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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 '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 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