Improving the Technical Quality of New Approach Methodologies (NAMs) and Nanotoxicity Methods

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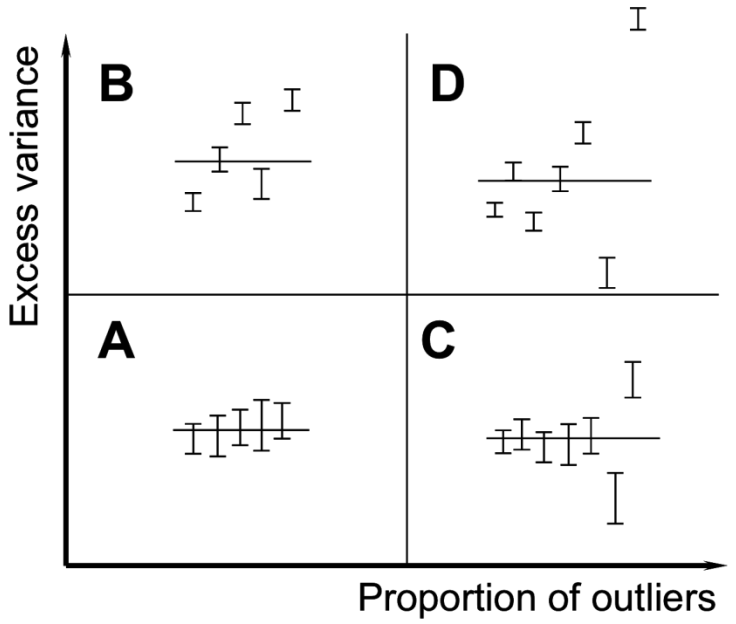
National Institute of Standards and Technology (NIST)

Design for Comparison Data

- Required:
  - quantitative numeric measurand
    - "Y-axis" values
  - quantitative measurement uncertainty estimates
    - error bars
- Useful:
  - estimate of correct value…
    - either
      - "reference" value or range
      - Orthogonal measurement
    - or
      - estimate of reference from the population of data



"CCQM Guidance Note: Estimation of a consensus KCRV and associated Degrees of Equivalence"
Draft 2010-03-01, Stephen LR Ellison, LGC and Maurice Cox, NPL

Technical Framework Manuscript

ALTEX, accepted manuscript
published July 15, 2022
doi:10.14573/altex.2205081

Bench Marks!

Technical Framework for Enabling High-Quality Measurements in New Approach Methodologies (NAMs)

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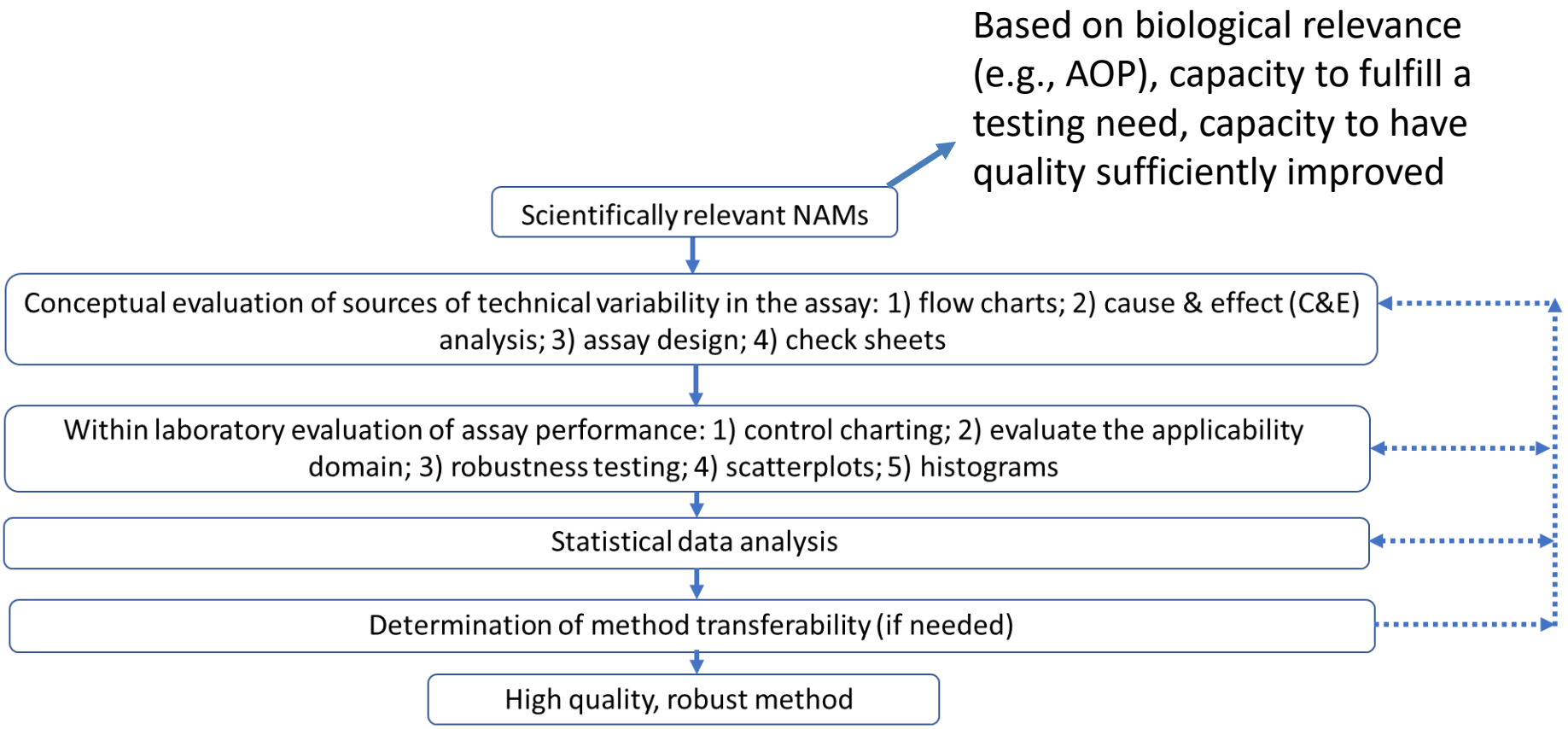
Technical Framework for High Quality NAMs

Collaborative project with CPSC, NICEATM, DOD, EMPA, NIST

- To yield reproducible NAM results across time and among laboratories, the framework includes a series of inter-related steps that describe
  - How to apply basic quality tools (cause-and-effect analysis, flow charts, control charts, etc) to improve confidence in NAMs
  - Approaches for adding statistical confidence to decisions based on NAM results
  - There may be tradeoffs though with more controls potentially leading to higher costs

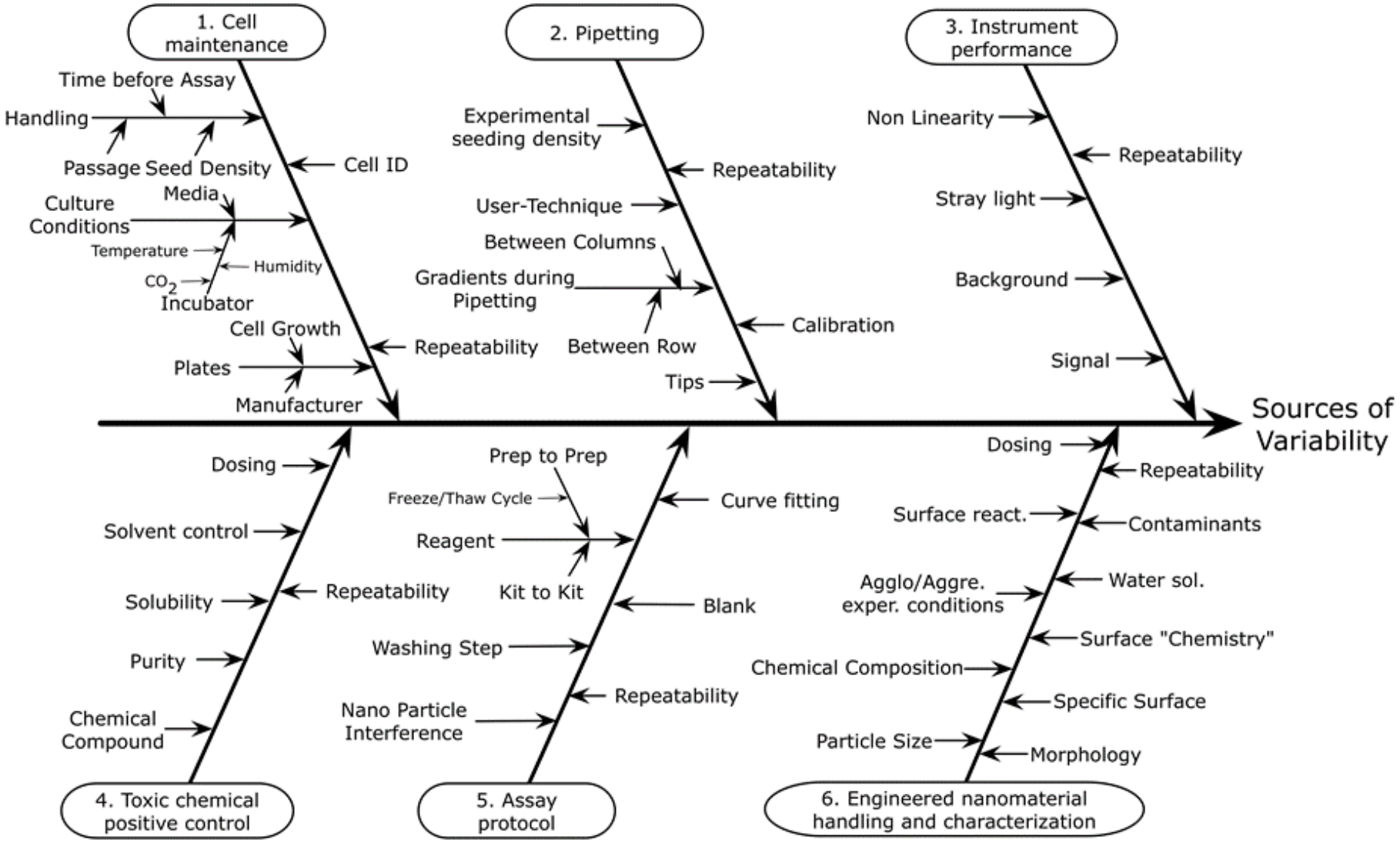
Petersen, E. J., Elliott, J. T., Gordon, J., Kleinstreuer, N., Reinke, E, Roesslein, M., Toman, B. 2022, Altex, in press. https://doi.org/10.14573/altex.2205081

Technical Framework For High Quality NAMs



Petersen, E. J., Elliott, J. T., Gordon, J., Kleinstreuer, N., Reinke, E, Roesslein, M., Toman, B. 2022, Altex, in press. https://doi.org/10.14573/altex.2205081

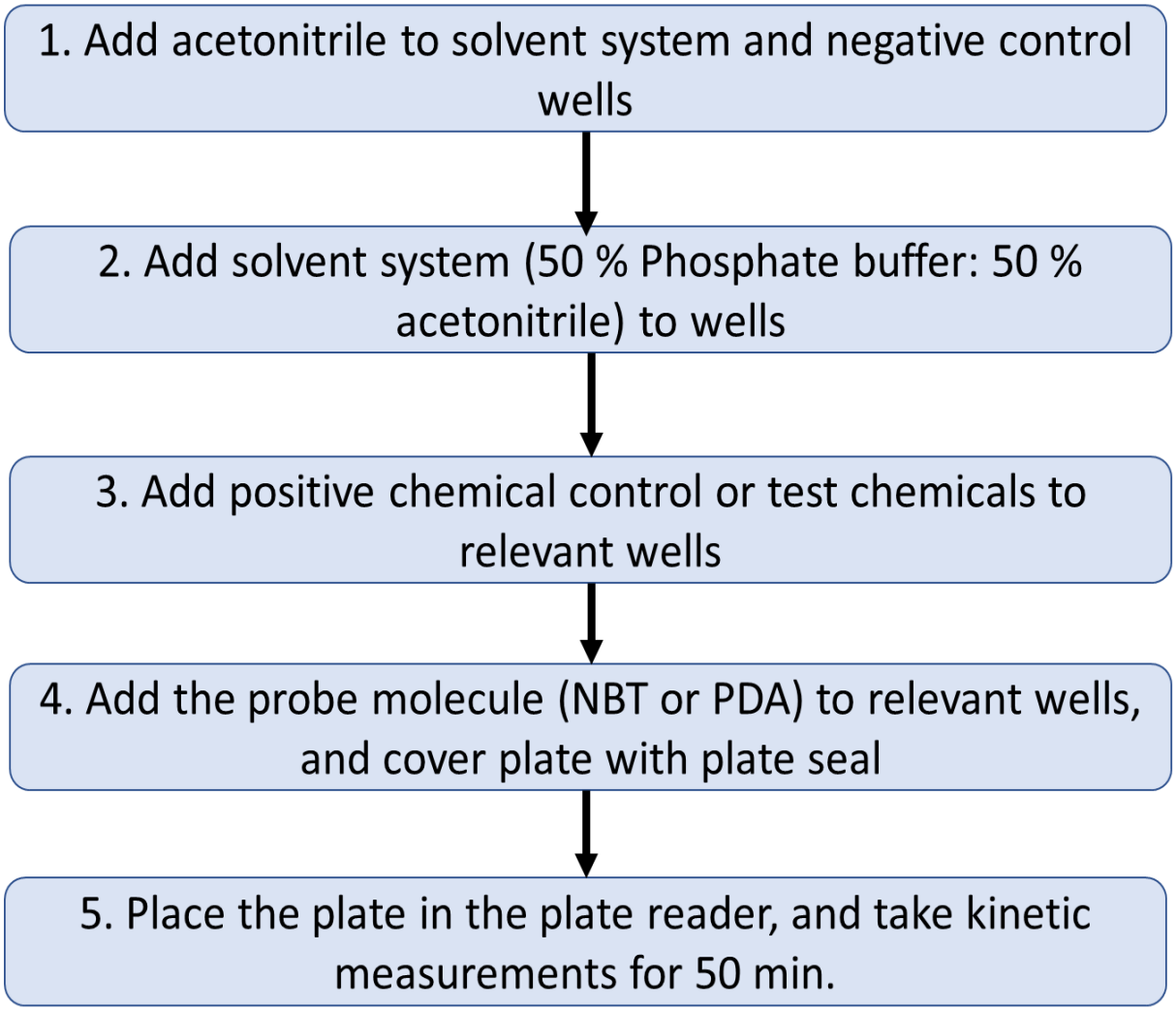
Example: cause-and-effect analysis



Robustness testing can evaluate each of the branches

Rosslein, M., Elliott, J. T., Salit, M., Petersen, E. J., Hirsch, C., Krug, H. F., Wick, P. The use of cause-and-effect analysis to design a high-quality nano-cytotoxicity assay, Chemical Research in Toxicology, **2015,** 28(1), 21-30.

Example: flow chart



Control measurements should cover each step in the flow chart

Petersen, E.J., Uhl, R., Toman, B., Elliott, J.T., Strickand, J., Truax, J., Gordon J. Development of a 96-Well Electrophilic Allergen Screening Assay for Skin Sensitization Using a Measurement Science Approach. Toxics, **2022**, 10(5), article number 257.

Example: plate design

SS - Blank (Solvent System)

NC - Negative Control

PC - Positive Control (serial dilution)

TC TC TC TC TC TC TC - Test chemicals

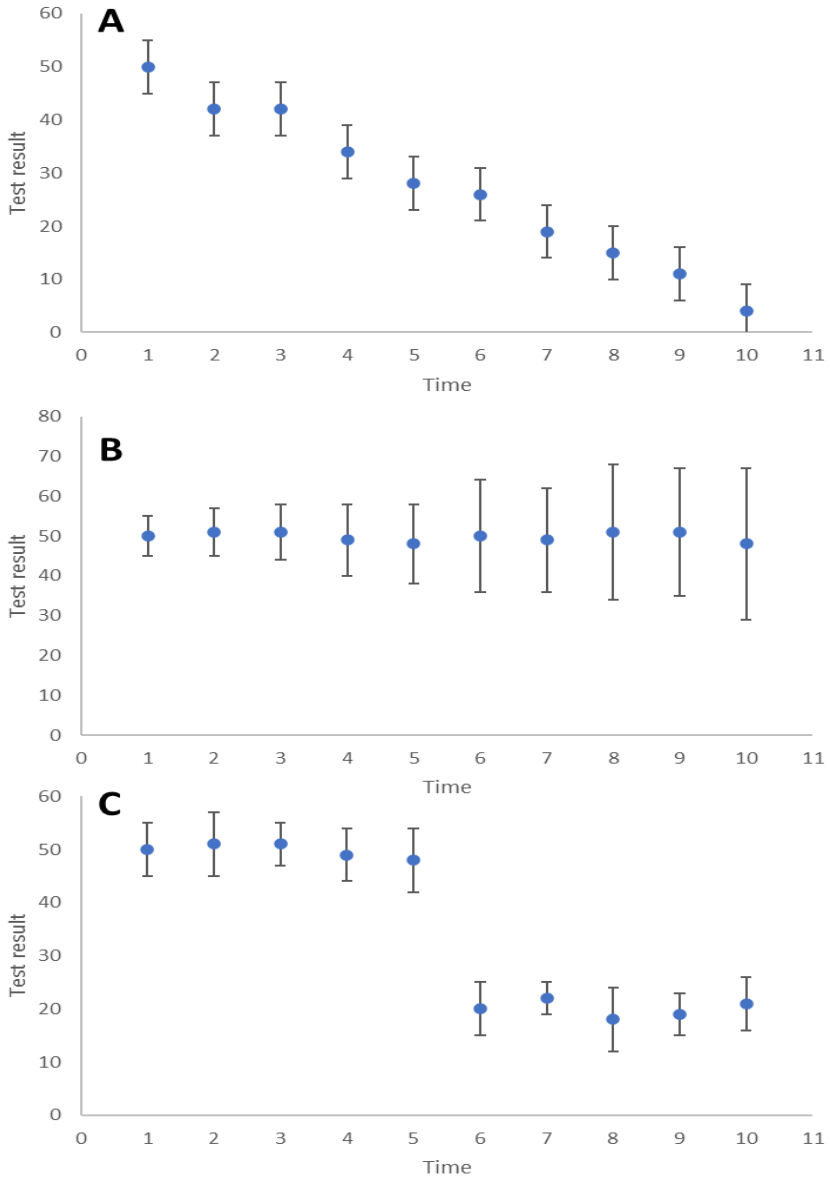
I - Test chemical interference wells

● - Wells without added reagents

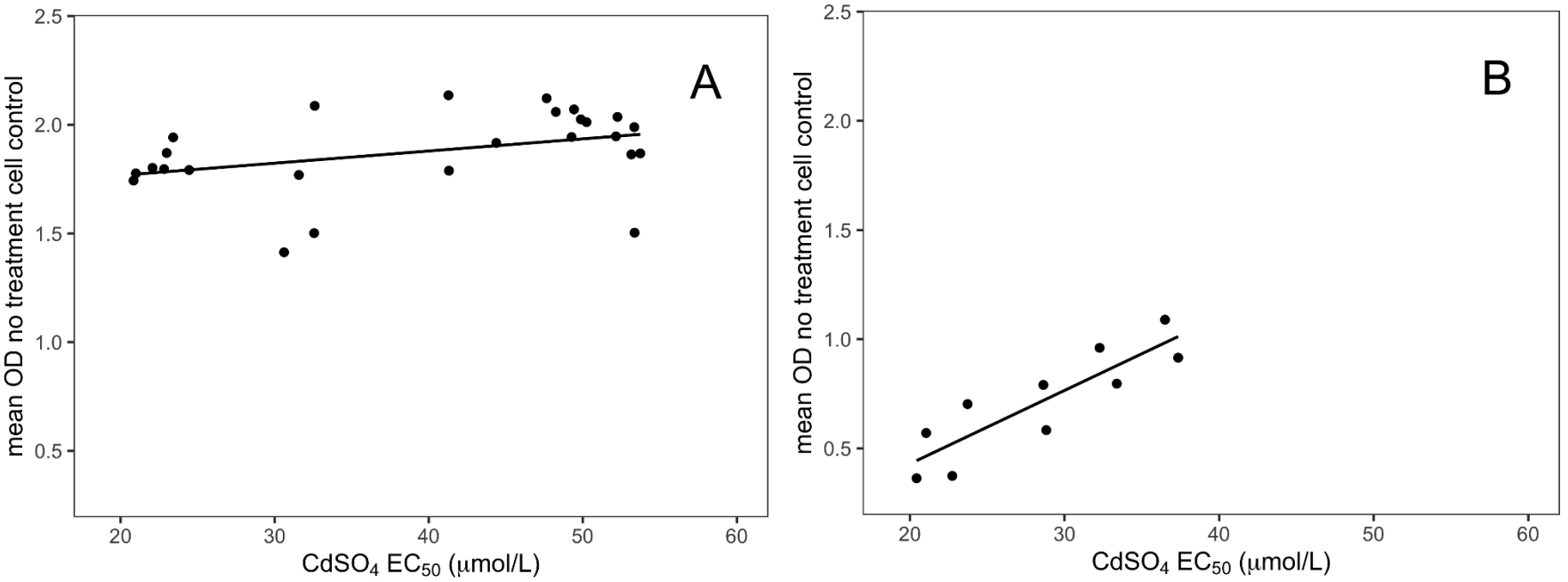
Control measurements evaluate key sources of variability each time the assay is performed

Petersen, E.J., Uhl, R., Toman, B., Elliott, J.T., Strickand, J., Truax, J., Gordon J. Development of a 96-Well Electrophilic Allergen Screening Assay for Skin Sensitization Using a Measurement Science Approach. Toxics, **2022**, 10(5), article number 257.

Example: control charting



Example: scatter plot

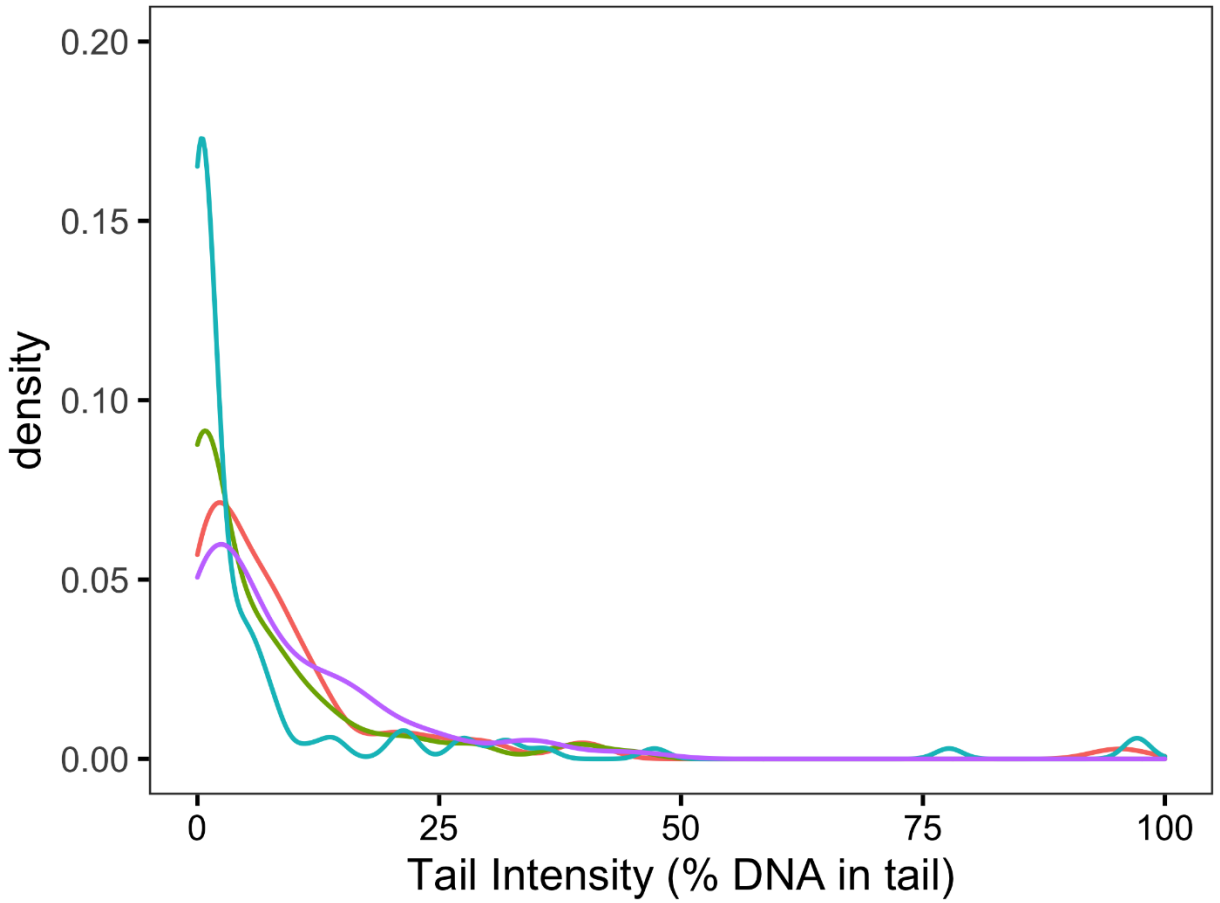


There is either a lack of an interaction between the EC_{50} values (part A) or an interaction (part B) depending upon the range of mean OD values which reflect the number of cells.

Ranges in specifications can be set to avoid interactions among variables

Elliott, J. T., Rosslein, M., Song, N. W., Toman, B., Kinsner-Ovaskainen, A., Maniratanachote, R., Salit, M. L., Petersen, E. J., Sequeira, F., Lee, J., Kim, S. J., Rossi, F., Hirsch, C., Krug, H. F., Suchaoin, W., Wick, P. Toward achieving harmonization in a nano-cytotoxicity assay measurement through an interlaboratory comparison study, **2017**, Altex, 34(2), 201-218.

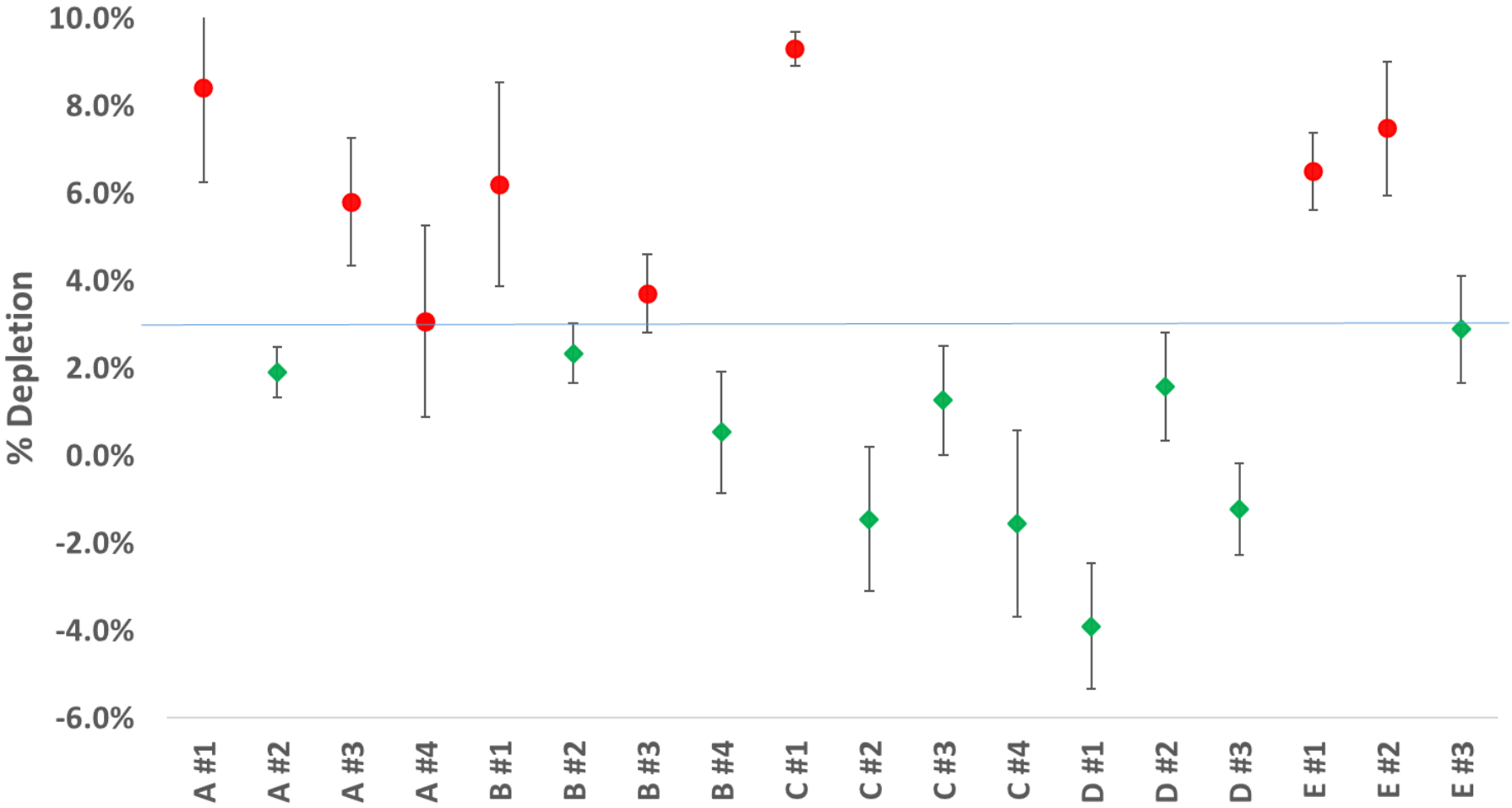
Example: histogram



If the data do not have a Gaussian distribution, different statistical approaches may be needed

Cassano, J. C., Roesslein, M., Kaufmann, R. et al. (2020). A novel approach to increase robustness, precision and high-throughput capacity of single cell gel electrophoresis *ALTEX - Alternatives to animal experimentation 3*, 95-109. http://dx.doi.org/10.14573/altex.1906252

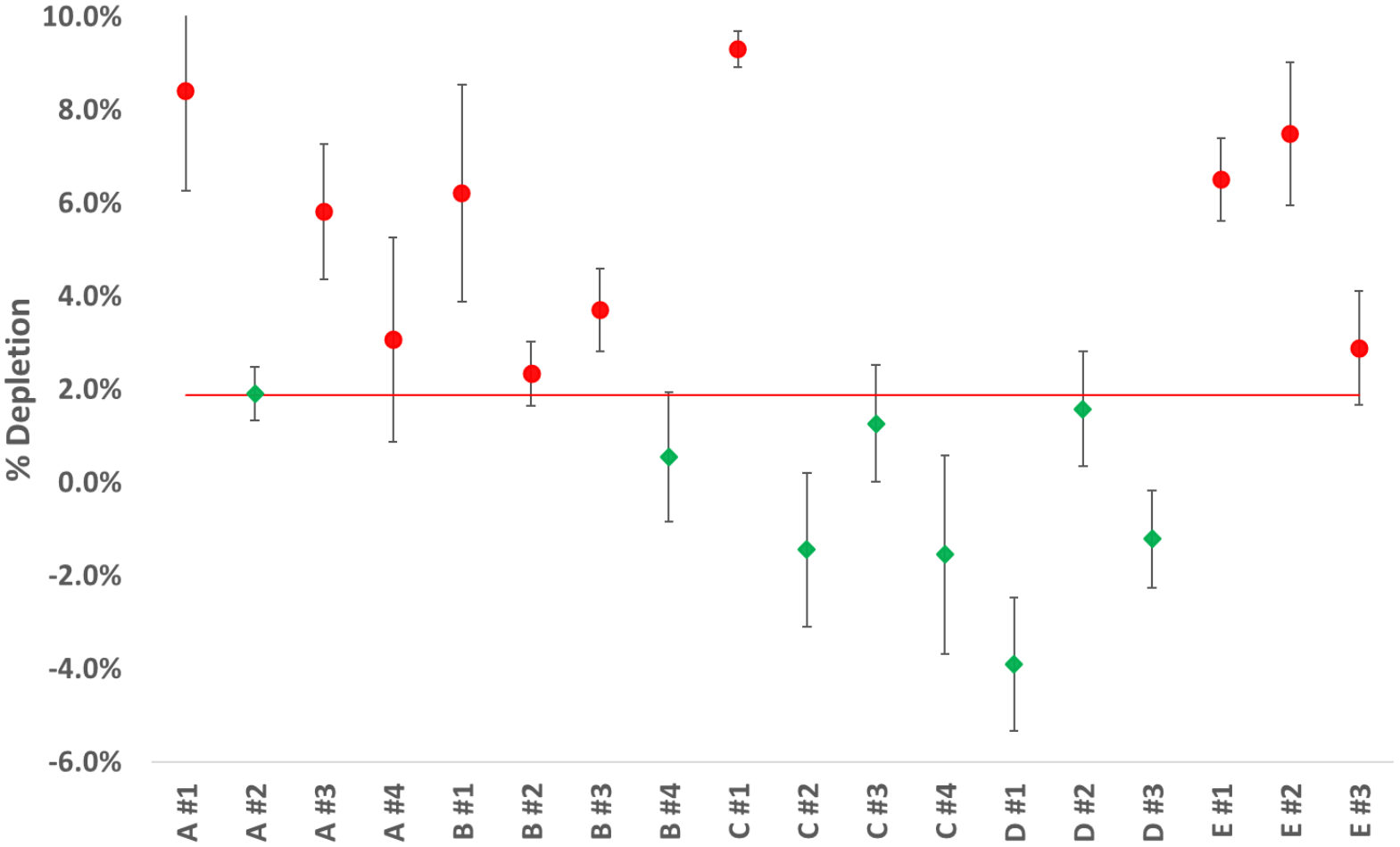
Statistical approaches: static call line



The call line is based on a set amount, in this case 3 %, regardless of the experimental uncertainty.

Data from https://doi.org/10.3390/toxics10050257; figure from https://doi.org/10.14573/altex.2205081

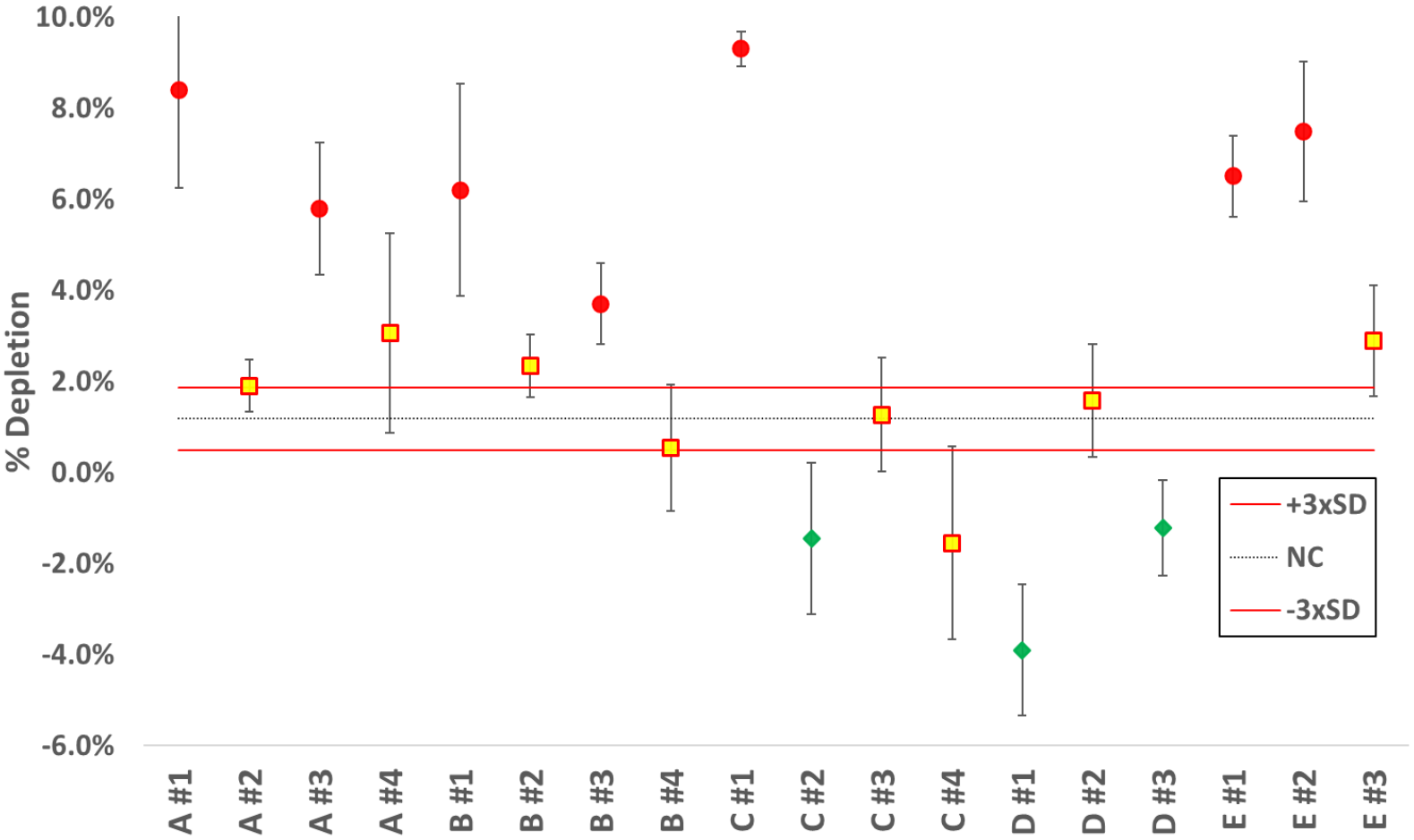
Statistical approaches: call line based on negative control uncertainty



The call line is based on the mean + 3 times the standard deviation of the negative control.

Data from https://doi.org/10.3390/toxics10050257; figure from https://doi.org/10.14573/altex.2205081

Statistical approaches: call line based on negative control uncertainty



The call line is based on mean ± 3 times the standard deviation of the negative control. If the 95 % confidence interval of the chemical in a run overlaps with the uncertainty band for the negative control, the data is called “borderline.”

Data from https://doi.org/10.3390/toxics10050257; figure from https://doi.org/10.14573/altex.2205081

Statistical evaluation

A T-score is calculated by taking the “Effect” and dividing by the standard error. In order to take all uncertainty into account, all sources of variability must be included in the calculation. In this case, we took into account the variability of the Negative Control, the NC/PC Blank, the test compound and the test compound Blank.

NC – Negative Control
S – NC/PC Blank
TC – Test Compound
TC_B – Test Compound Blank
sd – standard deviation
n – number of replicates

$$T = \frac{(\overline{NC} - \overline{S}) - (\overline{TC} - \overline{TC_B})}{\sqrt{\frac{sd_{NC}^2}{n_{NC}} + \frac{sd_S^2}{n_S} + \frac{sd_{TC}^2}{n_{TC}} + \frac{sd_{TC_B}^2}{n_{TC_B}}}}$$

Effect (or in our case Depletion)

Cumulative Uncertainty

Statistical approaches: call line based on t- value

The call line ($t_{critical}$ value for α=0.005) changes for every run based on propagated uncertainty in that run.

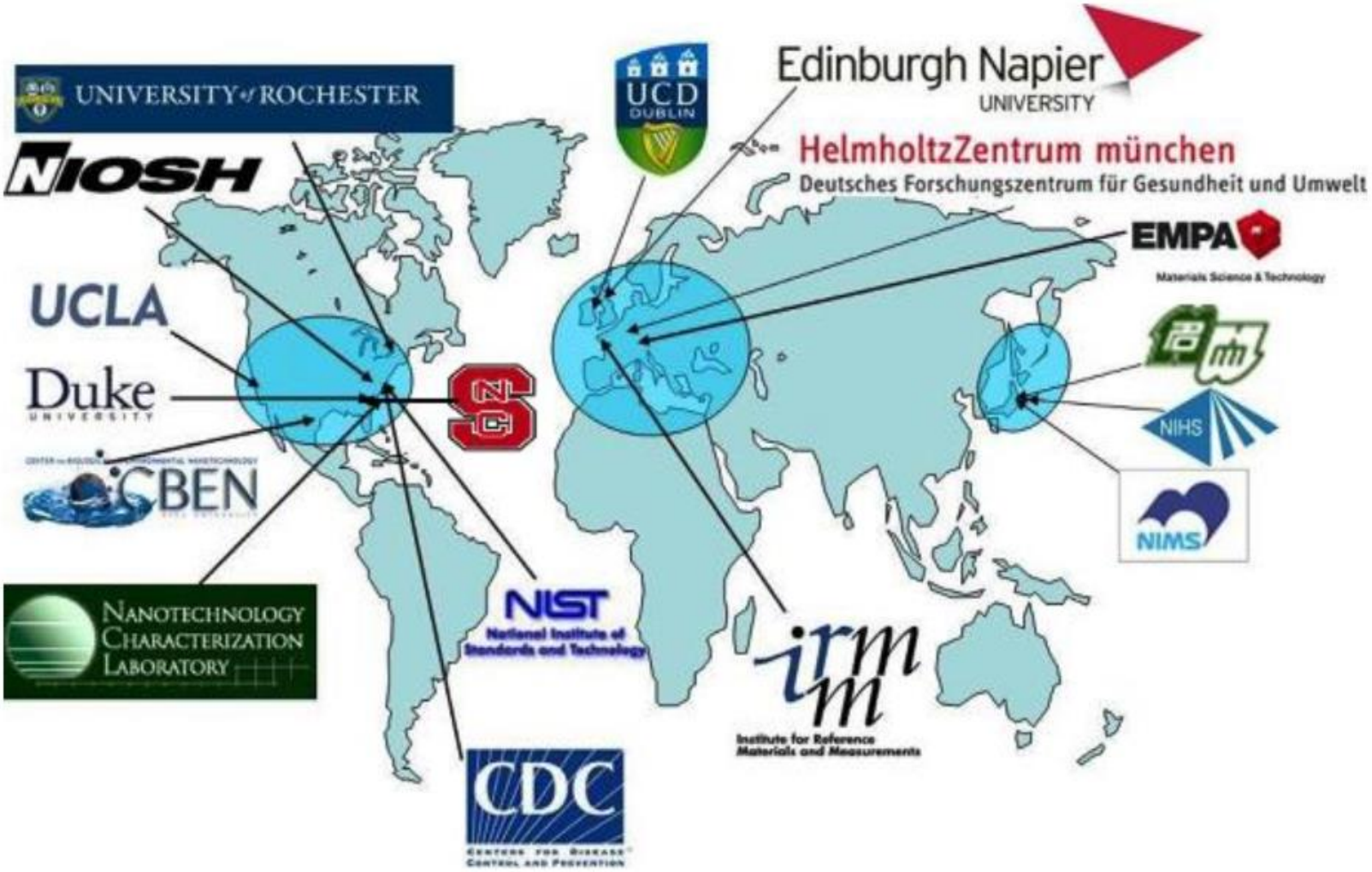
Data from https://doi.org/10.3390/toxics10050257; figure from https://doi.org/10.14573/altex.2205081

Summary

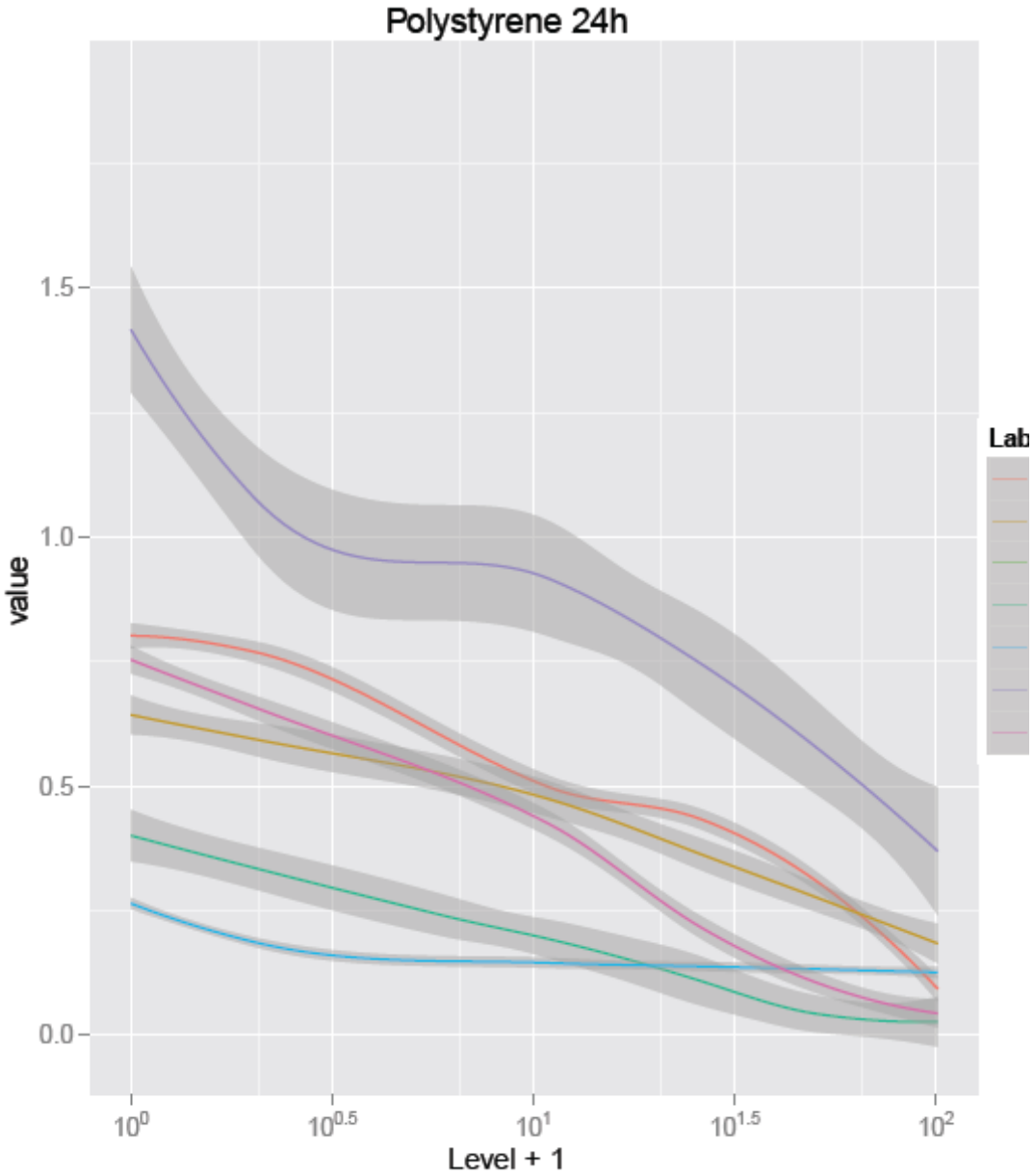
- Quality tools enable more confidence in measurement systems
- Technical framework focused on quality in NAMs
- Plate design allows direct encoding of control measurements for each test sample
- Statistical evaluation can yield a call with the likelihood of false positive/false negative decisions
- Possibly facilitates standardization and adoption of test methods

Harmonizonation of the MTS Nanocytotoxicity Assay

IANH International Alliance for NanoEHS Harmonization



Polystyrene nanoparticle 24 h results



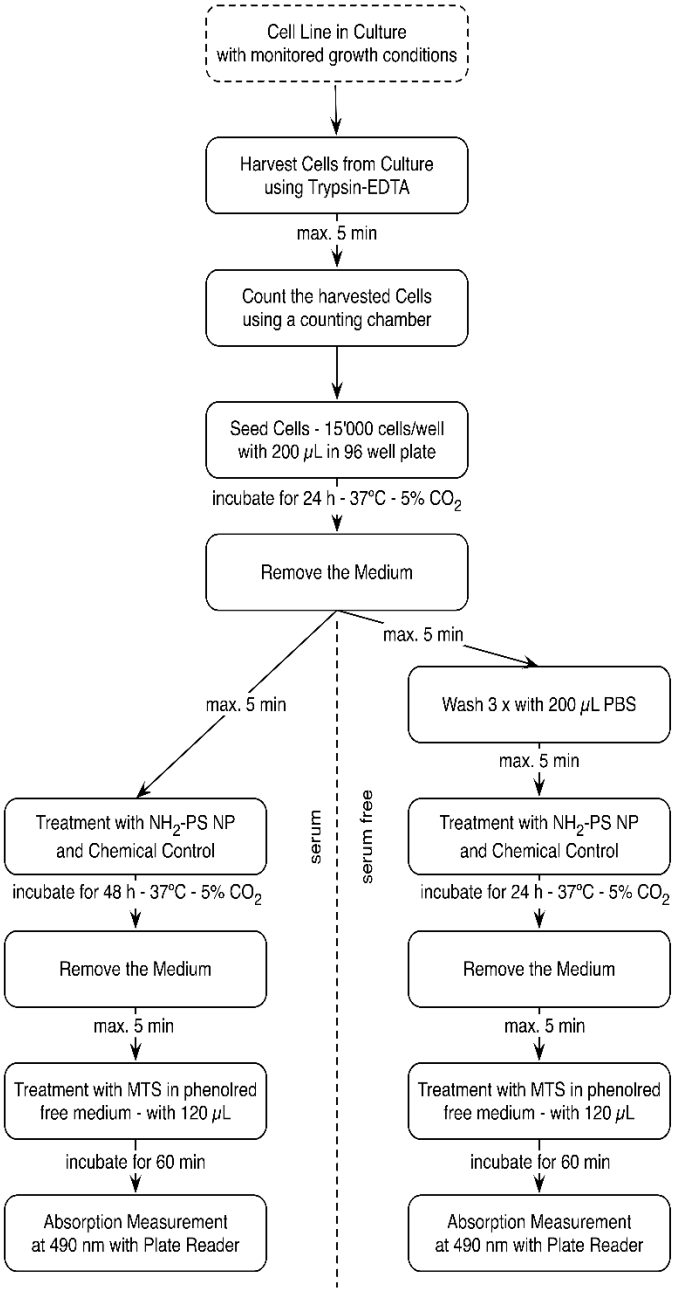
- Variations in absolute absorbance.
- Variations in response shape.
- All do show a “toxic” trend.

Interlaboratory comparison

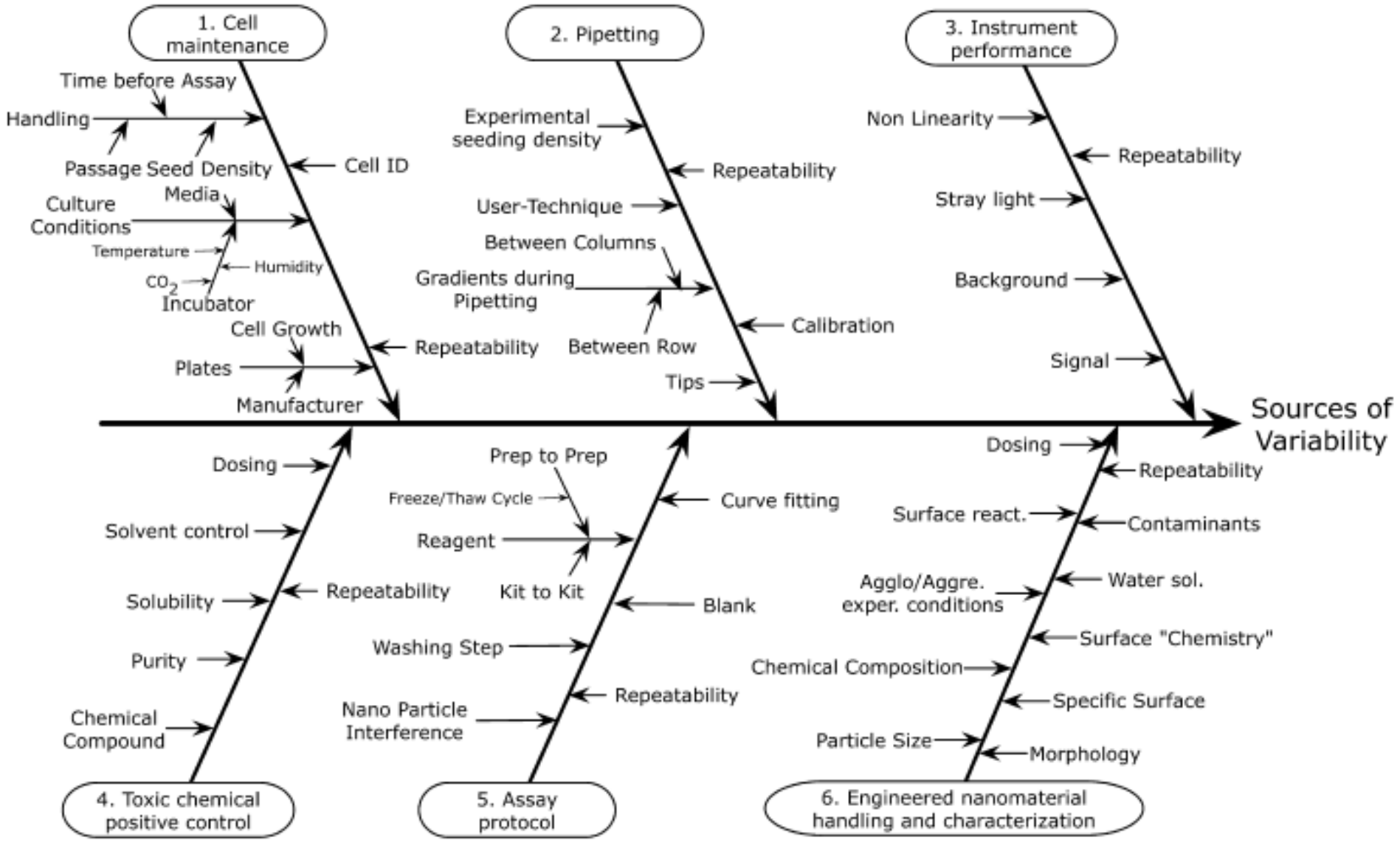
- 5 national metrology institutes were involved in the interlaboratory comparison
- Experimental design:
  - Share two A549 cell lines from ATCC and EMPA
  - Serum from local provider
  - Reagents from local provider
  - Serum and serum-free tests
  - Multiple replicates
  - Share nanoparticles (+ve PS) and chemical control ($CdCl_2$)

Elliott et al., 2017, Altex, 34, 201-218.

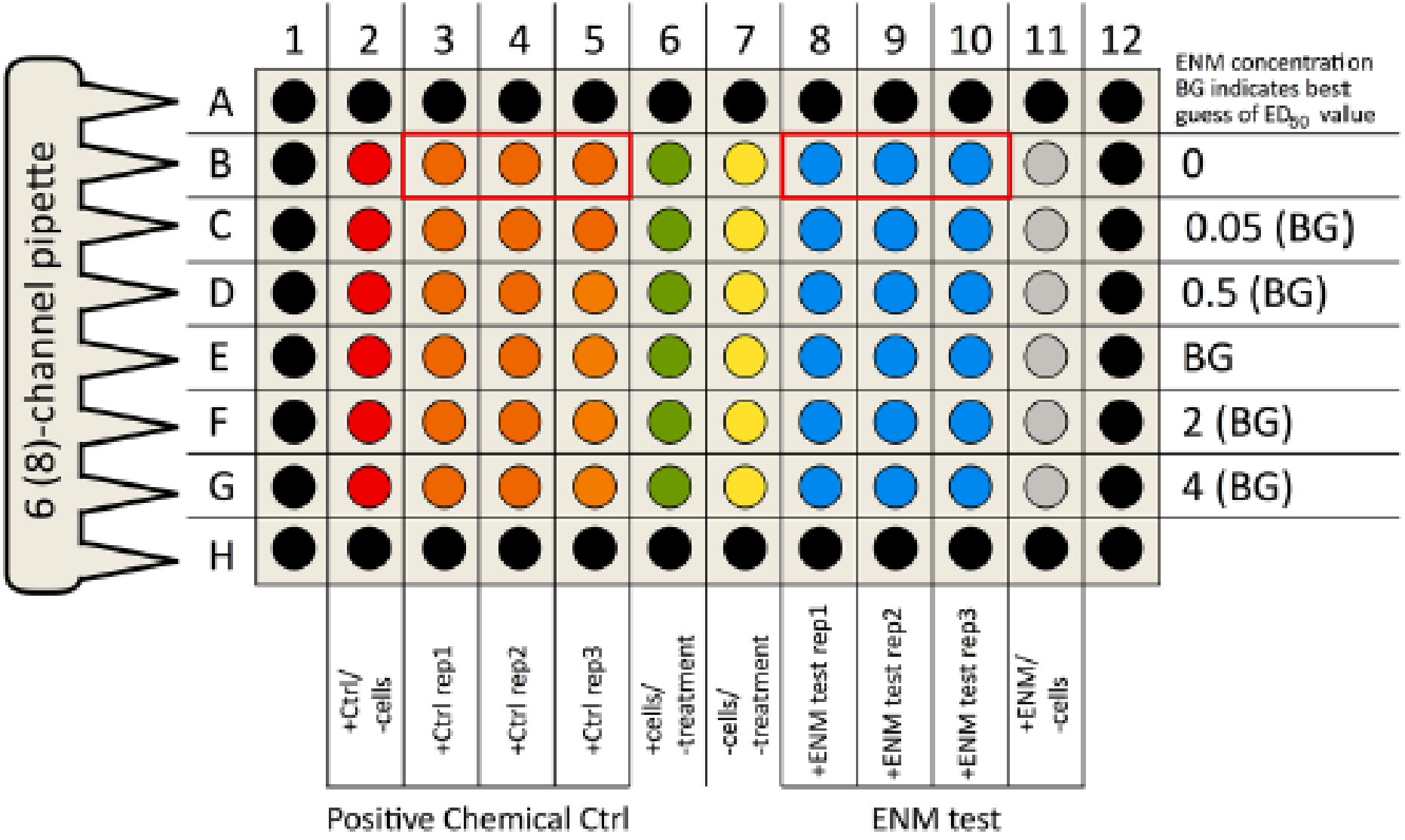
Flowchart with the main process steps of the MTS Assay



Cause & Effect Analysis of MTS Assay



Novel 96 well plate layout with control experiments

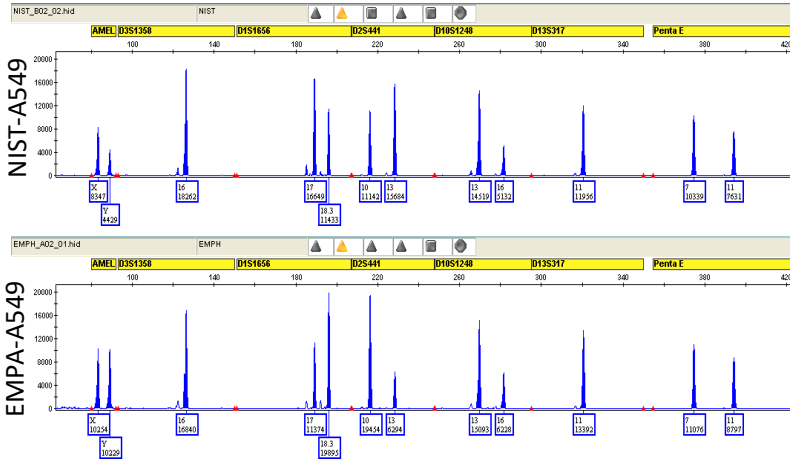


Two cell lines were tested in the interlab comparison

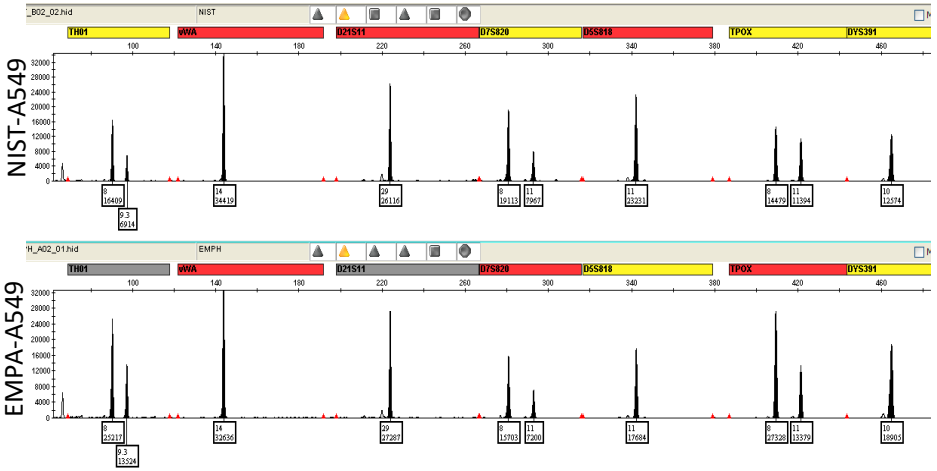
Cell line	**Cell cycle time (h)**	**Medium volume (um^3)[1]**	**Short Tandem Repeat (STR) analysis[2]**
A549-A	22.5±2.4	2047±90	In agreement with ATCC
A549-B	22.6±2.2	2327±94	Missing allele 12 (CSF1PO)

STR analysis of the two cell lines

a. FAM dye channel

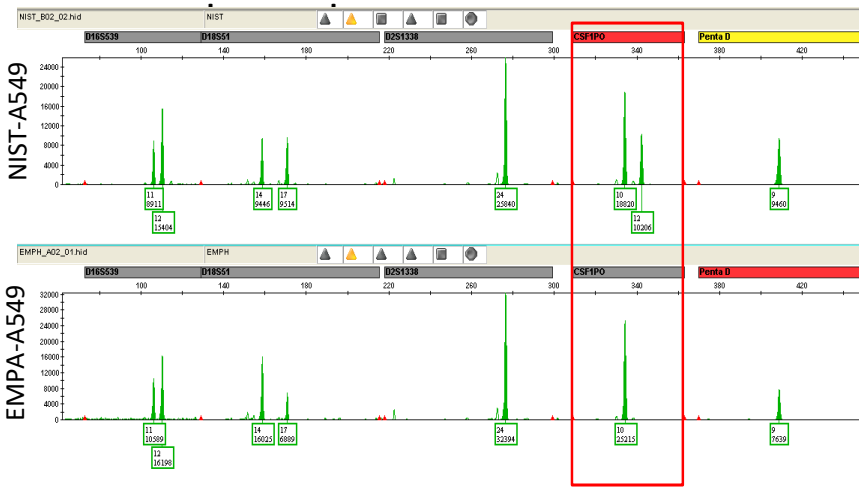


b. NED dye channel

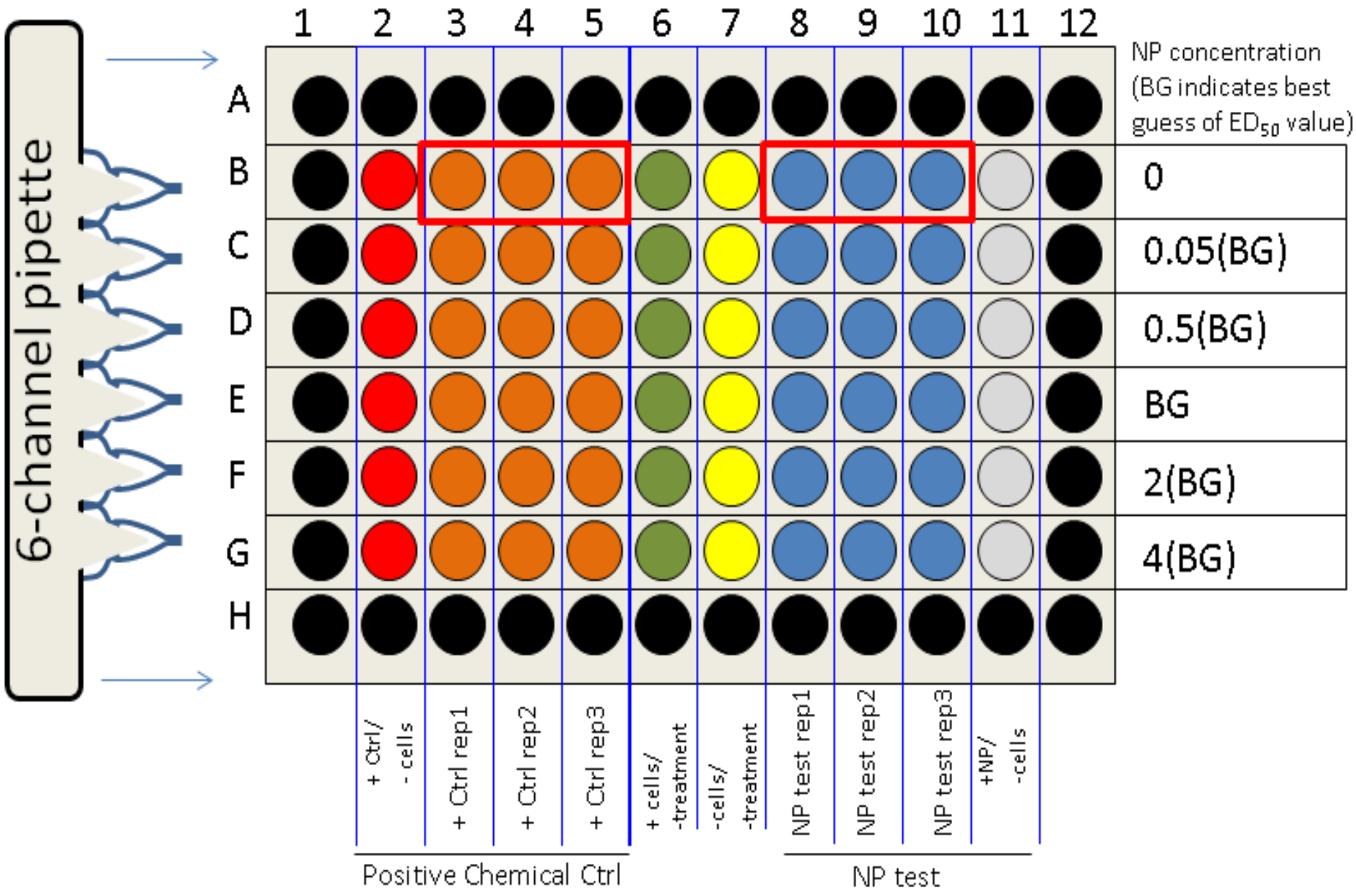


c. PET dye channel

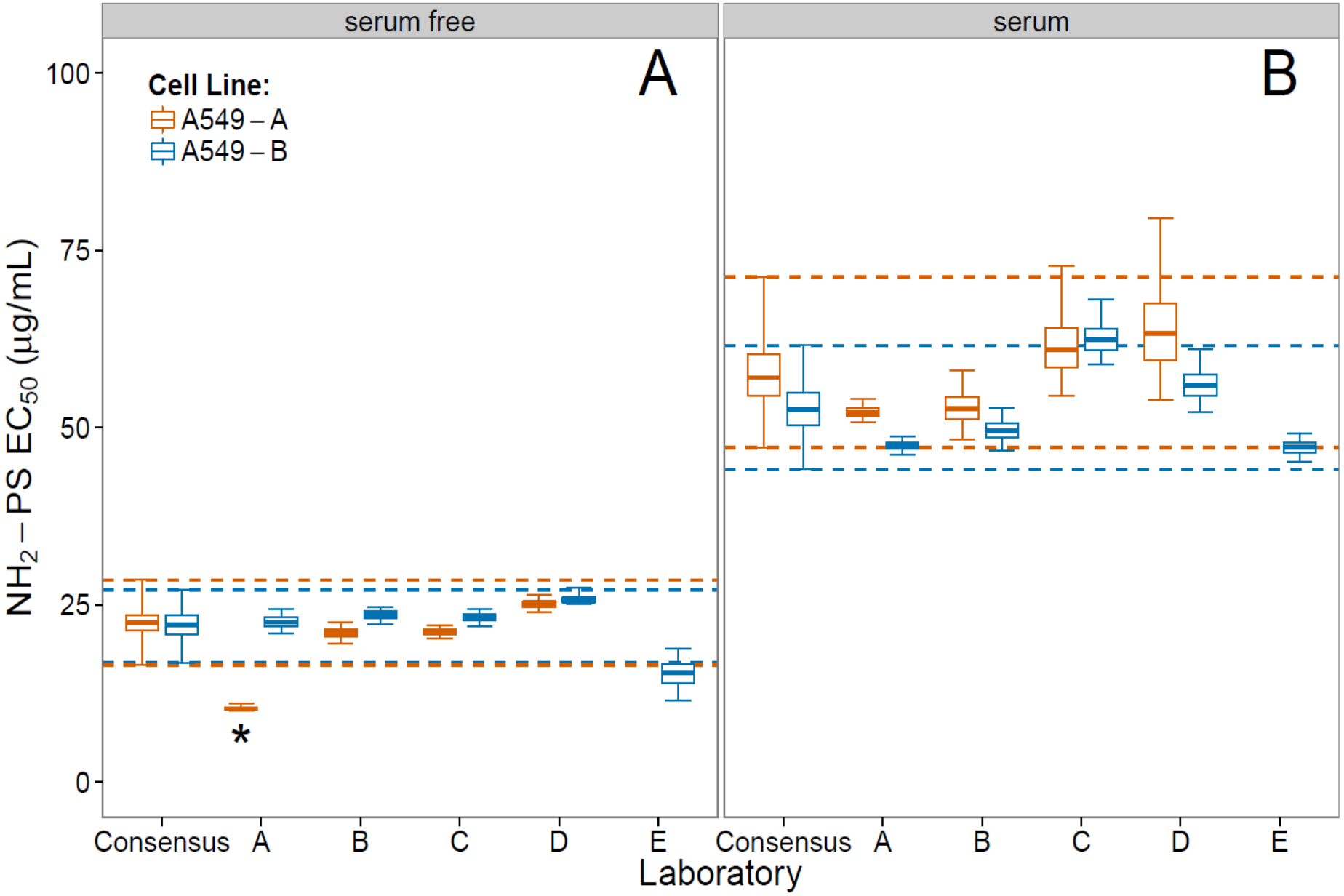
d. VIC dye



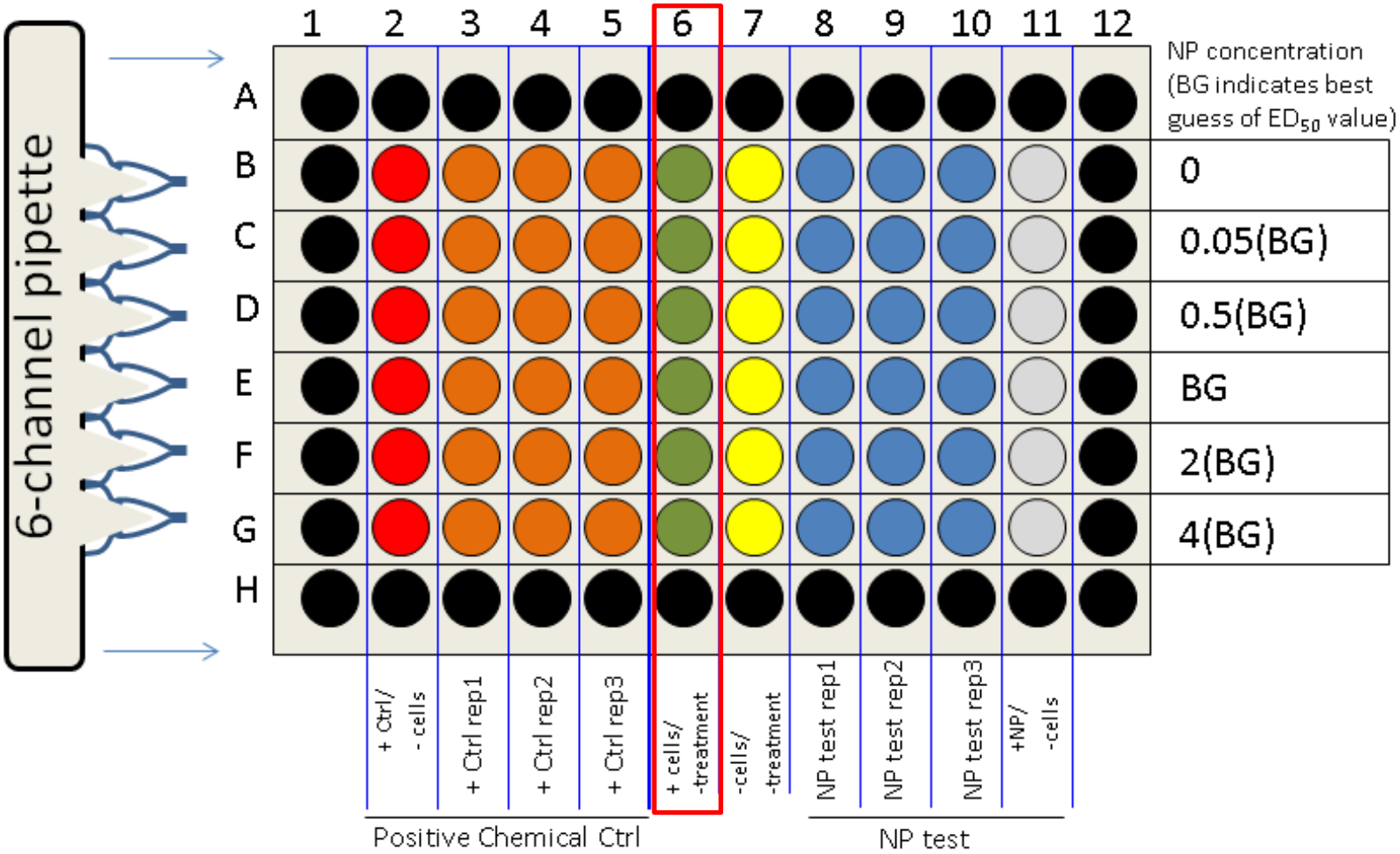
Dosing plate layout



Interlaboratory Agreement with Positively Charged Polystyrene Nanoparticles

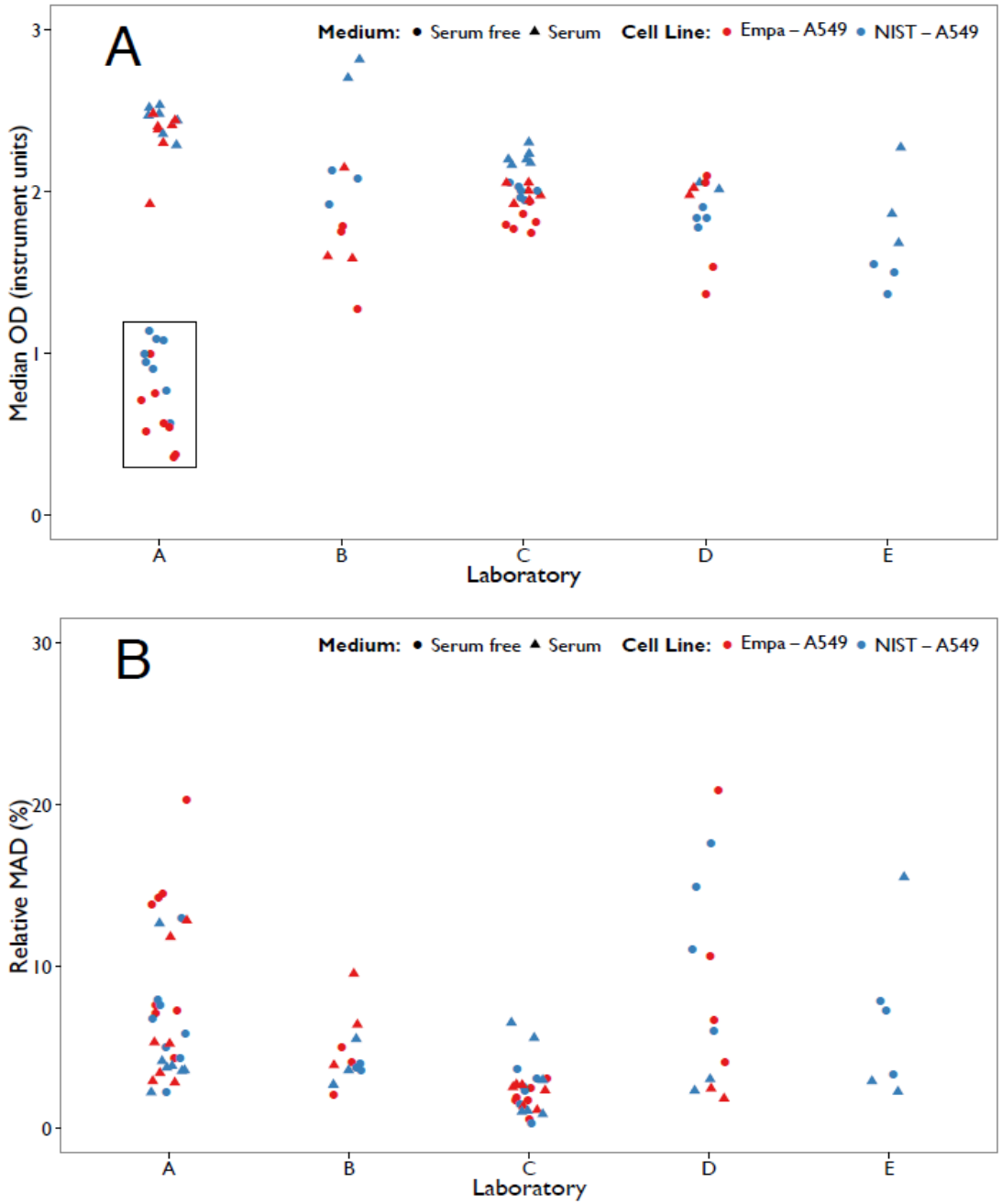


Design Element 1: Within Multichannel Pipette (MCP) Seeding Density

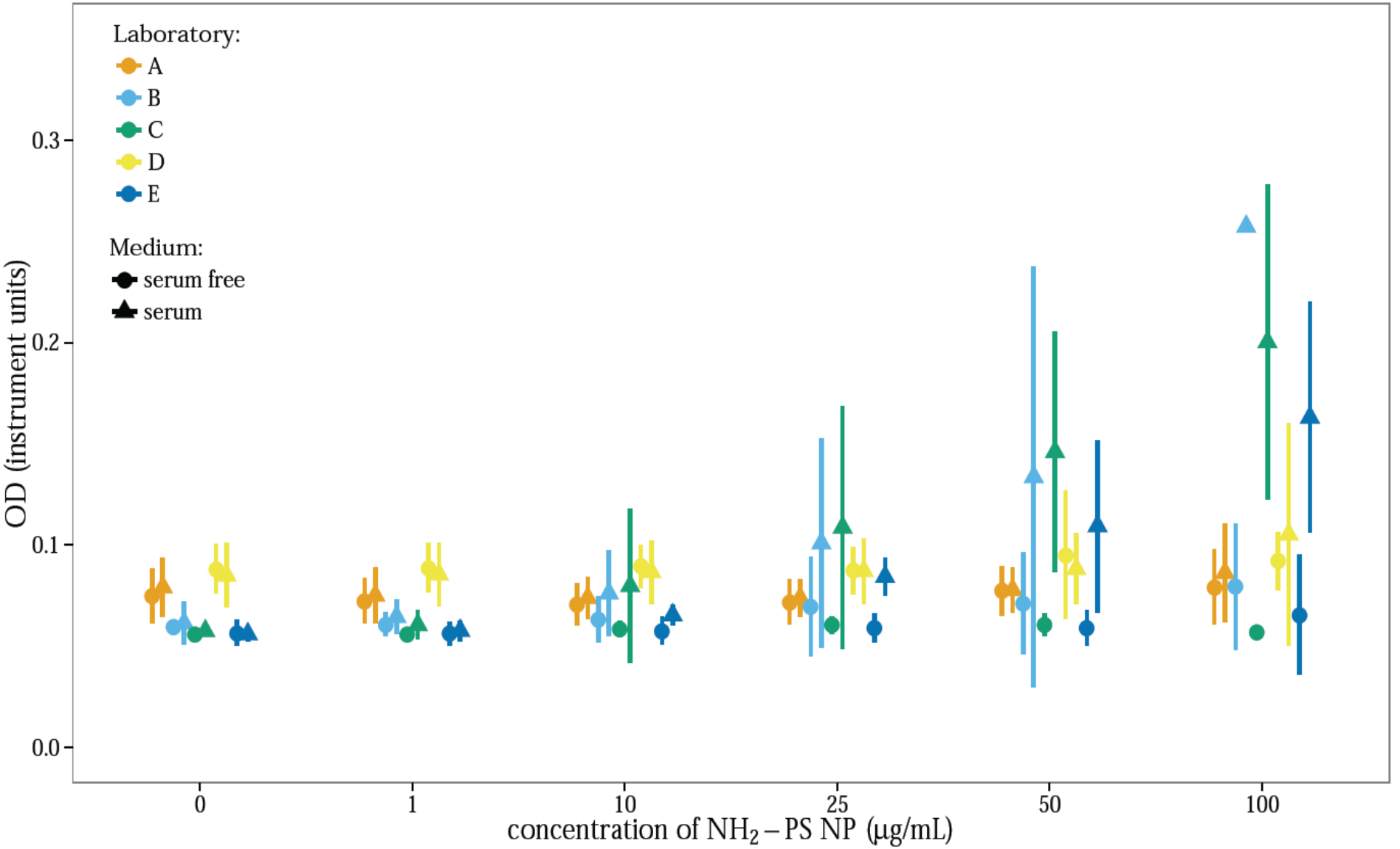


Assesses **within** multichannel pipetting variance. Non-treated cells seeded with a single multichannel pipette ejection step. Absolute absorbance measurement provides insight on nominal cell growth. Indicates technical problems with the pipette.

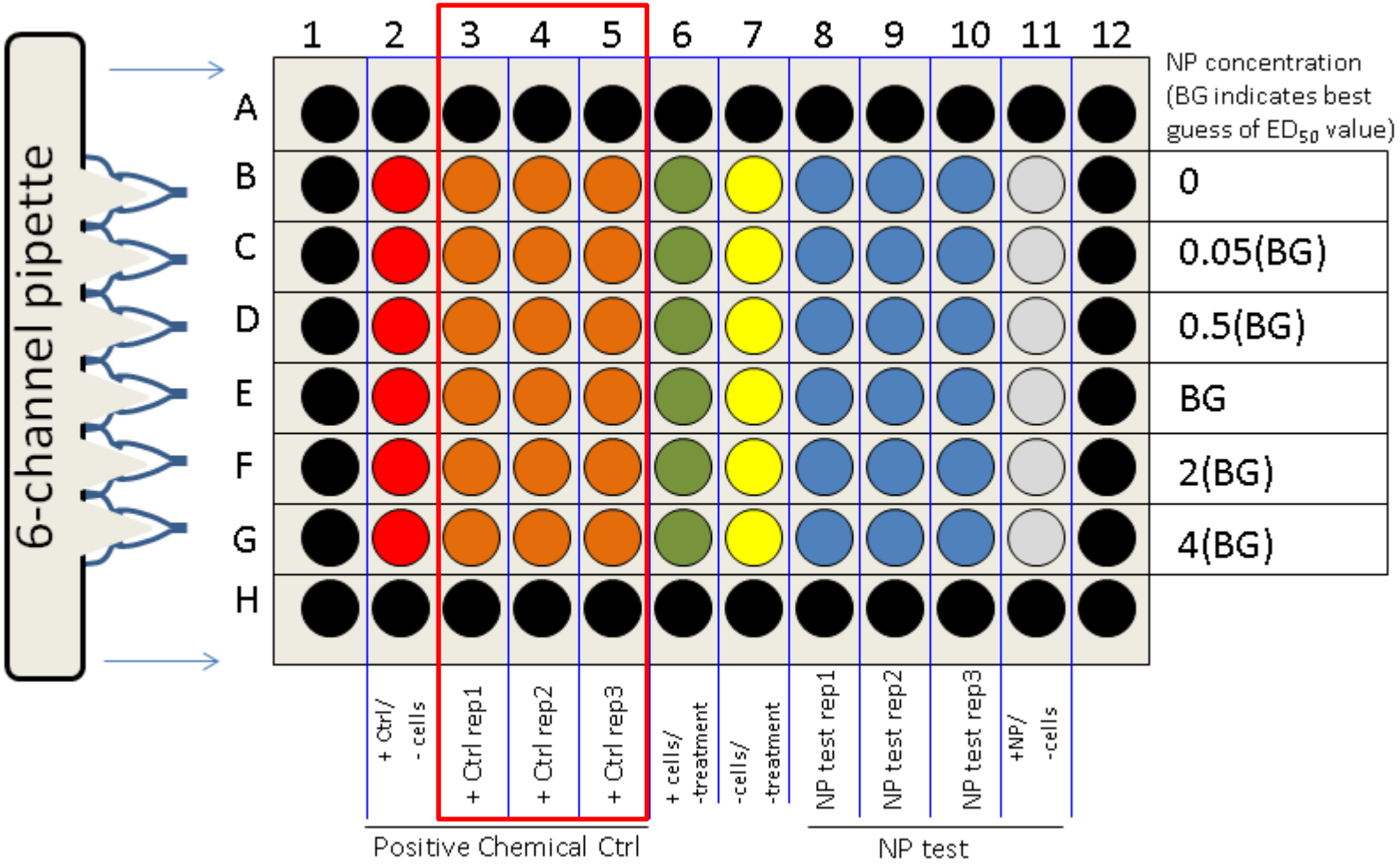
Design Element 1: Within Multichannel Pipette (MCP) Seeding Density



Design Element 4: Nanoparticle influence on assay readout (after rinsing)

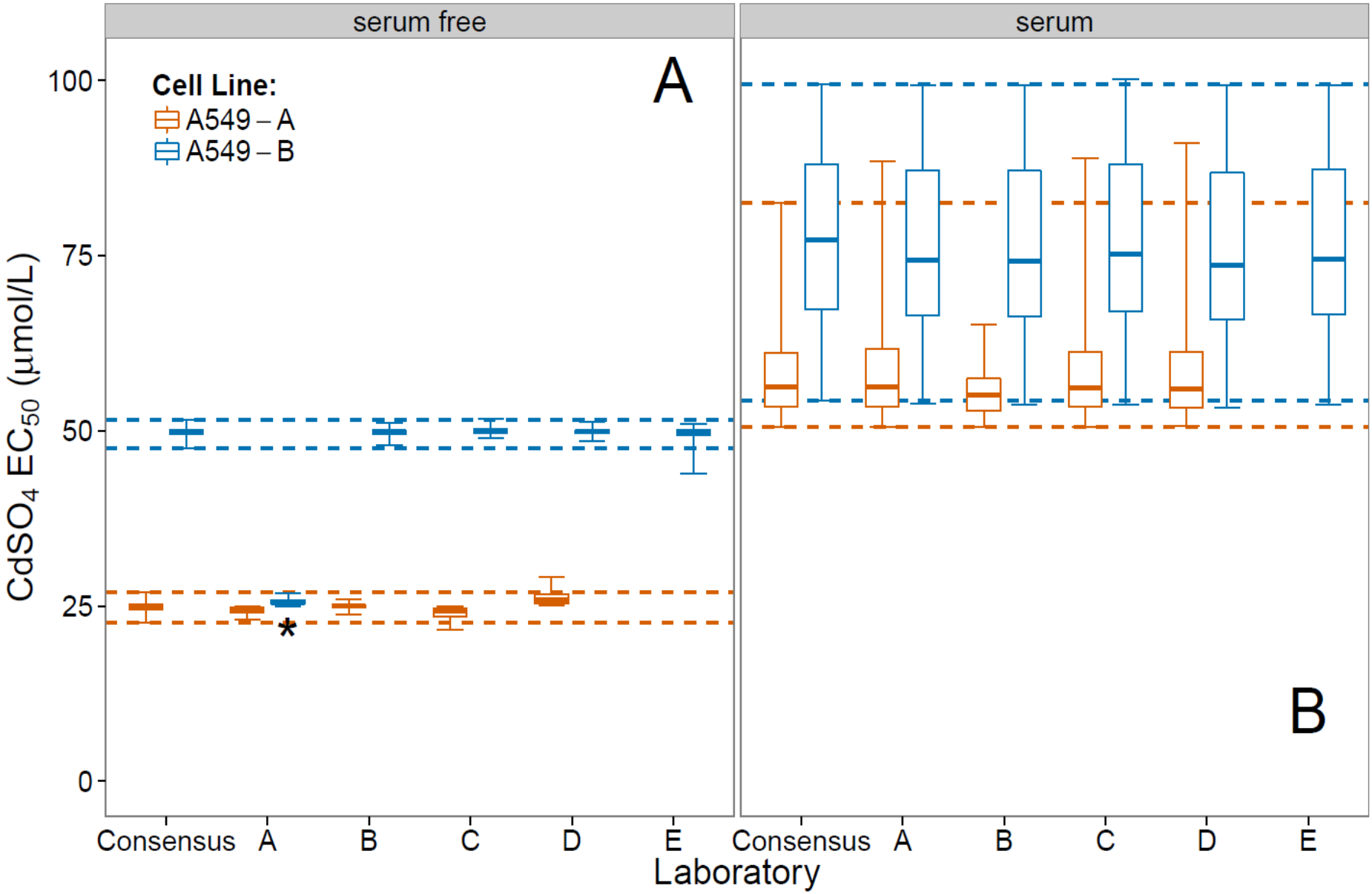


Design Element 5: Chemical Control dose response

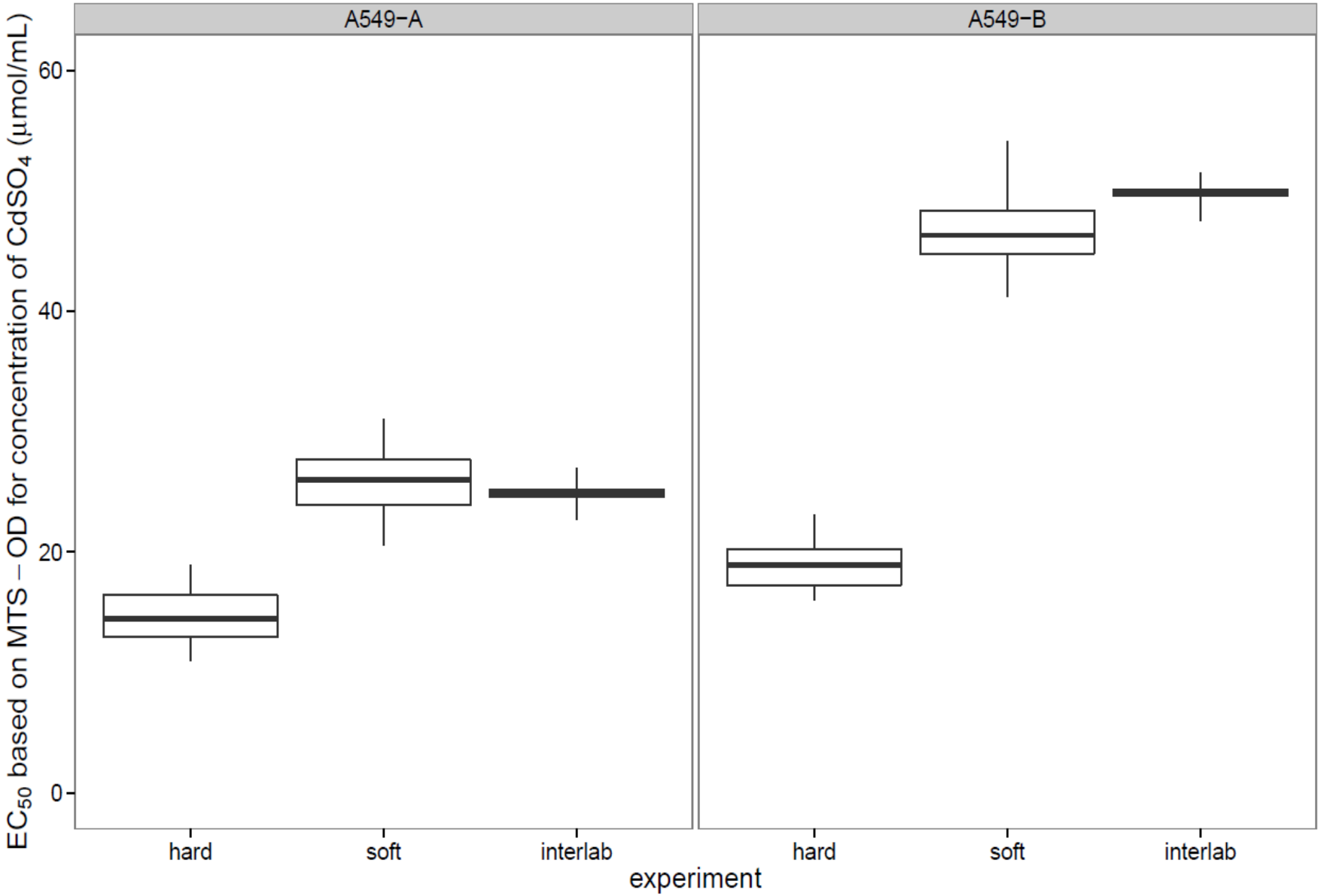


Triplicate reference chemical control. Shows that the assay worked as expected.

Design Element 5: Chemical Control dose response



Design Element 5: Chemical Control dose response



Outcomes

- Prepare large excess of cells and liquid when using a multichannel pipette
- Pipetting procedure (based on replicate details) is required to minimize variability
- Cell line differences may impact cytotoxicity results event if they are the same cell line
- Cell rinsing procedure in the serum free condition may need more exact specifications to minimize interlaboratory variability and limit outliers
- Good harmonization was obtained with polystyrene NPs and principles for obtaining reproducible nanocytoxicity results were developed

Developing a guidance document for aquatic toxicity testing

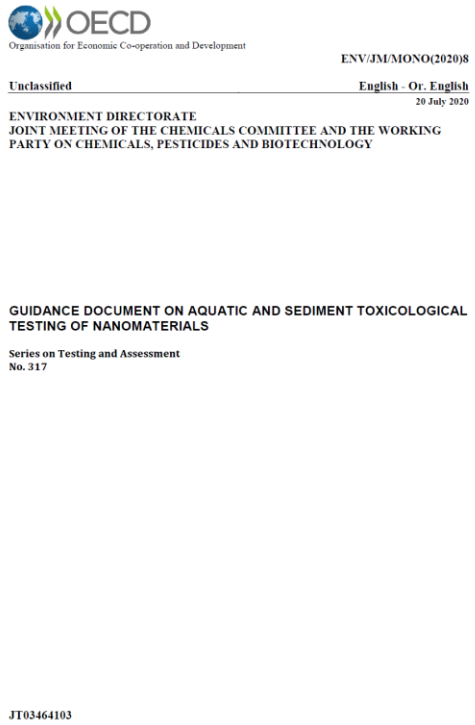
Contributions from over twenty colleagues from eight countries

Meetings that led to this guidance document

- February 2014 – Vienna, Austria (University of Vienna)
- July 2014 – Washington, DC (EPA, 23 experts from seven countries) – meeting exclusively focused on GD
- January 2015 – Dessau, Germany (German Environment Agency (UBA))
- November 2016 – Paris, France (Prosafe meeting)

Guidance document submission and revisions

- First draft submitted to OECD September 2017
- Revised drafts submitted to OECD August 2018, March 2019, September 2019
- OECD GD 317 published on-line July 2020: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2020)8&doclanguage=en


OECD
Organisation for Economic Co-operation and Development

ENV/JM/MONO(2020)8

Unclassified — English - Or. English

20 July 2020

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

GUIDANCE DOCUMENT ON AQUATIC AND SEDIMENT TOXICOLOGICAL TESTING OF NANOMATERIALS

Series on Testing and Assessment
No. 317

JT03464103

Petersen, E. J., Goss, G. G., von der Kammer, F., Kennedy, A. J. New guidance bring clarity to environmental hazard and behavior testing of nanomaterials. **2021**. Nature Nanotechnology, 16(5), 482-483.

Overview of Sections

1. Introduction
2. Scope
3. Background
4. Analytical and measurement techniques
5. Test dispersion preparation
6. Conduct of the test
7. Data analysis and reporting (Nanomaterial-specific)

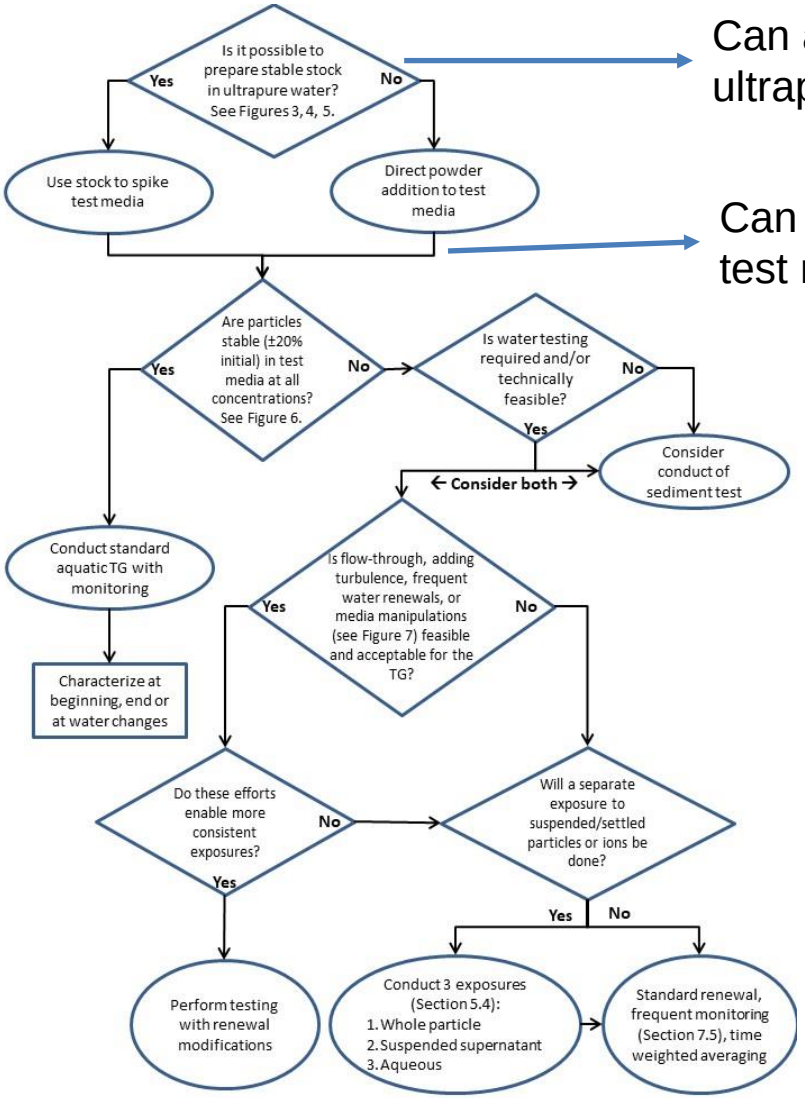
Key topics covered in the guidance document

- Characterization of the as-produced test material and the test material in stock and test dispersions
- Robust monitoring of exposure concentration and consistency (e.g., if the concentration remains within 20 %) during the experiment to determine need for water exchanges, time-weighted averages, etc.
- Test dispersion preparation approaches for materials with different levels of stability in suspension
- A hierarchy of modifications to the test media (e.g., pH, ionic strength, addition of natural organic matter) for particles that are not sufficiently stable in suspension

Key topics covered in the guidance document

- Discussion of potentially relevant control experiments to avoid artifacts and, if needed, understand mechanism of toxicity
- Detailed suggestions for additional assays-specific modifications for a range of OECD test guidelines
- Methods for spiking sediments for sediment exposures
- Key issues related to data analysis and reporting including different dose metrics

Flowchart for selecting method to suspend nanomaterial



Can a stock suspension be prepared in ultrapure water?

Can a stable suspension be prepared in the test media?

1 of 6 flowcharts

Topics for further refinement in future versions of the guidance document to improve testing robustness

(1) whether a single test media can be proposed for a specific test method to potentially improve the interlaboratory agreement of test results

(2) whether advances in analytical methods should lead to the recommendation of alternative exposure metrics (e.g., particle number concentration) instead of, or in addition to, the mass concentration

Topics for further refinement in future versions of the guidance document to improve testing robustness

(3) whether settled particles should be included in the exposure and dosimetry

Sub option	Description of approach	Objective of testing
(i) hazard testing of whole MN sample (suspended particles, settled particles, dissolved fraction)	Spiking of MN into each individual test media concentration (since serial dilution would introduce inconsistencies) and testing the hazard through monitoring of suspended particle and dissolved concentrations and settled particles (if possible)	This is the recommended default option for testing of MNs, with the potential to also conduct tests focused on determining the cause of toxicity under more controlled scenarios
(ii) hazard testing of suspended sample (suspended particles, dissolved fraction)	Testing the supernatant of the particles after a prescribed settling period determined from discretionary pre-tests, after which the supernatant is removed and tested (settled material is excluded)	When the goal is to reduce variability in the test and only the portion of the MN that remains in the water column for ecotoxicity assessment is considered (note: this approach will not address the effects of the unstable particulate fraction)
(iii) hazard testing of the dissolved fraction only	Testing after removing any undissolved particulate material using appropriate separation techniques (e.g., ultracentrifugation or ultrafiltration) and testing the hazard of the supernatant (or filtrate)	When determining the contribution of the dissolved fraction is needed for comparison to established toxicity thresholds established for dissolved chemicals (note: this approach will not address the effects of the particulate fraction)

NNI
National Nanotechnology Initiative

Public Meeting: Refreshing the NNI's Environmental, Health, and Safety Research Strategy

May 31–June 1, 2023
Online and L'Enfant Plaza SW, Washington, D.C.

Contact: Rhema Bjorkland
(rbjorkland@nnco.nano.gov)
Visit: https://www.nano.gov/ehsstrategymeeting