Development, Qualification, Validation, and Application of the Ames assay in a Vitrocell® Smoke Exposure System

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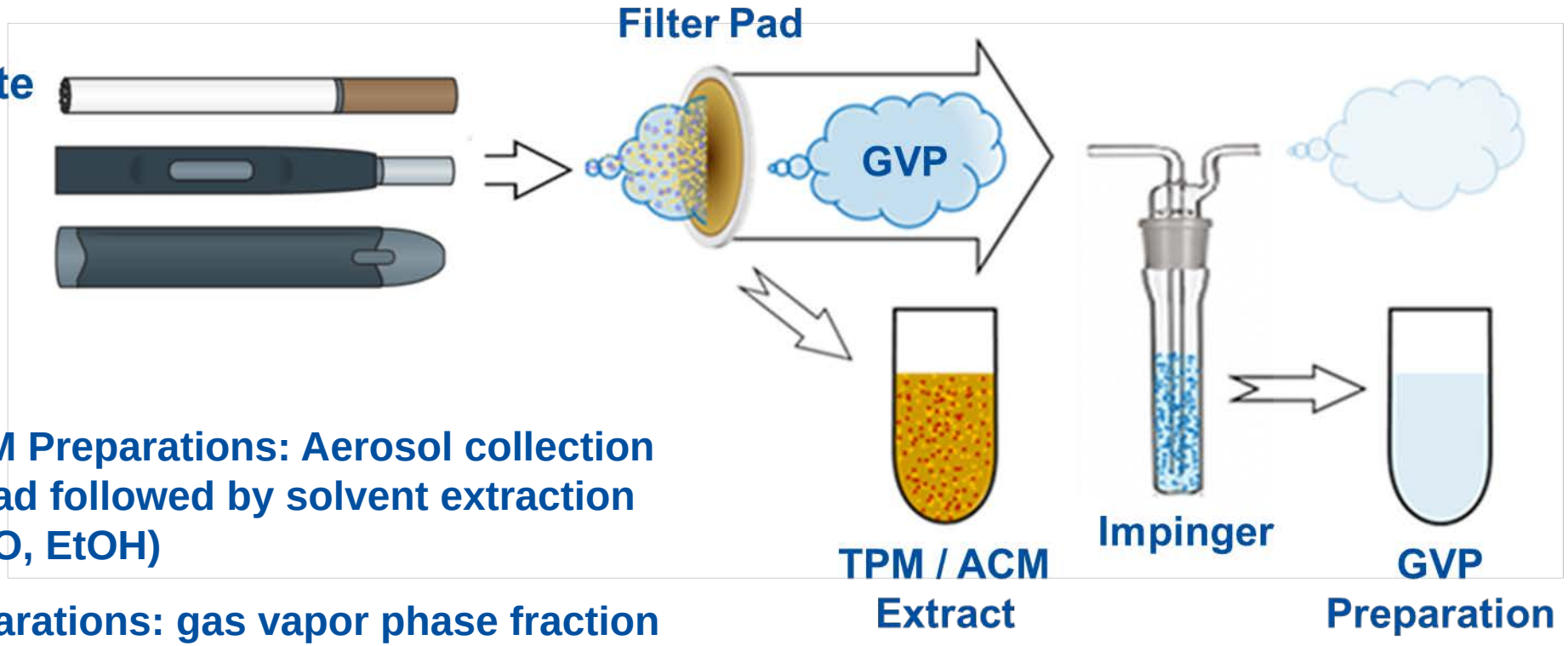
Introduction

- Ames has historical use in the assessment of potential mutagenicity of tobacco products
- Ames data has been an integral part in PMTA submissions
- Challenges of tobacco product testing in the Ames Assay
  - How to test aerosols from combustible and next generation products (NGPs)?
    - Historically have tested combustible cigarettes (CC) by the assessment of collected total particulate matter (TPM) and gas vapor phase (GVP) fractions
    - Adaptation of combustible cigarette sample collection methods to NGPs
      - Heated Tobacco Products (HTP)
      - Electronic Nicotine Delivery Systems (ENDS)
  - Prefer to assess unfractionated Whole Aerosol (WA), but how to expose whole aerosols in the Ames assay?

Aerosol Sample Collection Methods



TPM / ACM Preparations: Aerosol collection on filter pad followed by solvent extraction (e.g. DMSO, EtOH)

GVP Preparations: gas vapor phase fraction bubbled through solvent (e.g. PBS, EtOH) using an in-line impinger

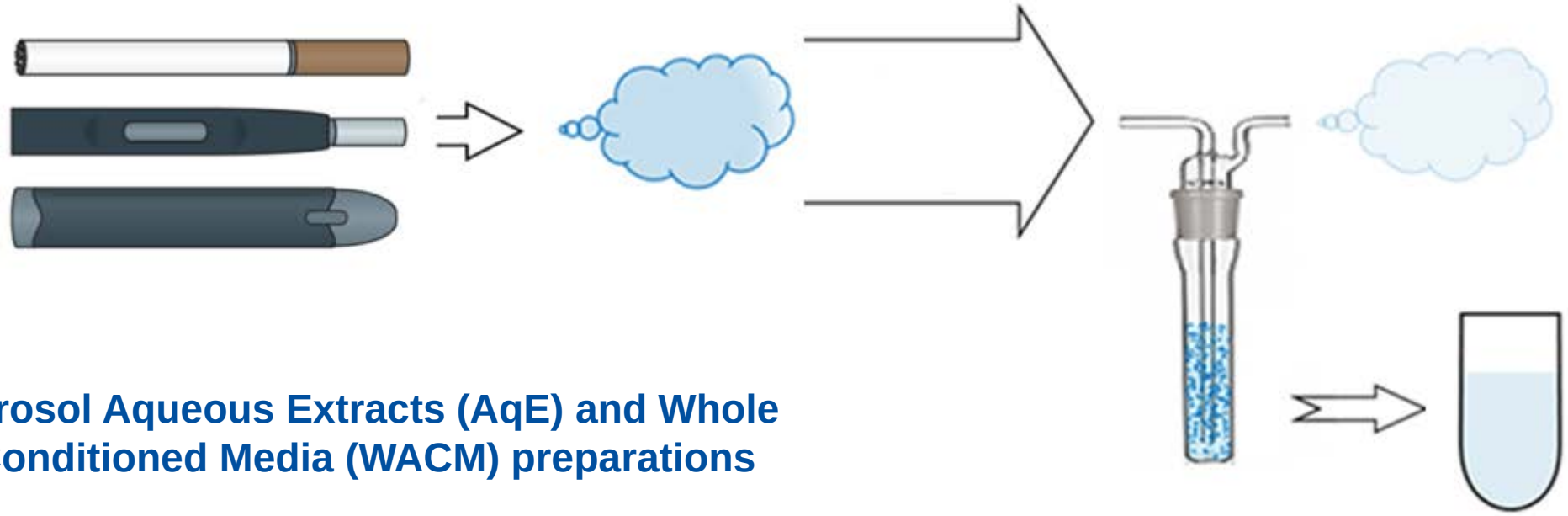
Figure adapted, with modifications, from IIVS Workshop; Key Challenges for Testing Tobacco Products, Feb 2020.

Aerosol Sample Collection Methods

Cigarette

HTP

ENDS



Whole Aerosol Aqueous Extracts (AqE) and Whole Aerosol Conditioned Media (WACM) preparations

AqE
WACM
Preparation

Figure adapted, with modifications, from IIVS Workshop; Key Challenges for Testing Tobacco Products, Feb 2020.

Bozhilova et al. (2020) Optimization of aqueous aerosol extract (AqE) generation from e-cigarettes and tobacco heating products for *in vitro* cytotoxicity testing. *Toxicology Letters* 335, 51–63

Taylor et al. (2020) *In vitro* biological assessment of the stability of cigarette smoke aqueous aerosol extracts. *BMC Res Notes* 13:492

Whole Aerosol Exposure

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Whole Aerosol Exposure*

- **Exposure system?**
  - **Develop a new system?**
  - **Commercially available system?**
- **What approach to expose tester strains to whole aerosol?**
  - **In suspension?**
  - **At the air-agar-interface (AAI)?**
- **Assay development based on OECD 471**

*Fowler et al. (2018) Development, qualification, validation and application of the Ames test using a VITROCELL® VC10® smoke exposure system. *Tox Reports*, 5; 542-551

Smoke Generation, Dilution and Exposure Systems

Aerosol Generation Systems

- At the time of development, there was a variety of commercial aerosol generating and exposure systems considered:
  - Borgwaldt (RM20S)
  - Burghart (Mimic Smoker)
  - Vitrocell® (VC10®)

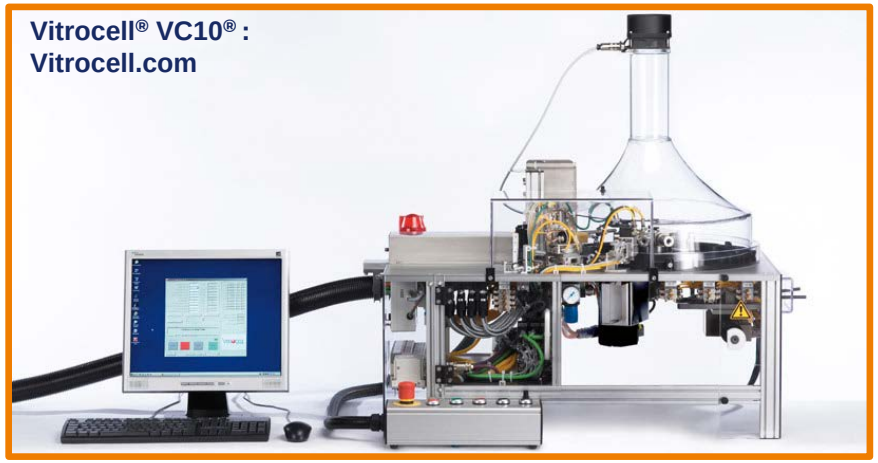
In Vitro Exposure System

- Smoke Dilution System
- Standard Ames Exposure Modules

Figure 50. The rotary Borgwaldt RM20S (108) generates and dilutes smoke from four different cigarettes simultaneously for *in vitro* cell culture testing. Klus et al. (2016)

Klus et al. (2016)

Figure 54. The five independent piston pumps of the Mimic Smoker MSB-01 (518) produce smoke for *in vitro* exposure of cells in a 96-microwell plate.



Vitrocell® VC10® : Vitrocell.com

Ames 3: Vitrocell.com

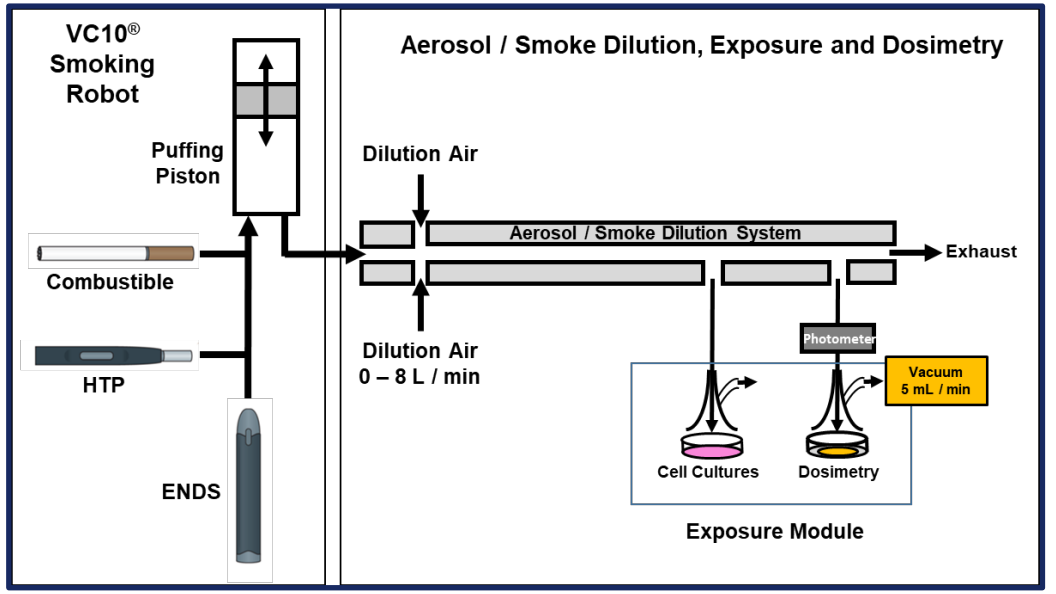
Vitrocell® Dilution System

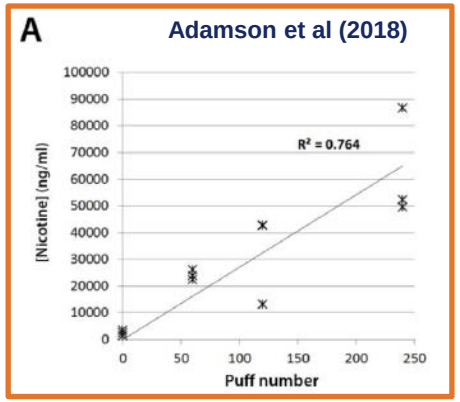
Klus et al. (2016) Cigarette Mainstream Smoke: The Evolution of Methods and Devices for Generation, Exposure and Collection. *Beiträge zur Tabakforschung*; 27,4 DOI: 10.1515/cttr-2016-0015

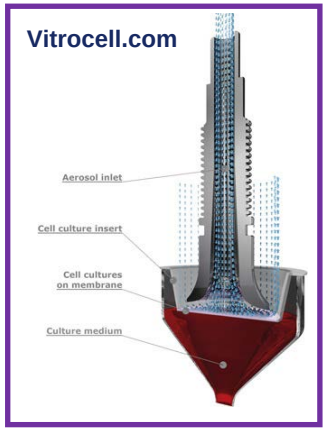
Whole Aerosol Exposure

Where to start?

- Aerosol generator
- Aerosol delivery
  - Path length
  - Losses within the system
- Aerosol dilution or delivery of undiluted[1] aerosol
  - Humidification[2]
- Aerosol deposition within exposure modules
- Dosimetry[3]
  - Quartz Crystal Microbalance (QCM)[4]
  - Photometers
  - Fluorescence of captured particulate matter[5]
  - Analytical Chemistry







[1]Adamson et al. (2018) Characterisation of the Borgwaldt LM4E system for in vitro exposures to undiluted aerosols from next generation tobacco and nicotine products (NGPs) Food and Chemical Toxicology 113, 337–344

[2]Zavala et al. (2017) Regulating temperature and relative humidity in air–liquid interface *in vitro* systems eliminates cytotoxicity resulting from control air exposures. *Toxicology Research* 6, 448

[3]Miller-Holt et al. (2022) Key challenges for in vitro testing of tobacco products for regulatory applications: Recommendations for dosimetry. *Drug Test Anal*; 1-14

[4]Adamson et al. (2017) Nicotine quantification in vitro: a consistent dosimetry marker for e-cigarette aerosol and cigarette smoke generation. Applied In Vitro Tox; 3 (1), 14-27

[5]Keyser et al. (2022a) Characterization of Aerosol Deliveries from Combustible Cigarettes, Heated Tobacco Products, and Electronic Nicotine Delivery Systems Using the Vitrocell® Ames 48, Applied In Vitro Toxicology 8 (2), 39-49

Whole Aerosol Ames Assay*

- **Qualification of the Vitrocell® VC10®**
  - **Installation and Operational Qualification (IOQ)**
  - **Performance Qualification (PQ)**
- **Whole Aerosol Ames Assay**
  - **Development**
  - **Pre-validation**
  - **Validation**

*Fowler et al. (2018) Development, qualification, validation and application of the Ames test using a VITROCELL® VC10 ® smoke exposure system. *Tox Reports*, 5; 542-551

Whole Aerosol Ames Assay*

- **IOQ**
  - **Proper operating environment**
  - **Demonstrate through testing and documentation system was suitable for intended use throughout operating range**
- **PQ**
  - **System performed as intended**
  - **Met predetermined acceptance criteria**
  - **Biannual system assessment**

*Fowler et al. (2018) Development, qualification, validation and application of the Ames test using a VITROCELL® VC10® smoke exposure system. *Tox Reports*, 5; 542-551

Whole Aerosol Ames Assay*

- **Assay Conditions**
  - **Tester strains: TA97, TA98, TA100, TA102, TA1535 (±S9)**
  - **Seeding density (2 x 10^7 / plate) on 35mm plates**
  - **S9 concentration (10%)**
  - **Culture stability under flowing air conditions**
  - **Spontaneous revertant ranges for each strain**

Table 1
Historical Control Range–Observed spontaneous revertant frequencies at the AAI.

Salmonella typhimurium test strain	Activation condition (revertants/plate)	
	absence of S9	presence of S9
TA97	5–21	13–32
TA98	2–13	2–16
TA100	11–41	8–26
TA102	19–47	19–60
TA1535	0–20	0–5

Thirty data points were collected in two independent experiments per strain per experimental condition.

*Fowler et al. (2018) Development, qualification, validation and application of the Ames test using a VITROCELL® VC10 ® smoke exposure system. *Tox Reports*, 5; 542-551

Whole Aerosol Ames Assay*

- **Smoke Generation**
  - ISO puffing regimen (ISO 3308; 2012)
  - Dilution flow rates (up to 12 L/min)
  - Puff exhaust duration (8 seconds)
  - Trumpet height (2 mm above plate surface)
  - Vacuum (5 ml/min)
- **Evaluation and Acceptance Criteria**
  - Spontaneous revertant counts within established historical ranges
  - Positive controls ≥ 2-fold over concurrent AAI controls
  - Concentration related increase in revertant counts
    - p ≤ 0.01 vs AAI control (Dunnett's test)
    - reproduceable

Table 2
Appropriate positive control treatment concentrations for test strains.

Strain	Metabolic activation	Chemical	Concentration (μg/plate)	Responses observed[a]
TA97	–	AAC	12.5	40.2 ± 16.7[b]
	+	AAN	0.8	139.9 ± 32.1
TA98	–	2-NF	0.4	85.6 ± 17.3
	+	B[a]P	0.8	60.7 ± 14.4
TA100	–	NaN_3	0.4	154.6 ± 13.9
	+	AAN	0.4	190 ± 55.4
TA102	–	MMC	0.1	163.5 ± 37.6
	+	AAN	5.0	219.1 ± 27.5
TA1535	–	NaN_3	0.8	117.3 ± 82.9
	+	AAN	0.8	34.7 ± 5.2

AAC = 9-aminoacridine, AAN = 2-aminoanthracene, 2-NF = 2-nitrofluorene, B[a]P = benzopyrene, NaN_3 = sodium azide, MMC = mitomycin C.

[a] Mean ± SD for 6 experiments, except for TA98 + S9 where 8 experiments were conducted.

[b] Although the positive control showed > 2-fold the UTC responses for TA97 -S9, the magnitude of the response was deemed to be low. Further testing with TA97 using acridine mutagen ICR191 (0.2–3 μg/plate) −S9 showed significant increases in revertant numbers (~5-fold to 27-fold the UTC value).

*Fowler et al. (2018) Development, qualification, validation and application of the Ames test using a VITROCELL® VC10® smoke exposure system. *Tox Reports*, 5; 542-551

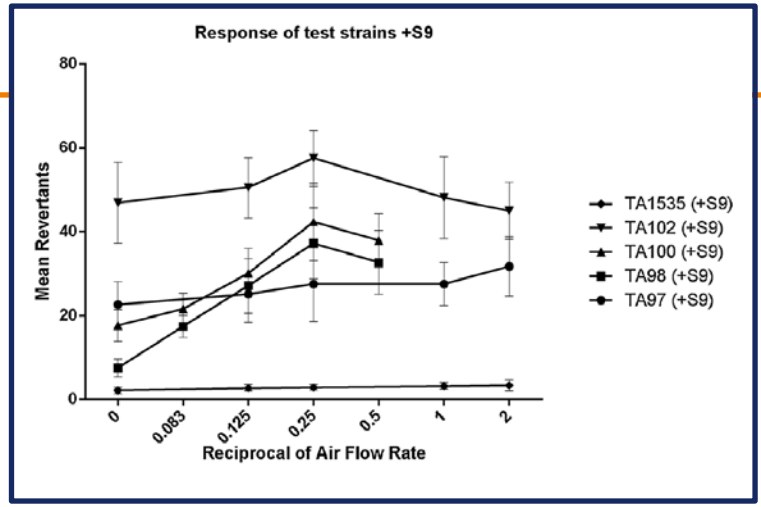
Whole Aerosol Ames Assay*

Validation Results (n=6)

Table 3
Responses of test strains to whole smoke from 3R4F cigarette.

		Strain (activation condition)[a]									
Air Flow Rate (L/min)	Reciprocal of Air Flow Rate	TA97 (−S9)	TA97 (+S9)	TA98 (−S9)	TA98 (+S9)	TA100 (−S9)	TA100 (+S9)	TA102 (−S9)	TA102 (+S9)	TA1535 (−S9)	TA1535 (+S9)
Untreated Control		15.9 ± 6.3	21.2 ± 5.8	4.7 ± 1.7	7.6 ± 2.6	20.8 ± 2.8	15.6 ± 2.7	29.2 ± 5.0	42.0 ± 9.1	3.4 ± 2.4	2.1 ± 0.7
AAI	0	13.8 ± 3.8	22.6 ± 5.5	5.5 ± 1.6	7.5 ± 2.1	22.1 ± 3.5	17.6 ± 3.8	33.2 ± 6.7	46.9 ± 9.6	3.1 ± 1.7	2.1 ± 0.8
12	0.083	NT	NT	NT	17.4 ± 2.6	NT	21.6 ± 3.7	NT	NT	NT	NT
8	0.125	13.1 ± 4.5	25.1 ± 4.5	4.4 ± 1.4	27.2 ± 8.9	20.5 ± 3.0	30.1 ± 3.4	29.9 ± 5.3	50.5 ± 7.2	3.1 ± 1.8	2.7 ± 0.8
4	0.25	17.1 ± 9.8	27.5 ± 9.0	6.1 ± 1.7	37.2 ± 8.5	23.7 ± 3.2	42.3 ± 9.2	33.8 ± 10.0	57.5 ± 6.7	3.9 ± 2.6	2.8 ± 0.7
2	0.5	NT	NT	NT	32.7 ± 7.6	NT	37.9 ± 6.4	NT	NT	NT	NT
1	1.0	15.6 ± 7.8	27.5 ± 5.1	4.3 ± 1.5	NT	22.8 ± 6.8	NT	31.4 ± 8.0	48.1 ± 9.8	3.2 ± 2.1	3.2 ± 0.8
0.5	2.0	16.5 ± 4.6	31.7 ± 7.1	3.6 ± 0.7	NT	22.0 ± 4.3	NT	23.6 ± 6.6	45.0 ± 6.8	2.7 ± 1.2	3.3 ± 1.4

NT–not tested.
[a] Mean ± SD for 6 experiments, except for TA98 + S9 where 8 experiments were conducted.



*Fowler et al. (2018) Development, qualification, validation and application of the Ames test using a VITROCELL® VC10 ® smoke exposure system. *Tox Reports*, 5; 542-551

Whole Aerosol Ames: High Throughput

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Aerosol Generation and Exposure Systems

Aerosol Generation Systems

- Increased variety of commercial aerosol generating and exposure systems available
  - Koerber (Borgwaldt), CH Technologies, Cerulean, SCIREQ, Vitrocell®
- Industry and laboratory developed systems
  - SEIVS (Imperial)
  - Havel et al. (2017) *Nicotine & Tobacco Research*, 1224–1231
  - Chandiramohan et al. (2021) *ERJ Open Res*; 7: 00705-2020
- Adaptability to variety of tobacco products
  - Single-port, linear, rotary
  - Pad-collection, impingers, whole aerosol exposure

Cerulean SPS-Shisha
Cerulean.com

LM4E Modular Vaping Machine
Borgwaldt.com

CH Technologies JB2090
chtechusa.com

Vitrocell VC1:
Vitrocell.com

Vitrocell 6/48: Vitrocell.com

Cultex-technology.com

expoCube®: SCIREQ.com

Vitrocell® VC 1/7: Vitrocell.com

Klus et al. (2016) Cigarette Mainstream Smoke: The Evolution of Methods and Devices for Generation, Exposure and Collection. *Beiträge zur Tabakforschung*; 27,4 DOI: 10.1515/cttr-2016-0015

Behrsing et al. (2017) *In Vitro* Exposure Systems and Dosimetry Assessment Tools for Inhaled Tobacco Products: Workshop Proceedings, Conclusions and Paths Forward for In Vitro Model Use. *ATLA* 45, 117–158.

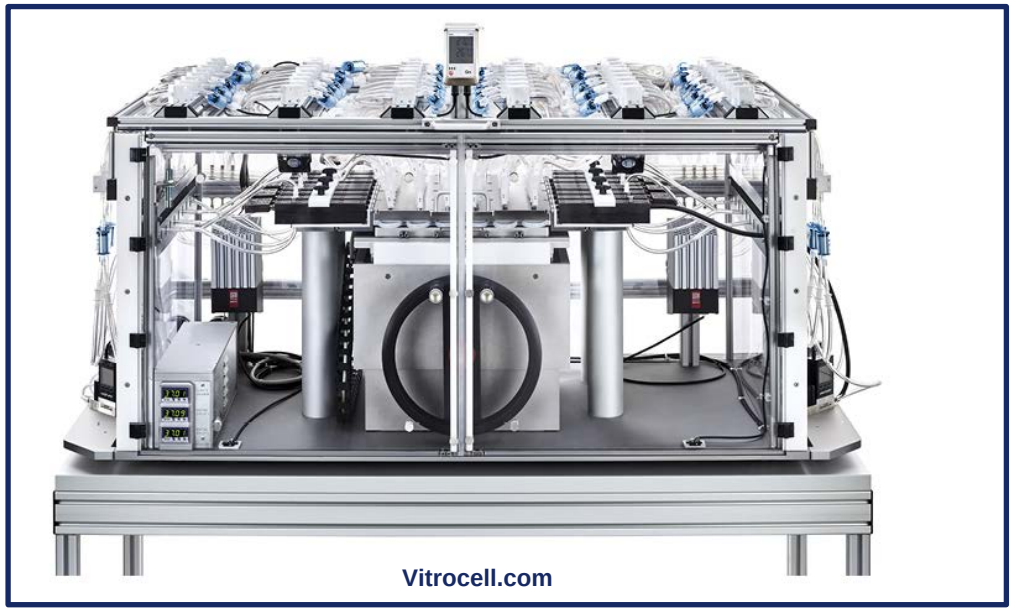
Whole Aerosol Ames Assay

Limitations

- Standard Ames exposure modules limited to 3 plates per dose per exposure
- Vitrocell® VC10® rotary smoke machine only allows 4 smoke concentrations per experiment (ISO regimen, with 8 second puff exhaust)
- Decreases to 2 smoke concentrations per experiment if using ISO intense regimen (ISO 20778; 2018)

Need to increase throughput if necessary to conduct a comprehensive Ames study

Vitrocell® Ames 48 Exposure Module



Whole Aerosol Ames 48 Module Validation*

Ames 48 Module Aerosol Deposition

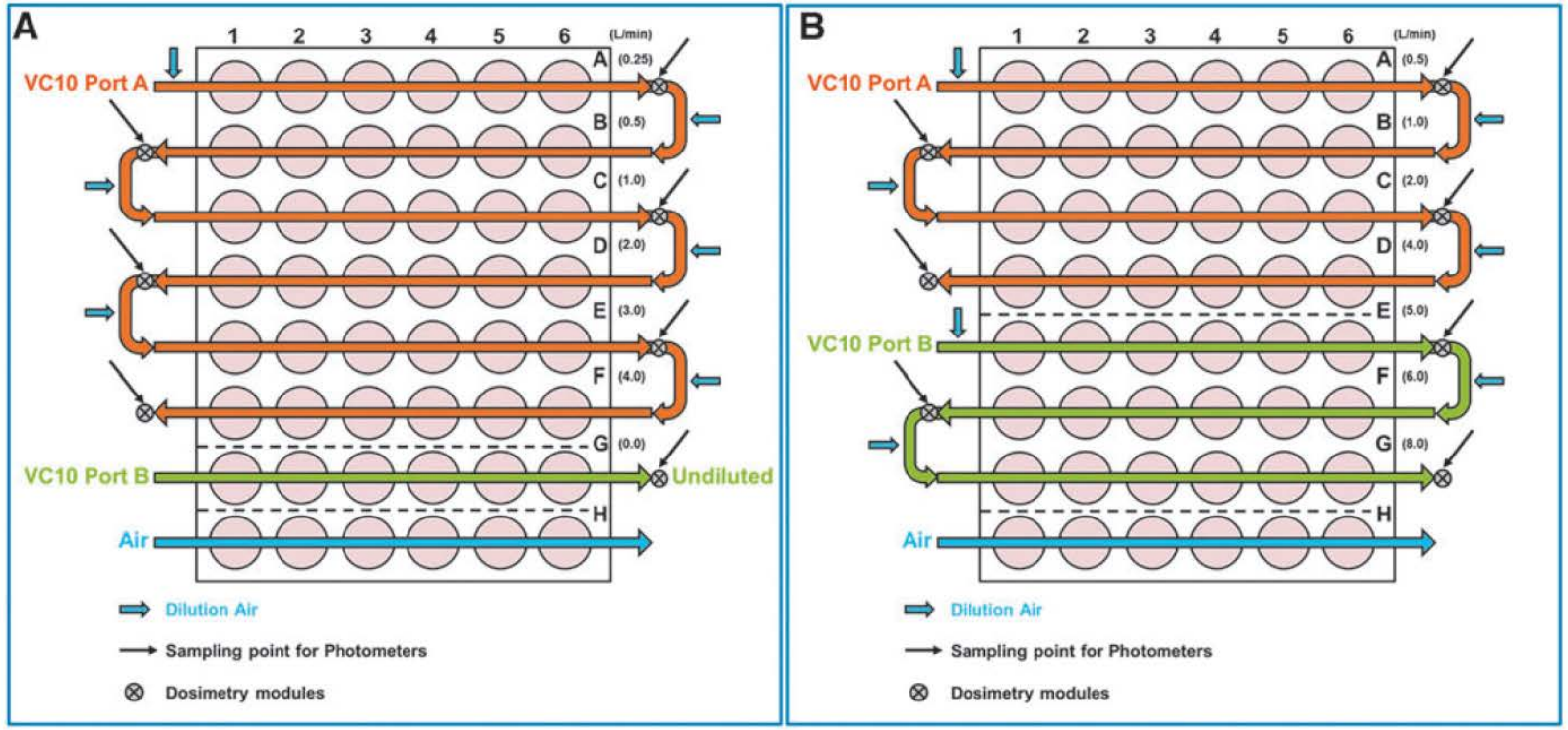
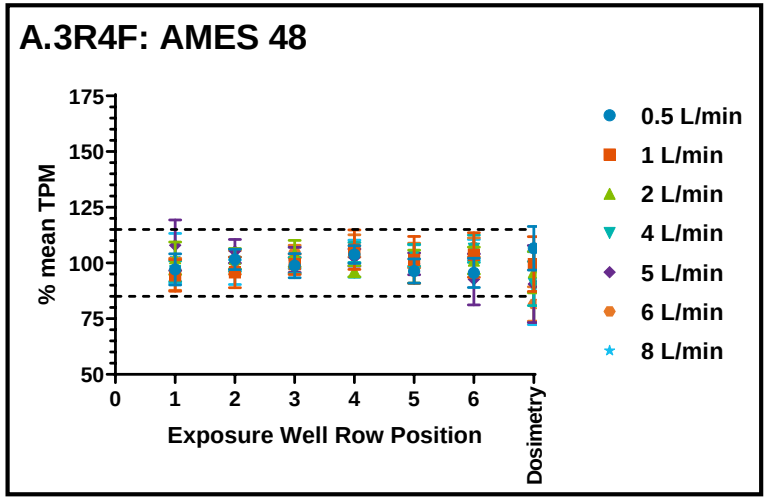


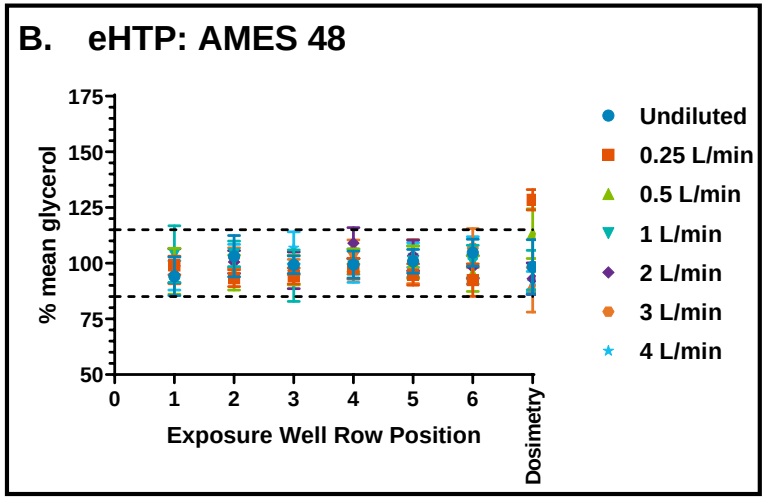
FIG. 2. **(A)** Schematic representation of airflows for eHTP and ENDS; diluting airflows from rows A to F corresponding to 0.25 to 4 L/min and undiluted airflow in row G. **(B)** Schematic representation of airflows for combustible cigarettes; diluting airflows from rows A to D for 0.5 to 4 L/min, and rows E to G for 5 to 8 L/min. eHTP, electrically heated tobacco product; ENDS, Electronic Nicotine Delivery System.

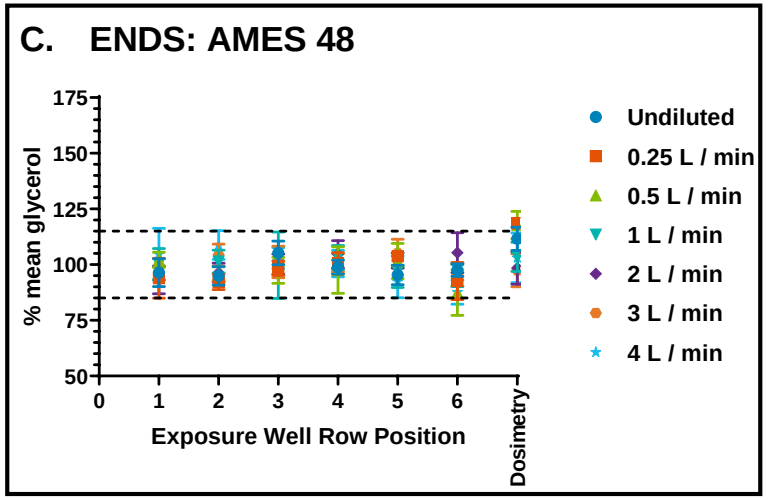
*Keyser et al. (2022) Characterization of Aerosol Deliveries from Combustible Cigarettes, Heated Tobacco Products, and Electronic Nicotine Delivery Systems Using the Vitrocell® Ames 48, *Applied In Vitro Toxicology* 8 (2), 39-49

Whole Aerosol Ames 48 Module Validation*

Ames 48 Module Aerosol Deposition:





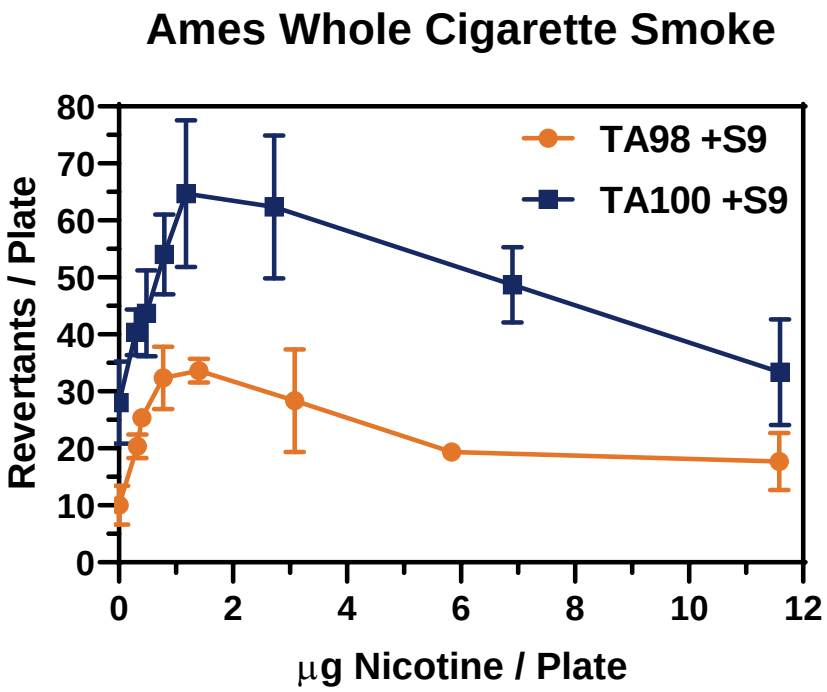


Deposition of 3R4F TPM (A), eHTP (B) and ENDS (C) glycerol within each well position per row (well 7 = dosimetry module) was determined by extrapolation of the Ex 355 / Em 485 fluorescence of smoke-exposed DMSO and glycerol deposition in PBS to their respective standard curves. Values are presented as % of the overall mean (± SD) for each air dilution. Dashed lines (--) are ± 15%.

*Keyser et al. (2022) Characterization of Aerosol Deliveries from Combustible Cigarettes, Heated Tobacco Products, and Electronic Nicotine Delivery Systems Using the Vitrocell® Ames 48, *Applied In Vitro Toxicology* 8 (2), 39-49

Whole Aerosol Ames 48 Module Validation

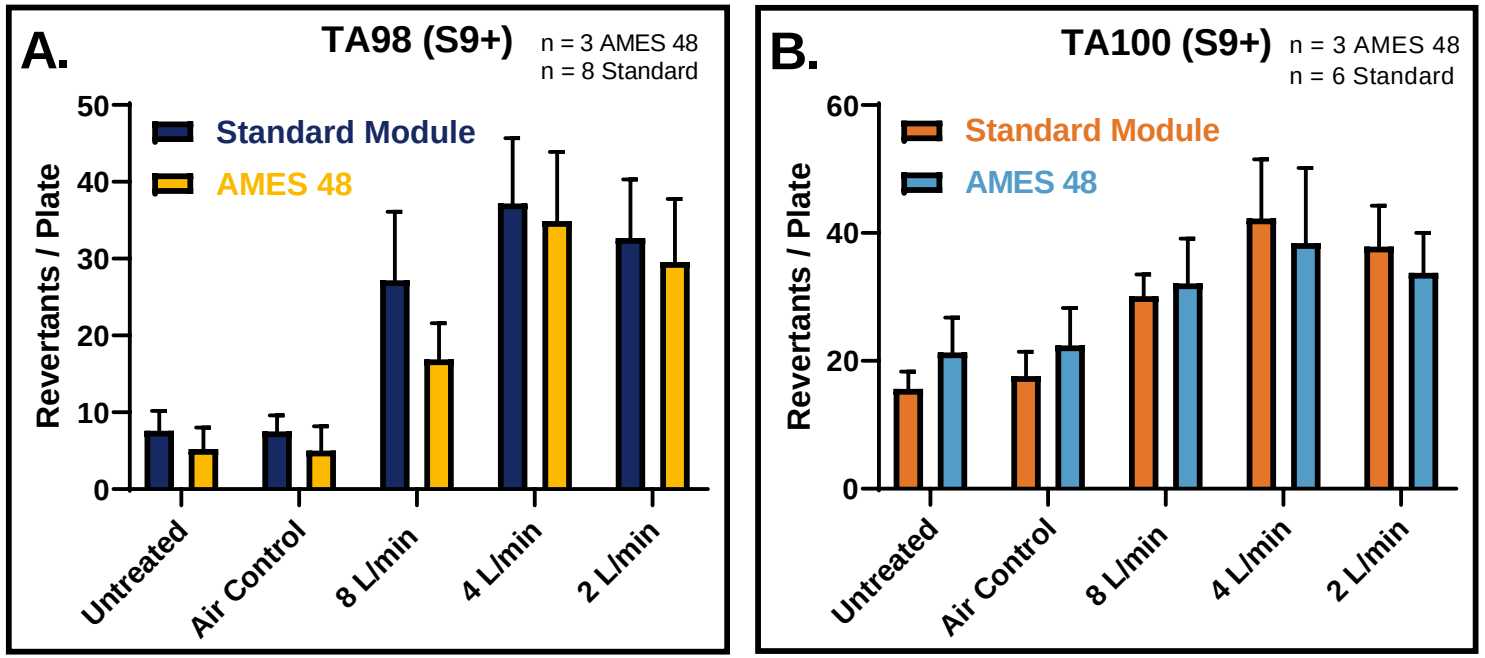
Ames 48 Module Ames Results (TA98 & TA100, +S9)



TA98 (+S9) and TA100 (+S9) were exposed to Marlboro whole smoke generated under HCI conditions from 2 cigarettes. Dose (µg Nicotine) determined by analysis of smoke-exposed PBS

Whole Aerosol Ames 48 Module Validation

Ames 48 Module Ames Results Compared to Standard Modules:



TA98 (A) and TA100 (B) were exposed to 3R4F whole smoke generated under ISO conditions (ISO 3308; 2012) from 8 cigarettes. Standard module data taken from Fowler et al. (2018). Revertant counts compared at equivalent dilution air flows.

System Characterization

Understand your system, from aerosol generation, collection or delivery (published examples)

- Adamson et al. (2018) Characterisation of the Borgwaldt LM4E system for in vitro exposures to undiluted aerosols from next generation tobacco and nicotine products (NGPs) *Food and Chemical Toxicology* 113, 337–344
- Adamson et al. (2014) An inter-machine comparison of tobacco smoke particle deposition in vitro from six independent smoke exposure systems. *Toxicology in Vitro* 28, 1320–1328
- Aufderheide et al. (2017) Improvement of the CULTEX® exposure technology by radial distribution of the test aerosol. *Experimental and Toxicologic Pathology* 69, 359–365
- Behrsing et al. (2018) Characterization of a Vitrocell VC1 Using Nicotine Dosimetry: An Essential Component Toward Standardized In Vitro Aerosol Exposure of Tobacco and Next Generation Nicotine Delivery Products. *Applied In Vitro Toxicology* 4:2, 159-166
- Keyser et al. (2022a) Characterization of Aerosol Deliveries from Combustible Cigarettes, Heated Tobacco Products, and Electronic Nicotine Delivery Systems Using the Vitrocell® Ames 48, *Applied In Vitro Toxicology* 8 (2), 39-49
- Keyser et al. (2022b) Characterization of smoke and aerosol deliveries from combustible cigarettes, heated tobacco products and electronic nicotine delivery systems in the Vitrocell® Mammalian 6/48 exposure module, *Toxicology Reports* 9, 1985-1992.
- Lucci et al. (2018) Characterization and modeling of aerosol deposition in Vitrocell® exposure systems - exposure well chamber deposition efficiency. *Journal of Aerosol Science* 123, 141–160
- Oldham et al. (2020) Deposition efficiency and uniformity of monodisperse solid particle deposition in the Vitrocell® 24/48 Air–Liquid-Interface in vitro exposure system, *Aerosol Science and Technology* 54:1, 52 – 65
- Thorne et al. (2021) An interlaboratory in vitro aerosol exposure system reference study. *Toxicology Research and Application* 5, 1–16
- Thorne et al. (2013) Characterization of a Vitrocell® VC 10 in vitro smoke exposure system using dose tools and biological analysis. *Chemistry Central Journal* 7, 146
- Thorne et al. (2018) Extreme testing of undiluted e-cigarette aerosol in vitro using an Ames air-agar-interface technique *Mutation Research*, 828
- Chandiramohan et al. (2021) Development and validation of an open-source, disposable, 3D-printed *in vitro* environmental exposure system for Transwell® culture inserts. *ERJ Open Res*; 7 (1): 00705-2020

Whole Aerosol Ames: Recommendations

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Recommendations

- The aerosol generation, delivery and exposure system should be well characterized and demonstrate reproducibility.
- Utilize smoke machines adaptable for different products, performance requirements, controlled parameters, and any regulatory requirements
  - Computer controlled for all puffing parameters
  - Confirm and document machine performance
- Document product conditioning, puffing parameters and testing atmosphere
  - Standard product conditioning and testing atmosphere parameters (ISO 3402; 1999)
  - Standard puffing regimens (e.g., ISO[1], Health Canada[2]) should be incorporated where possible, and nonstandard regimens should be described to a level of detail to allow replication.

[1]ISO 20778, 2018; Cigarettes-Routine analytical cigarette smoking machine-Definitions and standard conditions with an intense smoking regime

[2]ISO 20768, 2018; Vapour products-Routine analytical vaping machine-Definitions and standard conditions

Recommendations: Dosimetry*

- Methods used by laboratories to quantify delivered dose should be thoroughly documented, standardized, and/or validated and be capable of adaptation to a variety of *in vitro* whole-aerosol exposure systems to allow comparisons across laboratories, other *in vitro* and ALI exposure systems, and *in vivo* and human studies.
- System characterization should be conducted using identical aerosol and experimental conditions (temperature, humidity, dilutions, etc.) to demonstrate acceptable uniformity of aerosol delivery within the *in vitro* exposure system.
- Characterization should also include deposition efficiency and uniformity across the cell culture insert.
- Dosimetry parameters should be representative of the aerosol being assessed biologically *in vitro*.
- Measured aerosol constituent(s) should represent the specific aerosol phase (particle or gas vapor phase) being studied, or in which phase the biological activity is thought, or found to reside.
- Dosimetry parameter(s) must be measured in a representative manner during each experimental run using chemical, physical, and *in vitro* methods.

*Miller-Holt et al. (2022) Key challenges for *in vitro* testing of tobacco products for regulatory applications: Recommendations for dosimetry. *Drug Test Anal*.; 1-14

Issues Needing Additional Research*

- Which aerosol constituents adequately represent each aerosol phase (particulate and gas vapor) from next generation products and combustible cigarettes for specific biological endpoints.
- Any analytical methods developed should be simple and have as minimum an associated cost as minimal as possible to allow incorporation by a wide variety of laboratories.
- Collaborative studies are essential to facilitate interlaboratory comparisons for standardization purposes.
- Determine the differences in the hardware profiles for the variety aerosol generating systems and understand how those differences may impact aerosol constituents
  - Aerosol aging
  - Constituent losses within the system
  - Run-to-run and day-to-day variability

*Miller-Holt et al. (2022) Key challenges for *in vitro* testing of tobacco products for regulatory applications: Recommendations for dosimetry. *Drug Test Anal*.; 1-14

Questions?

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