The CORESTA *In Vitro* Test Battery for Combustible Tobacco Products: Update from the 2004 Rationale and Strategy Report

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ABSTRACT

In 2004, the CORESTA *In Vitro* Toxicity Task Force issued a rationale and strategy report, commonly known as "the CORESTA *in vitro* test battery guideline." The goals were to: 1) develop the rationale and strategy for conducting *in vitro* toxicity testing of tobacco smoke and 2) identify key procedures based on internationally recognized guidelines, adapted to accommodate the unique properties of tobacco smoke. The Task Force [now the Subgroup (IVTSG; *In Vitro* Toxicity Subgroup) since 2015] performed a series of proficiency trials based on the guideline.

Considering the time passed, the IVTSG has reviewed the guideline to: 1) re-evaluate the relevance of the initial rationale and strategy for *in vitro* testing of combustible tobacco products, 2) identify recent and comparable regulatory testing guidelines and examples in publications, and 3) provide a pragmatic summary of key features of each recommended assay. The endeavor confirmed the continued usage and reference of the 2004 CORESTA *in vitro* test battery, especially where standardized and validated testing is required (e.g., regulatory submission), upholding that the overall strategy and rationale remain valid and relevant. Sometimes these standardized testing results are supplemented with newer and exploratory *in vitro* assays (e.g., air-liquid-interface testing with fresh whole smoke), however the CORESTA *in vitro* test batteries are continuously used in comparative product testing, such as evaluating the biological impact of changes in ingredients or product designs as part of a weight-of-evidence toxicity evaluation.

In the updated 2019 guideline, the IVTSG recommends where standardized *in vitro* toxicity testing is desired, the following test battery for combustible tobacco products: 1) cytotoxicity (Neutral Red Uptake) assay with mammalian cells, 2) bacterial reverse mutation (Ames) assay in *Salmonella typhimurium*, and 3) mammalian cell cytogenetics/mutation assays (the *in vitro* micronucleus assay, the mouse lymphoma assay, or the chromosome aberration assay). The IVTSG reiterates that the biological significance of the *in vitro* results must be evaluated in conjunction with all available chemical and exposure/dosimetry data, in the context of the overall product risk assessment.

BACKGROUND

- The *in vitro* testing framework is built upon standardized and internationally recognized assays that are widely used for tobacco products (see references, including CORESTA proficiency reports).
- Currently no single *in vitro* assay can provide comprehensive information on toxicity or biological activity. Cytotoxicity assays can be used to support estimation of starting *in vivo* doses for acute toxicity testing and as part of other *in vitro* assays where the results are used to select the doses for genotoxicity evaluation. Representative cytotoxicity endpoints are cell viability and cellular growth rate.
- Genotoxicity testing is used to evaluate DNA damage or gene mutation and structural or numerical chromosome aberration. Because no single genotoxicity assay provides comprehensive information on various types of genetic damages, and in order to minimize false positives, a paired test is recommended (e.g., bacterial Ames mutation test and mammalian cell assay detecting chromosomal damage).
- Combustible tobacco test materials (e.g., total particulate matter [TPM]) are already demonstrated as cytotoxic and genotoxic. One of objectives is to do a comparative assessment, if a product change of interest has resulted in meaningful modification (no change, increase, or decrease) of biological activity *in vitro* compared with the product without specific change.

REFERENCES

Selected below. Full references included in the 2019 Report (https://www.coresta.org/groups/vitro-toxicity-testing)**:**

- CORESTA (2015). Technical Report - NRU Assay Proficiency Study
- CORESTA (2016a). Technical Report - Ames Assay Proficiency Study
- CORESTA (2019). Technical Report - *In vitro* MN Assay Proficiency Study
- CORESTA (2019). Technical Report - *In vitro* MLA Assay Proficiency Study (PENDING)
- Health Canada (2017a). Official method T-501, bacterial reverse mutation assay
- Health Canada (2017b). Official method T-502, neutral red uptake assay
- Health Canada (2017c). Official method T-503, *in vitro* micronucleus assay
- ISO 10993-5 (2009). Biological evaluation of medical devices – Part 5: Tests for *in vitro* cytotoxicity.
- OECD (1997). TG471: Bacterial reverse mutation test.
- OECD (2010). TG129: using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests.
- OECD (2016a). TG487: *In Vitro* Mammalian Cell Micronucleus Test.
- OECD (2016b). TG490: *In Vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene.
- Sobol Z, Homiski ML et al. (2012). Mutation Research 746(1), 29-34.

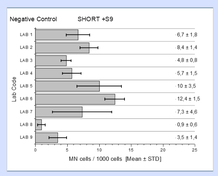
RECOMMENDED ASSAYS AND EXAMPLES

For studies that may be submitted to regulatory agencies and where *in vitro* toxicity testing is deemed appropriate, the CORESTA IVTSG recommends a test battery of:

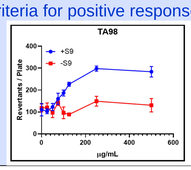
1. **Neutral Red Uptake (NRU) assay**: cytotoxicity with mammalian cell lines
2. **Ames test**: bacterial mutagenicity using *Salmonella typhimurium* strains, and
3. **Cytogenetics/mutation assay** with mammalian cell lines: the *in vitro* micronucleus (MN) assay, the mouse lymphoma assay (MLA), or the chromosome aberration (CA) assay.

SELECTED POINTS	NEUTRAL RED UPTAKE (NRU) ASSAY - EXAMPLES*
Cell lines	BALB/c3T3, A549, CHO, BEAS-2B, HepG2
Cell density for seeding	3,000 – 10,000 cells/well (96-well plate)
Medium volume	100 - 200 µL
Pre-culture	20 - 24 hours
Exposure time	24 to 65-70 hours
Conc. of serum	- Serum free medium (e.g., Promocell C-21060 + C-39165); - Up to 10% calf serum in medium
Conc. of DMSO	0.45 - 2 % (v/v)
Conc. of NR dye	25 - 66 mg/L
Duration for NR incorporation	3 hours
Duration for NR extraction	10 – 60 minutes
Positive control	Sodium dodecyl sulfate (SDS)
Endpoints	>20-30% cytotoxicity at Max. dose (ISO 10993-5, 2009)
	EC_{50} (50% reduction in viability) & dose-response curve fit

* Ref: OECD TG129 (2010), HC T-502 (2017b), CORESTA NRU proficiency (2015)

SELECTED POINTS	MICRONUCLEUS (MN) ASSAY - EXAMPLES*
Cell line	CHO, V79, CHL, L5178Y, TK6, blood lymphocytes
Cell density	Cultures that are sub-confluent should be used
Exposure time/condition	Short term (3-6 hours) ±S9; long term (20-48 hours, ~2 normal cell cycles) -S9; cytokinesis blocker (Cyto-B; optional)
Conc. of DMSO	Up to 1% of the culture volume (according to historical control)
Cytotoxicity	Not to exceed 60%
N of cultures/concentration	Minimum of 2
MN staining	Acridine orange, Giemsa or other DNA-specific dyes
N of cells to score	Minimum of 2,000 per concentration
N of conc (for MN reading)	Minimum of 3
Positive controls	Methyl methanesulfonate; Mitomycin C; 4-Nitroquinoline-N-oxide; Cytosine arabinoside; Benzo(a)pyrene; Cyclophosphamide; Colchicine; Vinblastine
Criteria for positive response	 Positive control responses are compatible with the laboratory historical positive control database and produce a statistically significant increase compared to the concurrent negative control. At least one of the test concentrations shows a statistically significant increase compared to the concurrent negative control; the increase is dose-related in at least one experimental condition when evaluated with an appropriate trend test; any of the results are outside the distribution of the historical negative control data (e.g., Poisson based 95% control limits; Sobol et al., 2012).

* Ref: OECD TG487 (2016a), HC T-503 (2017c); Example figure from the CORESTA MN proficiency report (2019)

SELECTED POINTS	AMES ASSAY - EXAMPLES*
Strain	TA98, TA100, TA1537 (or TA97), TA1535, TA102
Cell volume	10^9 cells/plate
Metabolic activation	Absence and presence of S9
Pre-incubation period	20 - 60 minutes (if direct plate incorporation is not used)
Solvent control	DMSO; ethanol; PBS
Maximum solvent control	Up to 4% for DMSO (HC T-501, 2004a); 10% for PBS
Max. conc of TPM to test	Up to 5,000 µg/plate
Replicates/concentration	Minimum of 3
Positive control (+S9)	One of: 9,10-Dimethylanthracene; 7,12-Dimethylbenzanthracene; Congo Red; Benzo(a)pyrene; Cyclophosphamide; 2-Aminoanthracene
Positive control (-S9)	TA98: 2-Nitrofluorene; TA100 & 1535: Sodium azide TA1537 (and TA97): 9-Aminoacridine or ICR191 TA102: Cumene hydroperoxide or Mitomycin C
Criteria for positive response	 Lab to maintain the historical control ranges for each positive control per strain to demonstrate proficiency. The positive controls induce a statistically significant increase in the number of revertants relative to the vehicle control; and the strain-specific fold-increase is acceptable. A concentration-related increase over the range tested and/or a reproducible increase compared to the vehicle control at one or more concentrations tested in at least one strain in the presence & absence of S9. Statistical methods (e.g., Dunnett's test) may aid evaluating the test results; however, statistical significance should not be the only determining factor for a positive response.

* Ref: OECD TG471 (1997), HC T-501 (2017a); Example figure from the CORESTA Ames proficiency report (2016a)

SELECTED POINTS	MOUSE LYMPHOMA ASSAY (MLA) - EXAMPLES*
Cell line	L5178Y *tk* +/- (3.7.2C)
Culture medium	RPMI 1640 Fishers medium / Horse serum (heat-inactivated)
Culture preparation	At least 1 x 10^7 cells (3 hours) or 4 x 10^6 (24 hours) for treatment
Metabolic activation	Co-factor supplemented S9 fraction Final concentration 1-2% in culture medium
Treatment period	3 and 24 hours -S9, 3 hours +S9
Conc. of solvent	1% (v/v) organic, 10% (v/v) aqueous
Conc. selection	Minimum 4 concentrations
Expression period	48 hours
Selective agent	Trifluorothymidine (TFT)
Incubation period	10 – 14 days
Positive controls	(-S9) Methyl methanesulfonate, 4-nitroquinoline N-oxide; (+S9) Benzo(a)pyrene, Cyclophosphamide, 7,12-Dimethylbenz[a]anthracene (+S9)
Criteria for positive control	Absolute increase in mutant frequency of at least 300 x 10^{-6} (minimum 40% small colony formation) or an increase in small colony mutant frequency of at least 150 x 10^{-6} compared to the vehicle control.
Mutagenic response	A dose-related increase in the mutant frequency exceeds the vehicle control plus the global evaluation factor (126x10^{-6} for the microwell or 90x10^{-6} for agar method)

* Ref: OECD TG490 (2016b), CORESTA MLA proficiency (pending)

REPRESENTATIVE TEST MATERIALS

TEST MATERIALS*	RECOMMENDATIONS
Total Particulate Matter (TPM) Also referred as: - Particulate Phase (PP) - Cigarette smoke condensates (CSC)	- Cambridge filter pads after smoking are kept at room temperature for ≤1 hour. Pads are extracted immediately or stored at <-70°C. Pads and TPM extracts are stored at <-70°C after collection for up to 2 years. Pads and TPM extracts should not be re-frozen once thawed. - TPM extracts are aliquoted into individual vials prior to freezing if multiple assays are needed. The TPM extract should be analyzed at minimum for nicotine.
Gas/Vapor Phase (GVP) - Mainstream smoke vapor/aerosol containing permanent gasses and volatilized compounds where TPM has been filtered out/removed	- GVP is collected by bubbling smoke into ice-cold calcium and magnesium-free Phosphate Buffered Saline (PBS), the preferred solvent, in an impinger or equivalent vessel. Particulate matter is filtered out by passing smoke through a filter pad. - GVP should be administered to test systems within 1 hour of collection. The filter pads are retained, in order to calculate equivalent deposited particulate matter.
Whole Smoke (WS) Also referred as: - TPM & GVP combined (TPM+GVP)	- WS should be administered to test systems within 1 hour of collection - Equal amounts of TPM and GVP should be mixed together such that the combined preparation contains equal amounts of particulate matter or equivalent from the TPM and GVP.

* In addition to the above, various other test materials are reported, for example: whole smoke sampling with bubbler (cigarette smoke extract) and diluted whole smoke directly delivered to Air-Liquid-Interface (ALI) *in vitro* exposure systems; whole smoke bubbling in bacteria suspension in Ames test)

There is no widely agreed consensus on the definitions and collection methods of test materials for tobacco smoke in vitro testing. The IVTSG recommends to report the actual collection methods and analytical characterization of the test materials as part of in vitro testing results.

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*CORESTA Website: https://www.coresta.org/groups/vitro-toxicity-testing