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# 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

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ABSTRACT

To date there are no widely accepted methods for the toxicological testing of complex gaseous mixtures and aerosols, such as cigarette smoke, although some modifications to the standard regulatory methods have been developed and used. Historically, routine testing of cigarettes has primarily focused on the particulate fraction of cigarette smoke. However, this fraction may not accurately reflect the full toxicity and mutagenicity of the smoke aerosol as a whole, which contains semi-volatiles and short-lived products of combustion. In this study we have used a modified version of the bacterial reverse-mutation (Ames) assay for the testing of mainstream smoke generated from 3R4F reference cigarettes with a Vitrocell® VC 10 exposure system. This method has been evaluated in four strains of *Salmonella typhimurium* (TA98, TA100, YG1024 and YG1042) and one strain of *Escherichia coli* (WP2 *uvrA* pKM101) in the absence and presence of a metabolic activation system. Following exposure at four concentrations of diluted mainstream cigarette-smoke, concentration-related and reproducible increases in the number of revertants were observed in all four *Salmonella* strains. *E. coli* strain WP2 *uvrA* pKM101 was unresponsive at the four concentrations tested. To quantify the exposure dose and to enable biological response to be plotted as a function of deposited mass, quartz-crystal microbalances were included *in situ* in the smoke-exposure set-up. This methodology was further assessed by comparing the responses of strain YG1042 to mainstream cigarette-smoke on a second VC 10 Smoking Robot. In summary, the Ames assay can be successfully modified to assess the toxicological impact of mainstream cigarette-smoke.



## 1. Introduction

The bacterial reverse-mutation assay, also known as the Ames assay [1], is widely used for the initial genotoxicity screening of pharmaceuticals and chemicals. The methodology is dictated by clear international regulatory guidelines (e.g. OECD, ICH) to ensure consistent testing across laboratories [2,3]. Although these guidelines are suitable for the testing of compounds as solutions or suspensions, modifications are required to enable the testing of pure gases [4]. The testing of complex gaseous mixtures and aerosols such cigarette smoke poses even greater challenges. A method capable of testing gases or aerosols would therefore be very beneficial, particularly to the tobacco industry where there is increased interest in performing toxicological testing on the entire smoke aerosol. The development of such methods for the testing of volatile tobacco products was discussed by the Committee on Mutagenicity in 2009 and the absence of an adequately validated methodology was noted [5].

Cigarette smoke is a complex aerosol made up of both a particulate fraction and a vapour phase (VP), making it exceptionally difficult to test this mixture by use of standard methods. To date, most toxicological testing of cigarette smoke has relied heavily on testing the particulate fraction with standard assays [6,7]. To capture the particulate matter (PM), cigarettes can be smoked onto a Cambridge filter pad and the PM extracted with dimethyl sulfoxide

*Abbreviations:* AAN, 2-aminoanthracene; B[a]P, Benzo(a)pyrene; DMSO, dimethyl sulphoxide; FI, fold increase; ISO, international standards organisation; MR, mean revertants; $NaN_3$, sodium azide; NQO, nitroquinoline oxide; PBS, phosphate buffered saline; PM, particulate material; QCM, quartz crystal microbalance; VC 10, Vitrocell® smoking robot; VP, vapour phase; 2NF, 2-nitrofluorene.

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(DMSO), which can then be treated as a standard test-article [8–10]. The results of such testing have demonstrated significant concentration-related increases in cytotoxicity and mutagenicity in several standard genotoxicity assays [11–14]. The particulate phase, however, is a small fraction of the whole-smoke aerosol [15] and testing of the particulate fraction alone does not account for semi-volatiles or gases found in the vapour phase of cigarette smoke. Some attempts have been made to test a more representative sample of whole smoke by bubbling the resulting vapour phase through phosphate-buffered saline (PBS) and testing both the particulate and vapour fractions either independently or as a mixture [8,16]. However, collecting and testing of particulate and vapour fractions independently is not ideal as it does not account for insoluble compounds or short-lived chemicals resulting from combustion. Due to the complexity of potential chemical interactions within and between phases, it is important for future toxicological testing of cigarettes to develop methods that enable testing of the entire smoke aerosol.

Recent technological advancements have seen the introduction of in vitro smoking machines, paired with exposure modules that allow exposure of cells to the whole-smoke aerosol at an air–liquid or air–agar interface. One example is the Vitrocell® VC 10 Smoking Robot which dilutes mainstream cigarette-smoke into a constant flow of air. A sample of this diluted smoke is pulled by vacuum into the exposure module where it is delivered to each chamber across the module [17]. The flow rate of the diluting air can be adjusted to alter the concentration of smoke delivered.

Dosimetry tools are also being developed that can be used in conjunction with whole-smoke exposure systems to quantify the deposition of smoke particulate mass [18]. Characterisation of quartz-crystal microbalances (QCMs) with the VC 10 Smoking Robot has demonstrated that this technology can be used to quantify particle deposition during whole-smoke exposure across a wide range of smoke dilutions [17]. The VC 10 together with QCM technology represents an appropriate system to modify the standard regulatory assays for exposure to aerosols such as whole smoke. In addition, the use of QCMs provides a valuable opportunity to monitor machine performance and present data against a quantitative dosimetry measure that could be translatable between laboratories.

In this study, we have applied a modified plating method, comparable to that reported by Araki et al. [19] and Aufderheide and Gressman [20], with minor modifications to adapt the standard regulatory Ames assay to exposure to gaseous aerosols on the Vitrocell® VC 10 exposure system. This exposure method has been evaluated with mainstream cigarette-smoke generated from 3R4F reference cigarettes on the Vitrocell® VC 10 Smoking Robot. The standard regulatory Ames assay may include a total of five bacterial strains, each of which identifies mutagens of a different chemical class [21]. The current OECD guidelines [2] recommend a total of five bacterial strains. Four strains of *Salmonella typhimurium* (TA1535; TA1537 or TA97 or TA97a; TA98 and TA100) are proposed, which between them detect both frameshift and base-pair substitution mutations, with GC base pairs at the primary reversion-site. A fifth strain is recommended to detect mutagens that may not be captured by the previous strains, such as certain hydrazines, oxidizing mutagens and cross-linking agents. Either *Salmonella typhimurium* strain TA102 or an *Escherichia coli* (*E. coli*) strain (WP2 *uvrA* or WP2 *uvrA* (pKM101)) are accepted as the fifth strain, each of which have an AT base-pair at the primary reversion site [2].

We tested the genotoxicity of whole smoke with five strains of bacteria. These included three strains accepted under the regulatory guidelines, i.e. *Salmonella typhimurium* TA98 and TA100, and *Escherichia coli* strain WP2 *uvrA* (pKM101). In addition, derivatives of TA98 (YG1024) and TA100 (YG1042) were selected based on their increased sensitivity to nitroarenes and aromatic amines [22,23], which are known mutagens present in cigarette smoke [24–26].

In the standard plate-incorporation method, bacteria are embedded in a top agar together with the test material. However, for the testing of whole smoke, it is important that the bacteria are fully exposed to both particulate and gaseous components that cannot be incorporated into the top agar and, as a result, exposure must be facilitated at the agar surface. The spread-culture method [19,20] was therefore selected for this study.

In this study we demonstrate that a modified Ames assay, similar to that used by Aufderheide and Gressman [20], with some modifications can work successfully in conjunction with the Vitrocell® VC 10, a commercially available aerosol-based exposure system. We have tested five bacterial strains with different sensitivities to mainstream cigarette-smoke. In combination with QCM technology, this method could provide an appropriate system for measurement of the toxicological impact of whole mainstream cigarette-smoke, rather than relying on testing of particulate matter and vapour phase as independent fractions.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals and reagents were obtained from Sigma–Aldrich (Gillingham, UK) unless otherwise stated.

### 2.2. Reference cigarettes

3R4F reference cigarettes were obtained from the University of Kentucky, Kentucky, USA. 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 of Standardisation (ISO) 3402:2000).

### 2.3. Bacterial strains and culture conditions

The details of the bacterial strains used in this study are presented in Table 1.

Frozen cultures of each strain were checked for strain characteristics according to Maron and Ames and De Serres and Shelby [28,29]. As is standard procedure within Covance Laboratories, overnight cultures were prepared from frozen stocks in 30 mL nutrient broth containing appropriate antibiotics to maintain plasmids, and grown in a shaking incubator at 37 °C for 8 h.

### 2.4. Vitrocell® VC 10 setup and whole-smoke exposure

The Vitrocell® VC 10 Smoking Robot (Serial Number VC10/090610) was used to expose bacteria to mainstream cigarette-smoke generated from 3R4F reference cigarettes. Cigarettes were smoked according to ISO 3308:2000 (one 35-mL puff per 60 s, over 2 s), with an 8-s exhaust. Triplicate bacterial plates were exposed in Vitrocell® AMES 4 stainless steel exposure-modules with a QCM in the fourth position to record deposition of PM as a measure of dosimetry. The trumpet height in the modules was set to 2 mm above the agar or QCM surface. Mainstream cigarette-smoke was diluted into a constant stream of air with a fixed vacuum of 5 mL/min to pull the diluted smoke through the exposure module. Different concentrations of smoke were achieved by varying the flow rate of the diluting air (1.0, 4.0, 8.0 and 12.0 L/min). Modules were exposed to a total of three or eight cigarettes smoked over 24 or 64 min, respectively, with a continual flow of diluting air between each cigarette puff. At the end of the whole-smoke exposure period, the final deposited mass reading on each QCM was recorded once a plateau in the deposition curve was observed.

### 2.5. Ames reverse-mutation assay

The Ames assay used in this study differs from the standard method in several key aspects, for example: the agar-plate size has been scaled down from 85 to 35 mm to facilitate aerosol/whole-smoke exposure (incorporation of agar plates into the Vitrocell® Ames module, or commercially available equivalent). As a result, cofactor preparation is an exact scaled-down equivalent. Positive control concentrations per plate have also been factored and assessed for this new scaled-down plated version of the assay (Table 2). In addition, in our protocol we have included a drying step, where plates were wrapped in parafilm, vented and incubated at 37 °C for 3 days. Parafilm wrapping (with vent holes) was used to prevent agar shrinkage and dehydration. Finally, we have varied the diluting air within the exposure system to create smoke dilutions and ultimately a dose–response. This is in contrast to other studies that have used cigarette numbers to create a dose–response [20], but still achieving the same end.



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