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Development, qualification, validation and application of the Ames test using a VITROCELL® VC10® smoke exposure system

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ABSTRACT

The Ames test has established use in the assessment of potential mutagenicity of tobacco products but has generally been performed using partitioned exposures (e.g. total particulate matter [TPM], gas vapor phase [GVP]) rather than whole smoke (WS). The VITROCELL®VC10® smoke exposure system offers multiple platforms for air liquid interface (ALI), or air agar interface (AAI) in the case of the Ames test exposure to mimic *in vivo*-like conditions for assessing the toxicological impact of fresh WS in *in vitro* assays.

The goals of this study were to 1) qualify the VITROCELL®VC10® to demonstrate functionality of the system, 2) develop and validate the Ames test following WS exposure with the VITROCELL®VC10® and 3) assess the ability of the Ames test to differentiate between a reference combustible product (3R4F Kentucky reference cigarette) and a primarily tobacco heating product (Eclipse). Based on critical function assessments, the VITROCELL®VC10® was demonstrated to be fit for the purpose of consistent generation of WS. Assay validation was conducted for 5 bacterial strains (TA97, TA98, TA100, TA1535 and TA102) and reproducible exposure–related changes in revertants were observed for TA98 and TA100 in the presence of rat liver S-9 following exposure to 3R4F WS. In the comparative studies, exposure-related changes in *in vitro* mutagenicity following exposure of TA98 and TA100 in the presence of S9 to both 3R4F and Eclipse WS were observed, with the response for Eclipse being significantly less than that for 3R4F ($p < 0.001$) which is consistent with the fewer chemical constituents liberated by primarily-heating the product.

1. Introduction

Regulatory requirements for nonclinical test data to assess potential health effects of tobacco and related products have been implemented relatively recently [1–4]. However, nonclinical testing has historically been, and continues to be, a component of RAI Services' (RAIS) product stewardship testing strategy as part of the company's guiding principles. One component of this strategy, the Ames test, has a long established use in several regulatory sectors including screening of chemicals [5], medical devices [6], pharmaceuticals [7], and for modified risk tobacco products [4].

The bacterial reverse mutation (Ames) test [8] utilizes bacteria tester strains (*Salmonella typhimurium* or *Escherichia coli*) engineered to be deficient in the synthesis of an essential amino acid (histidine or tryptophan, respectively). The tester strains are therefore considered auxotrophs for an essential amino acid and, after exposure to a mutagen, this provides a method of selection for those bacteria that have mutated, or reverted back, to being autotrophic (self-feeding) for that specific essential amino acid required for growth. The Ames test typically uses a series of at least five tester strains of *Salmonella typhimurium* and/or *Escherichia coli* in order to detect deletion, base substitution or frameshift mutations, depending on the tester strain's engineered genotype.

Chemical substances sometimes require metabolic activation in order to become mutgenic. As the metabolic enzymes of bacteria used in the Ames test differ substantially from those in mammals, an exogenous metabolic activation system prepared from liver homogenate (S-9) is often added to mimic mammalian metabolism. In the standard Ames test, bacterial cells are exposed to the test substance (liquid or solid) in the presence or absence of liver homogenate (S-9) using either

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plate incorporation or preincubation methods followed by two or three days of incubation at 37 °C, after which revertant colonies are counted and compared to the number of spontaneous revertant colonies for solvent controls to establish the mutagenic response resulting from the test compound.

Although methods are well defined for the testing of liquids and solids using the Ames test [5,7], no such guidelines exist for the testing of complex gaseous mixtures, such as cigarette whole smoke, which provides many challenges, both technical and biological. Cigarette whole smoke is made up of both a particulate fraction (total particulate matter (TPM)) and a vapor phase component. This whole smoke mixture, consisting of more than 7000 chemicals [9], makes testing by standard methods extremely difficult, and to date, most testing has focussed on testing TPM using standard methodology in several toxicological endpoints [10–12]. These endpoints include the Ames reverse mutation test, the *in vitro* micronucleus assay (IVMN), the neutral red uptake assay (NRU) and the Mouse Lymphoma Assay (MLA) [11,13–15]. These assays are consistent with many of the guidelines developed by the International Conference on Harmonization [7], the Committee on Mutagenicity [16] and, for tobacco smoke, Health Canada [17]. In addition, the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) *in vitro* sub-group (previously 'taskforce') has also recommended a similar approach for analysis of tobacco products [12].

Testing of TPM has demonstrated consistent concentration related increases in genotoxicity and cytotoxicity in several standard assays (*e.g.* Ames, IVMN, MLA, NRU) [13,18–20]. However, the particulate phase represents only a small fraction of the whole smoke that is generated when a cigarette is combusted or heated [21]. Testing of only this phase does not account for the gases or semi volatiles found in the vapor phase of cigarette whole smoke, which makes up the majority of the smoke fraction [22,23] and contains known toxicants that are responsible for adverse health effects [21,24,25]. Previous work has been undertaken to test a more representative sample of whole cigarette smoke by bubbling cigarette smoke through phosphate buffered saline (PBS) or culture media and then testing both particulate and vapor fractions (either independently or as a mixture) [11,26]. However, this still does not account for insoluble compounds or short-lived chemicals resulting from combustion. Therefore, within the tobacco industry, there is increasing demand for toxicological testing of whole smoke and aerosol from next generation tobacco products. As cited in Kilford et al. [27], the absence of validated methodology was noted by the Committee on Mutagenicity in 2009 [28]. Due to the complexity of potential chemical interactions within and between phases, development of this type of testing is considered to be of paramount importance. Furthermore, improving *in vitro* methods for assessing the genotoxicity of chemicals within whole tobacco smoke is consistent with the general aims of TOX21 [29] for improving toxicology testing in the 21st century.

Generation and testing of whole smoke is technically challenging and over recent years a great deal of focus has been placed on the development of cigarette whole smoke exposure systems [30–34], which capture both phases of tobacco smoke together and presents a more relevant test compound for the assessment of human risk. Prior to 2010, RAIS had traditionally used an in-house cigarette smoke exposure technology. This system provided exposures in primarily submerged culture systems, and demonstrated reproducible results in a concentration-dependent manner for several test systems. However, the cigarette smoke exposure technology exposures required a large number of cigarettes, significant set-up and exposure time and the system was not commercially available. **RAIS** therefore evaluated alternative *in vitro* whole smoke systems with the introduction of *in vitro* smoking machines (*e.g.* Borgwaldt RM20S, Burghart Mimic Smoker and the VITROCELL® VC10® smoking robot), paired with exposure modules that allow exposure of cells to whole smoke at the air-liquid interface (ALI) or air-agar interface (AAI). The VITROCELL® VC10® smoking robot was selected as it met the user-required specifications that included, but were not limited to, controlling smoking parameters, applying various smoking regimes, and providing direct exposure of *in vitro* test systems at ALI/AAI. The VITROCELL® VC10® smoking robot uses a constant flow of compressed air to dilute cigarette whole smoke. A sample of this diluted smoke is pulled, by vacuum, into the exposure module where it is delivered to individual chambers [35]. The flow rate of the diluting air can be adjusted to alter the concentration of smoke or aerosol delivered.

The primary aims of this study were to demonstrate the suitability of the VITROCELL® VC10® smoking robot for exposures at the air liquid or agar interface and then develop an adapted exposure methodology, based on an existing Ames protocol, for the evaluation of cigarette whole smoke. Adaptation of the methodology is required as the existing Ames protocols are based around exposing bacteria cultures in solution; therefore, exposure procedures have been modified to allow assessment of whole smoke at the AAI using bacterial tester strains. The aims were accomplished *via* operational and performance qualification protocols followed by execution of development, pre-validation and validation protocols described herein.

The standard Ames test typically uses a battery of 5 tester strains: 1) *S. typhimurium* TA98, 2) *S. typhimurium* TA100, 3) *S. typhimurium* TA1535, 4) *S. typhimurium* TA102 or *E.coli* WP2 uvrA or *E.coli* WP2 uvrA (pKM101) and 5) *S. typhimurium* TA97 or TA97a or TA1537. In this work, six tester strains (*Salmonella typhimurium* TA97, TA98, TA100, TA102, TA1535 and TA1537) were initially evaluated during method development. Due to the low spontaneous revertant rate for TA1537, five strains (TA97, TA98, TA100, TA1535 and TA102) were taken through to intra-laboratory method validation. Two strains (TA98 and TA100) were selected for use in the whole smoke comparative assay as these strains responded well to testing with whole smoke, and are commonly used in the testing of whole smoke condensate, TPM, pharmaceuticals and medical devices, and evaluate the types of DNA damage (basepair mutation and frameshifts) which are considered to be relevant for tobacco whole smoke [36].

The findings from this study demonstrated the capability of the AAI exposure system used in tandem with the Ames test to detect differences in the mutagenicity of whole smoke generated from different products.

2. Materials and methods

2.1. Tester strains

TA97 was originally obtained from Professor Bruce Ames; TA98, TA1535 and TA1537 were originally obtained from the UK National Collection of Type Cultures (NCTC); TA100 and TA102 were originally obtained from Covance Laboratories Inc., USA. Inocula were taken from master plates or vials of frozen cultures which had been checked for strain genotypes of histidine dependence, *rfa* mutation (cell wall permeability), *uvrB* mutation (error-prone DNA repair) and resistance to appropriate antibiotics, according to established methods [8,37].

2.2. Chemicals and reagents

Chemicals and reagents were obtained from the following suppliers: nutrient broth from Oxoid Ltd. (Basingstoke, UK), water (CAS No.7732-18-5) from Baxter (Newbury, UK), glucose (CAS No. 50-99-7), magnesium sulphate (CAS No. 7487-88-9), potassium chloride (CAS No. 7447-40-7) and sodium phosphate buffer from Fisher Scientific (Loughborough, UK), magnesium chloride (CAS No. 7786-30-3) from VWR (Radnor, PA, USA), citric acid (CAS No. 77-92-9), d-biotin (CAS No. 58-85-5), glucose-6-phosphate (CAS No. 3671-99-6), histidine (CAS No. 71-00-1) and sodium ammonium phosphate tetrahydrate (CAS No. 7783-13-3) from Sigma-Aldrich Co. Ltd. (Poole, UK), nicotinamide adenine dinucleotide phosphate (NADP) (CAS No. 698999-85-8) and rat liver S9 from Molecular Toxicology Inc. (Boone, NC, USA), Bactoagar from Becton Dickinson and Co. (Oxford, UK), dipotassium phosphate (CAS No. 7758-11-4) from Camlab (Cambridge, UK).

Positive control chemicals included 9-aminoacridine (CAS No. 90-45-9), 2-aminoanthracene (CAS No. 613-13-8), benzo[*a*]pyrene (CAS No. 50-32-8), mitomycin C (CAS No. 50-07-7), 2-nitrofluorene (CAS No. 607-57-8) and sodium azide (CAS No. 26628-22-8) all from Sigma-Aldrich (Poole, UK). Antibiotics comprised ampicillin (CAS No. 69-53-4) and tetracycline (CAS No. 60-54-8) from Sigma-Aldrich (Poole, UK).

2.3. Cigarettes

3R4F reference cigarettes were obtained from the University of Kentucky, Kentucky, USA. Eclipse cigarettes were obtained from R J Reynolds Tobacco Company. Prior to smoking, cigarettes were conditioned for at least 48 h and no more than 10 days at 22 ± 1 °C and 60 ± 3% relative humidity, according to the International Organisation for Standardisation (ISO) guideline 3402:2000 [38].

Due to the non-contact lighting method of the VITROCELL® VC10® smoking robot and the tobacco heating design of the Eclipse cigarette, manual lighting with a butane flame was used to ignite the carbon rod of the Eclipse cigarette.

2.4. Smoke generation with VITROCELL® VC10® smoking robot

Whole smoke was generated with a VITROCELL® VC10® smoking robot using the International Organization for Standardization (ISO) 3308 puff regime (ISO regime; 35 mL volume, 2 s duration, 60 s puff interval) [39] or the Health Canada Intense puff regime (HCI regime; 55 mL volume, 2 s duration, 30 s puff interval, 100% vent blocking) [40] with 5 ml/min vacuum. Different concentrations of whole smoke were achieved by altering the diluting air flow.

2.5. Qualification of the VITROCELL® VC10® smoking robot

The VITROCELL® VC10® smoking robot, dilution system and exposure modules were supplied by VITROCELL® Systems GmbH, Waldkirch, Germany. The VC10® is a rotary style smoking machine with a single piston that delivers tobacco smoke into an airflow dilution system. Smoke dilution is achieved *via* mixing cigarette whole smoke with a continuous flow of compressed air within a stainless steel dilution bar. A subsample of this diluted smoke is then pulled *via* vacuum into stainless steel exposure modules. Different concentrations of smoke can be achieved by altering either the diluting airflow rate (L/min) or the vacuum rate (mL/min). As it is the vacuum rate that dictates the flow of smoke over the bacteria on the surface of the agar plate, the decision was made to maintain this vacuum flow at a fixed rate of 5 mL/min for all experiments and to alter the diluting airflow (L/min) to adjust smoke concentrations.

In order to ensure that the smoking robot and associated equipment required for whole smoke exposure were functioning as required, the system was subject to qualification by manner of installation, operational and performance qualification. Installation and operational qualification (IOQ) was performed to establish that the VITROCELL® VC10® smoking robot was installed in an environment that was equipped and suitable for the operation of the test system and to demonstrate, through testing and documentation, that the equipment was suitable for its intended use throughout the operating range. Following successful completion of IOQ, a performance qualification (PQ) was conducted. PQ was performed in order to establish that all aspects of the VITROCELL® VC10® smoking robot performed as intended, met predetermined acceptance criteria and were operating to the user specific requirements. The specific assessments and criteria for IQ, OQ and PQ have been previously described [41].

2.6. Method development and validation

Pre-validation of the Ames test was undertaken with the following parameters assessed to determine the experimental model: 1) Evaluation of candidate test strains (TA97, TA98, TA100, TA102, TA1535 and TA1537) for suitability in the system; 2) assessment of stability of bacterial cell cultures under flowing air conditions; 3) generation of historical ranges for spontaneous revertants in each strain in AAI controls (*i.e.* clean air controls) and 4) determination of smoke concentrations (dilution air flow rates) and appropriate positive controls for use in subsequent method validation and product comparison studies.

Pre-validation of the experimental conditions included optimization of the incubation time to achieve exponential growth prior to exposure (optical density measurements and plating for viability conducted hourly from 4 to 11 h after inoculation of bacterial cells into nutrient broth cultures) for each candidate test strain. The following variables were also investigated for each candidate test strain in the absence and presence of a rat liver metabolic activation system (S9): viability of bacterial cells exposed to dilution air (flowing at up to 12 L/min) for 64 min; spontaneous revertant frequencies in AAI controls (using 0.2 L/min airflow and vacuum rate of 5 mL/min on all dilution bars) and smoke exposures with dilution air flow rates of 12, 8, 4, 1 and 0.5 L/min were evaluated, using triplicate plates per concentration and S9 condition for each test strain in 2 independent experiments. Once the pre-validation work was completed, six independent experiments were conducted in the validation protocol and five independent experiments were conducted in the product comparison protocol according to the procedures outlined in the following sections.

2.7. Bacteria culture and conditions

For all experiments, bacteria were cultured at 37 ± 1 °C for 8 h in nutrient broth containing antibiotics as required. Treatments began within 2 h of the end of the overnight culture incubation. Incubation was carried out with shaking at 120 rpm in an anhydric incubator, set to turn on using a timer switch. At least 10^9 bacteria/mL (approximately 2×10^7 bacteria/plate - scaled down from standard [100 mm plate] Ames test based on surface area) were plated, where possible onto 35-mm Vogel Bonner agar plates, following standard methods [42]; seeded plates were dried in an anhydric incubator at 37 ± 1 °C prior to treatment. For all experiments, untreated controls (UTC), AAI controls and positive controls were included. UTC and positive control cultures were left at room temperature for the duration of the treatment. AAI controls were exposed to a dilution air flow of 0.2 L/min. Data from cells subject to whole smoke treatment were compared to the AAI controls. All experiments were performed in the absence and presence of S9, which was obtained from Molecular Toxicology Incorporated, USA, where it was prepared from male Sprague Dawley rats induced with Aroclor 1254, and used at a final concentration of 10%.

2.8. Whole smoke generation for comparative study

The VITROCELL® VC10® smoking robot was used to expose bacterial cells to cigarette whole smoke generated from 3R4F reference cigarettes and Eclipse cigarettes with the following parameters:

Puff volume: 35 mL; Puff duration: 2 s Puff frequency: 60 s; Puff profile: Bell shaped

<u>Exposure Parameters:</u>

Puff exhaust duration: 8 s; Number of puffs per 3R4F cigarette: 8; Number of puffs per 3R4F exposure: 64 (from 8 cigarettes); Length of 3R4F exposure: 64 min

Number of puffs per Eclipse cigarette: 14

Number of puffs per Eclipse exposure: 56 (from 4 cigarettes)

Length of Eclipse exposure: 56 min

For each experiment, triplicate plates were exposed in VITROCELL®

Ames stainless steel exposure modules. The trumpet height in the modules was set to 2 mm above the agar (6 mm above the base). Cigarette whole smoke was diluted *via* a constant stream of air and delivered into the exposure modules with a fixed vacuum of 5 mL/min. Different concentrations of smoke were achieved by varying the flow rate of the diluting air in order to achieve a response in the test system or to the operating limits of the smoking robot. Modules were exposed as detailed above. Following exposure, all plates were dried, wrapped in parafilm (to prevent the plates from overdrying), inverted and incubated at 37 ± 1 °C for up to 3 days.

Samples to assess sterility (for S9 mix and phosphate buffer) (pre- and post-exposure) and viability (serial dilutions to final levels of 10^{-5} and 10^{-6} of the test culture prior to exposure) were taken and plated out on nutrient agar plates for scoring after incubation for 2 days at 37 ± 1 °C. Viability plates were used to calculate whether appropriate numbers of bacterial cells had been plated (at least 2×10^7 per plate). Toxicity was assessed by examination of the background bacterial lawn (thinning or presence of microcolonies). Revertant colonies were generally counted electronically (Sorcerer Colony Counter, Perceptive Instruments) or manually, where confounding factors such as low spontaneous revertant frequencies affected the accuracy of the automated counter.

2.9. Evaluation and acceptance criteria

Each individual experiment was considered valid if the following criteria were met:

1. For AAI control treatments, revertant counts fell within the historical control ranges
2. The positive control chemicals induced increases in revertant numbers over the concurrent AAI controls of ≥2-fold confirming discrimination between different strains and an active S9 preparation

For valid data, the test material was evaluated as mutagenic if the following criteria were met:

1. A concentration-related increase in revertant numbers was observed which was significant ($p \leq 0.01$) when assessed using Dunnett's test
2. The positive trend / effects described above were reproducible

The test article was considered to be non-mutagenic if none of the above criteria were met.

2.10. Statistical analysis

The mutagenic response was defined as the slope of the linear portion of the concentration-response curve. The linear portion of the concentration-response curve was determined by fitting a generalized linear model with Poisson distribution and identity link function, with the number of revertants as the response and the reciprocal of the airflow (L/min) as a fixed effect. The dose level for the AAI control was taken as zero. In addition, a separate parameter was fitted in the model for the "top dose" (highest reciprocal airflow). The portion of the concentration-response curve was considered to be linear where the "top dose" term of the model was non-significant ($p \geq 0.05$) or greater than zero. The slope from the linear portion of the concentration-response curve was then determined by fitting a generalized linear model with Poisson distribution and identity-link function, with the number of revertants as the response and the reciprocal of the airflow (L/min) as a fixed effect. At least three non-zero concentrations were used in generating the slope value.

For the product comparison protocol, the mean slopes from 5 experiments were compared using a two-sample *t*-test; this comparison was performed separately for each bacterial strain and activation condition. Levene's test for variances between the cigarettes was performed and where this showed evidence of heterogeneity ($p \leq 0.01$), the slopes were rank-transformed prior to analysis. Cigarettes were compared against each other in order to determine if there was a significant difference in their response in this test system.

3. Results

Through critical function and assay assessments *via* the installation, operational and performance qualification, the VITROCELL® VC10® smoking robot and associated whole smoke exposure equipment was deemed fit-for-purpose [41]. Whole smoke, generated using a VITROCELL® VC10® smoking robot, was evaluated for mutagenicity to bacterial cells using the Ames test. A subsequent comparison was made between whole smoke from a tobacco heating and a combustible cigarette.

3.1. Method development results

Growth curve assessments showed the appropriate incubation time to be 8 h for achieving exponential growth for all bacterial strains prior to plating (data not shown). Static and flowing air (up to 12 L/min) experiments were conducted at a vacuum rate of 5 mL/min for exposures up to approximately 64 min. The data from the static and flowing air experiments confirmed that there was no difference in revertant numbers between the static or flowing air exposures and the untreated controls (data not shown). Exposures to flowing air for up to 64 min were therefore considered acceptable for subsequent experiments. Of the six Ames *Salmonella typhimurium* strains which were investigated during method development (Table 1), TA98, TA1535 and TA1537 showed low spontaneous revertant rates and were scored manually. TA97 was also scored manually due to the presence of microcolonies, which were not considered to be true revertants. For TA1535 and TA1537, the standard deviation between triplicate plates exceeded the mean revertant number on some occasions. Since TA97 is an acceptable alternative test strain to TA1537, strain TA1537 was not included for further method development.

Appropriate positive control treatment concentrations were determined for TA97, TA98, TA100, TA102 and TA1535 (duplicate experiments, 64 min exposure) in the absence and presence of S9 and are shown in Table 2:

Following initial exposures with whole smoke generated from 3R4F Kentucky Reference cigarettes and subsequent observations of toxicity (slight thinning of background bacterial lawn), airflow dilutions of 8, 4, 1 and 0.5 L/min were selected for subsequent method validation for strains TA97, TA98, TA100 and TA102, in the absence of S9, for TA97 and TA102 in the presence of S9 and for TA1535 in the absence and presence of S9. Concentrations of 12, 8, 4 and 2 L/min were selected for method validation with TA98 and TA100 in the presence of S9. Despite low and variable numbers of spontaneous revertants (which required manual scoring) which were obtained for strain TA1535 following

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.

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).

exposure to whole smoke in the method development, this strain was selected for inclusion in the method validation work since this strain is a requirement of OECD [5].

3.2. Validation results

Following completion of the method development study, six experiments were conducted with 3R4F cigarettes to confirm reproducibility of the adapted protocol using the selected strains (TA97, TA98, TA100, TA1535 and TA102). For each experimental day, a separate smoke run was performed prior to the biological exposure, and total particulate matter (TPM) was collected for analysis of nicotine and water as a check for consistency in the smoke generation (data not shown). The results of the method validation work are shown in Table 3 and Fig. 1. These data showed no notable response to 3R4F whole smoke for TA97 in the absence of S9 or for TA1535 and TA102 in the absence or presence of S9. TA97 in the presence of S9 showed some evidence of mutagenic activity of whole smoke from 3R4F cigarettes but the response was weak. Test strains TA98 and TA100 showed no evidence of mutagenicity of 3R4F whole smoke when tested in the absence of S9. In contrast, both TA98 and TA100 showed reproducible induction of increases in revertant frequencies in the presence of S9. Hence, these data were used for validation of appropriate statistical analysis methods and TA98 and TA100 were selected for use in the comparative experiments.

3.3. Statistical analysis

For each strain and experiment, the mutagenic response was defined as the slope of the linear portion of the concentration-response curve (Table 4). Analysis was performed using an identity link function, where the slope from the linear portion of the concentration-response curve was determined by fitting a generalized linear model with Poisson distribution and identity link function, with the number of revertants as the response and the reciprocal of the dose (L/min) as a fixed effect.

In addition, a separate parameter was fitted for the highest dose. The portion of the concentration-response curve was deemed to be linear where this parameter was non-significant ($p > 0.05$). The AAI control was included in the analysis.

Based on the point rejection approach of Bernstein and colleagues [43], it is considered that fitting the model to untransformed revertant counts is more appropriate for this type of data.

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

Air Flow Rate (L/min)	Reciprocal of Air Flow Rate	Strain (activation condition)[a]									
		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.

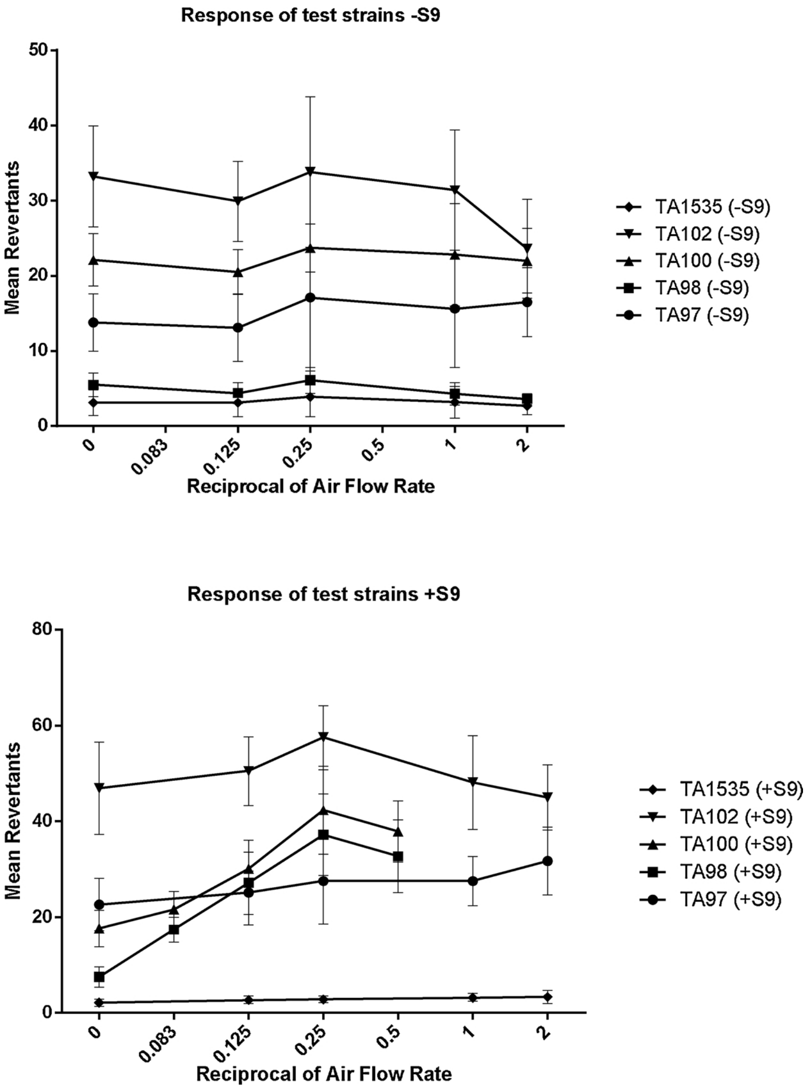

Fig. 1. Responses of 5 standard *Salmonella typhimurium* strains (TA97, TA98, TA100, TA1535 and TA102) to 3R4F whole smoke. The bacterial strains were exposed to whole smoke from 3R4F cigarettes over a concentration range of 0.083 to 2 (L/min)$^{-1}$ (expressed as reciprocal of the diluting air-flows 12–1 L/min) for 64 min in the absence (−S9) and presence (+S9) of exogenous metabolic activation (Aroclor-induced rat liver S9). The exposures were conducted at AAI using a VITROCELL® VC10® smoke exposure system, and the data is represented as mean induced revertants. Exposures were assessed in triplicate for each treatment and were conducted in six independent experiments.

Table 4
Statistical analysis[b]–Identity link function.

Strain	Slope	Concentrations in the Linear Range (L/min)	Variance of Slopes	Mean Slope	SD	%CV
TA98[a]	94.4	Air, 12, 8, 4	2160	134	46.5	35
	87.7	Air, 12, 8, 4				
	132.6	Air, 12, 8, 4				
	197.7	Air, 12, 8				
	112.4	Air, 12, 8, 4				
	113.6	Air, 12, 8, 4				
	119.6	Air, 12, 8, 4				
TA100	121.1	Air, 12, 8, 4	1280	99.2	35.7	36
	142.8	Air, 12, 8, 4				
	52.0	Air, 12, 8, 4, 2				
	60.3	Air, 12, 8, 4				
	104.5	Air, 12, 8, 4				
	114.5	Air, 12, 8, 4				

[a] For strain TA100, 6 experiments were conducted. For strain TA98, 8 experiments were conducted in order to acquire at least six experiments for analysis, because 1 experiment showed no linear portion of the curve.

[b] The analysis was conducted using data generated in the presence of S9 only.

3.4. Comparative study results

For the comparative study, whole smoke was generated from a primarily tobacco heating cigarette (Eclipse) and compared to whole smoke generated from 3R4F reference cigarettes. Both cigarette types were conditioned and smoked according to the International Organisation for Standardisation (ISO) smoking regime. One range-finder and five main experiments were performed in total for each cigarette type. *Salmonella* typhimurium strains TA98 and TA100 were treated with whole smoke concentrations (expressed as diluting air-flow) within the range of 12–0.5 L/min in the absence or presence of S9. Consistent with the validation work, a separate smoke run was performed prior to the biological exposure on each experimental day for collection of TPM which was subsequently analysed for TPM, nicotine and water as a check for consistency in the smoke generation (data not shown). All controls gave valid responses in all experiments when compared to the acceptance criteria. For each strain and S9 condition, the mean slopes from the 5 experiments were compared using a two-sample *t*-test. Levene's test for variances between the cigarettes was performed and where this showed evidence of heterogeneity ($p < 0.01$), the slopes were rank-transformed prior to analysis. The cigarettes were compared against each other in order to determine if there was a significant difference in the response in the test system. The results are presented in Table 5 and Fig. 2 and an overall summary, with the statistical analysis, is presented in Table 6.

Table 5
Comparison of response to whole smoke from 3R4F and Eclipse in TA98 and TA100 −/+S9.

Air Flow Rate (L/min)	Reciprocal of Air Flow Rate	Strain (activation condition)							
		Eclipse				3R4F			
		TA98 (−S9)	TA98 (+S9)	TA100 (-S9)	TA100 (+S9)	TA98 (−S9)	TA98 (+S9)	TA100 (−S9)	TA100 (+S9)
Untreated Control		6.3 ± 7.8	5.4 ± 0.6	25.3 ± 3.5	24.0 ± 1.6	4.3 ± 1.0	5.6 ± 1.5	19.9 ± 4.3	23.0 ± 5.0
AAI	0	4.7 ± 1.3	5.1 ± 0.6	22.9 ± 3.0	24.0 ± 4.0	4.5 ± 1.2	5.8 ± 1.8	20.9 ± 5.2	20.8 ± 6.3
12	0.083	NT	NT	NT	NT	NT	8.2 ± 2.7	NT	19.6 ± 5.7
8	0.125	5.0 ± 1.6	6.1 ± 1.3	21.1 ± 3.4	25.3 ± 2.0	3.5 ± 0.9	18.7 ± 5.0	21.7 ± 4.5	32.4 ± 4.6
4	0.25	4.9 ± 1.0	9.4 ± 4.3	23.5 ± 5.5	23.3 ± 3.0	5.4 ± 1.4	41.1 ± 2.2	23.2 ± 4.7	45.0 ± 10.1
2	0.5	NT	NT	NT	NT	NT	41.1 ± 7.7	NT	NT
1	1.0	4.8 ± 1.7	23.0 ± 4.5	22.8 ± 4.9	31.6 ± 4.6	5.5 ± 1.5	NT	26.5 ± 5.7	37.8 ± 11.5
0.5	2.0	5.4 ± 1.1	22.3 ± 3.9	20.7 ± 5.5	39.6 ± 8.9	4.1 ± 3.5	NT	20.8 ± 2.8	NT
Positive controls (µg/plate)									
2NF 0.4		151.7 ± 89.3	–	–	–	154.7 ± 78.5	–	–	–
B[a]P 0.8		–	67.2 ± 12.6	–	–	–	68.5 ± 11.2	–	–
NaN_3 0.4		–	–	183.5 ± 16.7	–	–	–	148.3 ± 16.9	–
AAN 0.4		–	–	–	244.2 ± 51.4	–	–	–	211.2 ± 52.8

Mean ± SD for 5 experiments.
NT–not tested.

These data show statistically significant differences in revertants for whole smoke samples from 3R4F and Eclipse cigarettes, when tested in *Salmonella typhimurium* strains TA98 and TA100 in the presence of S9 using the Vitrocell® VC10® Ames test system.

4. Discussion and conclusion

Exposure systems such as the VITROCELL® VC10® have been developed for use in combination with biological endpoints to detect potential interactions at the ALI or AAI for a variety of inhaled materials including tobacco smoke [44–49], e-cigarettes [50], nanomaterials [51–53], therapeutic aerosols [54], airborne chemicals and pollutants [31,55–57]. In addition, the VITROCELL® VC10® system has been shown to be a robust and reproducible *in vitro* method through an inter-machine comparison of tobacco smoke particle deposition using six independent smoke exposure systems [44]. Furthermore, the Ames test is widely used in the evaluation of mutagenicity *in vitro* including assessments of cigarette smoke [45,58–69]. However, the World Health Organisation does not support the use of machine smoke emission data to support claims of reduced exposure or reduced risk to humans [70]. Development of validated test methods which can provide data on the relative genotoxicity of the total output (gas phase and particulate matter) from a range of modified and traditional tobacco products will facilitate aerosol-based research and relative hazard assessment.

The data presented in this publication show that application of the intra-laboratory validated methodology using the VITROCELL® VC10® smoke exposure system in combination with a 2 strain Ames test method allowed for differential detection of mutagenicity in *Salmonella typhimurium* TA98 and TA100 cells following exposure to a combustible reference cigarette (3R4F) and a primarily tobacco heating product (Eclipse) in the presence of an exogenous metabolic activation system (S9). We observed that the Eclipse response generally required a lower (8–0.5 L/min) dilution air flow range than that for testing 3R4F cigarettes (mainly 12–2 L/min), indicating a reduced potency of induced mutagenic response. This is consistent with findings from previously published research, which report lower genotoxicity [45,60,64,71], cytotoxicity [61,64,72], DNA adduct formation [73,74], clinical bronchiolar inflammation [75] and urine mutagenicity [76] following exposure to tobacco heating (*e.g.* Eclipse or TOB-HT) smoke and condensate rather than reference cigarette smoke.

Our data are also consistent with reports that investigations using the VITROCELL® VC10® (or other *in vitro* exposure machines) and Ames test system showed the capability to analyze the effects of native whole smoke, to detect dose-dependent induction of revertants and to allow comparison with other nicotine / tobacco products [27,42,48,77–80]. In work cited by Leverette et al. 2012 [81], correlations between whole smoke-induced mutations in bacterial strains (*e.g.* TA98 and TA100) were shown to be consistent with TPM–induced mutations in the same strains. Equivalent levels of TPM were used to indicate the comparability of the WS-exposure adapted assay to liberate a mutatgnic response relevant to a standard format of the assay.

It should be noted that high-throughput usage of the module used herein is limited. However, this may be alleviated by the development, validation and use of modules designed to provide an increased number of wells and accommodate more exposure doses per run. Characterization of the aerosol is another area of keen research for the industry and may be most effectively addressed through collaborative efforts. Currently, some labs [49] have conducted chemical analyses for individual constituents; however, broader information from inline tools may require further development and new methods.

The findings from this body of work indicate that the VITROCELL® VC10® is capable of consistent generation of whole smoke. Method development for exposure of 6 strains of *Salmonella typhimurium* cells (TA97, TA98, TA100, TA1535, TA1537 and TA102) at the AAI was successful and the subsequent method validation in 5 strains (TA97, TA98, TA100, TA1535 and TA102) followed by selection of 2 strains (TA98 and TA100) for use in the comparator study indicated the ability to induce exposure-related changes in *in vitro* mutagenicity following exposure to whole smoke in the presence of S9. It is concluded that the methodology developed can be used to assess the mutagenicity of existing and novel tobacco products. Furthermore, it is concluded that the protocol which was developed is sufficiently sensitive to allow potential comparative analysis between whole smoke generated from cigarettes with markedly different smoke profiles (composition and/or yield). Hence, it is concluded that the available data support the potential use of the VITROCELL® VC10® and multi-strain Ames test system for assessment of the relative mutagenicity of whole smoke.

Transparency document

The Transparency document associated with this article can be found in the online version.

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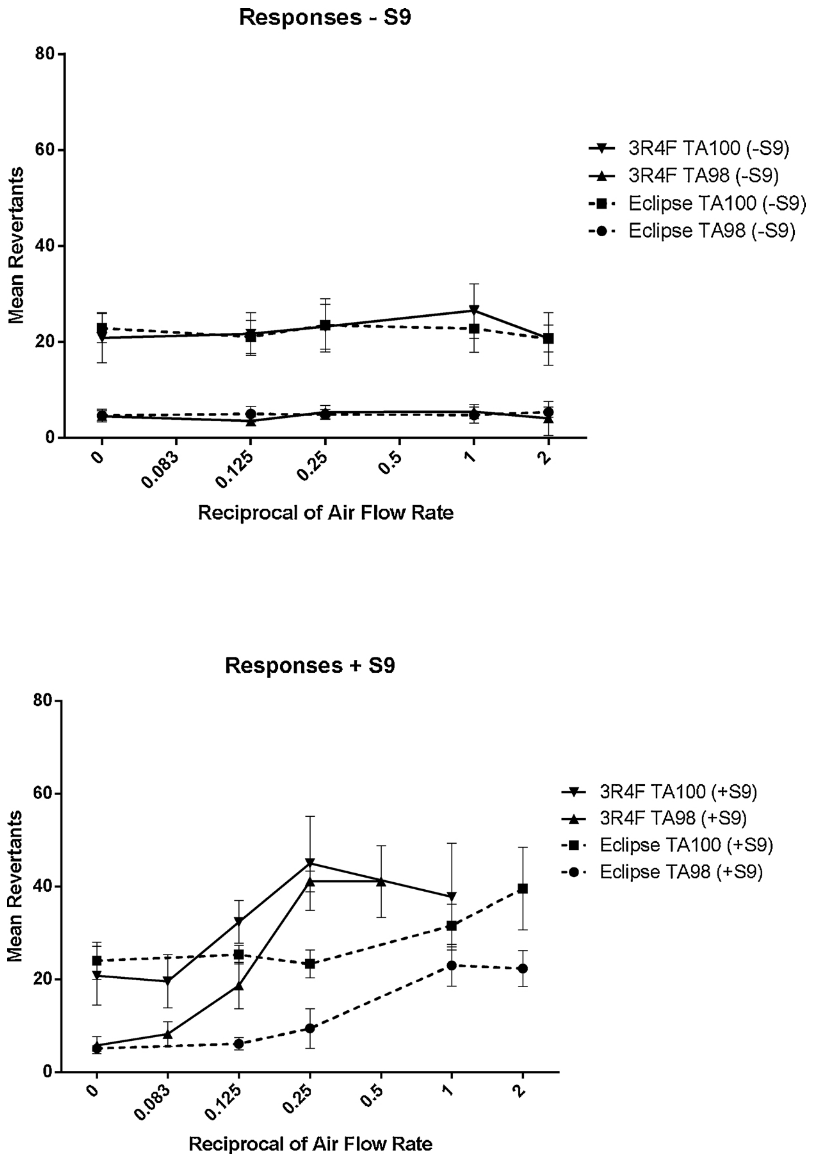

Fig. 2. Comparative assessment of mutagenic responses following 3R4F and Eclipse exposures. Standard Ames test strains *Salmonella typhimurium* TA98 and TA100 were exposed to whole smoke from 3R4F and Eclipse in the absence (−S9) or presence (+S9) of exogenous metabolic activation (Aroclor-induced rat liver S9) at AAI using a VITROCELL® VC10® smoke exposure system. The data is represented as mean induced revertants. Exposures for the respective test articles were assessed in triplicate for each treatment and were conducted in five independent experiments.

Table 6
Comparison of mutagenic response to 3R4F and Eclipse whole smoke.

Cigarette	Strain	S9	Concentration-related increase	Statistical significance at 1% level	Assessment	Mean slope	Statistical comparison
Eclipse	TA98	−	No	No	Not mutagenic	0.2	No statistical difference
3R4F	TA98	−	Yes (in a single experiment)	in single experiment	Biological relevance uncertain	−0.1	
Eclipse	TA98	+	Yes	Yes	Mutagenic	17.7	Significant difference
3R4F	TA98	+	Yes	Yes	Mutagenic	120.4	3R4F > Eclipse
Eclipse	TA100	−	No	No	Not mutagenic	−0.8	No statistical difference
3R4F	TA100	−	No	No	Not mutagenic	11.8	
Eclipse	TA100	+	Yes (in range-finder only)	Yes (in RF plus 2 experiments)	Biological relevance uncertain	8.0	Significant difference 3R4F > Eclipse
3R4F	TA100	+	Yes (in RF plus 4 experiments)	Yes (in RF plus 3 experiments)	Mutagenic	95.7	

RF = Ranger finder.

For each strain and treatment condition (−/+S9), the mean slope was generated using values from 5 independent experiments, each of which included a negative control and 4 test concentrations.

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References

[1] European Union, Directive 2014/40/EU of the European Parliament and of the Council of 3 April 2014 on the Approximation of the Laws, Regulations and Administrative Provisions of the Member States Concerning the Manufacture, Presentation and Sale of Tobacco and Related Products and Repealing Directive 2001/37/EC. O.J. L 127, 29.04.2014, (2014), pp. 1–38 (Accessed 5 March 2018), http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:JOL_2014_127_R_0001.

[2] U.S. Food and Drug Administration, Family Smoking Prevention and Tobacco Control Act. U.S. Government Public Law 111-31-June 22 2009, (2009) (Accessed March 5, 2018), http://www.gpo.gov/fdsys/pkg/PLAW-111publ31/pdf/PLAW-111publ31.pdf.

[3] U.S. Food and Drug Administration, 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 Distribution of Tobacco Products and Required Warning Statements for Tobacco Products. Final rule, Fed. Regist. 81 (90) (2016) 28973–29106.

[4] Institute of Medicine, Scientific Standards for Studies on Modified Risk Tobacco Products, Committee on Scientific Standards for Studies on Modified Risk Tobacco Products; Institute of Medicine, 2011 ISBN: 978-0-309-22398-0.

[5] Organisation for Economic Co-operation and Development, Bacterial Reverse Mutation Test, OECD Guideline for the Testing of Chemicals 471, (1997) (Adopted 21 July 1997).

[6] International Organization for Standardization, Biological Evaluation of Medical Devices–Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity, ISO 10993-3, Third edition 2014-10-01. ISO Copyright Office, Case postale 56, CH-1211, Geneva 20 (2014).

[7] International Council on Harmonisation, Guidance for Industry. S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use, June 2012 U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), 2012 (Accessed 5 March 2018), http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm074931.pdf.

[8] D.M. Maron, B.N. Ames, Revised methods for the Salmonella mutagenicity test, Mutat. Res. 113 (1983) 173–215.

[9] Centers for Disease Control and Prevention, The Health Consequences of Smoking—50 Years of Progress. A Report of the Surgeon General, US Department of Health and Human Services, 2014 (Accessed 5 March 2018), http://www.surgeongeneral.gov/library/reports/50-years-of-progress/full-report.pdf.

[10] I. Crooks, D.M. Dillon, J.K. Scott, M. Ballantyne, C. Meredith, The effect of long term storage on tobacco smoke particulate matter in in vitro genotoxicity and cytotoxicity assays, Regul. Toxicol. Pharmacol. 65 (2013) 196–200.

[11] W.S. Rickert, A.H. Trivedi, R.A. Momin, W.G. Wright, J.H. Lauterbach, Effect of smoking conditions and methods of collection on the mutagenicity and cytotoxicity of cigarette mainstream smoke, Toxicol. Sci. 96 (2007) 285–293.

[12] CORESTA In Vitro Toxicology Task Force, The Rationale and Strategy for Conducting in Vitro Toxicology Testing of Tobacco Smokemay Rationale and Strategy for Conducting *in Vitro* Toxicology Testing of Tobacco Smoke, (2004) (Accessed 8 March 2018), https://www.coresta.org/sites/default/files/technical_documents/main/IVT_TF_Rationale-IVT-Testing-Tob.-Smoke_Report_Jun04.pdf.

[13] C. Andreoli, D. Gigante, A. Nunziata, 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 17 (2003) 587–594.

[14] S. Bakand, C. Winder, C. Khalil, A. Hayes, Toxicity assessment of industrial chemicals and airborne contaminants: transition from in vivo to in vitro test methods: a review, Inhal. Toxicol. 17 (2005) 775–787.

[15] M.D. Johnson, J. Schilz, M.V. Djordjevic, J.R. Rice, P.G. Shields, Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless tobacco, Cancer Epidemiol. Biomark. Prev. 18 (2009) 3263–3304.

[16] Committee on Mutagenicity, Guidance: A Strategy for Testing Chemicals for Genotoxicity, (2011) (Accessed 7 March 2018), https://www.gov.uk/government/publications/a-strategy-for-testing-of-chemicals-for-genotoxicity.

[17] Health Canada, Regulations Amending the Tobacco Reporting Regulations, (2005) (Accessed 8 March 2018), http://www.gazette.gc.ca/rp-pr/p1/2017/2017-05-27/html/reg4-eng.html.

[18] D.J. Doolittle, C.K. Lee, J.L. Ivett, J.C. Mirsalis, E. Riccio, C.J. Rudd, G.T. Burger, A.W. Hayes, Comparative studies on the genotoxic activity of mainstream smoke condensate from cigarettes which burn or only heat tobacco, Environ. Mol. Mutagen. 15 (2012) 93–105.

[19] E. Roemer, T.H. Ottmueller, V. Zenzen, S. Wittke, F. Radtke, I. Blanco, R.A. Carchman, Cytotoxicity, mutagenicity, and tumorigenicity of mainstream smoke from three reference cigarettes machine-smoked to the same yields of total particulate matter per cigarette, Food Chem. Toxicol. 47 (2009) 1810–1818.

[20] H. Schramke, T.J. Meisgen, F.J. Tewes, W. Gomm, E. Roemer, The mouse lymphoma thymidine kinase assay for the assessment and comparison of the mutagenic activity of cigarette mainstream smoke particulate phase, Toxicology 227 (2006) 193–210.

[21] A. Thielen, H. Klus, L. Muller, Tobacco smoke: unraveling a controversial subject, Exp. Toxicol. Pathol. 60 (2008) 141–156.

[22] M.F. Dube, C.R. Green, Methods of collection of smoke for analytical purposes, Recent Adv. Tob. Sci. 8 (1982) 42–102.

[23] C.R. Green, A. Rodgman, Methods of collection of smoke for analytical purposes, Recent Adv. Tob. Sci. 22 (1996) 131–133.

[24] J. Fowles, E. Dybing, Application of toxicological risk assessment principles to the chemical constituents of cigarette smoke, Tob. Control 12 (2003) 424–430.

[25] D. Hoffmann, I. Hoffmann, The changing cigarette, 1950–1995, J. Toxicol. Environ. Health 50 (1997) 307–364.

[26] E. Roemer, F.J. Tewes, T.J. Meisgen, D.J. Veltel, E.L. Carmines, Evaluation of the potential effects of ingredients added to cigarettes. Part 3: in vitro genotoxicity and cytotoxicity, Food Chem. Toxicol. 40 (2002) 105–111.

[27] J. Kilford, D. Thorne, R. Payne, A. Dalrymple, J. Clements, C. Meredith, D. Dillon, A method for assessment of the genotoxicity of mainstream cigarette-smoke by use of the bacterial reverse-mutation assay and an aerosol-based exposure system, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 769 (2014) 20–28.

[28] Committee on Mutagenicity, Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environmen, www.archives.gov Accessed March 8, 2018 (2009).

[29] National Institutes of Health, Tox21–Toxicology in the 21st Century, US Department of Health and Human Services, National Institutes of Health, National Center for Advancing Translational Sciences, 2015 (Accessed 5 March 2018), http://www.ncats.nih.gov/tox21/about.

[30] M. Aufderheide, J.W. Knebel, D. Ritter, Novel approaches for studying pulmonary toxicity in vitro, Toxicol. Lett. 140–141 (2003) 205–211.

[31] M. Aufderheide, J.W. Knebel, D. Ritter, An improved in vitro model for testing the pulmonary toxicity of complex mixtures such as cigarette smoke, Exp. Toxicol. Pathol. 55 (2003) 51–57.

[32] K. Okuwa, M. Tanaka, Y. Fukano, H. Nara, Y. Nishijima, T. Nishino, In vitro micronucleus assay for cigarette smoke using a whole smoke exposure system: a comparison of smoking regimens, Exp. Toxicol. Pathol. 62 (2010) 433–440.

[33] J. Phillips, B. Kluss, A. Richter, E. Massey, Exposure of bronchial epithelial cells to whole cigarette smoke: assessment of cellular responses, Altern. Lab Anim. 33 (2005) 239–248.

[34] M.J. Scian, M.J. Oldham, D.B. Kane, J.S. Edmiston, W.J. McKinney, Characterization of a whole smoke in vitro exposure system (Burghart Mimic Smoker-01), Inhal. Toxicol. 21 (2009) 234–243.

[35] J. Adamson, D. Thorne, A. Dalrymple, D. Dillon, C. Meredith, Assessment of cigarette smoke particle deposition within the vitrocell® exposure module using quartz crystal microbalances, Chem. Cent. J. 7 (2013) 50.

[36] G.P. Pfeifer, M.F. Denissenko, M. Olivier, N. Tretyakova, S.S. Hecht, P. Hainaut, Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers, Oncogene 21 (2002) 7435–7451.

[37] F.J. de Serres, M.D. Shelby, Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay, Mutat. Res. 64 (1979) 159–165.

[38] International Organization for Standardization, Tobacco and Tobacco Products–Atmosphere for Conditioning and Testing, ISO 3402:2000 (2000) (Accessed 8 March 2018), https://www.iso.org/standard/28324.html.

[39] International Organization for Standardization, Routine Analytical Cigarette-Smoking Machine–Definitions and Standard Conditions, ISO 3308:2012 (2012) (Accessed 8 March 2018), https://www.iso.org/standard/60404.html.

[40] Health Canada, Test Method T-115: Determination of Tar, Water, Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke, 1999-12-31 (1999).

[41] W. Fields, K. Fowler, V. Hargreaves, L. Reeve, B. Bombick, Development, qualification, validation and application of the neutral red uptake assay in Chinese Hamster Ovary (CHO) cells using a VITROCELL® VC10® smoke exposure system, Toxicol. In Vitro 40 (2017) 144–152.

[42] M. Aufderheide, H. Gressmann, A modified Ames assay reveals the mutagenicity of native cigarette mainstream smoke and its gas vapour phase, Exp. Toxicol. Pathol. 58 (2007) 383–392.

[43] L. Bernstein, J. Kaldor, J. McCann, M.C. Pike, An empirical approach to the statistical analysis of mutagenesis data from the Salmonella test, Mutat. Res. 97 (1982) 267–281.

[44] J. Adamson, D. Thorne, G. Errington, W. Fields, X. Li, R. Payne, T. Krebs, A. Dalrymple, K. Fowler, D. Dillon, F. Xie, C. Meredith, An inter-machine comparison of tobacco smoke particle deposition in vitro from six independent smoke exposure systems, Toxicol. In Vitro 28 (2014) 1320–1328.

[45] S. Ishikawa, Y. Kanemaru, H. Nara, K. Erami, Y. Nagata, Assessing the mutagenic activities of smoke from different cigarettes in direct exposure experiments using the modified Ames Salmonella assay, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 803–804 (2016) 13–21.

[46] A.R. Iskandar, F. Martin, M. Talikka, W.K. Schlage, R. Kostadinova, C. Mathis, J. Hoeng, M.C. Peitsch, Systems approaches evaluating the perturbation of xenobiotic metabolism in response to cigarette smoke exposure in nasal and bronchial tissues, BioMed Res. Int. 2013 (2013) 512086.

[47] W.K. Schlage, A.R. Iskandar, R. Kostadinova, Y. Xiang, A. Sewer, S. Majeed, D. Kuehn, S. Frentzel, M. Talikka, M. Geertz, C. Mathis, N. Ivanov, J. Hoeng, M.C. Peitsch, In vitro systems toxicology approach to investigate the effects of repeated cigarette smoke exposure on human buccal and gingival organotypic epithelial tissue cultures, Toxicol. Mech. Methods 24 (2014) 470–487.

[48] D. Thorne, J. Kilford, M. Hollings, A. Dalrymple, M. Ballantyne, C. Meredith, D. Dillon, The mutagenic assessment of mainstream cigarette smoke using the Ames assay: a multi-strain approach, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 782

(2015) 9–17.

[49] D. Thorne, J. Kilford, R. Payne, J. Adamson, K. Scott, A. Dalrymple, C. Meredith, D. Dillon, Characterisation of a vitrocell® VC 10 in vitro smoke exposure system using dose tools and biological analysis, Chem. Cent. J. 7 (2013) 146.

[50] L. Neilson, C. Mankus, D. Thorne, G. Jackson, J. DeBay, C. Meredith, Development of an in vitro cytotoxicity model for aerosol exposure using 3D reconstructed human airway tissue; application for assessment of e-cigarette aerosol, Toxicol. In Vitro 29 (2015) 1952–1962.

[51] E. Frohlich, G. Bonstingl, A. Hofler, C. Meindl, G. Leitinger, T.R. Pieber, E. Roblegg, Comparison of two in vitro systems to assess cellular effects of nanoparticles-containing aerosols, Toxicol. In Vitro 27 (2013) 409–417.

[52] J.S. Kim, T.M. Peters, P.T. O'Shaughnessy, A. Adamcakova-Dodd, P.S. Thorne, Validation of an in vitro exposure system for toxicity assessment of air-delivered nanomaterials, Toxicol. In Vitro 27 (2013) 164–173.

[53] S.G. Klein, T. Serchi, L. Hoffmann, B. Blomeke, A.C. Gutleb, An improved 3D tetraculture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung, Part. Fibre Toxicol. 10 (2013) 31.

[54] U. Deschl, J. Vogel, M. Aufderheide, Development of an in vitro exposure model for investigating the biological effects of therapeutic aerosols on human cells from the respiratory tract, Exp. Toxicol. Pathol. 63 (2011) 593–598.

[55] S.E. Anderson, L.G. Jackson, J. Franko, J.R. Wells, Evaluation of dicarbonyls generated in a simulated indoor air environment using an in vitro exposure system, Toxicol. Sci. 115 (2010) 453–461.

[56] R. Gminski, T. Tang, V. Mersch-Sundermann, Cytotoxicity and genotoxicity in human lung epithelial A549 cells caused by airborne volatile organic compounds emitted from pine wood and oriented strand boards, Toxicol. Lett. 196 (2010) 33–41.

[57] C. Persoz, C. Leleu, S. Achard, M. Fasseu, J. Menotti, P. Meneceur, I. Momas, F. Derouin, N. Seta, Sequential air-liquid exposure of human respiratory cells to chemical and biological pollutants, Toxicol. Lett. 207 (2011) 53–59.

[58] B. Bombick, J. Avalos, K. Putman, D. Bowman, J. Mabe, K. Fowler, D. Bombick, W. Morgan, D.J. Doolittle, Comparative studies on the genotoxic and cytotoxic potential of mainstream smoke condensate from menthol and non-menthol cigarettes which burn or primarily heat tobacco, Environ. Mol. Mutagen. 37 (S32) (2001) 23.

[59] B.R. Bombick, J. Avalos, P.R. Nelson, F.W. Conrad, D.J. Doolittle, Comparative studies on the mutagenicity of environmental tobacco smoke from cigarettes which burn or primarily heat tobacco, Environ. Mol. Mutagen. 27 (9) (1996).

[60] B.R. Bombick, J.T. Avalos, P.R. Nelson, F.W. Conrad, D.J. Doolittle, Comparative studies of the mutagenicity of environmental tobacco smoke from cigarettes that burn or primarily heat tobacco, Environ. Mol. Mutagen. 31 (1998) 169–175.

[61] B.R. Bombick, H. Murli, J.T. Avalos, D.W. Bombick, W.T. Morgan, K.P. Putnam, D.J. Doolittle, Chemical and biological studies of a new cigarette that primarily heats tobacco. Part 2. In vitro toxicology of mainstream smoke condensate, Food Chem. Toxicol. 36 (1998) 183–190.

[62] D. Bombick, K. Putnam, B.R. Bombick, W. Morgan, P. Ayres, D. Doolittle, In vitro assessment of biological activity of mainstream smoke from cigarette which burn or primarily heat tobacco, Int. Toxicol. 7 (1995) 73.

[63] D.W. Bombick, B.R. Bombick, P.H. Ayres, K. Putnam, J. Avalos, M.F. Borgerding, D.J. Doolittle, 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. 39 (1997) 11–17.

[64] J.W. Foy, B.R. Bombick, D.W. Bombick, D.J. Doolittle, A.T. Mosberg, J.E. Swauger, A comparison of in vitro toxicities of cigarette smoke condensate from Eclipse cigarettes and four commercially available ultra low-"tar" cigarettes, Food Chem. Toxicol. 42 (2004) 237–243.

[65] C.J. Smith, D.W. Bombick, B.A. Ryan, W.T. Morgan, D.J. Doolittle, Urinary mutagenicity in nonsmokers following exposure to fresh diluted sidestream cigarette smoke, Mutat. Res. 470 (2000) 53–70.

[66] C.J. Smith, S.C. McKarns, R.A. Davis, S.D. Livingston, B.R. Bombick, J.T. Avalos, W.T. Morgan, D.J. Doolittle, Human urine mutagenicity study comparing cigarettes which burn or primarily heat tobacco, Mutat. Res. 361 (1996) 1–9.

[67] R.H. Steele, V.M. Payne, C.W. Fulp, D.C. Rees, C.K. Lee, D.J. Doolittle, 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. 342 (1995) 179–190.

[68] D.L. Bowman, C.J. Smith, B.R. Bombick, J.T. Avalos, R.A. Davis, W.T. Morgan, D.J. Doolittle, Relationship between FTC' tar' and urine mutagenicity in smokers of tobacco-burning or Eclipse cigarettes, Mutat. Res. 521 (2002) 137–149.

[69] T.A. Chepiga, M.J. Morton, P.A. Murphy, J.T. Avalos, B.R. Bombick, D.J. Doolittle, M.F. Borgerding, J.E. Swauger, A comparison of the mainstream smoke chemistry and mutagenicity of a representative sample of the US cigarette market with two Kentucky reference cigarettes (K1R4F and K1R5F), Food Chem. Toxicol. 38 (2000) 949–962.

[70] World Health Organization, WHO TobLabNet Official Method SOP 01. Standard Operating Procedure for Intense Smoking of Cigarettes, World Health Organisation, 2012 (Accessed 5 March 2018), http://apps.who.int/iris/bitstream/10665/75261/1/9789241503891_eng.pdf.

[71] W.R. Fields, R.M. Leonard, P.S. Odom, B.K. Nordskog, M.W. Ogden, D.J. Doolittle, Gene expression in normal human bronchial epithelial (NHBE) cells following in vitro exposure to cigarette smoke condensate, Toxicol. Sci. 86 (2005) 84–91.

[72] K.P. Putnam, D.W. Bombick, P. Ayres, J. Corn, Use of the Neutral Red Cytotoxicity Assay to Compare the Cytotoxic Potential of Whole Smoke from Eclipse 9-014, 1R4F and 1R5F Cigarettes, (1999) (Accessed 8 March 2018), https://www.industrydocumentslibrary.ucsf.edu/docs/#id=mxgy0225.

[73] B. Brown, J. Kolesar, K. Lindberg, D. Meckley, A. Mosberg, D. Doolittle, Comparative studies of DNA adduct formation in mice following dermal application of smoke condensates from cigarettes that burn or primarily heat tobacco, Mutat. Res. 414 (1998) 21–30.

[74] E.M. Lee, J.L. Malson, E.T. Moolchan, W.B. Pickworth, Quantitative comparisons between a nicotine delivery device (Eclipse) and conventional cigarette smoking, Nic. Tob. Res. 6 (2004) 95–102.

[75] S.I. Rennard, T. Umino, T. Millatmal, D.M. Daughton, L.S. Manouilova, F.A. Ullrich, K.D. Patil, D.J. Romberger, A.A. Floreani, J.R. Anderson, Evaluation of subclinical respiratory tract inflammation in heavy smokers who switch to a cigarette-like nicotine delivery device that primarily heats tobacco, Nic. Tob. Res. 4 (2002) 467–476.

[76] D.J. Doolittle, C.A. Rahn, G.T. Burger, R. Davis, J.D. deBethizy, G. Howard, C.K. Lee, S.C. McKarns, E. Riccio, J. Robinson, J. Reynolds, A.W. Hayes, Human urine mutagenicity study comparing cigarettes which burn or only heat tobacco, Mutat. Res. 223 (1989) 221–232.

[77] M. Aufderheide, H. Gressmann, Mutagenicity of native cigarette mainstream smoke and its gas/vapour phase by use of different tester strains and cigarettes in a modified Ames assay, Mutat. Res. 656 (2008) 82–87.

[78] M. Aufderheide, U. Mohr, A modified CULTEX system for the direct exposure of bacteria to inhalable substances, Exp. Toxicol. Pathol. 55 (2004) 451–454.

[79] D. Breheny, F. Cunningham, J. Kilford, R. Payne, D. Dillon, C. Meredith, Application of a modified gaseous exposure system to the in vitro toxicological assessment of tobacco smoke toxicants, Environ. Mol. Mutagen. 55 (2014) 662–672.

[80] M. Misra, R.D. Leverette, B.T. Cooper, M.B. Bennett, S.E. Brown, 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 11 (2014) 11325–11347.

[81] R.D. Leverette, Utilization of a whole smoke exposure system for the comparison of mainstream cigarette smoke, gas vapor phase and tar mutagenicity, The Toxicologist 51 (2012) 2091.