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

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1 RAI Services Company bears stewardship responsibility for each of RAI’s tobacco-manufacturing operating companies, namely R.J. Reynolds Tobacco Company (RJRT), American Snuff Co., LLC (ASC), and Santa Fe Natural Tobacco Company, Inc. (SFNTC). RAI Services Company is a wholly owned subsidiary of Reynolds American Inc., which is a wholly owned subsidiary of British American Tobacco plc.

A R T I C L E   I N F O

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A B S T R A C T

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 functional integrity 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 mutagenic. 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
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 in 2009 [28] and, for tobacco smoke, Health Canada [17]. In addition, the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) in vitro sub-group (previously ‘task-force’) 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 airagar 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

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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-aminolevulinic acid (CAS No. 90-45-9), 2-aminoanthracene (CAS No. 613-13-8), benz[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 (HCl 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 IOQ, 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, TA1537 and TA1535) 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 10⁸ bacteria/mL (approximately 2 × 10⁵ 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
- Exposure Parameters:
  - 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 × 10⁷ 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 ≤ 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 ≥ 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 ≤ 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 |  |
|----------|--------------------------------------------------------------------------------|---|
| Strain   | 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.
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.
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 12–1 L/min in the absence (−S9) and presence (+S9) of exogenous metabolic activation (Arochlor-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.

![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 (Arochlor-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.](image)

**Table 4**

| Strain  | Slope | Concentrations in the Linear Range (L/min) | Variance of Slopes | Mean Slope | SD | %CV |
|---------|-------|-------------------------------------------|--------------------|------------|----|-----|
| TA98a   | 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                             |                    |            |     |     |
|         | 60.3  | Air, 12, 8, 2                             |                    |            |     |     |
|         | 104.5 | Air, 12, 8                                |                    |            |     |     |
|         | 114.5 | Air, 12, 8                                |                    |            |     |     |

*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.
null response. This is consistent with
ettes (mainly 12
potential interactions at the ALI or AAI for a variety of inhaled materials
cytotoxicity \[ 61,64,72\], DNA adduct formation \[ 73,74\], clinical
(S9). We observed that the Eclipse response generally required a lower
reference cigarette (3R4F) and a primarily tobacco heating product
Salmonella typhimurium
strains TA98 and TA100 cells following exposure to a combustible
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 compar-
ability of the WS-exposure adapted assay to liberate a mutagenic re-
sponse 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\textsuperscript{®} 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 \textit{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 ex-
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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 mutagen | 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 mutagen | −0.8       | No statistical difference |
| 3R4F      | TA100  | −  | No                              | No                                 | Not mutagen | 11.8       |                         |
| Eclipse   | TA100  | +  | Yes (in range-finder only)      | Yes (in RF plus 2 experiments)     | Biological relevance uncertain | 8.0        | Significant difference  |
| 3R4F      | TA100  | +  | Yes (in RF plus 4 experiments)  | Yes (in RF plus 3 experiments)     | Mutagenic   | 95.7       | 3R4F > Eclipse          |

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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