Measurement science in ICCVAM

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Nitric oxide nanotoxicity assay

How the assay works

- Macrophage cells (RAW 264.7) are seeded on 24-well plates
- The amount of nitrite they produced is quantified
- Nitrite production is a surrogate for nitric oxide, but that cannot be easily measured given its short half life

NIST collaborators: Robert Gutierrez, Ana Barrios, Elijah Petersen, Bryant Nelson, John Elliott, TJ Cho, Alex Tona, Aaron Johnston-Peck

Nitric oxide nanotoxicity assay

Motivation

- Assay was nominated by FDA (Nanotechnology Core Facility, NCTR) as a priority for nanomedicine development in ASTM E56
- Based on a protocol developed by the Nanotechnology Characterization Laboratory (NCL)
- Potential role in multiple adverse outcome pathways related to inflammation

Work performed at NIST

- Evaluated > 10 different key potential sources of variability in the assay
- Identified recommendations to further refine the assay
- Potential future interlab testing
- A collaborative manuscript with FDA is under review

Standard

- Accepted as a standard test method by ASTM in 2022

Oral Mucosal Tissue Irritation Assay

Human Oral Epithelium

- TR146 cells (derived from a **squamous cell carcinoma** of the buccal mucosa) **EpiOral**
- 8-11 layers of cells per construct
- Mattek EpiOral chosen as a case study

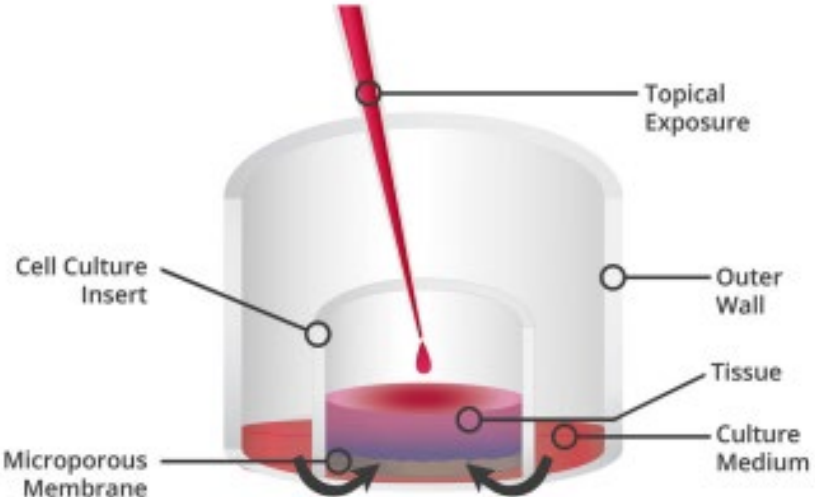
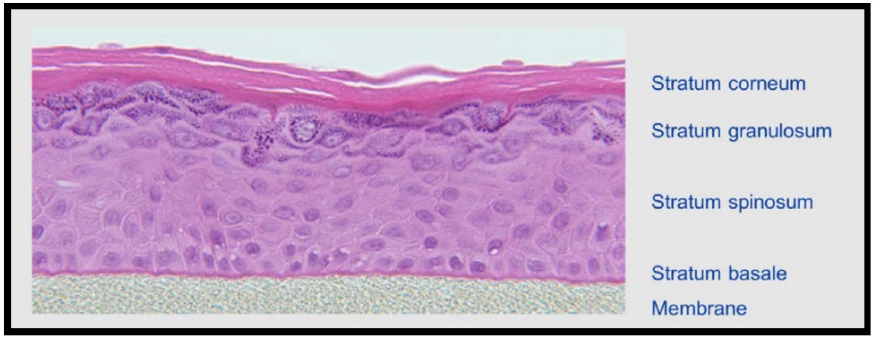


Figure. Schematic of culture at the air-liquid interface (ALI)

Test

- Material Irritants in polar or nonpolar solvents
- Described in ISO 10993:23 for skin irritation using human epidermal tissue



Oral Mucosal Tissue Irritation Assay

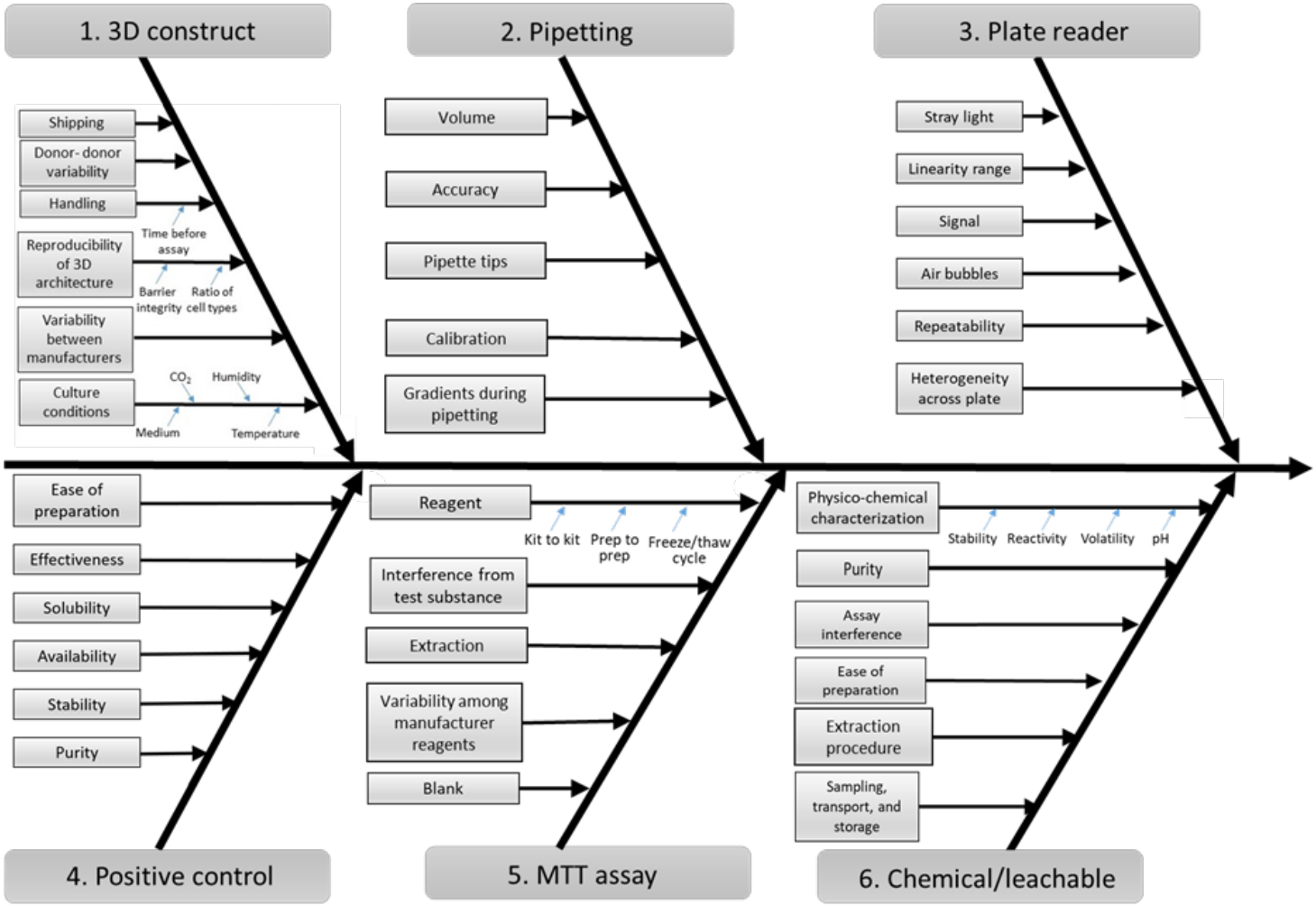
Motivation

- Assay recommended during a 2022 NIST/NIDCR workshop
- Fulfilled a task in NIST/NIDCR IAA (2020-2023)
- Skin irritation model has been standardized (ISO 10993 biocompatibility series) and is accepted worldwide for regulatory use (except for medical devices in US)
- Expanded our work into a new type of assay (3D constructs)

Work performed at NIST

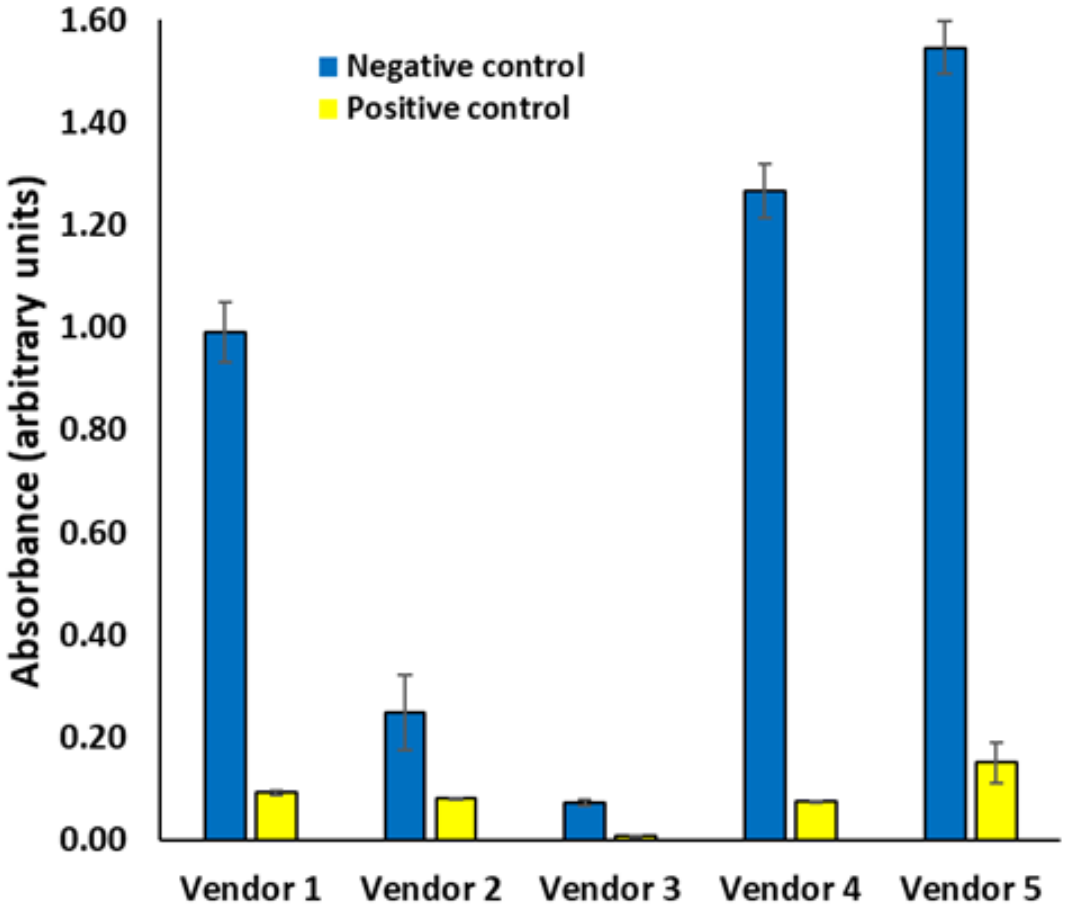
- Performed extensive robustness testing
- Monitored assay performance across time for key control measurements
- Thorough technical characterization of key sources of uncertainty
- Statistical model built
- Recommendations for a protocol for potential standardization

Oral Mucosal Tissue Irritation Assay



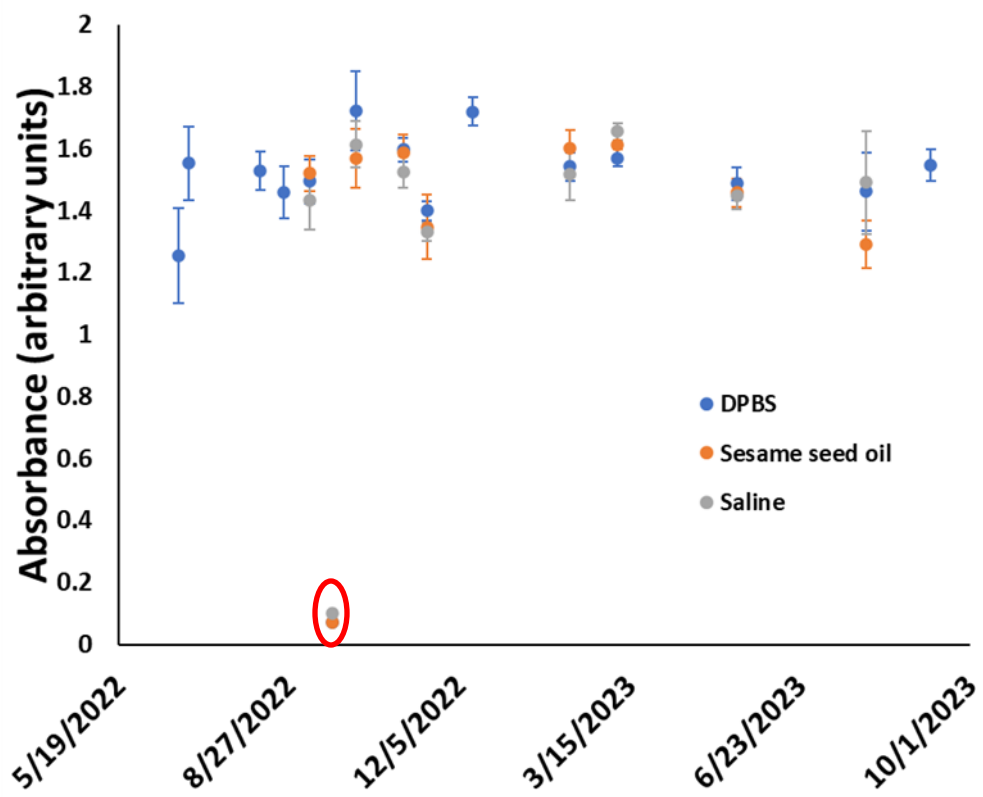
Gutierrez, R., Toman, B., Ma, Y., Elliott, J. T., Petersen, E. J. Sensitivity analysis and quality indicators for an *in vitro* oral irritation assay. Altex, 2024, 41(4), pp. 633-646. doi: 10.14573/altex.2405071.

Robustness testing: different MTT vendors



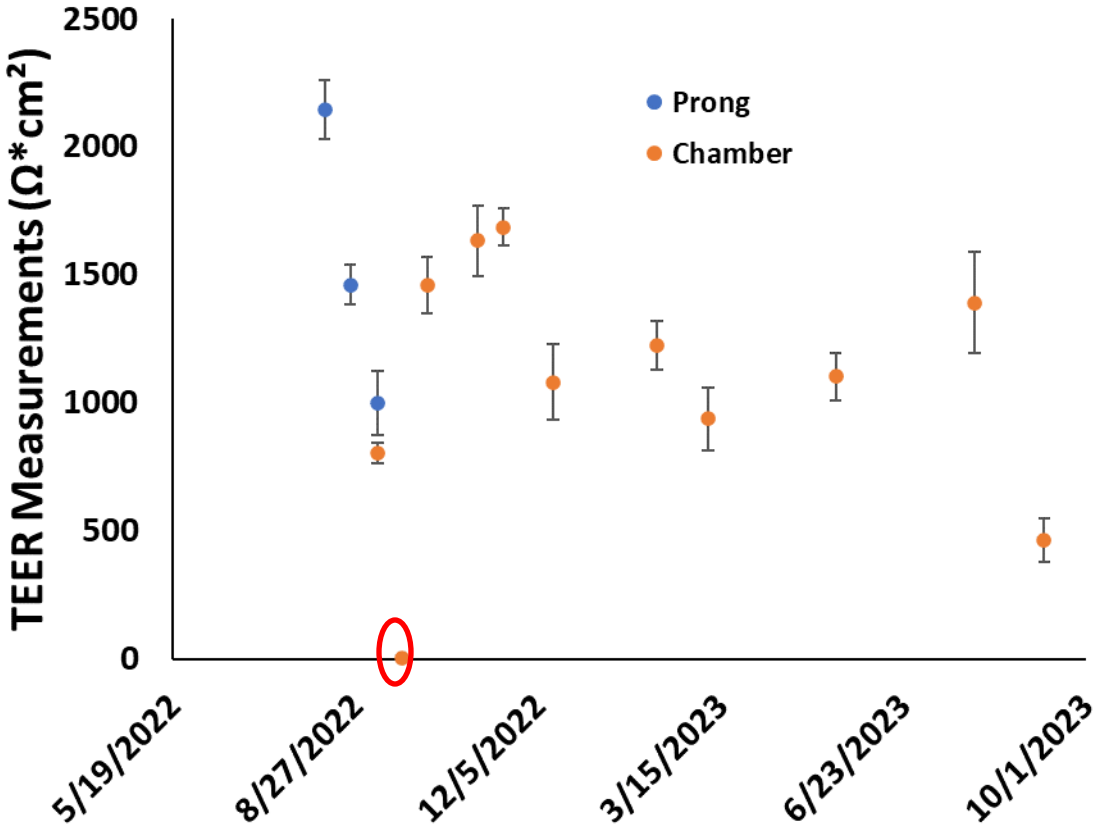
- There were substantial differences among vendors for the absorbance values
- The Z-factor values were more similar ranging from 0.70 to 0.86 for four of the vendors, while the value for vendor 2 was -0.33
- Specifications could be based on the Z-factor, not the absorbance

Robustness testing: control charting of MTT assay for negative and vehicle controls



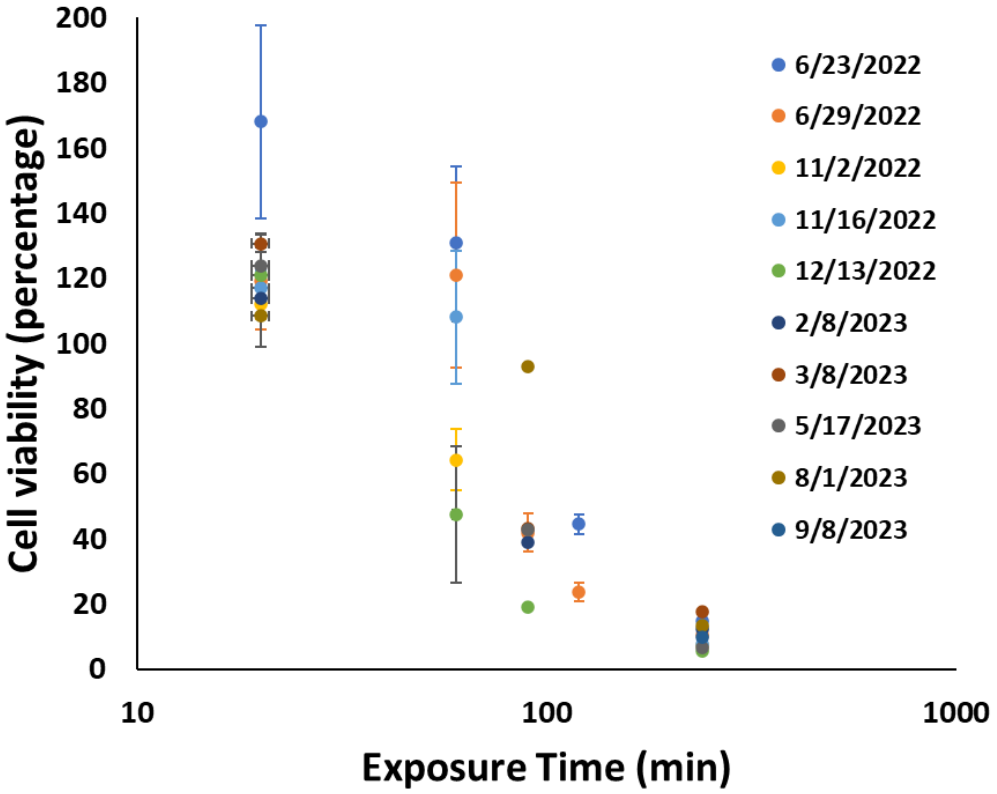
- 4-hour exposure
- Relatively consistent results were observed for the DPBS, sesame seed oil, and saline solutions across approximately 1.5 years
- Outlier results were observed once

Robustness testing: control charting of TEER assay for negative controls



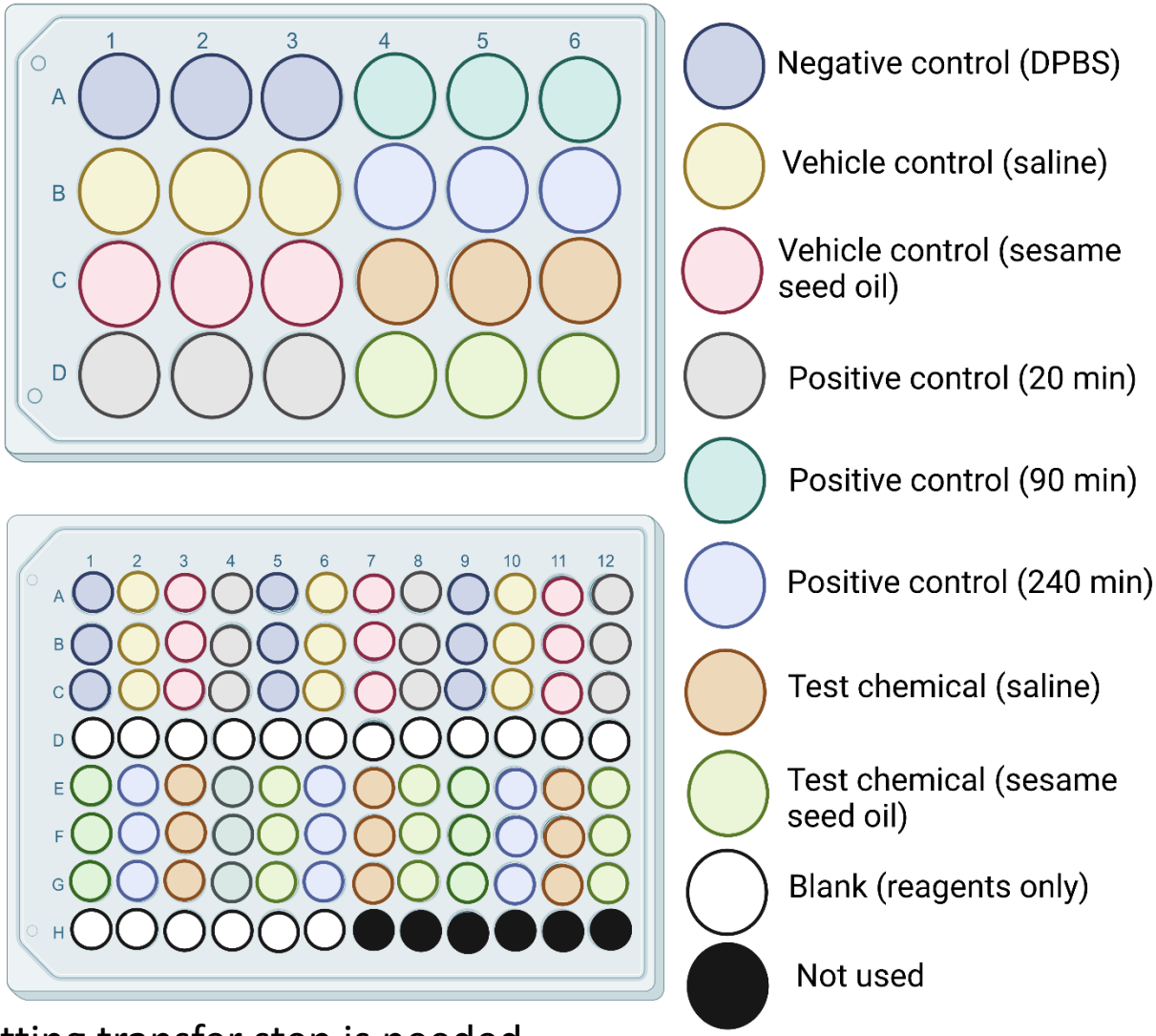
- TEER results were more variable than those for the MTT assay
- Outlier results were observed on the same experiment as for the MTT assay
- TEER is a helpful complementary measurement to confirm the integrity of the constructs

Robustness testing: control charting of MTT assay for positive control



- Data at 20 min and 240 min were very consistent
- Data at 60 min had the most variability and is maybe not suitable for a specification
- Data at 90 min was less variable than for 60 min
- Overall, triton X-100 fulfills many of the criteria sought after in a positive control (Petersen et al., 2021; doi.org/10.14573/altex.2102111)

Plate layout



- A pipetting transfer step is needed
- A pipetting procedure is described to reduce variability
- Staggering the transfer of samples for a test condition is recommended to minimize random errors

Statistical model for evaluating test chemicals taking into account multiple subsamples

$$PC,TC\ \%\ Cell\ viability\ = \left(\frac{\overline{TC} - \overline{blank}}{\overline{NC} - \overline{blank}}\right) x100\%$$

$$Depletion = \overline{NC} - \overline{TC}$$

Null hypothesis: % Viability = 100 % or NC=TC or Depletion=0
Alternative hypothesis: Viability < 100 % or NC > TC or Depletion > 0
Variability from the blank was << variability from sample and excluded

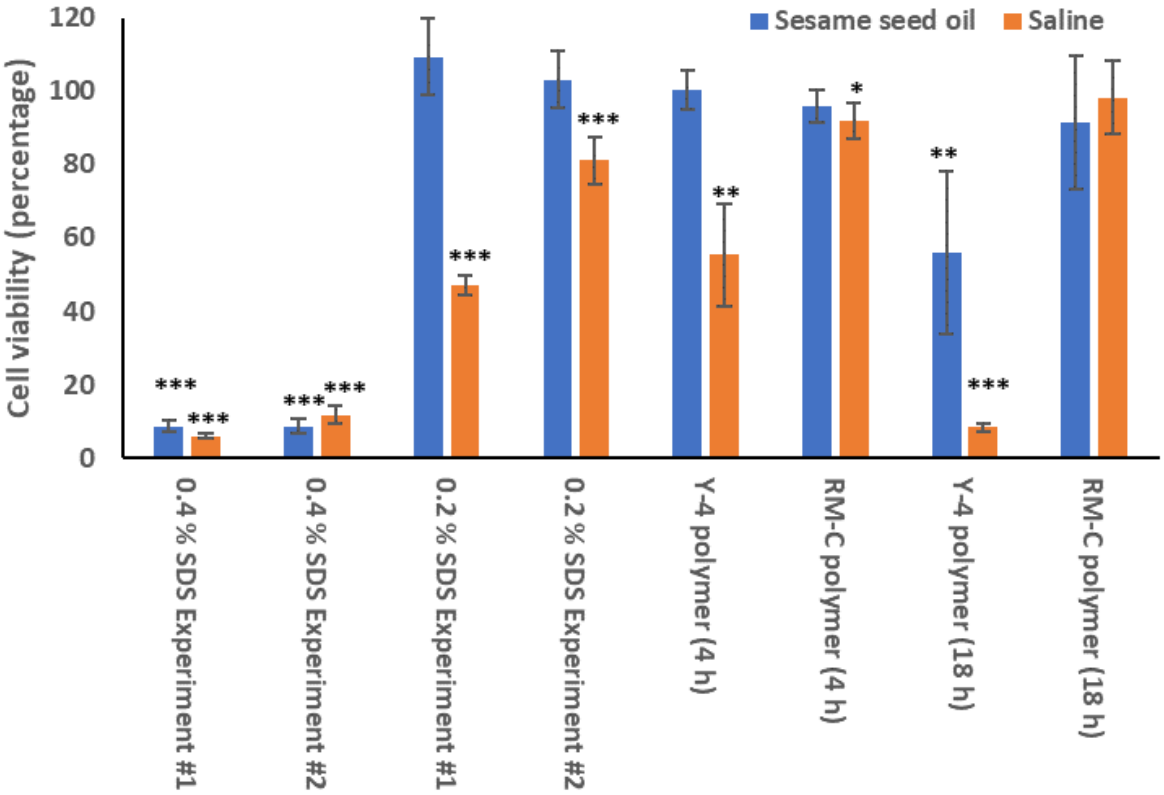
$$V(\overline{NC}) = \frac{1}{n_{sNC}\ x\ n_{rNC}}(n_{rNC}\, s_{NC}^2 + s_{rNC}^2) \qquad V(\overline{TC}) = \frac{1}{n_{sTC}\ x\ n_{rTC}}(n_{rTC}\, s_{TC}^2 + s_{rTC}^2)$$

where n refers to the number of pipetting aliquots (n_r) or samples (n_s) and s refers to the standard deviations

$$t = \frac{\overline{NC} - \overline{TC1}}{\sqrt{V(\overline{NC}) + V(TC)}} \qquad Limit\ of\ detection = t_{critical}\sqrt{V(\overline{NC}) + V(TC)}$$

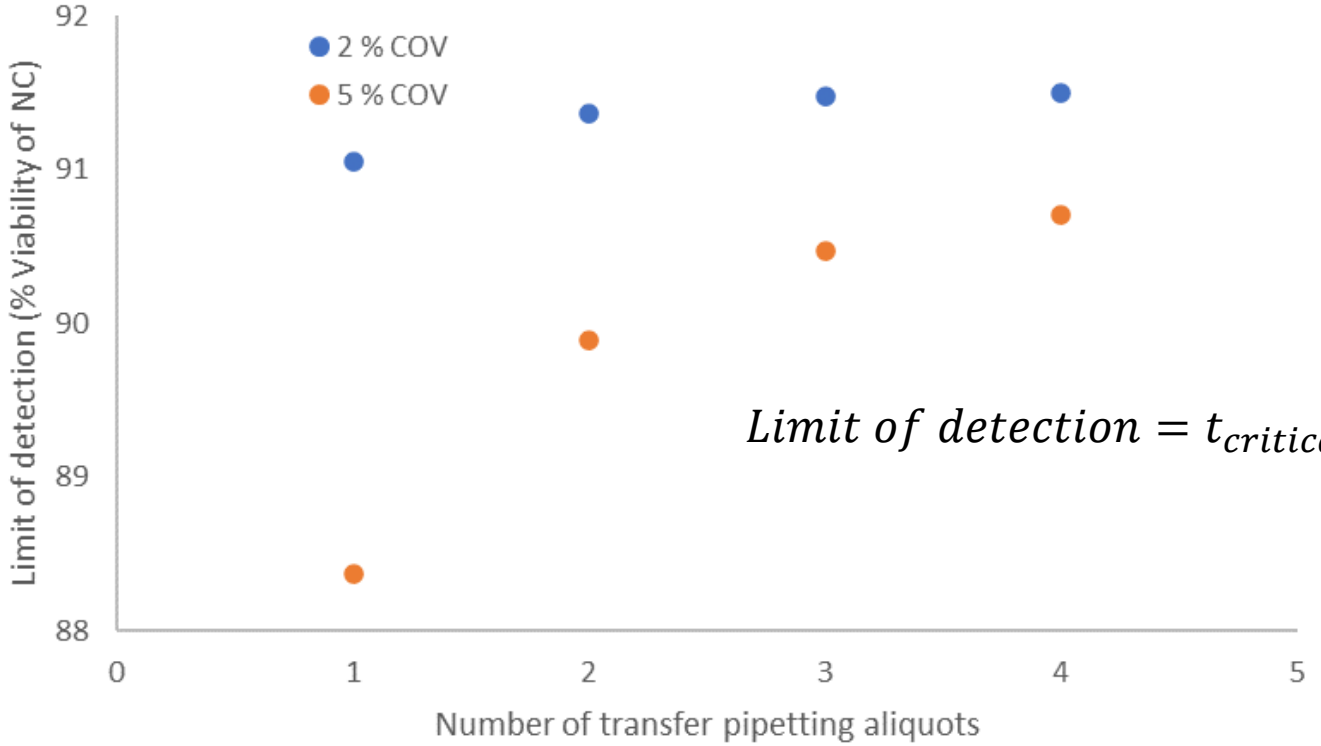
These equations can be used to make a positive or negative call with an associated statistical probability

Robustness testing: test chemical and polymers



- Results mostly yielded the expected results with SDS at 0.2 % and 0.4 % and the Y-4 polymer yielding decreased cell viability while the RM-C polymer did not have an effect
- Repeated experiments yielded similar results
- Cell viability decreased differently between the two vehicle controls

Detection limit calculation: Impact of number of pipetting aliquots



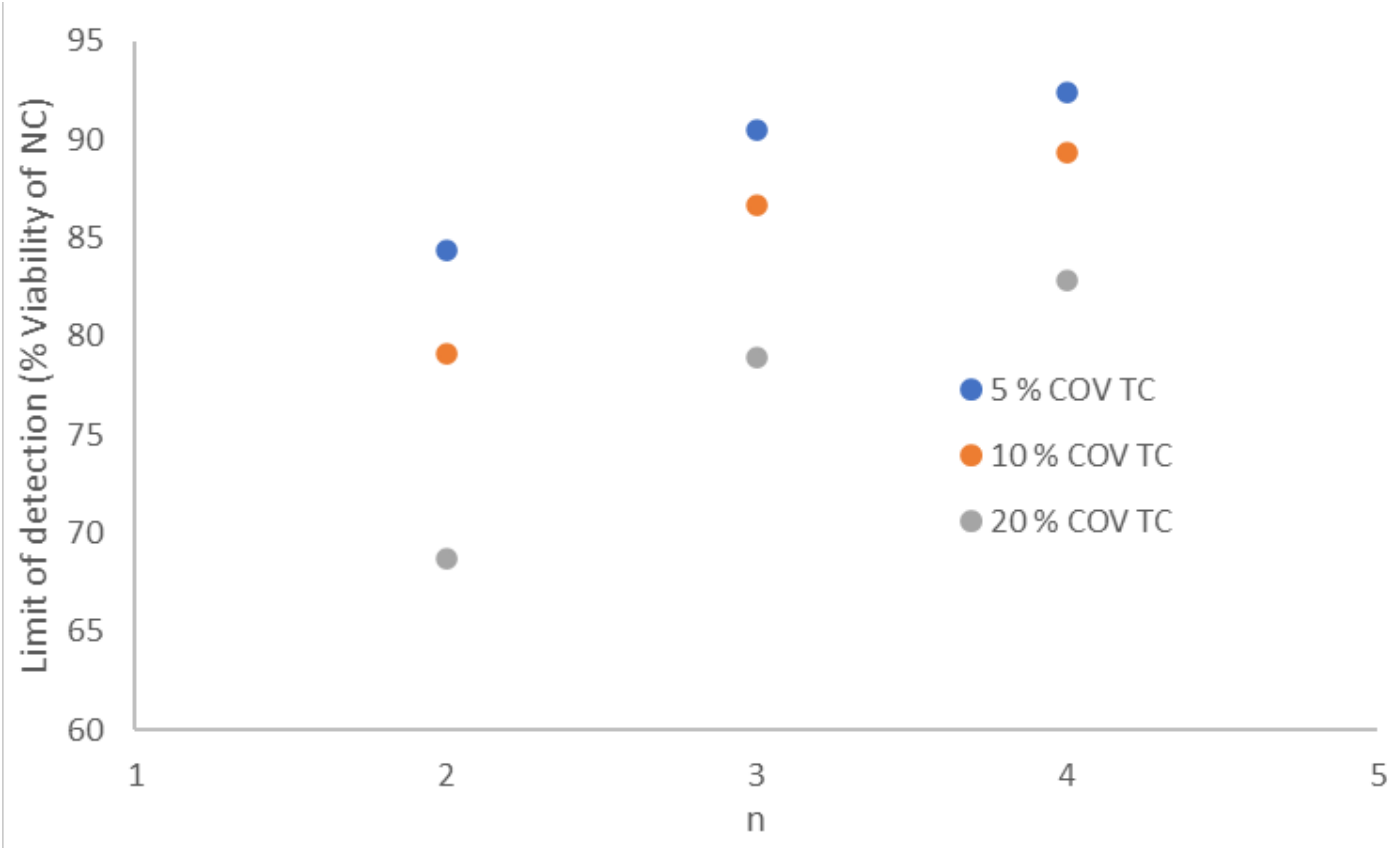
$$Limit\ of\ detection = t_{critical}\sqrt{V(\overline{NC}) + V(TC)}$$

Parameters: n=3 for TC and NC; COV for TC and NC=5 %;alpha=0.05; pipetting aliquot COV is varied

Limit of detection (y-axis) indicates the minimum amount of viability loss for the TC compared to the NC before a significant difference

The number of transfer pipetting aliquots and COV of the pipetting has a minimal impact

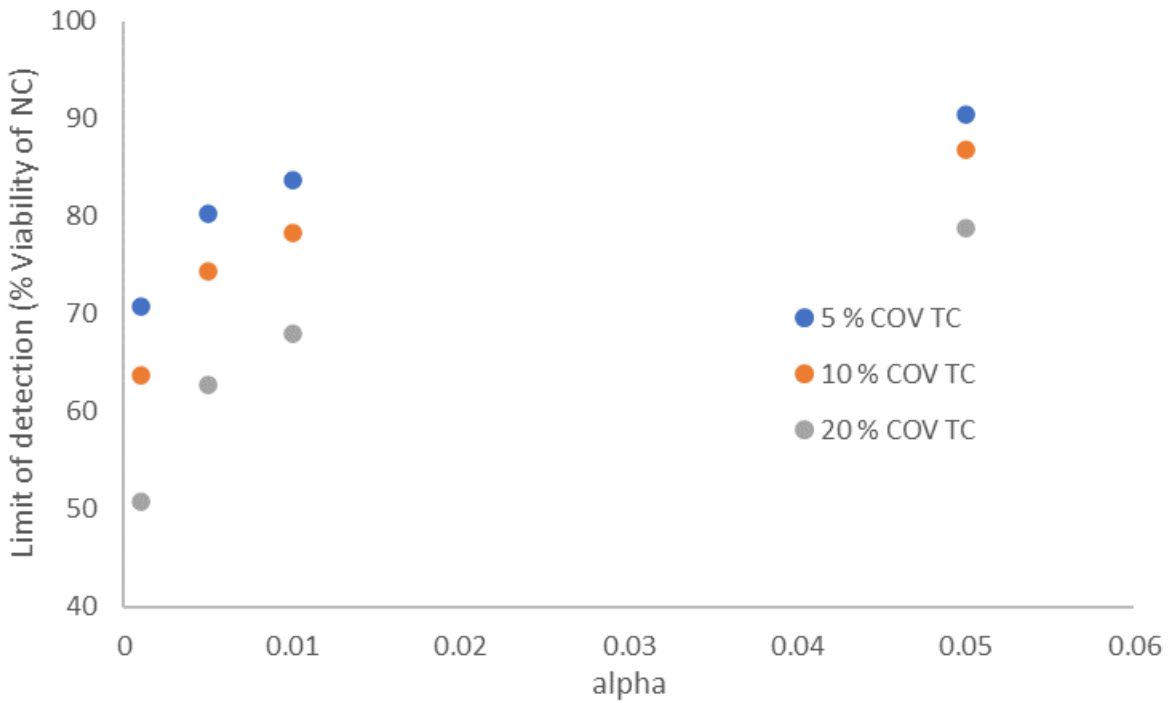
Detection limit calculation: Impact of number of biological samples



Parameters: n for TC and NC is varied; alpha=0.05; pipetting COV=2%; 3 aliquots; COV for NC is 5%; COV for the TC is varied

The number of samples has a substantial impact by impacting $t_{critical}$

Detection limit calculation: Impact of alpha



Parameters: n for TC and NC=3; pipetting COV=2%; 3 aliquots; COV for NC is 5%; COV for TC is varied, alpha is varied: 0.001, 0.005, 0.1, and 0.05

Alpha has a substantial impact

Conclusions

- A thorough investigation of sources of uncertainty has been performed
  - Helps de-risk interlaboratory studies since unexpected sources of uncertainty are less likely to be found
- This testing can be relevant for other assays that have shared branches in their cause-and-effect diagrams
- Limited data is publicly available for comparison to human or *in vivo* oral irritation
- Potential for future standardization