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Perspective

Use of Cause-and-Effect Analysis to Optimize the Reliability of *In Vitro* Inhalation Toxicity Measurements Using an Air–Liquid Interface

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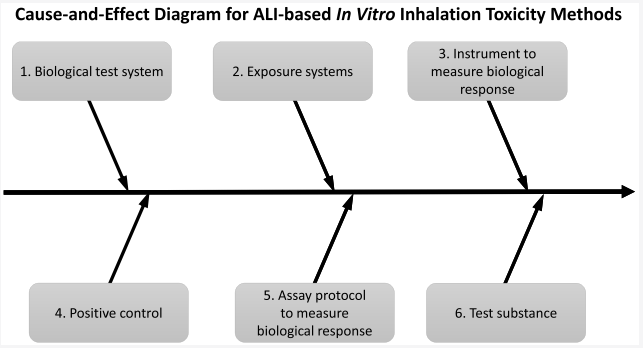
ABSTRACT: *In vitro* inhalation toxicology methods are increasingly being used for research and regulatory purposes. Although the opportunity for increased human relevance of *in vitro* inhalation methods compared to *in vivo* tests has been established and discussed, how to systematically account for variability and maximize the reliability of these *in vitro* methods, especially for assays that use cells cultured at an air–liquid interface (ALI), has received less attention. One tool that has been used to evaluate the robustness of *in vitro* test methods is cause-and-effect (C&E) analysis, a conceptual approach to analyze key sources of potential variability in a test method. These sources of variability can then be evaluated using robustness testing and potentially incorporated into in-process control measurements in the assay protocol. There are many differences among *in vitro* inhalation test methods including the use of different types of biological test systems, exposure platforms/conditions, substances tested, and end points, which represent a major challenge for use in regulatory testing. In this manuscript, we describe how C&E analysis can be applied using a modular approach based on the idea that shared components of different test methods (e.g., the same exposure system is used) have similar sources of variability even though other components may differ. C&E analyses of different *in vitro* inhalation methods revealed a common set of recommended exposure systems and biological in-process control measurements. The approach described here, when applied in conjunction with Good Laboratory Practices (GLP) criteria, should help improve the inter- and intralaboratory agreement of *in vitro* inhalation test results, leading to increased confidence in these methods for regulatory and research purposes.



INTRODUCTION

Many agencies are moving toward the use of non-animal methods to fulfill regulatory testing requirements. The United States Environmental Protection Agency (EPA) plans to eliminate the requests and funding for all mammalian studies by 2035.[1] Animal studies are already forbidden for the development of cosmetic products in Europe in agreement with guideline 2010/63/EU for the protection of animals used for scientific purposes and the regulation of cosmetic products.[2] There are currently several examples from European and U.S. legislation that require, or strongly encourage, the replacement of animal testing.[1−7]

Inhalation toxicity testing is one area where *in vivo* animal tests[8−12] are still routinely used for regulatory decision making. However, due to monetary, ethical, and scientific concerns associated with these tests, there has been substantial interest in optimizing non-animal approaches that can replace the *in vivo* tests.[13−18] A number of *in vitro* methods are available to assess the respiratory toxicity potential of a variety of substances in human cells.[13,19−22] One key factor for potential regulatory application of these methods is their reliability, which can be demonstrated by interlaboratory reproducibility, intralaboratory repeatability, and robustness[23] (i.e., resistance to measured change in the assay resulting from unintended variations in experimental reagents or protocols).

Understanding the reliability of *in vitro* methods depends on identifying the potential sources of variability. Cause-and-effect

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(C&E) analysis has been recently used to categorize sources of variability in a range of toxicological assays.[24−27] This approach uses a fishbone diagram to visually illustrate all expected major causes of variability that can impact the overall assay result. C&E diagrams provide a visual display of these sources of variability which can then guide assay protocol development, such as which in-process control measurements to incorporate into the protocol, and robustness testing. To fully understand the potential sources of variability in an assay, it is necessary to evaluate each of the branches shown in the C&E analysis either in one-time preliminary experiments or each time the assay is performed.

The use of *in vitro* methods to assess potential respiratory effects of substances on the lung requires consideration of several features including the biological test system (e.g., monoculture systems, coculture systems, and three-dimensional [3D] constructs), mode of exposure (submerged or air−liquid interface [ALI]), characterization of test materials, appropriate controls, and assays to measure the biological response. This manuscript provides a conceptual evaluation of the aforementioned potential sources of variability for *in vitro* inhalation toxicity assays that use cells cultured at the ALI. The approach was designed to be general so that the sources of variability identified were not specific to an individual assay, material, or test system. Based on practical experience, these sources of variability are discussed in detail, and control measurements are suggested to improve assay reproducibility. One key value of these control measurements is that they can reveal information about the analytical assay performance (e.g., is the sensitivity of the biological test system similar to previous assays?). This can be useful to deconvolute the measured assay variability, because multiple factors can contribute to the overall variability. This information can also lead to refinement of the assay by identifying and decreasing the largest sources of variability. Ultimately, the goal of this manuscript is to describe how to maximize the reliability of ALI cell culture methods for use in current experiments as well as any future interlaboratory comparisons and method standardization.

CAUSE-AND-EFFECT DIAGRAMS FOR *IN VITRO* INHALATION TOXICITY ASSAYS

To evaluate the potential sources of variability in an assay, the main stages of the assay protocol can be diagramed using a flowchart, shown as steps a−e in Figure 1. The first step in an ALI cell culture assay relates to the preparation of the biological test system and varies in complexity based on the system used. The second step involves preparation of the test substance for exposure and varies in complexity depending on the characteristics of the test substance. The preparation of the exposure system (step c) is the least complicated when the test substance is pipetted onto the biological test system. For more complex ALI exposure systems, the functioning of the nebulization/aerosolization and the in-line characterization instrument (e.g., quartz crystal microbalance [QCM] or scanning mobility particle sizer [SMPS]) need to be evaluated prior to exposing the biological test system to ensure that the system is performing properly. The fourth step involves the exposure of the biological test system to the test substance using the exposure system, and the last step is choosing and performing the biological assay (e.g., evaluation of changes in cell viability or the production of cytokines by the biological test system).

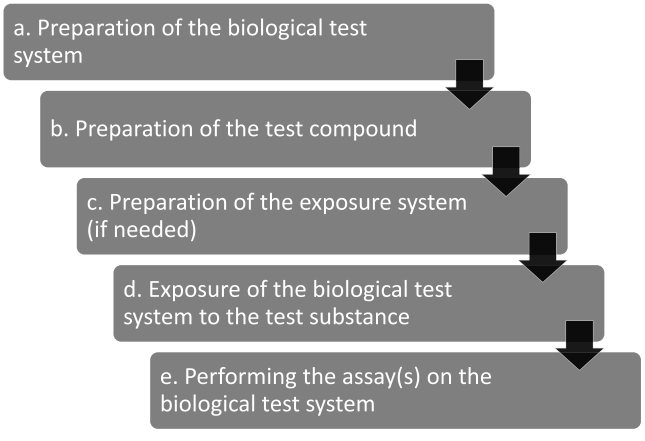

Figure 1. Flowchart detailing the main steps in an *in vitro* inhalation toxicity assay.

One challenge in making C&E diagrams that span the five steps discussed above is that ALI-based *in vitro* inhalation toxicity assays can vary substantially based on the study requirements. Nevertheless, there are some steps that will be shared among assays. Thus, it is possible to design modular C&E diagrams to identify potential sources of variability for specific branches based upon which assay is used (i.e., all assays with monoculture of cells as the biological test system would share a branch). In addition, certain assays may share some of the same sources of variability. For example, it has been shown that no modifications are needed in some branches of the 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS) assay for use in other assays, such as the Comet assay or a flow cytometry-based apoptosis/necrosis assay, because those assays share common sources of variability.[27]

Figure 2 presents a generalized C&E diagram for ALI cell culture inhalation assays. The sources of variability for branches 1−6 are shown in Figures 3−8, respectively. Combinations of these branches can yield an overall C&E diagram for a particular assay, as depicted in Figure 9.

BRANCH 1: BIOLOGICAL TEST SYSTEM

Different types of biological test systems are used to model the lung for ALI exposures, including mono- or cocultures or 3D organotypic tissues. They differ from each other in complexity and physiological relevance, and the choice of which system to use will depend on the purpose of the study. Therefore, evaluation of newer cell lines, which may introduce for instance a barrier function, including comparison with well-tested cell lines is crucial.[28] Minimization of inter- and intralaboratory variability related to these systems requires consideration of parameters that are generally applicable to all cell-based systems, including identification and characterization of cells, such as the cell ID (including the supplier [e.g., ATCC], source type [i.e., cell line or primary culture], origin [e.g., species], and history of cells [e.g., cell passage number, freezing protocol, phenotypic and genotypic verification, and mycoplasma contamination]), donor variability, general handling and maintenance, and culture conditions (see Figure 3). The aforementioned parameters have been described in several manuscripts and guidance documents, such as the Guidance Document on Good *In Vitro* Method Practices (GIVIMP), which suggest ways to optimize the reliability and robustness and to reduce the variability of *in vitro* methods.[13,29−34] In

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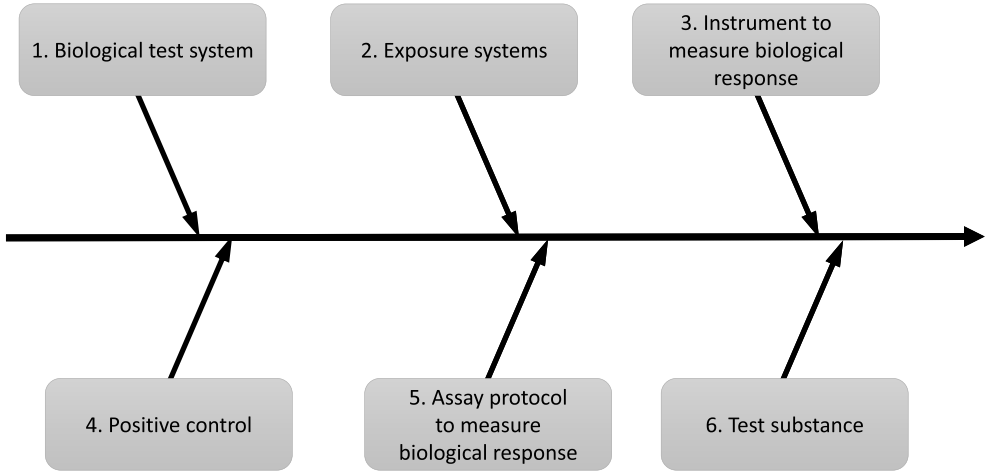

Figure 2. C&E diagram of *in vitro* ALI inhalation toxicity assays. Sources of variability for the branches are shown in subsequent figures (Figures 3–8).

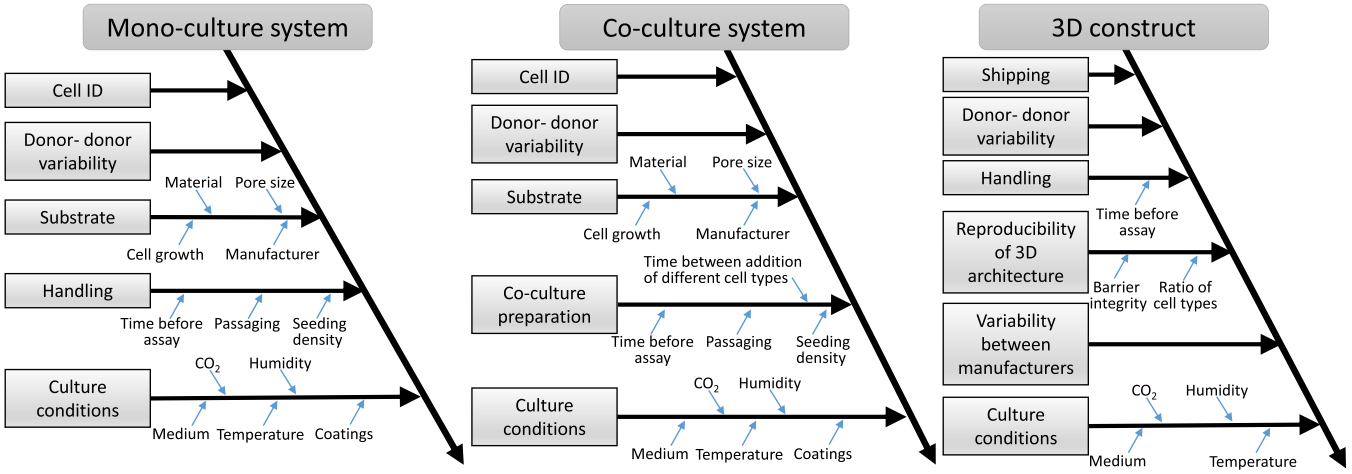

Figure 3. Branches for the "biological test system" branch in Figure 2. The branch for "monoculture system" is modified and reprinted with permission from ref 27. Copyright 2020 American Chemical Society. Note that some branches may have slightly different sub-branches, for example, handling of a monoculture system includes time before assay, passaging, and seeding density, while the user handling a purchased commercially available 3D construct does not need to consider passage and seeding density.

addition to the general topics that are discussed in these documents, other potential sources of variability for the biological test system have to be taken into account when working with ALI test systems. Some of these potential sources of variability are shared among all types of ALI test systems, while others are specific to certain test systems. Both are described below.

Sources of Variability Common to All Types of Biological Test Systems. *Substrate.* To expose cells at the ALI, especially for exposure times longer than a few minutes, the cells or tissues need to be cultured on a substrate that allows them to receive nutrients from the basolateral side (i.e., the side that is in contact with the cell culture medium). The most commonly used substrates are microporous membrane-based inserts that can differ in geometry, type of material, thickness, and pore size and density. Changes in these properties may affect the attachment of cells, absorption, and adsorption of substances (e.g., proteins), direct cell-to-cell contact, migration of cells to the other side of the membrane, or changes in the cell barrier properties.[35,36] Variabilities between inserts attributed to the manufacturing process cannot be completely avoided; however, choosing the same type of cell culture insert from the same manufacturer for the entirety of an experiment, and including the catalogue number and manufacturer information in publications, minimizes those variabilities.

Culture Conditions. Most cell types used for ALI exposure assays will only adhere to cell culture inserts with special coatings (e.g., biological [e.g., extracellular matrix proteins] or synthetic polymers [e.g., polylysine]). Various potential sources of variability arise from different coatings. While the use of commercially available precoated inserts offers a solution to reduce variability related to coating, it can be expensive. Therefore, many laboratories choose to coat the inserts in-house. Use of synthetic coatings and controlling the composition,[37] pH,[38] temperature, ionic strength,[39] and storage conditions of the coating solution[40,41] will help achieve homogeneous and reproducible coating on the inserts. Additionally, the use of animal-derived components (such as serum, growth factors, and hormones) in the cell culture media is a known source of variability in *in vitro* experiments. The use of chemically defined medium, instead of an undefined mixture (e.g., media with fetal bovine serum), can greatly reduce the variability related to culture medium.[42]

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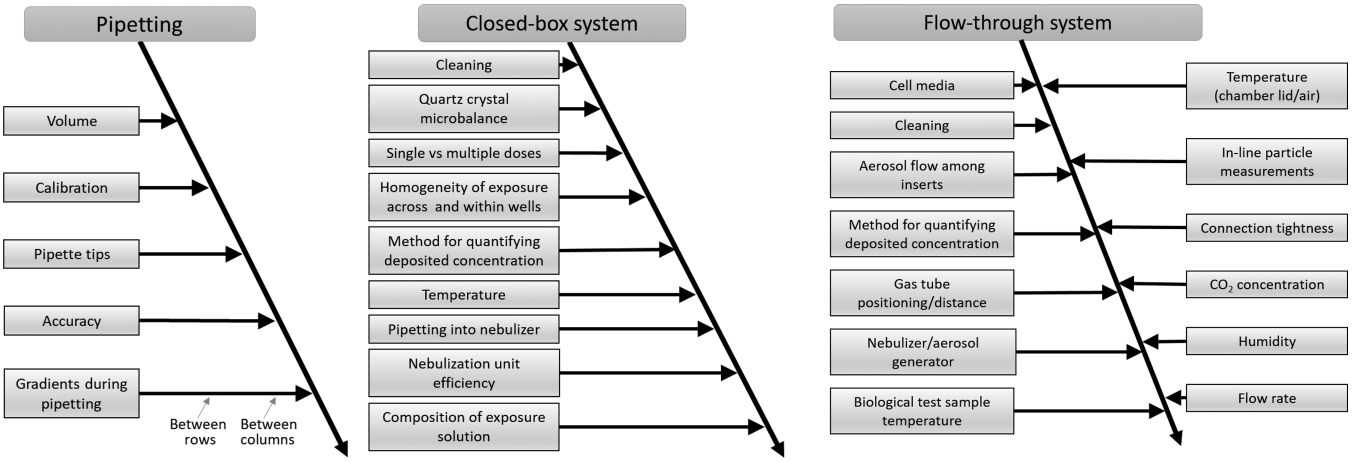

Figure 4. Branches for the "exposure of biological test system" branch in Figure 2. The " pipetting" branch is modified and reprinted with permission from ref 27. Copyright 2020 American Chemical Society.

Handling. The way the cells/tissues are prepared before exposures can change the study outcome and is important to monitor. For example, the timing of transitioning the cells from submerged to ALI conditions has to be carefully considered. Certain cells need more time to adapt to the new environment than others. Besides, cells and tissues grown at ALI often produce an epithelial lining fluid, such as mucus or surfactant.[13,43] The timing of the transitioning to ALI, of apical washes, or trans-epithelial electrical resistance (TEER) measurements influences whether this epithelial lining fluid is (partially) present during the ALI exposure. In addition, the culture media may or may not be added back to the cells after exposure to the test substance. Therefore, all of these details should be thoroughly documented in laboratory notebooks and publications.

TEER measurements evaluate the integrity and permeability of biological barriers. TEER measurements can serve as a quality control for cell/tissue well-being before the start of an experiment, and only samples that reach a previously defined threshold should be used.[44] While useful, TEER measurements should be conducted carefully and sporadically, because frequent measurements can potentially stress the cells and will remove the epithelial lining fluid because liquid is added to, and removed from, the apical side of the cells.

Sources of Variability Related to Adherent Monocultures. When choosing a cell type, it is important to know whether it can be cultured at the ALI for prolonged periods and whether the cells are contact inhibited or will grow in multiple layers. While working with cells that are not contact-inhibited (e.g., A549 cells), it is important to make sure that the cell culture insert is covered with a single layer of cells. A multilayer of cells is not desirable for multiple reasons: it may not represent *in vivo* conditions, it hinders estimating the number of cells, and only the top layer is directly exposed to the test substance. These sources of variability, which are also relevant for coculture systems, can be reduced by optimizing the timing and seeding density of cells that are not contact inhibited, or by using cells that are contact inhibited and will stop proliferating once confluent.

Sources of Variability Related to Coculture Systems. When two or more cell types are cultured together, one cell type may outgrow the other(s), and the secreted molecules (e.g., growth hormones, or pro- and anti-inflammatory mediators) from one cell type can influence another cell type (paracrine signaling). For example, a pulmonary epithelial-endothelial coculture increased the barrier tightness of epithelial cells,[45] and culturing epithelial cells with fibroblasts increased the wound-healing abilities of the epithelial cells.[46] Therefore, the timing and density of seeding different cell types are critical and should be carefully documented in laboratory notebooks and publications.

Another potential source of variability is the cell culture medium used. Most cells require culture medium that is tailored to their needs and changes in the constituents of the medium can influence their phenotype or genotype. For cocultures, two or more types of media are often mixed to support the different types of cells. Therefore, each cell type should be investigated with the new cell culture medium and given enough time to adapt to it before any further steps are performed.

Sources of Variability Related to 3D Constructs. When cultured at the ALI under specific conditions, some types of epithelial cells can differentiate into a 3D tissue that closely represents human physiology. Due to its complexity, the differentiation process is prone to variability; use of commercially available fully differentiated 3D tissues can reduce this variability. If the cells are differentiated in-house, then standardized protocols should be used (if available) and the method should be carefully documented. Timing of tissue orders from suppliers requires attention to avoid hold-ups due to weekends or public holidays. Upon receipt, and if not already performed by the supplier prior to shipping, a few random tissues should be histologically analyzed (e.g., for tissue thickness, composition of cell types, and abnormalities) as a quality control measure. After shipping, 3D tissues need time to recover and adapt to the new environment. A predefined TEER threshold value is a useful quality control for the barrier property. This measurement should be performed a few days ahead of the ALI exposure, if the presence of the epithelial lining fluid is desired.

It is recommended to follow manufacturer-provided protocols while handling and using 3D tissues, when possible. For example, during the development and optimization of the EpiAlveolar tissue model (MatTek Life Sciences), protocols and training videos were shared with the testing laboratories to demonstrate proper tissue handling.[47] Any details specific to a chosen end point or deviations from the standard protocols (e.g., adjustments to suit a specific study design), if needed,

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should be documented in laboratory notebooks and publications. For instance, when treated with substances that elicit pro-fibrotic responses, the EpiAlveolar tissues can sometimes detach from the membrane insert, resulting in false negative readings; therefore, care should be taken when washing these tissues after exposure to such substances.[47]

BRANCH 2: EXPOSURE SYSTEMS

Cells cultured at an ALI can be exposed either to a liquid by pipetting or to an aerosol/gas/vapor using an ALI exposure system (Figure 4). The selection of the exposure approach should be carefully considered depending upon the purpose of the measurements. Particle deposition from aerosols is predominately performed in closed-box or flow-through systems.

Sources of Variability Related to Exposure by Pipetting. Pipetting is the simplest way to test an adverse effect of a test substance using an *in vitro* system. Physical properties of the matrix containing the test substance, such as its viscosity and surface tension, can contribute to the variability of the exposure concentration. These sources of variability would also apply to other steps in the protocol involving pipetting of liquids and may also apply to protocols using aerosol exposure. Under ALI conditions, it is possible to add a small amount of test substance directly onto the apical side of the biological test system, in which case the entire pipetted volume should come into contact with the cells. It is also possible to pipet into the basal medium, but only a fraction of the amount added will reach the cells. This fraction depends upon diffusion of the substance to the cells. For particles, sedimentation may also decrease the concentration that reaches the cells. This contrasts with exposure of submerged cells located at the bottom of a well, where sedimentation of particles from the overlying medium would increase the cellular exposure. Whether exposing to the apical or basal side of the ALI culture, the ALI on top of the biological test system should be preserved.[48−50] Addition or removal of any liquid to or from the apical side of the biological test system may disturb the epithelial lining fluid and, therefore, affect the biological response. Interactions of the test substance with the media or the epithelial lining fluid may induce changes to the test substance's surface, such as formation of a corona around an engineered nanomaterial (ENM), which might affect the test result.[51]

Sources of Variability Related to Aerosol, Vapor, or Gas Exposure. For ALI exposure to aerosols, vapors, or gases, additional parameters (such as the inlet air flow rate and the distance between inlet and biological test system) need to be considered and can be system specific. In general, by minimizing the contact between test substance and cell media, ALI exposure systems help to avoid chemical reactions between media and test substance or formation of a corona for particulate test substances. When using an ALI exposure system, it is crucial to characterize and quantify the test atmosphere to ensure consistency among experiments and to calculate the deposited dose fraction.[52] Thorough cleaning of the systems when using different test substances is essential to avoid contamination.

Sources of Variability Related to Closed-Box Systems. Exposure in closed-box systems is based on sedimentation of the aerosolized test substance onto the biological test system, often in a defined chamber. Sources of variability in the exposure dosage for this exposure system include the test substance's properties within the test media (e.g., viscosity), which can impact the aerosol generation and its potential to contaminate the nebulizer. Furthermore, changes in humidity causing condensation on the chamber walls needs to be prevented, because sample in the condensate will decrease the intended exposure concentration. How much deposition occurs is impacted by the test substance's liquid matrix. For particulate test substances, a high deposition rate within in a short period can be achieved.[53−56] This could lead to complete coverage of the biological test system's apical side, which could potentially alter the epithelial lining fluid. Therefore, a more detailed assessment of deposited dose and inclusion of quality-control measurements based on specific parameters (e.g., required nebulization time, fluid volume used to generate the aerosol, and method used to measure the deposited dose [e.g., QCM]) is advised.[53] A QCM is often integrated in newer closed-box systems and, therefore, should be incorporated into studies to monitor reproducible nebulization and deposition.[57] When using the QCM to determine deposited mass, time to dry off the microbalance after exposure should be documented. In addition, different nebulization efficiency, due to physicochemical matrix effects of different samples, can be taken into account to ensure similar dosage. Similarly, coupling to a condensation particle counter (CPC) and/or a SMPS to assess the generated aerosols, especially for particle aerosols, and to characterize their particle concentration and size distribution helps ensure repeatability and comparability of experiments. Depending on the system's setup, potential interference with the exposure experiments due to the aerosol sampling has to be considered. It may also be possible to use a sample port to collect deposited samples on an electron microscopy grid for further analysis.

Sources of Variability Related to Flow-through Systems. In flow-through ALI exposure systems, a very low sample flow rate is directed to the apical side of the biological test system. Due to the stagnation flow slowing the aerosol plume and the resulting slow aerosol movement, deposition occurs mainly via diffusion.[13,58,59] ALI flow-through systems are available that utilize other deposition methods (e.g., thermophoresis, electrostatic deposition), different setups (e.g., sample flow parallel to the apical side), and parameters (e.g., flow rates, constant or pulsed electrophoresis).[60,61] Nevertheless, differences in exposure procedure and exposure setup characterization (e.g., humidity, flow rate, distances, and exposure duration) for all flow-through ALI systems (stagnation flow-based and other models) can result in variabilities for the deposited dose and the response to the exposure system negative control.[62] Quality control measurements of the system and determination of the deposited dose, for example, via QCM or an alternate appropriate method, are advised. In flow-through ALI systems, the QCM is only installed in a separate ALI chamber. Although online assessment of the deposition on the cells directly is not feasible, the QCM can be used to monitor the aerosol and its deposition by stagnation flow in the sytem in general. Additional coupling with online aerosol measurements (e.g., CPC, SMPS) helps to assess the particulate character of the aerosol. As sampling flow rates of the measurement instruments are significantly higher than typical ALI sample flow rates, the isokinetic aerosol behavior may be affected. Nevertheless, aerosol characterization offers possibilities to support the comparability of different experiments. Again, it

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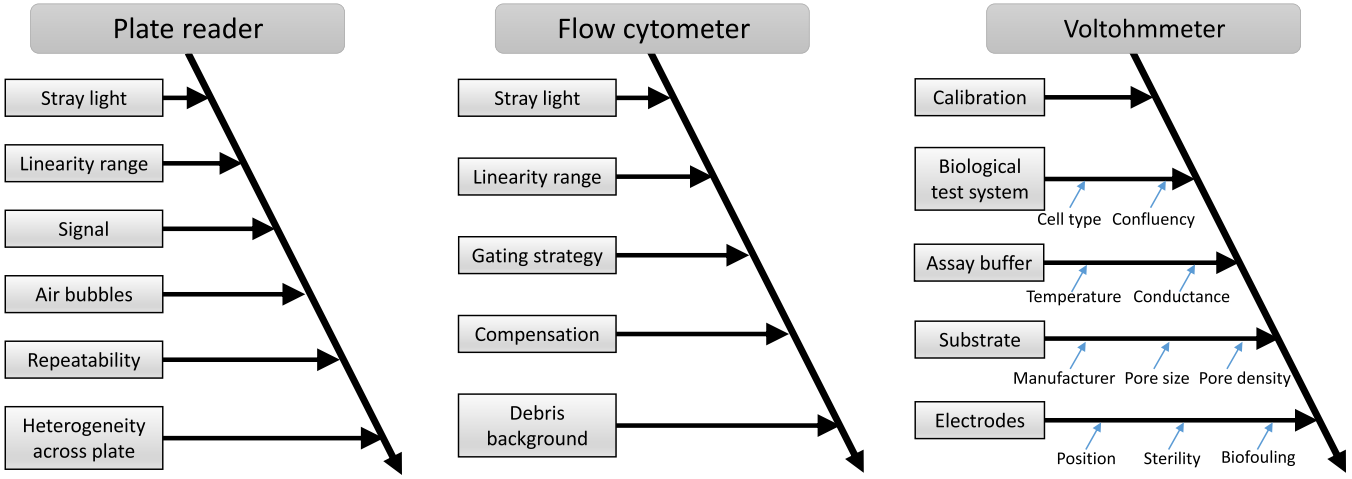

Figure 5. Branches for the "instrument to measure biological response" branch in Figure 2. The "plate reader" branch is modified and reprinted with permission from ref 27. Copyright 2020 American Chemical Society.

may be possible to use a sample port to collect deposited particles on an electron microscopy grid for additional analysis.

BRANCH 3: INSTRUMENT TO MEASURE BIOLOGICAL RESPONSE

Several instruments are used to measure the biological responses of different types of *in vitro* systems. Figure 5 shows three examples of instruments, plate reader, flow cytometer, and voltohmmeter, along with the parameters that need to be considered to reduce variability. A voltohmmeter is used to determine TEER, which is used to assess barrier integrity of cells that form tight junctions and generally applies to all three types of biological systems described in Branch 1. The control measurements are often specific to the instrument and should be considered for reliability.

BRANCH 4: POSITIVE CONTROL

There are two main types of positive test substance controls to consider when using an ALI exposure system for *in vitro* inhalation toxicity testing: incubator positive controls and exposure system positive controls. Incubator positive controls are conducted in the incubator and not the exposure system. A substance that is known to yield the biological effect under investigation (i.e., from previously obtained *in vivo* or epidemiological data) is delivered to the cells by pipetting directly onto the cell insert or by addition to the basal media. This can reveal information about numerous key dimensions for the assay performance. For example, this control can potentially provide information about the cell pipetting and rinsing procedures based on the magnitude of the response observed; lower numbers of cells are often more strongly impacted during cytotoxicity assays with lower effective concentrations causing a 50% effect (i.e., EC_{50} values).[24] The exposure system positive control measurement yields important information by also testing the performance of Branch 2 (the exposure system), in addition to the components of the assay tested by the incubator positive control. This provides information about the consistency of biological responses from the exposure system. By performing the incubator and exposure system positive control measurements concurrently, it is possible to isolate if a problem is occurring with, for example, the number of cells in the biological test system, in which case both positive controls are impacted. Alternately, if there is a problem with the exposure system, only the exposure system positive control is impacted. The exposure system positive control may, for some exposure systems, need to be performed before or after the exposure of the test substance given the limited number of samples that can be exposed concurrently.

Numerous characteritics should be considered when selecting the positive control, including its stability in relevant media (i.e., cell media or in air), interference with assay reagents, purity, commercial availability, ease of preparation in the desired matrix, and capacity to elicit the intended biological effect (Figure 6).[63] Some compounds that may work well as

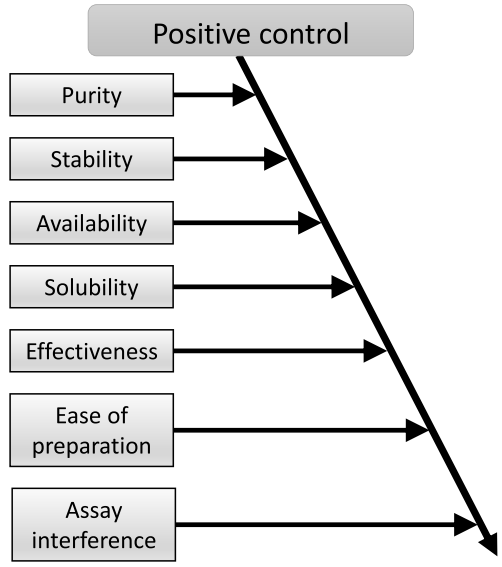

Figure 6. Branch for the "positive control' in Figure 2. These characteristics are relevant for both the incubator positive control and the exposure system positive control.

the incubator positive control (e.g., surfactants) may be problematic for use as the exposure system positive control. Thus, it may be necessary to have different positive control substances for the incubator and exposure system. In addition to positive controls, adequate negative controls need to be considered in the study design, such as an incubator control

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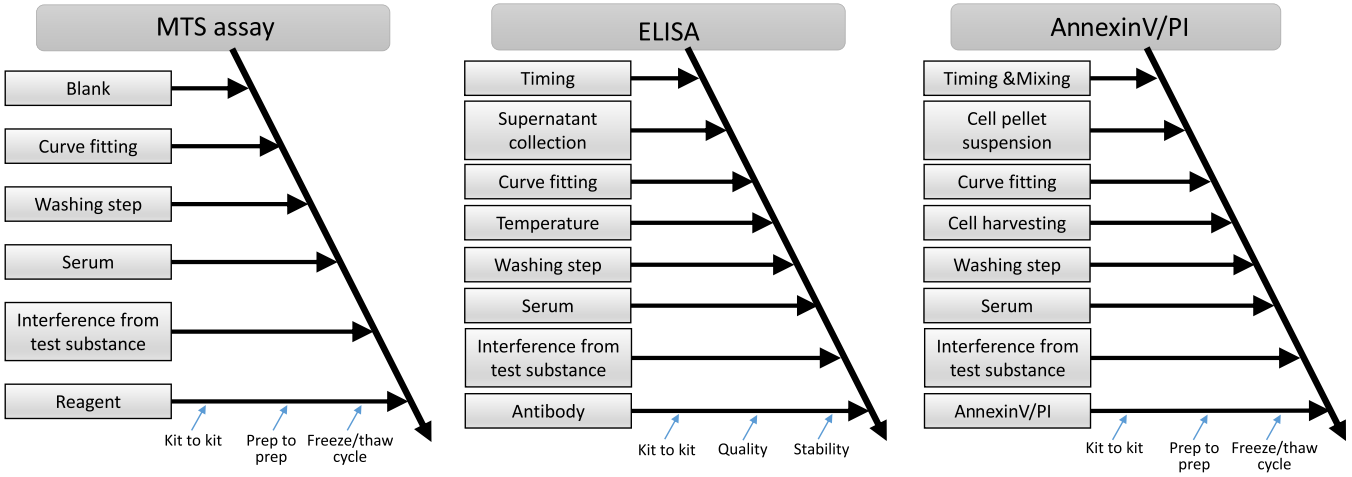

Figure 7. Branches for the "assay protocol to measure biological response" branch in Figure 2. All branches are reprinted with permission from ref 27. Copyright 2020 American Chemical Society. Abbreviations: ELISA, enzyme-linked immunosorbent assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); Prep, preparation; PI, propidium iodine.

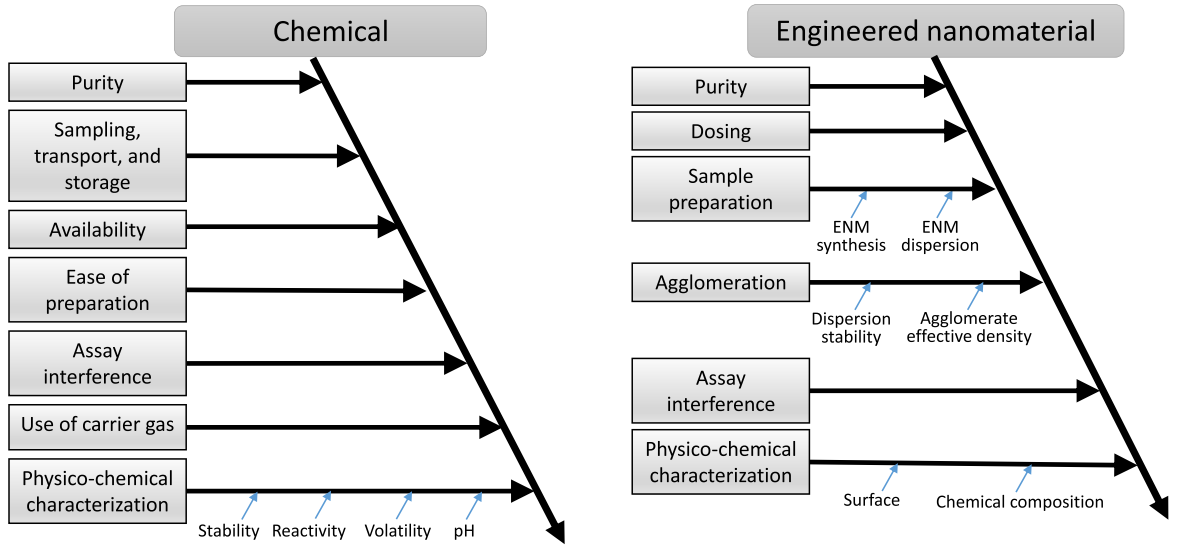

Figure 8. Branches for the "test substance" branch in Figure 2. The "Engineered nanomaterial" branch is reprinted with permission from ref 27. Copyright 2020 American Chemical Society. Abbreviations: ENM, engineered nanomaterial.

and exposure controls (e.g., clean air or sodium chloride/phosphate buffered saline solutions).

Another key topic to consider for the positive control is the number of concentrations tested. It is preferable, when possible, to test a sufficient number of concentrations to determine the assay's maximal response, and thus, its dynamic range, and also the dose–response function. Evaluating the dose–response function can provide information about the magnitude of effects observed for specific concentrations of the positive control chemical and enable comparability of the results obtained on different days within a single laboratory or among laboratories. Unlike the exposure system positive control, where there may be limits to the number of samples that can be concurrently exposed, testing a larger number of concentrations is more straightforward for the incubator positive control. Thus, it may be necessary for some exposure systems to test the exposure system positive control periodically rather than concurrently with every experiment.

BRANCH 5: ASSAY PROTOCOL TO MEASURE BIOLOGICAL RESPONSE

Biological response is measured using assays that generally include processing of the cell system, for instance, to collect cellular lysate or supernatant or incubation with assay chemicals. Figure 7 shows three examples of assays, MTS assay, enzyme-linked immunosorbent assay, and Annexin V/propidium iodide assay, which are commonly used in toxicological studies. Each of these assays and their sources of potential variability have been recently described in detail.[27] In general, parameters such as interference of test substance and cell culture reagents (especially serum), timing, and washing steps are common among these assays, but there are other parameters that are specific to individual assays and should be considered for reliability.

BRANCH 6: TEST SUBSTANCE

For every study, sufficient characterization of the test substance is required. Only with sufficient knowledge about the substances' properties (e.g., form or presence of impurities)

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can correlations be made to the observed effects. Uncertainties regarding the test substance can stem from variability among batches or the preparation of dispersions for particles. Sample preparation or aging while in storage can also cause changes affecting the test substance's properties and toxicity.

While sources of variability for chemicals are well-known and ENMs have been studied in recent years, there are two new types of particulate material that are the focus of increasing research efforts: advanced materials and microplastics. Because both are novel and complex materials to test, they have not been included in the C&E diagram for this branch (Figure 8), but the following discussion focuses on expected key sources of variability for testing of these materials that can help guide future testing.

Sources of Variability Related to Chemicals. For chemical test substances, and any formulation derived from them, sampling, transport, and storage have the greatest influence on statistical and systematic errors.[64,65] The substance's physical state can affect the variability during its sampling (e.g., weighing for solid materials, pipetting for liquids, or aspiration for gaseous samples). For example, solid substances that are challenging to weigh (e.g., oily solids) or liquids that are viscous will have higher variability than other substances. The physicochemical properties (e.g., dissolution, homogeneity) of the chemical have to be considered for sample preparation (e.g., by digestion, separation, purification, or enrichment/dilution), because they can also impact the exposure variability.

Furthermore, key physicochemical parameters that could influence the variability when testing chemical test formulations are stability/reactivity, volatility, and pH. The solution stability of the chemical test formulation should be ensured for at least 24 h to avoid interferences from degradation products and changes to the actual applied dose. Likewise, loss of the test substance due to its volatility, or from its enrichment in the administered forumulation due to other volatile components (e.g., solvents), should be considered and avoided to ensure a stable concentration. In this context, the initial pH value should be monitored and kept constant during the application phase.

Sources of Variability Related to Engineered Nanomaterials. "Nanomaterials" are defined by the International Organization for Standardization (ISO) as "materials with any external dimension in the nanoscale [i.e., 1−100 nm] or having internal structure or surface structure in the nanoscale".[66] Because ENMs are frequently not tested in their as-received form, the process used to modify them prior to testing can add variability. If they are tested after suspension in a liquid, the dispersion process can introduce variability, such as what fraction is suspended and if the particles are changed during the process.[26] Furthermore, the quantity of energy applied during sonication can potentially change the agglomeration state of the ENM and modify the surface coatings and chemistry.[67,68] As the ENM remains in suspension, changes can occur to the ENM physical properties, such as the size distribution due to agglomeration and/or particle dissolution, surface chemistry, and form (e.g., Ag particles turning to AgCl).[69−71] These changes can, in turn, impact the ENMs' chemical reactivity and likely also the toxicity.[70] Changes in the suspension caused by agglomeration and sedimentation can affect the performance of analytical methods (e.g., dynamic light scattering),[72] and the exposure process (e.g., nebulization or aerosolization). Therefore, the use and characterization of an appropriate dose metric, considering all the aforementioned possible changes, are required.[72,73] It is important to note that relevant characterization methods of the ENMs, such as mass and size, typically are highly dependent upon the type of ENM evaluated.[72,74−76] In addition to sources of variability for the ENM itself, the preparation, handling, batch-to-batch variability,[77] and storage of the formulations used for ENM dispersion can introduce additional variabilities similar to those for chemical formulation. Morever, some ENMs can cause interferences in some biological assays.[27] When available, standardized methods should be used.

Advanced Materials. There is no current consensus definition of "advanced materials", but this topic is under discussion by groups, such as the Organization for Economic Co-operation and Development (OECD) and ISO and may include ENMs in addition to other types of materials and technologies. One definition, which is used in legal contexts, describes these substances, which often consist of multiple materials, as follows: "Advanced materials are materials with technical properties created by the development of specialized processing and synthesis technologies, including ceramics, metals with high added value, electronic materials, composites, polymers, and biomaterials".[78] In addition, Kennedy et al.[79] defined "advanced materials" as "novel materials with unique or enhanced properties relative to conventional materials".

Nevertheless, the described C&E analysis should generally be applicable to these newly emerging materials yet may need adaptation as more is learned. One specific foreseeable challenge in testing advanced materials is that they often release complex mixtures of different types of substances. For example, 3D printers or printing pens may release volatiles, plastic particles, and potentially ENMs, if present in the printing filament. Thus, it may be challenging to have consistent doses of the components among experiments and possibly even replicates within a single experiment. A second potential challenge arises if different components of mixtures have unique properties (e.g., electromagnetic field)[80] when they are present together in a certain configuration, because preparing the samples for exposure could potentially alter this configuration.

Microplastics. "Microplastics" are generally defined as particles of any plastic material with one dimension <5 mm.[81−83] They are released either already at this size parameter or are formed as a result of the degradation of larger plastic materials (secondary microplastics).[81] A potential source of secondary microplastics with relevance for daily human exposure is represented by 3D printer and pen emissions.[84] Despite their almost ubiquitous occurrence in the environment, especially in water and soil but also in the atmosphere,[85] analysis is difficult and often requires a combination of techniques to assess the variability in sizes and materials in complex matrices.[81,83] Depending on their size, microplastics, especially any nanoparticulate fraction, can be inhaled and may reach deep in the lungs. Different toxicity modes of action have been proposed and may even overlap: (1) effects based on the particulate property itself, (2) effects due to any adsorbed substance on the microplastic particle (carrier effect), and (3) effects based on leaching of potential toxic substances from the microplastic particle's matrix.[82,86] Many of the aforementioned sources of variability for ENMs may also be applicable to microplastics, such as the handling of the test substance, colloidal stability, and purity. For instance, any leaching or carrier effect is likely to be affected by the

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Table 1. Exposure System Control Measurements

	Control measurement	Branches on C&E diagram (Figure 2)	Steps in flow chart (Figure 1)	How it works	What information it provides
Exposure system cellular controls	Exposure system negative control	1, 2, 3, 5	a, d, e	Expose the biological test system in the exposure system to air (no exposure to test substances) and then perform the biological assay	Reveals information about performance of the biological assay, exposure system, and the biological test system; shows whether toxicity could be caused from the exposure system (e.g., residues from previous experiments)
	Exposure system negative chemical control	1, 2, 3, 4, 5	a, d, e	Expose the biological test system in the exposure system to a negative chemical control and then perform the biological assay	Provides information similar to the exposure system negative control and also reveals if the production of the aerosol would cause toxicity
	Exposure system positive chemical control	1, 2, 3, 4, 5	a, d, e	Expose the biological test system in the exposure system to a positive chemical control and then perform the biological assay	Provides information similar to the incubator positive chemical control (Table 2) and also reveals how consistently the exposure system is functioning
Exposure system technical controls	Flow rate	2	c, d	Use a sensor to measure the flow rate for flow-through exposure systems	Ensures that the gas flow rate is consistent across experiments and potentially also among different exposure chambers
	Temperature	1,2	c, d	Use a sensor to measure temperature through the exposure system	Checks that a constant temperature is maintained across experiments since temperature can impact cell viability
	Humidity measurements	2	c, d	Use a humidity sensor to evaluate the relative humidity of the gas for flow-through exposure systems	Evaluates the relative humidity of the air prior to passing over the cells since the air humidity can impact cell viability
	In-line characterization instrument(s) performance	2	c	Perform measurements to assess if the in-line characterization instrument(s) (e.g., QCM or CPC-SMPS) are functioning within specifications to measure the concentration before and during the exposure period	Evaluates the in-line characterization instruments used to monitor whether the test substance concentration is working properly prior to exposing the biological test system
	Homogeneity of deposition	2	c	Perform measurements of the deposition distribution on a substrate (e.g., electron microscopy grids)	Evaluates the homogeneity of test substance deposition within one well and among different wells

Table 2. Biological Control Measurements

	Control measurement	Branches on C&E diagram (Figure 2)	Steps in flow chart (Figure 1)	How it works	What information it provides
Biological controls	Incubator negative control	1, 3, 5	a, e	Maintain biological test system in the incubator without chemical exposure and perform the biological assay	Reveals information about performance of the biological assay and the biological test system
	Incubator positive chemical control	1, 3, 4, 5	a, e	Maintain biological test system in the incubator, expose it to a positive chemical control, and perform the biological assay	Reveals information about the biological test system preparation and if the biological assay is working as expected with a consistent sensitivity
	Microscopic analysis of biological test system (no exposure)	1	a	Analyze the biological test system microscopically prior to any exposure	Evaluates the condition of the biological test system (e.g., number of cells, type of cells, and 3D architecture) before any exposure occurs
	Microscopic analysis of biological test system (after exposure to positive chemical control or test chemical)	1, 2	a, d	Analyze the biological test system microscopically after exposure to a positive chemical control or test chemical	Shows whether exposure to the positive control or test chemical causes a heterogenous toxicological effect on the biological test system (e.g., decreased viability in the middle of the well but not on the periphery)
	Quantification of test substance concentration on biological test system	2	c	Quantify the concentration of test substance deposited on the biological test system	Provides quantitative information about the deposition rate of the test substance and the exposure concentration for the biological test system
	Transepithelial electrical resistance (TEER)	1	a	Measure the electrical resistance of the cell barrier	Provides information about the tight junctions between the cells and whether the cell barrier is compromised

surface of the microplastic particle. When assessing the mechanism(s) for toxicity, control experiments may be needed to determine which component of the microplastic is the cause of toxic effects observed.[87] Another challenge, which may also

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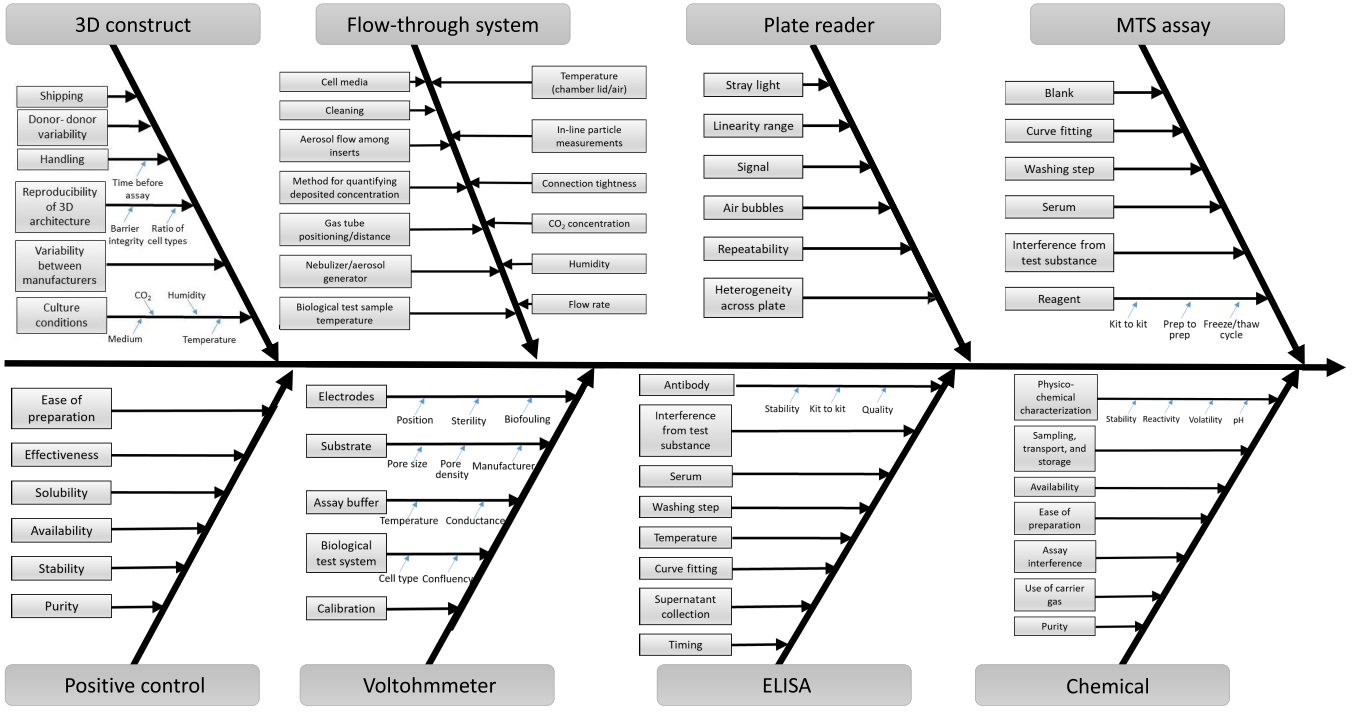

Figure 9. Example of a complete C&E diagram for a hypothetical study that includes exposing a chemical to a 3D tissue model at ALI using a flow-through system and assessing biomarkers such as barrier integrity (using a voltohmmeter), cytotoxicity (MTS assay), and inflammatory response (using ELISA and a plate reader) relative to a positive control.

pose a problem for larger ENM agglomerates, is that there may be an upper size limit for analytical instruments to quantify the aerosol size distribution as well as for transport through the exposure system itself. The size distribution of the microplastic fraction that reaches the biological test system may vary among exposure systems and may differ from that of the initial material.

POTENTIAL IN-PROCESS CONTROL MEASUREMENTS

In addition to the exposure system and incubator positive control experiment described in Branch 4, the C&E analyses revealed additional potential in-process control measurements (Tables 1 and 2). These measurements help to ensure that the assay system is working as expected by providing quantitative information about the assay performance and are used to develop specifications for the allowable ranges for key assay parameters. For some of the in-process control measurements, an unexpected result (e.g., the TEER measurements falling outside of the specification range) can cause the experiment to be stopped, while for others, such as the gas flow rates, it may be possible to make changes prior to the biological test system exposure to allow for the experiment to continue. The in-process control measurements listed in Tables 1 and 2 cover all five steps of the generic assay protocol (Figure 1) and also all branches of the generic C&E diagram (Figure 2). Which in-process controls are needed to ensure assay reliability will depend upon the specific protocol. While some of these in-process control measurements are described in the literature, such as the incubator negative control, other in-process control measurements are less consistently used (or reported), such as the exposure system and incubator positive controls. Thus, one recommendation for scientists developing methods in this area is to carefully consider the best way to incorporate the positive control measurements, because they are critical for ensuring inter- and intralaboratory comparability.

EXAMPLES OF USING C&E DIAGRAMS

C&E analysis can be added to any study and can provide a quick visual approach for identifying parameters that may be sources of variability. As a hypothetical example, Figure 9 provides a C&E diagram for a study that includes exposing a chemical to a 3D tissue model using a flow-through system and assessing biomarkers, such as barrier integrity, cytotoxicity, and inflammatory response. While the specific results will vary depending upon the protocol, it is critical to evaluate the impact of different parameters on the results for the in-process control measurements and carefully report these results and the parameters used when exposing cells to test substances. Moreover, it is also helpful to include troubleshooting guidance learned during assay development, when possible.

As an example of how a C&E diagram can help guide robustness testing, a recent study applied this approach in the design and evaluation of a cytotoxicity (tetrazolium salt-based WST-1) assay using a monoculture system (A549 cells) and a flow-through ALI exposure system (VITROCELL 6/4).[88] After conducting a C&E analysis, the impact of five parameters (exposure duration, humidity, flow rate, the addition of CO_2, and temperature) from branch 2 on the exposure system negative control cells was evaluated. All of these parameters were found to potentially impact the cell viability results, and, for this particular example, several key problematic issues and potential solutions are provided in Table 3. While the initial conditions in this exposure system yielded cell viability of approximately 40%, results for the optimized system were increased to nearly 90% using a flow rate of 5 mL/min and were not statistically different from the incubator negative control.

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Table 3. Examples of Troubleshooting Topics for the Cytotoxicity Method Described in Leibrock et al.[88] and Potential Solutions

Issue	Branch 2: Exposure system (Parameter(s))	Potential solutions
Decreased cell viability in the flow-through exposure system negative control cells	• Temperature (chamber lid/air) • Flow rate • CO_2 concentration • Relative humidity	• Increase relative humidity and/or air flow temperature • Decrease flow rate • Supply gas flow with 5 % CO_2
Heterogeneity in cell viability in the exposure system control in a flow-through exposure system	• Uneven aerosol flow among inserts • Trumpet positioning/distance • Toxic residuals from cleaning solution • Uneven access of cells to cell culture media • Connection tightness • Variable cell density	• Check whether the exposure chamber is leveled to ensure proper medium supply to all inserts • Check whether the chamber is fully closed • Verify aerosol inlet height in each slot • Revise cleaning procedure to ensure no residue maintains in the slot • Check homogeneity of the cell monolayer (Branch 1, culture conditions) • Verify cell confluence via microscope and use same growth protocols for all experiments
Low deposition of the test substance on the exposed cells in the flow-through exposure system	• Flow rate • Trumpet positioning/distance • Method for quantifying deposited concentration	• Increase concentration of the test substance • Increase flow rate (without loss in cell viability) • Verify aerosol inlet height in each slot • Extend exposure time (without loss in cell viability) • Check method for quantifying deposited concentration (sensitivity, limit of detection, limit of quantification)
Heterogeneous deposition in the flow-through exposure system among replicates	• Aerosol flow among inserts • Trumpet positioning/distance • Connection tightness	• Set vacuum pump to equal flow rates for all experiments • Verify if exposure chamber is fully closed → connect all gas exit tubes together → control flow rates → utilized flow rate should be equal to the sum of the three insert flow rates

STATISTICAL CONSIDERATIONS FOR EXPERIMENTAL PLANNING AND INTERPRETATION

Given the broad range of *in vitro* inhalation toxicity assays and purposes for which these assays are performed, it is not possible to provide definitive guidance on the number of technical replicates (measurements made within a single experiment) and repetitions (performing the same experiment multiple times such as on different days) for the experiments; additionally, it may be important to perform robustness testing to evaluate a similar test sample with cells from different donors for primary cells or with different cell passage numbers. In general, repeated testing is critical to understand day-to-day variability among experiments. The number of repetitions needed depends upon the precision of the assay results, how close the results are to relevant thresholds (e.g., the results from a statistical test to the selected α value), and the overall goal of the experiment. For terms such as the percentage depletion that are calculated using several variables, it is important that the uncertainties in each variable (e.g., incubator negative control, blank control [no cells or chemicals added] and cells exposed to the test chemicals) are incorporated to understand the cumulative uncertainty using a propagation of error frequentist approach or Bayesian analysis.[24,88]

PUTTING IT INTO PRACTICE: IMPLEMENTATION OF *IN VITRO* INHALATION METHODS

In order for regulatory agencies to adopt methods as part of their hazard and risk assessment process, they need to be applied in a way that will allow for both scientific reliability and quality to be assured. This can be achieved through the use of C&E analysis in conjunction with Good Laboratory Practices (GLP). Furthermore, the application of the findability, accessibility, interoperability, and reusability (FAIR) principles[89] can help enable wider use of generated data.

After identification of potential sources of variability through C&E analysis, key in-process control measurements and their

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specifications should be incorporated into a test method to ensure consistent assay performance. Through GLP, these in-process control measurements and specifications are monitored and recorded to support the reliable performance of the assay. GLP help with planning, performing, monitoring, recording, archiving, and reporting of data in studies intended for regulatory purposes and can also assist in tracking problems with the methods that may arise. For instance, if a test method suddenly stops working properly with some in-process control measurements falling outside of the specifications, proper documentation could allow for identifying the problem source. Incorporation of C&E analysis, GLP, and FAIR principles will ensure that protocols are well documented, robust methods are used, and the generated data are accessible for use by machines to support broader usage of the data.

CONCLUSION

This manuscript describes how C&E analysis can help minimize the variability of ALI assays to assess the toxicity of inhaled materials *in vitro*. Although ALI-based assays can vary substantially depending on the study requirements, there are some components that are shared among assays. In this manuscript, parameters are identified that are generally applicable to *in vitro* inhalation assays and can lead to variability in the overall outcome of the study. C&E analysis provides a means to thoroughly document potential sources of experimental variability in a visual way that can be easily shared and understood. Together with the usage of GLP and future standardization, C&E analysis of ALI-based assays could facilitate regulatory testing. In-process control measurements have been described that cover each branch of the C&E diagram and that can help identify sources of assay variability and maximize overall robustness of *in vitro* inhalation toxicity studies.

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Notes

Certain commercial products or equipment are described in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or by the Consumer Product Safety Commission, nor does it imply that it is necessarily the best available for the purpose. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute of Standards and Technology. These findings and conclusions similarly do not necessarily represent the official position of the Consumer Product Safety Commission. This work has not been reviewed or approved by and does not necessarily represent the views of, the Commission.

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Amy J. Clippinger: Dr. Amy Clippinger is the president of PETA Science Consortium International. She received her doctorate in Cellular and Molecular Biology and completed a postdoctoral fellowship in the Cancer Biology Department at the University of Pennsylvania. In 2012, Dr. Clippinger joined the Science Consortium where she collaborates with industry, academia, and regulatory

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John Gordon: Dr. Gordon is currently a toxicologist at United States Consumer Product Safety Commission (CPSC) and performs risk assessments relating to exposure from consumer products. He received his doctorate in Biochemical Genetics from West Virginia University (WVU). He then did post-doctoral work in multidrug resistance in breast cancer and leukemia at both the MUSC Hollings Cancer Center in Charleston, SC and IUPUI Cancer Center in Indianapolis, IN. He then worked in industry for 12 years developing commercial bioassays. He then worked for the United States Department of Defense with the Medical Countermeasures Group at Fort Detrick before joining CPSC. He is the CPSC representative on Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and is co-chair of ICCVAM's Validation Work Group.

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Lars Leibrock: Lars Leibrock holds a master's degree in Biomedical Sciences from the University of Applied Sciences Albstadt-Sigmaringen. After completing his masters, he worked at the German Federal Institute for Risk Assessment (BfR) as a Ph.D. student. At BfR, his research focused on nanotoxicology and *in vitro* inhalation toxicity studies. In 2020, he joined the Department of Hand Surgery, Plastic Surgery, and Aesthetic Surgery of Prof. Giunta at Ludwig-Maximilians-University (LMU) in Munich as a research assistant. At LMU, Lars investigates new therapeutic applications in bone and cartilage regeneration based on adipose-derived stem cell approaches.

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Frank Bierkandt: Dr. Bierkandt is a scientific researcher at the German Federal Institute for Risk Assessment since 2017. Currently he is working in the Product Research and Nanotechnology unit. As an analytical chemist, his tasks comprise the characterization and quantification of nanomaterials and aerosols in consumer-related products as well as the application and development of spICP-MS, LA-ICP-MS, and ALI techniques. From 2016 to 2017, Dr. Bierkandt worked as a medical representative for Sanofi Genzyme at the +49 GmbH. He studied chemistry at the Humboldt University of Berlin and worked on LA-ICP-MS quantification methods at the Federal Institute for Materials Research and Testing.

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