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Workshop Series to Identify, Discuss, and Develop Recommendations for the Optimal Generation and Use of *In Vitro* Assay Data for Tobacco Product Evaluation: Phase 1 Genotoxicity Assays

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Abstract

Introduction: The Institute for In Vitro Sciences is sponsoring a workshop series to identify, discuss, and develop recommendations for optimal scientific and technical approaches for conducting *in vitro* assays—focusing first on genotoxicity assays—used for assessing potential toxicity within and across tobacco and electronic nicotine delivery product categories (hereafter referred to as tobacco products).
Materials and Methods: Workshops provide a unique opportunity for invited expert stakeholders to share experiences and to develop recommendations that may serve as a resource for developing optimal testing approaches and data interpretation. It is envisioned that some recommendations would form the basis for the generation of guidance documents and/or serve as authoritative reference publications to support regulatory submissions.
Results and Discussion: During the first workshop (November 27–28, 2018), workgroup members identified important issues for using *in vitro* genotoxicity assays for evaluating tobacco products. These issues were triaged into three priority categories that will provide the basis for selecting high-priority topics for subsequent workshops. To provide background for future workshops and to serve as a scientific community resource, the workgroup developed a tabulated referenced summary of the types of tobacco product test samples that have been evaluated using the regulatory genotoxicity assays and the types of regulatory questions that have been addressed. A touch-base meeting was held March 7, 2019, and a second workshop June 4–5, 2019 to discuss ongoing issues and to further organize workgroup activities.
Conclusion: The current publication lists priority topics and background summary information for using regulatory genotoxicity assays to evaluate tobacco products.

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Introduction

GENETIC TOXICOLOGY TESTS have been widely used to assess potential hazard in the development of a variety of products (e.g., new chemicals, pesticides, pharmaceuticals, food contact substances, and food additives). The Organization for Economic Cooperation and Development (OECD) first developed and adopted test guidelines (TGs) for genetic toxicology tests in 1983. Since that time, new TGs have been added and older TGs have been revised or deleted (for example, TG479 for the *in vitro* sister chromatid exchange assay was deleted in 2014). Recently, all the OECD genetic toxicology TGs were considered for revision and most were revised. It should be noted that the Ames test guideline (TG471) has not been revised, and thus, the 1997 version of the TG is still applicable. However, a new OECD project initiative for updating TG471 is currently being discussed.

Regulatory requirements for genetic toxicology evaluations, including recommended test batteries related to consumer products, have been developed and used for many years.[1–6] The primary focus of genetic toxicology testing has generally been on hazard identification and the health outcomes of concern related to these tests, including cancer and heritable germ cell mutation.

Genetic toxicology assays for tobacco products

Historically, tobacco products have not been covered by the same regulatory frameworks as other consumer products. However, during the same timeframe (starting in the 1970s), in which regulatory recommendations were developed and genetic toxicology tests were being applied to a broad spectrum of products, tobacco companies and other organizations were also using these genetic toxicology tests to assess potential and relative hazards from tobacco products. The questions addressed, however, were somewhat different in scope as the products themselves (particularly combusted cigarettes) were recognized as genotoxic (and carcinogenic). Tobacco product stewardship has focused on (1) evaluating the impact of additives on overall product genotoxic hazard, (2) comparing the genotoxic potency of various combusted cigarette components and designs, (3) comparing the genotoxic potency of different categories of tobacco products (e.g., combusted cigarettes versus heat-not-burn products [tobacco-heating products, THPs]), and (4) comparing results from various types of product sample preparations (e.g., condensates, aqueous solutions, extracts, and aerosols). More recently, electronic nicotine delivery systems (ENDS), including e-cigarettes and e-liquids, were tested using these assays to understand their potential genotoxicity.

In 2004, the *In Vitro* Toxicity Testing Sub Group (IVTSG) of the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), an association founded to promote international tobacco research, evaluated potential options for an *in vitro* genetic toxicology battery for tobacco products and published its recommendations.[7] The CORESTA *in vitro* battery consists of a bacterial mutation test (the Ames test) and an *in vitro* mammalian cell test (either the chromosome aberration assay, the mouse lymphoma gene mutation assay using the thymidine kinase locus, or the micronucleus assay). This *in vitro* battery is consistent with the recommended *in vitro* regulatory batteries for consumer products in many countries. Specifically, this is the same *in vitro* battery that is recommended by the International Committee for Harmonization (ICH) for pharmaceuticals intended for human use.[6,8] Over and above the genetic toxicology tests, CORESTA also recommends an *in vitro* test for general cytotoxicity, the neutral red uptake (NRU) assay.

Current regulatory landscape for tobacco products

Unlike the consumer products mentioned above, regulations recommending or requiring toxicological testing for tobacco products have only recently been established, and only in some jurisdictions. In 2005, Canadian Tobacco Reporting Regulations began to require annual *in vitro* toxicity testing (Ames test, NRU assay, and the *in vitro* micronucleus assay) on cigarette emissions for mainstream tobacco smoke. These Canadian Guidelines have been recently updated.[9–11]

In 2009, the United States Food and Drug Administration (FDA) gained regulatory authority over tobacco products with the passage of the Family Smoking Prevention and Tobacco Control Act (FSPTCA), an amendment to the Federal Food, Drug, and Cosmetic Act.[12] Subsequently, the FDA has issued guidance for regulatory pathways to market for tobacco products that include substantial equivalence to a similar product already on the market, or for a new product, draft guidance for a Premarket Tobacco Product Application (PMTA).[13,14] ENDS were deemed as ''tobacco products'' in 2016[15] and therefore also subject to FDA regulation; a draft guidance document for Premarket Applications for ENDS was issued in 2016 and finalized in 2019.[16] FDA also issued a draft guidance document outlining recommendations for submitting a Modified Risk Tobacco Product Application (MRTPA) for claims of modified health risk or reduced exposure to harmful and potentially harmful constituents in tobacco products and tobacco smoke.[8] These guidance documents include endorsement of toxicological testing, and some of the guidance documents specifically list genetic toxicology tests as a part of the recommendations. The FDA Center for Tobacco Products (CTP) website includes links to the latest versions of all their guidance documents (https://www.fda.gov/tobacco-products/rules-regulations-and-guidance/guidance).

Within the European Union, tobacco companies have provided genotoxicity test data as part of a submission package under the Tobacco Product Directive, with more recent examples, including the data package provided as part of the assessment for priority additives per the requirements in the EU Tobacco Products Directive (2014/40/EU).[17,18]

Workshop Rationale

Clearly, there is an increasing emphasis on the use of genetic toxicology tests for evaluating the toxicity of tobacco products (including combusted cigarettes, ENDS, THPs, smokeless, and cigars). While there is a need to evaluate the genotoxicity of single chemicals that are tobacco product

constituents or additives, the fact that the tobacco product test material is generally a complex mixture, adds significant complexity to the situation. For many tobacco products, the hazard assessment requires an understanding of the genotoxic potential of smoke/aerosols, rather than (or in addition to) the more commonly studied solids or liquids. Thus, the application of genetic toxicology tests to tobacco products involves several technical challenges that are not encountered when evaluating single chemicals. For instance, the route of exposure for combusted cigarettes, THPs, and ENDS is inhalation, and the exposure dosimetry of such complex mixtures is complicated. The fact that the products are combusted before use, or heated (but not combusted) adds complexity to the generation of appropriate test samples, the evaluation of exposure, and the interpretation of the biological responses. In addition to the inhaled products, there are products that are orally consumed (e.g., smokeless tobacco products). This requires the development of approaches to compare products across these diverse exposure categories. Issues that become important in making these comparisons include the generation of appropriate product-category-specific test samples, evaluation of exposure, and relevant methods to express exposure so that meaningful product category hazard comparisons can be developed.

As already indicated, tobacco companies and other organizations have historically generated a substantial amount of *in vitro* (and some *in vivo*) genetic toxicology research. While there are many published studies, including summaries/reviews of the literature,[19–25] much of the research data and technical expertise required to evaluate tobacco products resides within the individual tobacco companies and/or within contract testing laboratories. It is important to recognize that while much of these specific data are proprietary, as with other types of consumer products, it is possible to benefit from the lessons learned by sharing general expertise and nonproprietary information. With the new international regulatory requirements for tobacco product toxicological assessments (see above), there is a need to develop consensus recommendations for the conduct of *in vitro* genetic toxicology assays and the interpretation of data that are based on this wealth of information and expertise of the various stakeholders.

Similar stakeholder discussions for other types of chemicals/consumer products have resulted in many of the recommendations that are currently used for genetic toxicology regulatory testing. The International Workshop for Genotoxicity Testing (IWGT) meeting series over the past two decades is an excellent example of stakeholder discussions that resulted in recommendations now included in OECD TGs for genetic toxicology assessments.[26–30] Based on the general IWGT format and experience, The Institute for In Vitro Sciences (IIVS), a nonprofit organization focused on developing and promoting the use of *in vitro* assays, is organizing a series of workshops specifically focused on developing recommendations for the technical aspects of conducting *in vitro* studies, focusing first on genetic toxicology research for the regulatory evaluation of tobacco products.

Workshop Series Overview

Before this current series of workshops whose initial focus is regulatory *in vitro* genetic toxicology assays, IIVS held and published the results from workshops that addressed *in vitro* models for chronic obstructive pulmonary disease and *in vitro* exposure systems and dosimetry assessment.[31,32] These two workshops highlighted the need for further in-depth discussions of the allocation of traditional *in vitro* genetic toxicology assays to the complex problems associated with testing complex materials such as tobacco and other nicotine containing aerosols. Therefore, the objective of the current IIVS workshop series is to provide a forum for stakeholders to identify, discuss, and develop recommendations for the optimal generation of test samples and use of *in vitro* assays to support product regulatory requirements. This series will capitalize on the extensive historical experience with the ''standard'' regulatory *in vitro* genetic toxicology assays and will evaluate the technical details involved in generating samples, defining appropriate exposure methods, obtaining experimental results, and interpreting the results. These workshops can be distinguished from (but coordinated with) other complementary efforts such as those of CORESTA's IVTSG. For example, CORESTA has conducted a series of proficiency trials for the regulatory *in vitro* genetic toxicology assays (https://www.coresta.org/groups/vitro-toxicity-testing) and information from these trials will be considered in the IIVS workshop deliberations.

Invited experts for the IIVS workshops include scientists from tobacco companies, contract research organizations, US regulatory agencies, and other *in vitro* assay experts with tobacco product experience. The format for this workshop series is primarily discussion, which provides an environment to tackle issues in detail. Participants are expected to actively participate by collecting relevant published and unpublished nonproprietary research information to offer experiences and expert opinions and to actively share with the workgroup members. While the focus will be on the widely used regulatory *in vitro* genetic toxicology and cytotoxicity assays, it is important to note that much of the discussion will be applicable to all *in vitro* assays. As a part of the workshop discussion, data gaps will be identified and included in the publications. Thus, in addition to recommendations based on current information, this workshop series will provide key research questions that need to be addressed by the scientific community. This will provide a useful roadmap for research that can have a direct impact on the regulation of tobacco products and on protecting human health related to consumer use of tobacco products. The product of these workshops will be a series of scientific publications that can be used by all stakeholders.

Workshop 1

The first workshop in this series was held at the IIVS facility in Gaithersburg, Maryland on November 27–28, 2018. The following were the goals of the first workshop:

1. Identify key issues to be addressed by the workshop series
2. Prioritize key issues for discussion in subsequent workshops into three areas:
   - Extensive information available; recommendations can be readily developed (Category 1)
   - Additional short-term (1–2 years) information/research required (Category 2)

Table 1. Initial List of Items to Be Discussed—Grouped by General Topics

Assays
- In addition to the genetic toxicology assays, is it within the workshop scope to include cytotoxicity assays in the discussion (i.e., NRU or other potential options)?
- Endorse the recommended *in vitro* battery of genetic toxicology assays (Ames test and MLA or *in vitro* MN or *in vitro* chromosome aberration assay)?
- Promising new *in vitro* assays for assessing genetic damage and recommendations for ''validating/qualifying'' them for routine use.

Sample preparation
- Recommended methods to prepare specific types of samples (i.e., condensates from combusted cigarettes, ENDS and THPs, aerosols, or smoke from combusted cigarettes, ENDS and THPs, and extracts from smokeless products).
- Recommendations for appropriate solvents used with specific sample types.
- Recommendation for ''puffing/vaping'' regime for generation of test material (i.e., condensates, aerosols, and smoke).
- Recommendations for test article characterization for GLP.
- Recommendations for how to assess stability and its impact on biology of the samples prepared for *in vitro* assays (recommendations on ''use by'' date/shelf life).

Assay conduct
- Recommended Ames test strains?
- Any recommendations for cell lines for the *in vitro* MN assay?
- Recommendations for top concentration when sample is not sufficiently cytotoxic (and the top concentration is limited by amount of solvent).
- Recommended methods to expose cells to aerosols, and smoke (from combusted cigarettes ENDS and THPs). This includes generation and handling of the aerosol, exposure and recovery of cells, exposure duration, appropriate cytotoxicity, and concentration range, dosimetry.
- Recommended experimental design providing appropriate data for quantitative comparisons (i.e., number of replicate cultures, concentration spacing, number of independent experiments, and so on).

Data comparisons and potency
- Recommended methods to express exposure, particularly when comparing different types of products (i.e., combusted cigarettes vs. smokeless vs. ENDS vs. THPs).
- Recommended methods to make quantitative comparisons for *in vitro* responses (i.e., potency, graphical, BMD, or another metric).
- What are the specific issues (and potential solutions) associated with evaluating and comparing relative potency of complex mixtures? What is the minimum amount of genotoxicity/toxicity that can be detected in a mixture?

Testing strategy
- Recommendations for follow-up tests when deconvoluting positive results—potentially generating a decision tree.
- Recommendations for evaluating large numbers of products (particularly ENDS) varying only in flavoring compounds.

General/Miscellaneous issues
- Extrapolation of *in vitro* results to *in vivo*.
- Use of bridging biomarkers from *in vitro* to human.
- Recommendations for validation of software for GLP.
- Recommendations on how to collect and present *in vitro* data for regulatory submission.

BMD, benchmark dose; ENDS, electronic nicotine delivery systems; GLP, good laboratory practice; NRU, neutral red uptake; THPs, tobacco-heating products.

   - Additional long-term (>2 years) information/research required (Category 3)
3. Develop corresponding strategies, timelines, and specific objectives to obtain information/research

Before the first workshop, the participants identified issues that are important to the use of genetic toxicology (and other *in vitro*) assays for evaluating tobacco products (Table 1). During the workshop, each issue was discussed and those issues that the workgroup plans to deal with were triaged into the three priority categories (described above). The focus of the discussion was intentionally macroscopic; the goal was to identify the questions and not try to provide answers to the questions. It was noted that any decisions made during the first workshop would be ''non-binding'' and that as we move forward, new information might change the strategy and the priorities. The discussion was managed by taking frequent opinion polls (voting by raising hands) with the goal of reaching workgroup consensus. Majority opinion was used as the driver for the direction of the group and the priorities. Confidentiality of the discussions was also addressed. While it was agreed that no participant would share confidential information with other members of the workgroup, it was also agreed that public dissemination of information from the meetings would be in the form of articles developed, authored, and reviewed by those workgroup members choosing to participate as coauthors and/or in scientific meeting presentations.

It was agreed that the emphasis for the workshop discussion would be the regulatory use of data, but that there might be other uses or applications for the data. For example, genetic toxicology information can be used not only for hazard identification but also to address questions of risk. It was also agreed that while there are *in vivo* genetic toxicology tests that can be used (and have been applied) to answer tobacco product questions, the focus of this workshop series would be on the *in vitro* assays. It was decided that future discussions of the priority topics would include the consideration of (1) validation of methods for their intended use, (2) fit-for-purpose (ability of the approach to address the regulatory question), (3) application of quality standards, and (4) the limitations of any methods. While the general focus will be on methods that can be used under good laboratory practice (GLP) and/or International Organization for Standardization (ISO) regulations or guidelines, there should be a consideration of ''non-GLP'' methods (but which follow good research and documentation practices). In considering the use of software under GLP, there is a need to include recommendations for having a quality standard for supporting data collection. As the individual assays are discussed, there is a need to have quality standards for software, which may be applied to each specific assay. There was also a suggestion that computational toxicology methods should be considered, particularly as a part of any new test systems (beyond the standard test battery). Computational methods are already in common use to assist in predicting genotoxicity outcomes for single chemicals or well-characterized mixtures of chemicals and their potential for use with more complex tobacco-related materials could be explored.

The workgroup decided that a survey of the types of tobacco products and test materials that have been evaluated in the regulatory *in vitro* genetic toxicology battery, with representative literature citations, would be useful background

TABLE 2. A SUMMARY OF THE TYPES OF TOBACCO PRODUCTS AND THE REGULATORY GENETIC TOXICOLOGY ASSAYS THAT HAVE BEEN UTILIZED TO EVALUATE THE VARIOUS PRODUCTS (WITH EXAMPLE REFERENCES)

	Products			
Assays	*Combusted cigarettes*	*ENDS (including e-cigarettes)*	*THPs (heat-not-burn)*	*Smokeless*
	Selected references			
Ames test	17,33–47	41,42,45–48	37,43,44,49–51	41,52–57
IVMN	17,38,40–42,45–47,58,59	41,45–47,60	42,60	41,56,57,61
IVCA	37,38,59,62		37,49	54,61,63
MLA	42,43,50,64–66	67	43,50,51,64,67	56

IVCA, *in vitro* mammalian cell chromosome aberration assay; IVMN, *in vitro* mammalian cell micronucleus assay; MLA, mouse lymphoma gene mutation assay using the thymidine kinase gene.

for the group's activities. This information is summarized in Tables 2 and 3. In addition, to provide a summary of the types of tobacco product research questions that have been addressed using the regulatory *in vitro* genetic toxicology assays, the workgroup developed a summary table highlighting a few of the many published studies (Table 4). This table includes a brief summary of the goals and results of the studies (as reported by the author of the publication). In addition to providing some insight into the broad scope of the reported studies, these tables also provide an overview of the organizations (government, academic institutions, contract testing organizations, and tobacco companies) that have conducted and contributed to the literature for tobacco product research.

Recommendations from Workshop 1

As they were readily addressed, workgroup consensus was reached for the first two issues listed in Table 1 during the first workshop. With regard to the first issue, it was noted that CORESTA has a three-endpoint/assay battery, which includes the NRU assay for cytotoxicity. While the initial focus of the workshops is on genetic toxicology, it was agreed that *in vitro* cytotoxicity assays such as the NRU should be included in the workshop series discussions, in part, based on recommendations by FDA CTP guidance documents (https://www.fda.gov/tobacco-products/rules-regulations-and-guidance/guidance), which include cytotoxicity as an endpoint.

The second issue listed in Table 1 is the endorsement of the current CORESTA- (and ICH-) recommended *in vitro* genetic toxicology battery and its use for tobacco product evaluation. The group noted that the chromosome aberration assay has decreased in use throughout the genetic toxicology community, while the use of the micronucleus assay has increased. This shift has occurred following the adoption of an OECD TG for the *in vitro* micronucleus assay, primarily because the micronucleus assay is technically much easier to conduct than the chromosome aberration assay, and, particularly when automated scoring methods are used, it can have

TABLE 3. A SUMMARY OF THE TYPES OF TOBACCO PRODUCT SAMPLES PREPARED AND SUCCESSFULLY EVALUATED IN THE STANDARD GENETIC TOXICOLOGY ASSAYS

	Inhalable tobacco products (e.g., combusted cigarettes, ENDS and THPs)			*Smokeless tobacco products*	*e-Liquids used in ENDS*
Sample type	*Pad collected material (particulates: TPM/CSC/NFDPM)*	*Gases captured in solvent*[a]	*Smoke/aerosol at air/liquid interface*[b]	*Extracts*	*e-Liquids*
	Selected references				
Ames test	35–43,45,49,51,68–70	46,47,71	36,43,44,48,70,71	41,52–57	41,54
IVMN monolayer cells	40–42,45,49,51,59,60,68,72	58,59	46,47,58	41,56	41,45,60
IVMN suspension cells	38,45			57	45
IVCA monolayer cells	37,38,59,62	59		54,63	
MLA	42,43,50,51,64,65,67–69	66,69		56	67

The *in vitro* cytogenetic assays are summarized to include either the use of monolayer cell lines or suspension cell lines. Example references are provided.

[a]Liquid trapped GVP or liquid trapped smoke or aerosol. Exposure can be to a stored sample or cells can be exposed to the smoke/aerosol which is freshly generated and bubbled through the exposure medium.

[b]Cells are directly exposed to freshly generated smoke/aerosol.

CSC, cigarette smoke condensate; GVP, gas-vapor phase; NFDPM, nicotine-free dry particulate matter; TPM, total particulate matter.

Table 4. Example Tobacco Product Research Questions That Have Been Addressed Using the *In Vitro* Standard Regulatory Genetic Toxicology Assays

Genotoxicity of tobacco product constituents or additives (tested as individual chemicals)		*References*
Ames test	Glycerol, an additive used in cigarette manufacture, was evaluated for mutagenicity in TA98, TA100, TA1535, TA1537, and TA1538 and found to be negative.	73
	N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were evaluated in the Ames test and found to be positive.	74
	N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were evaluated in the Ames test and found to be positive.	75
IVMN	Primary human lymphocytes were exposed to nicotine and evaluated by flow cytometry for the induction of micronuclei. An increase in the number of micronucleated cells was observed at concentrations that did not impact the frequency of apoptotic cells.	76,77
IVCA	Glycerol, an additive used in cigarette manufacture, was evaluated in the CHO chromosome aberration assay and found to be negative.	73
	N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were evaluated for the induction of chromosome aberrations in human peripheral blood lymphocyte cultures and found to be positive.	74
	Primary human lymphocytes, exposed to nicotine, and evaluated for the induction of chromosome aberrations. The results indicated that nicotine was clastogenic.	76
MLA	Five carcinogens found in cigarette smoke [4-aminobiphenyl (4-ABP), benzo[*a*]pyrene (BaP), cadmium (in the form of $CdCl_2$), 2-amino-3,4-dimethyl-3H-imidazo[4,5-f]quinoline (MeIQ), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)] were evaluated for mutagenic potency in the MLA with and without S9. $CdCl_2$ was the only chemical that was positive without S9. Positive dose-responses were observed for all chemicals with S9.	78
Does an individual additive or other modification impact genotoxic potency of the product?		*References*
Ames test	A cigarette with a novel carbon filter and low-nitrogen containing tobacco was compared to five other full-flavor low tar cigarettes (two prototypes and three commercially available). The low-nitrogen containing tobacco resulted in lower activity in TA98 with S9 when compared to CSC from the other test cigarettes.	36
	Test cigarettes containing 9.5%, 18.5% and 25% expanded shredded tobacco stems were compared with a control cigarette containing no shredded tobacco stems. Condensates were evaluated in TA98 and TA100 (both with S9). There were no differences in the mutagenic potency when the results were presented as either revertants per mg ''tar'' or as revertants per cigarette.	79
	Test cigarettes containing added diammonium phosphate and urea were compared with test cigarettes without the added ingredients. Condensates were evaluated in TA98 and TA100 (both with S9). There was no difference in the mutagenic potency of the test cigarettes that contained the additional ingredients. This was the case when the results were presented as revertants per mg of ''tar'' or revertants per cigarette.	80
	Test cigarettes containing 10 or 15% cast sheet tobacco were compared with cigarette made with standard tobacco. TA98 and TA100 (both with S9). When the number of revertants was expressed per mg of ''tar'' there was no difference in the mutagenic potency of the three test materials in either strain. When the results were expressed as revertants/cigarettes, there was a statistically significant difference (higher) in TA100 for the cigarette made with 15% cast sheet.	81
	THPs, with and without flavorings were evaluated and compared to 3R4F. Neostiks composed of reconstituted Virginia tobacco blend with glycerol (14.8%) with and without flavorings were evaluated. TPM was generated for all the products. TA98, TA1535, TA1537, TA100, and TA102 were used. While the TPM from 3R4F was mutagenic, none of the TPMs from the flavored or nonflavored Neostiks was mutagenic.	51
IVMN	Experimental cigarettes were designed to produce reduced levels of toxicants. Designs included the use of tobacco-substitute sheet containing glycerol, as well as the incorporation of blend-treated tobacco to reduce the levels of nitrogenous precursors and some polyphenols. Particulate matter from the experimental cigarettes was compared with particulate matter from reference combusted cigarettes using the *in vitro* MN assay and V79 cells. All of the samples were positive both with and without S9.	40
	THPs, with and without flavorings were evaluated (and compared to 3R4F). Neostiks composed of reconstituted Virginia tobacco blend with glycerol (14.8%) with and without flavorings were evaluated. TPM was generated for all the products. V79 cells were used for the MN assay and treatment times were 3 hours with and without S9 and 24 hours without S9. The TPM from 3R4F was positive, but TPM from the flavored or nonflavored Neostiks was negative.	51

(*continued*)

TABLE 4. (CONTINUED)

MLA	Experimental cigarettes were designed to produce reduced levels of toxicants. Designs included the use of tobacco-substitute sheet containing glycerol, as well as the incorporation of blend-treated tobacco to reduce the levels of nitrogenous precursors and some polyphenols. Particulate matter from the experimental cigarettes was compared with particulate matter from reference combusted cigarettes using the MLA. Dose-dependent responses were obtained for all samples; however, some were not sufficient to be positive. Overall, there was no consistent difference among the samples, when the test concentration was corrected for NFDPM content.	40
	THPs, with and without flavorings were evaluated (and compared to 3R4F). Neostiks composed of reconstituted Virginia tobacco blend with glycerol (14.8%) with and without flavorings were evaluated. TPM was generated for all the products. Treatment times were 3 hours with and without S9 and 24 hours without S9. The 3R4F TPM was mutagenic. The TPM from both the flavored and nonflavored Neostiks was not mutagenic.	51
Comparative genotoxic potency of products within an individual product class		*References*
Ames test	Condensates from experimental cigarettes made from Burley tobacco grown with 4 different amounts of nitrogen fertilizer and from bright-type tobacco, 15 commercial brands of cigarettes (7 American, 4 Japanese, 3 English, and 1 German) were compared for mutagenic potency using TA1538 with S9. The condensate from Burley tobacco was more mutagenic than the condensate from bright-type tobacco. There was a significant correlation between the nitrate content of the cigarette and the mutagenic potency.	34
	Reference cigarette 1R4F was compared with 73 marketed brands of combusted cigarettes to determine if 1R4F was a good ''representative'' model for US marketed combusted cigarettes. TA98 and TA100 were used with S9 and the authors concluded that 1R4F could be used as representative.	35
	Comparative mutagenicity evaluation was conducted for the condensates from 10 different cigarettes [2R4F, 6 commercial brands (full flavor to ultralight), 100% reconstituted tobacco experimental cigarette, 100% Burley experimental cigarette and 100% flue-cured experimental cigarette. All 10 condensates were mutagenic and the potencies varied 7-fold when the data were presented as revertants per μg of CSC; potencies varied 158-fold when expressed as revertants per mg nicotine. The potency rank order was the same in the two strains used (TA98 and YG1041, both with S9). The mutagenic potency did not correlate with the level of tar in the cigarettes.	38
	Comparison between reference 2RF4 and 4 commercial brands of combusted cigarettes with varying amounts of tar (1–10 mg). Whole smoke samples were used to directly expose the bacteria and many Ames strains (with and without S9) were compared. Strains used: TA1538, TA98, YG1021, YG1024, YG1041, TA1535, TA100, YG1026, YG1029, YG1042, TA1537, TA102, and *Escherichia coli* WP2*uvrA*-pKM101. Not all of the strains detected the mutagenicity and the mutagenicity was not directly related to the tar content of the cigarettes.	71
	Comparison among three types of commercially marketed brands: Virginia flue-cured full flavor, a mixture of tobacco types and marketed as ''blonde,'' a Virginia flue-cured marketed as light. The strains used (with S9) were TA98, YG1041 and YG5161. The mutagenic potency values were lowest in TA98 followed by YG5161 and then YG1041. The blonde cigarette was the most mutagenic; however, the mutagenic potency was not markedly different across the brands.	39
IVMN	Comparative clastogenicity was evaluated for the CSC from 10 different cigarettes [2R4F, 6 commercial brands (full flavor to ultralight), 100% reconstituted tobacco experimental cigarette, 100% Burley experimental cigarette and 100% flue-cured experimental cigarette]. L5178Y $TK^{+/-}$ 3.7.2C cells were exposed to condensates with S9. All 10 condensates induced positive MN responses with the responses varying by less than threefold when the data were expressed per μg condensate. It was noted that this range of responses was much less than the range observed with the other endpoints (Ames test and chromosome aberrations) evaluated.	38
IVCA	Comparative clastogenicity was evaluated for the condensates from 10 different cigarettes [2R4F, 6 commercial brands (full flavor to ultralight), 100% reconstituted tobacco experimental cigarette, 100% Burley experimental cigarette and100% flue-cured experimental cigarette]. CHO-K1 cells were exposed to condensates with S9. All but one of the condensates were positive and the range of responses observed was up to about fourfold when data were expressed per μg condensate.	38

(*continued*)

Table 4. (Continued)

MLA	Condensates from single tobacco type (bright, burley or oriental) cigarettes were compared. The mutagenic activity of the burley cigarette was ~40% of that of the bright and oriental cigarettes.	64
	Comparative mutagenicity was evaluated for 11 condensates from 10 different cigarettes [2R4F, 7 commercial brands (full flavor to ultralight), 100% reconstituted tobacco experimental cigarette, 100% Burley experimental cigarette and 100% flue-cured experimental cigarette]. Both the agar and microwell versions of the MLA (with S9) were used for the evaluation. All of the condensates were mutagenic. There was no relationship between the mutagenic potency and the tar yield or the nicotine concentration. The mutagenic potencies varied by about 4-fold when the data were expressed per μg condensate and 12 to 13-fold when the data were expressed per μg nicotine.	65
	Comparative mutagenicity was evaluated for six WSSs [20 or 60 commercial cigarettes of two different types smoked under two different smoking regimens (ISO and HCI)]. Five out of six WSSs treated with S9 were mutagenic, while only three WSSs were mutagenic without S9.	66
Comparative genotoxic potency of products across different product classes		*References*
Ames test	Comparison of combusted cigarettes, cigars and pipe tobacco with and without filters. TA98 and TA100 with and without S9. Multiple comparisons of mutagenic activity. The mutagenicity was not explained by the concentration of benzo(a)pyrene or nitroso compounds present in the test samples.	82
	Comparison between mainstream smoke condensates from combusted cigarettes and heat-not-burn test products using TA1535, TA1537, TA1538, TA98, and TA100 (with and without S9). The condensates from the combusteds were mutagenic but the condensates from the heat-not-burn test products were not.	49
	Comparison of particulate matter collected from cigarettes (five brands), cigars (two brands), cigarillos (two brands), bidis (two brands), and pipe tobacco (two brands). DMSO extracts of smokeless tobacco (six brands) were also used. All were tested with strains TA98 (with S9) and TA100 (with S9). Mutagenic responses were calculated based on nicotine and compared relative to cigarettes. A range of positive responses was observed for the various products except (in TA98) for smokeless tobacco products where the response was not positive. However, the response in TA100 for the extract from smokeless tobacco products was positive.	55
	Comparison of an electrically heated cigarette system with commercial and reference combusted cigarettes (Marlboro Lights, Marlboro Ultra Lights, Merit Ultima, and 2R4F). Various puffing protocols were used to generate test samples. Strains used were TA1537, TA98 and TA100 (with and without S9). The mutagenicity of the electrically heated cigarette was 70%–90% lower than the mutagenicity of the combusted cigarettes.	50
	Comparison of two commercially available THPs to Kentucky reference 3R4F. Strains used were TA98, TA100, TA1535, TA97, and TA102. Condensate exposure was performed with and without S9; whole-aerosol exposure was performed with S9. For the 3R4F condensate (with S9) clear positive responses were observed in TA98, TA100, and TA1537; 3R4F whole aerosol (with S9) was mutagenic in strains TA98 and TA100. The THPs were not mutagenic in either type of exposure.	43
	THPs, with and without flavorings, were evaluated (and compared to 3R4F). Neostiks composed of reconstituted Virginia tobacco blend with glycerol (14.8%) with and without flavorings were evaluated. TPM was generated for all the products. TA98, TA1535, TA1537, TA100, and TA102 were used. While the TPM from 3R4F was mutagenic, none of the TPMs from the flavored or nonflavored Neostiks was mutagenic.	51
IVMN	Comparison of condensates from Kentucky reference 3R4F, straight Burley and Virginia tobacco cigarettes and an e-liquid formulation. Both TK6 cells and V79 cells were used for the MN evaluation and quantitative responses were obtained for the condensates from cigarettes. The e-liquid was not positive for the induction of MN.	45
	THPs, with and without flavorings, were evaluated and compared to 3R4F. Neostiks composed of reconstituted Virginia tobacco blend with glycerol (14.8%) with and without flavorings were evaluated. TPM was generated for all the products. TA98, TA1535, TA1537, TA100, and TA102 were used. While the TPM from 3R4F was clastogenic, none of the TPMs from the flavored or nonflavored Neostiks was clastogenic.	51
	Comparative clastogenicity was evaluated from TPM from 3R4F reference cigarettes against a commercial e-liquid, and particulate matter generated from a commercial e-cigarette and THP. Three cell types (CHO, V79 and TK6) were used, and an extended recovery period of 1.5–2 cell cycles was used to increase assay sensitivity. Data were normalized against nicotine equivalents to enable comparisons between different test matrices and products. Negative responses were observed for all products except for 3R4F.	60

(*continued*)

Table 4. (Continued)

IVCA	A new cigarette that primarily heats tobacco was compared with Kentucky reference 1R4F and 1R5F combusted cigarettes. CHO cells were treated with condensates, both with and without S9. The condensates from the combusted cigarettes induced both increases in the % cells with aberrations and cytotoxicity. The condensate from the heat-not-burn cigarette was much less cytotoxic and did not induce a significant increase in the % cells with chromosome aberrations.	37
MLA	Condensates from eight U.S. commercially marketed cigarettes and reference 1R4F and 2R4F were compared with condensate from a prototype electrically heated cigarette smoking system. The mutagenic activity of the condensate from the heated cigarette was lower than that from the conventional cigarettes.	64
	An electrically heated smoking system was compared with commercial and reference combusted cigarettes (Marlboro Lights, Marlboro Ultra Lights, Merit Ultima, and 2R4F). The MLA was conducted both with and without S9. Various puffing protocols were used to generate test samples. Positive mutagenic responses were observed with all the test materials both with and without S9. When expressed on a per cigarette basis, the electrically heated smoking system was less mutagenic than the combusted cigarette.	50
	Comparison of two commercially available THPs to Kentucky reference 3R4F. Using condensate samples, the MLA was performed with and without S9 using 3-hour exposure times and without S9 using a 24-hour exposure. For the 3R4F condensate, all three exposure conditions induced clear positive responses. The THPs were not mutagenic under any of the exposure conditions.	43
	THPs, with and without flavorings, were evaluated and compared to 3R4F. Neostiks composed of reconstituted Virginia tobacco blend with glycerol (14.8%) with and without flavorings were evaluated. TPM was generated for all the products. TA98, TA1535, TA1537, TA100, and TA102 were used. While the TPM from 3R4F was mutagenic, none of the TPMs from the flavored or unflavored Neostiks was mutagenic.	51
	Comparative mutagenicity was evaluated from TPM from 3R4F reference cigarette against a commercial e-liquid, and particulate matter generated from a commercial e-cigarette and THP. Data are presented as a function of nicotine equivalents for comparisons between different test matrices and products. 3R4F demonstrated a mutagenic response where as no marked induction of mutations was observed for e-liquid, e-cigarette aerosol, or THP test articles.	67
Methods for relative potency comparisons		*References*
Ames test	Ten CSCs were prepared from commercial cigarettes (ultralow tar to full flavor), experimental cigarettes using a single type of tobacco, and reference 2R4F cigarettes. Strains TA98 and YG1041 were used with S9. The various samples were compared using mutagenic potencies that were calculated as the slope from the linear portion of the dose-response curves.	38
	Development of a statistical method using the minimum detectable difference (MDD) as a measure of the assay discriminatory power. Using data generated from a multiyear program of evaluating the impact of various ingredients added to cigarettes, the authors determined that the MDDs for the Ames test varied from 20% to 81%.	83
	Recommendation of a statistical method for conducting the Ames test for comparative studies. The publication also makes recommendation for the minimum number of replicates (4 to 10 depending upon the strain) required to ascertain a 30% difference in mutagenicity.	68
IVMN	Ten CSCs were prepared from commercial cigarettes (ultralow tar to full flavor), experimental cigarettes using a single type of tobacco, and reference 2R4F cigarettes. The induction of MN was evaluated using L5178Y $TK^{+/-}$ 3.7.2C cells and a 4-hour treatment with S9. The various samples were compared for their mutagenic potencies which were calculated as the slope of the linear portion of the dose-response curves.	38
	Recommendation of a statistical method for conducting the *in vitro* MN assay for comparative studies. The publication also makes recommendation for the minimum number of replicates (4) required to ascertain a 30% difference in clastogenicity.	68
IVCA	Ten CSCs were prepared from commercial cigarettes (ultralow tar to full flavor), experimental cigarettes using a single type of tobacco, and reference 2R4F cigarettes. The induction of CAs was evaluated in CHO-K_1 cells using 4-hour treatment and S9. The various samples were compared for their mutagenic potencies which were calculated as the slope from the linear portion of the dose-response curves.	38

(*continued*)

Table 4. (Continued)

MLA	Recommendation of a statistical method for conducting the MLA for comparative studies. The publication also makes recommendation for the minimum number of replicates (6) required to ascertain a 30% difference in mutagenicity.	68
	Five carcinogens found in cigarette smoke [4-aminobiphenyl (4-ABP), benzo[*a*]pyrene (BaP), cadmium (in the form of $CdCl_2$), 2-amino-3,4-dimethyl-3H-imidazo[4,5-f]quinoline (MeIQ), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)] were evaluated for mutagenic potency in the MLA with and without S9. $CdCl_2$ was the only chemical that was positive without S9. Positive dose-responses were observed for all chemicals when tested with S9. Various methods (including the BMD producing a 10%, 50%, 100%, or 200% increase of the background mutant frequency) were used to make potency comparisons. All of the metrics had similar rank order for potency, but the ratio of the greatest value to the least value for the chemicals varied from 16- to 572-fold depending upon the metric used.	78
	Comparative mutagenicity of 6 WSSs [20 or 60 of two commercial cigarettes from the same brand smoked under two different smoking regimens (ISO and HCI)]. Five of the six WSSs were mutagenic with S9, while only three WSSs were mutagenic without S9. Several quantitative approaches (BMD analysis, NOGEL, and mutagenic potency) generated a similar rank order, with potency increasing with the level of tar. Differences in potency were associated with the number of cigarettes smoked, the cigarette product smoked, and the smoking machine protocol used to prepare the sample. The confidence intervals generated by the BMD approach resulted in the most informative comparisons between the dose–responses.	66

ISO, International Organization for Standardization; NOGEL, no observed genotoxicity level; WSS, whole smoke solutions.

Table 5. Institute for In Vitro Sciences Workshop Recommended *In Vitro* Genetic Toxicology Test Battery for Tobacco Products

1. Bacterial gene mutation (Ames Test: OECD TG471)
2. Choice of at least one of the following mammalian cell assays:
Micronucleus assay (OECD TG487)
Mouse lymphoma gene mutation assay using the thymidine kinase locus (OECD TG490)
Chromosome aberration assay (OECD TG473)

OECD, Organization for Economic Cooperation and Development; TG, test guideline.

substantially more statistical power. Thus, very little chromosome aberration data appear to be generated by groups conducting tobacco product research. Regardless, the consensus of the workgroup members was to endorse all the assays (including chromosome aberration) listed in the *in vitro* genetic toxicology battery recommended by CORESTA and ICH. The *in vitro* battery endorsed by the IIVS workgroup is listed in Table 5.

The workgroup also agreed that it is important to have common definitions for the types of samples that can be generated and used in tobacco product research and developing consensus definitions will be a focus of the third workshop (to be held early 2020). While there have historically been three different types of samples used to evaluate combusted cigarettes, the specific methods for their generation and the terminology applied to each type of sample is somewhat variable among different researchers. The three basic types of

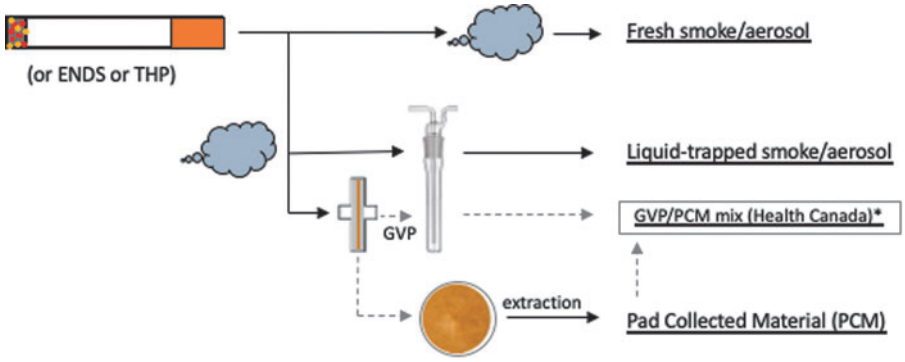

FIG. 1. Schematic for the types of samples that can be generated from inhaled tobacco products including combusted cigarettes, THPs, and e-cigarettes. These types of samples can be evaluated in most of the standard regulatory genetic toxicology assays (see Table 8). *Note that Health Canada recommends testing a combination of the GVP and the PCM for the NRU assay (Health Canada, 2017). GVP, gas-vapor phase; PCM, pad-collected material; THP, tobacco-heating product; ENDS, electronic nicotine delivery systems; NRU, neutral red uptake. Color images are available online.

Table 6. Discussion Items Identified as Category 1: Extensive Information Available; Recommendations Can Be Readily Developed

Issues discussed and resolved during the first workshop
• Consensus reached to include cytotoxicity assays in the workshop discussion • Consensus reached to endorse the ICH- and CORESTA-recommended in vitro battery of genetic toxicology tests including the Ames test, and at least one of the following mammalian genetic toxicology tests: the MLA, the in vitro micronucleus assay and the in vitro chromosome aberration assay
Issues identified as high priority for discussion at the second workshop
• Relevant test material matrix and test material preparation (renamed from ''sample preparation'') ○ Recommended methods to prepare specific types of samples (i.e., particulate matter, condensates or GVP from combusted cigarettes, ENDS and THPs, and extracts from smokeless). ○ The identification of the appropriate types of samples relative to product types and methods to prepare those samples. ○ Recommendations for appropriate solvents or matrix media for specific types of samples. ○ Recommendation for ''puffing/vaping'' profile for generation of test material (i.e. condensates/particulates, aerosols, and smoke). ○ Recommendations for test article characterization for GLP studies. ○ Recommendations for stability assessment and its impact on biology of samples prepared for in vitro assays (recommendations on ''use by'' date/shelf life). • Recommended Ames test strains
Issues identified for discussion at subsequent workshops
• Recommended cell lines for the in vitro micronucleus and in vitro chromosome aberration assays. • Recommendation for top concentration when a sample is not sufficiently cytotoxic (and the top concentration is limited by the amount of solvent).

CORESTA, Cooperation Centre for Scientific Research Relative to Tobacco; ICH, International Committee for Harmonization.

Table 7. Discussion Items Identified as Category 2: Additional Short-Term (<2 Years) Information/Research Required

• Promising new *in vitro* assays for genetic damage and recommendations for ''validating/qualifying'' them for routine use. • Recommended methods to expose cells to aerosols and smoke (combusted cigarettes, THPs and ENDS). This includes generation and handling of the aerosol, exposure and recovery of cells, exposure duration, appropriate cytotoxicity and concentration range, and dosimetry. • Recommendations for follow-up tests when deconvoluting positive results—potentially generating a decision tree. • Recommendations for evaluating large numbers of products (particularly ENDS) varying only in single additives such as flavoring compounds.

Table 8. Discussion Items Identified as Category 3: Additional Long-Term (>2 Years) Information/Research Required

• Recommended experimental design providing appropriate data for quantitative comparisons (i.e., number of replicate cultures, concentration spacing, number of independent experiments, and so on). • Recommended methods to express exposure, particularly with comparing different types of products (i.e., combusted cigarettes vs. smokeless vs. ENDS vs. THPs). • Recommended methods to make quantitative comparisons for *in vitro* responses (i.e., potency, graphical, BMD, or another metric). • What are the specific issues (and potential solutions) associated with evaluating and comparing relative potency of complex mixtures? What is the minimum amount of genotoxicity/toxicity that can be detected in a mixture? • Extrapolation of *in vitro* results to *in vivo*. • Use of bridging biomarkers from *in vitro* to human.

combusted cigarette samples include (1) particulates from cigarette smoke trapped on glass-fiber filters (generally captured on a Cambridge filter, or in early studies such as those by Kier et al., using a cold trapping method),[33] often referred to as total particulate matter, cigarette smoke condensate, and/or nicotine-free dry particulate matter; (2) gases from smoke that are captured in some type of solvent or medium (liquid-trapped gas-vapor phase [GVP] or liquid-trapped smoke/aerosol); and (3) smoke in its ''native'' form, including both the particulate and gas phases, used as it is generated by the smoking machine to directly expose cells. As the workgroup moves forward in its activities, it will be important that common terminology is agreed upon and used. It was noted that types of samples similar to those used for combusted cigarettes have been used to evaluate THPs and more recently to evaluate nontobacco containing e-cigarettes.

During the touch-base meeting (March 7, 2019) and the second workshop (June 4 & 5, 2019), the workgroup discussed and developed a simple graphic to illustrate these basic types of samples (Fig. 1). It is noted that Health Canada specifically requests that for the NRU assay, products be tested using both pad-collected material and liquid-trapped GVP.[11] In addition to the samples prepared from combusted cigarettes, extracts using both aqueous and organic solvents have been generated from smokeless tobacco products and evaluated for genotoxicity. The e-liquids that are the key components (or consumables) for ENDS products can also be applied directly to test cultures.

Triaged topics for future workshops

The first workshop discussion resulted in a list of topics triaged by priority category (see Tables 6–8). The highest priority topics, shown in Table 6, were further separated into three subcategories. The two highest priority topics were covered in the first workshop (see above). The second priority topics (shown in Table 6) included a literature-based summary of methods used to generate appropriate tobacco product test samples for *in vitro* genetic toxicology testing and a literature-based summary of the Ames test strains used in tobacco product evaluation. These topics

were the focus of a second workshop (held June 4 & 5, 2019) during which workgroup members presented initial literature search information followed by discussion. The workgroup decided that these topics should be further developed for additional discussion and recommendations during the third workshop (to be held early in 2020). Topics for subsequent workshops will be selected from Tables 6–8 by the workgroup.

Conclusion

The IIVS workshop series provides an opportunity for invited experts to share their experiences and knowledge to develop recommendations that will be useful to the broader scientific and regulatory communities seeking to evaluate the toxicity of tobacco products. The tables summarizing the types of tobacco products evaluated, the genetic toxicology tests used, and also the types of research questions addressed, provide a background summary for both the workgroup and the reader of this publication. The triaged list of topics developed in the first workshop has been prioritized for detailed discussions during subsequent workshops. The workgroup strategy is to collect information before each convened meeting in preparation for detailed workshop discussions. The goal of the workgroup discussions will be to share insights and to develop recommendations that will be shared in a series of publications and in presentations at scientific meetings.

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Authors' Contributions

Drs. M.M.M. and R.C., the cochairs of the workshop series facilitated the workshop discussion, managed between-meeting workgroup activities, and provided a first draft of the article. All authors contributed to the discussion, activities of the workgroup, and the content of the article. All authors read and approved the final article.

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Author Disclosure Statement

U.D., M.G., T.H., K.J., K.L., R.L., D.M., J.M., G.P., D.S., D.T., E.W., R.W., and K.Y. are employed by companies whose products may be tested by the methods discussed in the article. J.C., P.D., H.R. L.R., S.R., L.S., and R.C. are employed by companies who conduct genetic toxicology assays as a fee for service. M.M. provides consulting services to various clients, including tobacco companies. X.G. declares no conflicts of interest.

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References

1. Dearfield KL, Auletta AE, Cimino MC, et al. Considerations in the U.S. Environmental Protection Agency's testing approach for mutagenicity. Mutat Res 1991:258;259–283.
2. FDA/CFSAN. *Guidance for Industry and Other Stakeholders: Toxicological Principles for the Safety Assessment of Food Ingredients.* Redbook; 2000, updated 2007. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-and-other-stakeholders-redbook-2000 (accessed June 5, 2020).
3. Cimino MC. Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. Environ Mol Mutagen 2006:47;362–390.
4. COM. *Guidance on a Strategy for Genotoxicity Testing of Chemical Substances*. Committee on Mutagenicity of Chemicals in Food; 2011. Available at: https://www.gov.uk/government/publications/a-strategy-for-testing-of-chemicals-for-genotoxicity (accessed June 5, 2020).
5. EFSA. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA J 2011:9;2379.
6. ICH S2(R1). *Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use*. 2011. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/s2r1-genotoxicity-testing-and-data-interpretation-pharmaceuticals-intended-human-use (accessed June 5, 2020).
7. CORESTA. *The Rational and Strategy for Conducting In Vitro Toxicology Testing of Tobacco Smoke*. CORESTA In vitro Toxicology Task Force; 2004. Available at: https://www.coresta.org/rationale-and-strategy-conducting-vitro-toxicology-testing-tobacco-smoke-29237.html (accessed June 5, 2020).
8. FDA/CTP. Guidance for Industry: Modified Risk Tobacco Product Applications. Draft Guidance. 2012. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/modified-risk-tobacco-product-applications (accessed June 5, 2020).
9. Health Canada. Official Method T-501. *Bacterial Reverse Mutation Assay for Mainstream Tobacco Smoke*. 2017.
10. Health Canada. Official Method T-503. *In Vitro Micronucleus Assay for Mainstream Tobacco Smoke*. 2017. Available at: https://healthycanadians.gc.ca/en/open-information/tobacco/t500/micronucleus (accessed June 5, 2020).
11. Health Canada. Official Method T-502. *Neutral Red Uptake Assay for Mainstream Tobacco Smoke*. 2017. Available at: https://healthycanadians.gc.ca/en/open-information/tobacco/t500/assay (accessed June 5, 2020).
12. FDA. *Family Smoking Prevention and Tobacco Control Act (FSPTCA)*. 2009. Available at: https://www.fda.gov/tobacco-products/rules-regulations-and-guidance/family-smoking-prevention-and-tobacco-control-act-overview (accessed June 5, 2020).
13. FDA/CTP. Demonstration of Substantial Equivalence for Tobacco Products. 2011b. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/

section-905j-reports-demonstrating-substantial-equivalence-tobacco-products (accessed June 5, 2020).
14. FDA/CTP. Guidance for Industry: Applications for Premarket Review of New Tobacco Products. Draft Guidance. 2011a. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/applications-premarket-review-new-tobacco-products (accessed June 5, 2020).
15. FDA/CTP. Deeming Tobacco Products to be Subject to the Federal Food, Drug, and Cosmetic Act, as Amended by the Family Smoking Prevention and Tobacco Control Act; Restrictions on the Sale and Disctribution of Tobacco Products and Required Warning Statements for Tobacco Products. Federal Register. 2016. Available at: https://www.govinfo.gov/content/pkg/FR-2016-05-10/pdf/2016-10685.pdf (accessed June 5, 2020).
16. FDA/CTP. Premarket Tobacco Product Applications for Electronic Nicotine Delivery Systems. 2019. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/premarket-tobacco-product-applications-electronic-nicotine-delivery-systems-ends (accessed June 5, 2020).
17. Stabbert R, Ghosh D, Clark, et al. Assessment of priority tobacco additives per the requirements in the EU Tobacco Products Directive (2014/40/EU), part 2: smoke chemistry and in vitro toxicology. Reg Tox Pharm 2019:104;163–199.
18. Simms L, Clarke A, Paschke T, et al. Assessment of priority tobacco additives per the requirements of the EU Tobacco Products Directive. 2014/40/EU: Part 1: Background, approach, and summary of findings. Reg Tox Pharm 2019: 104;84–97.
19. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate. Mutat Res 1983:114;59–89.
20. Andreoli C, Gigante D, Nunziata A. A review of in vitro methods to assess the biological activity of tobacco smoke with the aim of reducing the toxicity of smoke. Toxicol In Vitro 2003:17;587–594.
21. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutat Res 2004:56;447–474.
22. Husgafvel-Pursiainen K. Genotoxicity of environmental tobacco smoke: a review. Mutat Res 2004:567;427–445.
23. Johnson MD, Schilz J, Djordjevic MV, et al. Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless tobacco. Cancer Epidemiol Biomarkers Prev 2009:18;3263–3304.
24. Wan J, Johnson M, Schilz J, et al. Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless tobacco. Cancer Epidemiol Biomarkers Prev 2009:18;3263–3304.
25. Li X. In vitro toxicity testing of cigarette smoke based on the air-liquid interface exposure: a review. Toxicol In Vitro 2016:36;105–113.
26. Kirkland DJ, Hayashi M, MacGregor JT, et al. Summary of major conclusions. Mutat Res 2003:540;123–125.
27. Kirkland DJ, Hayashi M, Jacobson-Kram D, et al. The International Workshops on Genotoxicity Testing (IWGT): history and achievements. Mutat Res 2007:627;1–4.
28. Kirkland DJ, Hayashi M, Jacobson-Kram D, et al. Summary of major conclusions from the 5th IWGT, Basel, Switzerland, 17–19 August 2009. Mutat Res 2011:723; 73–76.
29. Martus HJ, Hayashi M, Honma M, et al. Summary of major conclusions from the 6th Internationals Workshop on Genotoxicity Testing (IWGT), Foz do Iguacu, Brazil. Mutat Res 2015:783;1–5.
30. OECD. Overview of the set of OECD Genetic Toxicology Test Guidelines and updates performed in 2014–2015. Paris: Organisation for Economic Cooperation and Development. Series on Testing & Assessment, No. 238 – second edition. 2017. Available at: www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV-JM-MONO(2016)33/rev1&doclanguage=en. Accessed July 1, 2019.
31. Behrsing H, Raabe H, Manuppello J, et al. Assessment of in vitro COPD models for tobacco regulatory science: workshop proceedings, conclusions and paths forward for in vitro model use. Altern Lab Anim 2016:44;129–166.
32. Behrsing H, Hill E, Raabe H, et al. In vitro exposure systems and dosimetry assessment tools for inhaled tobacco products: workshop proceedings, conclusions and paths forward for in vitro model use. Altern Lab Anim 2017:45;117–158.
33. Kier LD, Yamasaki E, Ames BN. Detection of mutagenic activity in cigarette smoke condensates. Proc Natl Acad Sci 1974:71;4159–4163.
34. Mizusaki S, Takashima T, Tomaru K. Factors affecting mutagenic activity of cigarette smoke condensates in *Salmonella Typhimurium* TA 1538. Mutat Res 1977:48;29–36.
35. Steele RH, Payne VM, Fulp CW, et al. A comparison of the mutagenicity of mainstream cigarette smoke condensates from a representative sample of the U.S. cigarette market with a Kentucky reference cigarette (K1R4F). Mutat Res 1995:342;179–190.
36. Bombick DW, Bombick BR, Ayres PH, et al. Evaluation of the genotoxic and cytotoxic potential of mainstream whole smoke and smoke condensate from a cigarette containing a novel carbon filter. Fundam Appl Toxicol 1997: 39;11–17.
37. Bombick BR, Murli H, Avalos JT, et al. Chemical and biological studies of a new cigarette that primarily heats tobacco. Part 2. In vitro toxicology of mainstream smoke condensate. Food Chem Toxicol 1998:36;183–190.
38. DeMarini DM, Gudi R, Szkudlinska A, et al. Genotoxicity of 10 cigarette smoke condensates in four test systems: comparisons between assays and condensates. Mutat Res 2008: 650;15–29.
39. Yauk CL, Williams A, Buick JK, et al. Genetic toxicology and toxicogenomic analysis of three cigarette smoke condensates in vitro reveals few differences among full-flavor, blonde, and light products. Environ Mol Mutagen 2012:53; 281–296.
40. Combes R, Scott K, Crooks I, et al. The in vitro cytotoxicity and genotoxicity of cigarette smoke particulate matter with reduced toxicant yields. Toxicol In Vitro 2013:27;1533–1541.
41. Misra M, Leverette RD, Cooper BT, et al. Comparative in vitro toxicity profile of electronic and tobacco cigarettes, smokeless tobacco and nicotine replacement therapy products: e-liquids, extracts and collected aerosols. Int J Environ Res Public Health 2014:11;11325–11347.
42. Crooks I, Scott K, Dalrymple A, et al. The combination of two novel tobacco blends and filter technologies to reduce the in vitro genotoxicity and cytotoxicity of prototype cigarettes. Reg Tox Pharm 2015:71;507–514.
43. Thorne D, Breheny D, Proctor C, et al. Assessment of novel tobacco heating product THP1.0. Part 7: comparative in vitro toxicological evaluation. Reg Tox Pharm 2018:93; 71–83.

44. Fowler K, Fields W, Hargreaves V, et al. Development, qualification, validation and application of the Ames test using a Vitrocell VC10 smoke exposure system. Toxicol. Rep 2018:12;542–551.
45. Trelles SE, Wieczorek R, Simms L, et al. Use of human derived cell lines to increase the biological relevance of cigarette condensate, non-tobacco materials and e-liquid testing. The Toxicologist: Supplement to Tox Sci 2018: 57;231.
46. Wieczorek R, Trelles SE, Walele T, et al. Biological test procedure for fresh generated cigarette smoke and e-cigarette aerosols under air-liquid interface (ALI) exposure in 24 and 96 multi-well plates. CORESTA Congress, Kunming, 2018, Smoke Science/Product Technology Groups, STPOST 52.2018. Available at: https://www.coresta.org/abstracts/biological-test-procedure-fresh-generated-cigarette-smoke-and-e-cigarette-aerosols-under
47. Wieczorek R, Trelles SE, Simms L, et al. Biological test procedures for fresh generated smoke and aerosols. The Toxicologist: Supplement to Tox Sci 2018:57;228.
48. Thorne D, Hollings M, Seymour A, et al. Extreme testing of undiluted e-cigarette aerosol in vitro using an Ames air-agar-interface technique. Mutat Res 2018:828;46–54.
49. Doolittle DJ, Lee CK, Ivett JL, et al. Comparative studies on the genotoxic activity of mainstream smoke condensate from cigarettes which burn or only heat tobacco. Environ Mol Mutagen 1990:15;93–105.
50. Werley MS, Freelin SA, Wrenn SE, et al. Smoke chemistry, in vitro and in vivo toxicology evaluations of the electrically heated cigarette smoking system series K. Reg Tox Pharm 2008:52;122–139.
51. Crooks I, Neilson L, Scott K, et al. Evaluation of flavourings potentially used in a heated tobacco product: chemical analysis, in vitro mutagenicity, genotoxicity, cytotoxicity and in vitro tumor promoting activity. Food Chem Toxicol 2018:118;940–952.
52. Shah AS, Sarode AV, Bhide SV. Experimental studies on mutagenic and carcinogen effects of tobacco chewing. J Cancer Res Clin Oncol 1985:109;203–207.
53. Guttenplan JB. Mutagenic activity in smokeless tobacco products sold in the USA. Carcinogenesis 1987:8;741–743.
54. Jansson T, Romert L, Magnusson J, et al. Genotoxicity testing of extracts of a Swedish moist oral snuff. Mutat Res 1991:261;101–115.
55. Rickert WS, Wright WG, Trivedi AH, et al. A comparative study of the mutagenicity of various types of tobacco products. Reg Tox Pharm 2007:48;320–330.
56. Coggins CRE, Ballantyne M, Curvall M, et al. The in vitro toxicology of Swedish snus. Crit Rev Toxicol 2012:42;304–313.
57. Hurtado SB, Lallier B, Roberts DJ, et al. Validation of an *in vitro* test battery to evaluate smokeless tobacco products. Toxicologist 2019:58;135.
58. Okuwa K, Tanaka M, Fukano Y, et al. In vitro micronucleus assay for cigarette smoke using a whole smoke exposure system: a comparison of smoking regimens. Exp Toxicol Pathol 2010:62;433–440.
59. Roemer E, Zenzen V, Conroy LL, et al. Automation of the in vitro micronucleus and chromosome aberration assay for the assessment of the genotoxicity of the particulate and gas-vapor phase of cigarette smoke. Toxicol Mech Methods 2015:25;320–333.
60. Thorne D, Leverette R, Breheny D, et al. Genotoxicity evaluation of tobacco and nicotine delivery products: Part Two: *in vitro* micronucleus assay. Food Chem Toxicol 2019:132; 110546.
61. Jaju RJ, Patel RK, Bakshi SR, et al. Chromosome damaging effects of pan masala. Cancer Lett 1992:65;221–226.
62. Lafi A, Parry JM. The effects of ventilation and the dilution of smoke upon the clastogenic and aneugenic activity of tobacco particulate matter in cultured mammalian cells. Mutat Res 1991:264;51–57.
63. Trivedi AH, Dave BJ, Adhvaryu SG. Genotoxic effects of tobacco extract on Chinese hamster ovary cells. Cancer Lett 1993:15;107–112.
64. Schramke H, Meisgen TJ, Tewes FJ, et al. The mouse lymphoma thymidine kinase assay for the assessment and comparison of the mutagenic activity of cigarette mainstream smoke particulate phase. Toxicology 2006: 227;193–210.
65. Guo X, Verkler TL, Chen Y, et al. Mutagenicity of 11 cigarette smoke condensates in two versions of the mouse lymphoma assay. Mutagenesis 2011:26;273–281.
66. Guo X, Heflich RH, Dial SL, et al. Quantitative differentiation of whole smoke solution-induced mutagenicity in the mouse lymphoma assay. Environ Mol Mutagen 2018:59; 103–113.
67. Thorne D, Leverette R, Breheny D, et al. Genotoxicity evaluation of tobacco and nicotine delivery products: Part One: mouse lymphoma assay. Food Chem Toxicol 2019:132; 110584.
68. Scott K, Saul J, Crooks I, et al. The resolving power of in vitro genotoxicity assays for cigarette smoke particulate matter. Toxicol In Vitro 2013:27;1312–1319.
69. Schaller J-P, Keller D, Poget L, et al. Evaluation of the tobacco heating system 2.2. Part 2: chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol. Reg Tox Pharm 2016:81;S27–S47.
70. Thorne D, Crooks I, Hollings M, et al. The mutagenic assessment of an electronic-cigarette and reference cigarette smoke using the Ames assay in strains TA98 and TA100. Mutat Res 2016:812;29–38.
71. Aufderheide M, Gressmann H. Mutagneicity of native cigarette mainstream smoke and its vapor/gas phase by use of different tester strains and cigarettes in a modified Ames asssy. Mutat Res 2008:656;82–87.
72. Takahashi Y, Kanemaru Y, Fukushima T, et al. Chemical analysis and in vitro toxicological evaluation of aerosol from a novel tobacco vapor product: a comparison with cigarette smoke. Reg Tox Pharm 2018:92;94–103.
73. Doolittle DJ, Lee DA, Lee CK. The genotoxic activity of glycerol in an in vitro test battery. Food Chem Toxicol 1988:26;631–635.
74. Padma PR, Amonkar AJ, Bhide SV. Mutagenic and cytogenetic studies of N'-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Cancer Lett 1989:46;173–180.
75. Yim SH, Hee SS. Bacterial mutagenicity of some tobacco aromatic nitrogen bases and their mixtures. Mutat Res 2001:492;13–27.
76. Ginzkey C, Friehs G, Koehler C, et al. Assessment of nicotine-induced DNA damage in a genotoxicological test battery. Mutat Res 2013:751;34–39.

77. Smart DJ, Helbling FR, Verardo M, et al. Mode-of-action analysis of the effects induced by nicotine in the *in vitro* micronucleus assay. Environ Mol Mutagen 2019:60;778–791.
78. Guo X, Heflich RH, Dial SL, et al. Quantitative analysis of the relative mutagenicity of five chemical constituents of tobacco smoke in the mouse lymphoma assay. Mutagenesis 2016:31;287–296.
79. Theophilus EH, Pence DH, Meckley DR, et al. Toxicological evaluation of expanded shredded tobacco stems. Food Chem Toxicol 2004:42;631–639.
80. Stavanja MS, Curtin GM, Ayres PH, et al. Safety assessment of diammonium phosphate and urea used in the manufacture of cigarettes. Exper Tox Path 2008:59; 339–353.
81. Potts RJ, Bombick BR, Meckley DR, et al. A summary of toxicological and chemical data relevant to the evaluation of cast sheet tobacco. Exp Toxicol Path 2010:62;117–126.
82. Sato S, Seino Y, Ohka T, et al. Mutagenicity of smoke condensates from cigarettes, cigars and pipe tobacco. Cancer Lett 1977:3;1–8.
83. Oldham MJ, Haussmann H-J, Gomm W, et al. Discriminatory power of standard toxicity assays used to evaluate ingredients added to cigarettes. Reg Tox Phar 2012:62; 49–61.

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