The CORESTA In Vitro Test Battery for Combustible Tobacco Products: Update from the 2004 Rationale and Strategy Report LEE KM¹; JORDAN *KG*²; WIECZOREK R³; MOENNIKES O⁴; CLEMENTS J⁵; CROOKS I⁶; HASHIZUME T⁷; MILLER J⁸; WEBER E⁹; YOSHINO K⁷

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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.

- . Neutral Red Uptake (NRU) assay: cytotoxicity with mammalian cell lines
- 2. Ames test: bacterial mutagenicity using Salmonella typhimurium strains, and

SELECTED POINTS	NEUTRAL RED UPTAKE (NRU) A
Cell lines	BALB/c3T3, A549, CHO, BEAS-2B
Cell density for seeding	3,000 - 10,000 cells/well (96-well p
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., Promote of the serum in medium) 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)
Endnainta	>20-30% cytotoxicity at Max. dose
Endpoints	EC ₅₀ (50% reduction in viability) & 0
* Ref: OECD TG129 (2010), HC T-50	02 (2017b), CORESTA NRU proficiency (201

SELECTED POINTS	MICRONUCLEUS (MN) ASSAY - E
Cell line	CHO, V79, CHL, L5178Y, TK6, bloc
Cell density	Cultures that are sub-confluent sho
Exposure time/condition	Short term (3-6 hours) ±S9; long ter cell cycles) -S9; cytokinesis blocker
Conc. of DMSO	Up to 1% of the culture volume (acc
Cytotoxicity	Not to exceed 60%
N of cultures/concentration	Minimum of 2
MN staining	Acridine orange, Giemsa or other D
N of cells to score	Minimum of 2,000 per concentration
N of conc (for MN reading)	Minimum of 3
Positive controls Criteria for positive response	Methyl methanesulfonate; Mitomyci oxide; Cytosine arabinoside; Benzo Cyclophosphamide; Colchicine; Vin Positive control responses are com
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* Ref: OECD TG487 (2016a), HC T-503 (2017c); Example figure from the CORES

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 a TPM extracts are stored at <-70°C af TPM extracts are aliquoted into indivision 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 impinger or equivalent vessel. Particular GVP should be administered to test sparticulate matter.
Whole Smoke (WS) Also referred as: - TPM & GVP combined (TPM+GVP)	 WS should be administered to test sy Equal amounts of TPM and GVP sho equivalent from the TPM and GVP.

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.

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:

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.

Itate)Cell volume10° cells/plateMetabolic activationAbsence and presence of SMetabolic activationAbsence and presence of SSolvent controlDMSO; ethanol; PBSMaximum solvent controlUp to 4% for DMSO (HC T-5Max. conc of TPM to testUp to 5,000 µg/plateReplicates/concentrationMinimum of 3Positive control (-S9)One of: 9,10-DimethylanthraBenzo(a)pyrene; CyclophosTA38: 2-Nitrofluorene; TA10Positive control (-S9)TA1537 (and TA97): 9-AminTA102: Cumene hydropene; TA102: Cumene hydropene;Multa be usedImmonitorial the historical demonstrate proficiency. The interease is acceptable. A correproducible increase compiles test of nal least one strain in the number of reventants 1MA-specific dyes* Ref: OECD TG471 (1997), HC T-501 (2017a); Example figure from theSELECTED POINTSMOUSE LYMPHOMA ASSACell lineL5178Y tk +/- (3.7.2C)Culture mediumRPMI 1640 Fishers mediumCulture preparationAt least 1 x 107 cells (3 hournnConc. selectionMinimum 4 concentration 1-2% in original concent	SSAY - EXAMPLES*	SELECTED POINTS	AMES ASSAY - EXAMPLES
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are kept at room temperature for ≤1 hour. Pads are extracted immediately or stored at <-70°C. Pads and after collection for up to 2 years. Pads and TPM extracts should not be re-frozen once thawed. vidual vials prior to freezing if multiple assays are needed. The TPM extract should be analyzed at

into ice-cold calcium and magnesium-free Phosphate Buffered Saline (PBS), the preferred solvent, in an culate matter is filtered out by passing smoke through a filter pad. systems within 1 hour of collection. The filter pads are retained, in order to calculate equivalent deposited

systems within 1 hour of collection

ould be mixed together such that the combined preparation contains equal amounts of particulate matter or





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A97), TA1535, TA102

te incorporation is not used)

501, 2004a); 10% for PBS

acene; 7,12-Dimethylbenzanthracene; Congo Red; phamide; 2-Aminoanthracene

00 & 1535: Sodium azide

oacridine or ICR191 kide or Mitomycin C

al control ranges for each positive control per strain to e positive controls induce a statistically significant increase relative to the vehicle control; and the strain-specific foldoncentration-related increase over the range tested and/or a ared to the vehicle control at one or more concentrations in the presence & absence of S9. Statistical methods (e.g., uating the test results; however, statistical significance mining factor for a positive response.

CORESTA Ames proficiency report (2016a)

Y (MLA) - EXAMPLES*

/ Horse serum (heat-inactivated) s) or 4×10^6 (24 hours) for treatment

fraction culture medium

+S9

aqueous

ate, 4-nitroquinoline N-oxide;

ophosphamide, 7,12-Dimethylbenz[a]anthracene (+S9) frequency of at least 300 x 10⁻⁶ (minimum 40% small colony small colony mutant frequency of at least 150 x 10⁻⁶

ne mutant frequency exceeds the vehicle control plus the Sx10⁻⁶ for the microwell or 90x10⁻⁶ for agar method)

ACKNOWLEDGEMENT

IVTSG REPRESENTATIVES		
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Okolab, JT International Group, Austria	EW	
KT&G, Korea	CHP	
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