549-A and A549-B cells in serum-free media using “hard” or “soft” rinsing procedures from Laboratory A and comparing to the interlaboratory consensus values (“interlab”). Whisker error bars represent 95% confidence intervals. When the “soft” rinsing procedure was performed, the results from Laboratory A aligned with those from the interlaboratory comparison consensus values.