A modified Ames methodology for the assessment of mainstream cigarette smoke genotoxicity using an aerosol-based exposure system

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Introduction

The development of whole smoke exposure systems has been driven by the fact that traditional smoke exposure techniques are based on the particulate phase of tobacco smoke and not the complete whole smoke aerosol. To overcome these challenges, whole smoke exposure systems have been developed which expose cell cultures to diluted tobacco smoke and capture the full interactions of both smoke phases[1-2]. Furthermore, standard methodologies, governed by regulatory guidelines are not necessarily compatible with complex aerosols, such as cigarette smoke.

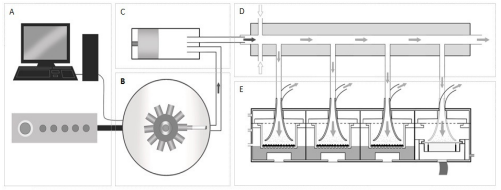
Aim

To develop a modified version of the Ames reverse mutation assay suitable for whole smoke exposure. For this study, five strains were selected and exposed to diluted 3R4F mainstream cigarette smoke using the Vitrocell® VC 10 Smoking Robot. Quartz crystal microbalances (QCM)[3] gave further confidence in the exposure system and enabled biological responses to be presented as a function of real-time obtained deposited mass.

Materials and Methods

Cigarette Smoke Generation

A Vitrocell® VC 10 Smoking Robot (Serial Number VC10/090610) was used to expose bacteria to mainstream cigarette smoke generated from 3R4F reference cigarettes **(Fig 1)**. Cigarettes were conditioned according to ISO 3402:2000 and smoked according to ISO 3308:2000, with an 8 second exhaust. Mainstream cigarette smoke was passed into a constant flow of diluting air set at varying flow rates (1-12 L/min) to achieve different doses. The diluted smoke was drawn through the modules using a constant vacuum of 5.0 mL/min for all experiments.



Robot carousel where cigarettes are loaded and smoked, enclosed within an extraction ventilation hood. For Gas Vapour Phase (GVP) studies a Cambridge filter pad was installed into the line between the smoking carousel and the piston, for removal of the particulate smoke fraction. **[C]** Piston/ syringe, which draws and delivers mainstream cigarette smoke to the dilution system. **[D]** Dilution, transit and delivery of whole smoke occurs in the dilution bar, of which multiple bars (up to five) can make up the complete dilution system. **[E]** Smoke is sampled from the dilution system into the exposure module through negative pressure applied via a vacuum pump at 5.0 ml/min.

Ames Assay

Four strains of *S. typhimurium* (TA98, TA100, YG1024 and YG1042) and one strain of *E. coli* (WP2 *uvrA* pKM101) were exposed to diluted mainstream smoke in the presence or absence of 10% S9. Approximately $2x10^7$ cells were plated onto 35mm Vogel-Bonner agar plates using spread plate methodology such that bacteria were exposed at an air-agar interface. Plates were exposed to a total of 3 cigarettes smoked over 24 minutes. Concurrent negative (air and untreated) and positive controls were included with each exposure. Following exposure, plates were incubated at 37°C for 3 days before revertant colony numbers were counted using an automated scoring system.

Measurement of Particulate Dose

A QCM **(Fig 2)** was placed in the fourth position of the exposure module for all whole smoke exposures in order to quantify the dose delivered by measuring deposition of particulate mass. 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[3].

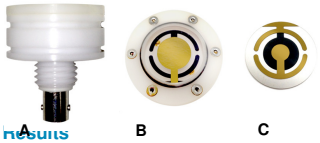


Figure 2: A QCM. Housing unit **[A]** side view and **[B]** top view; **[C]** QCM crystal (underside) 25 mm ø

Results

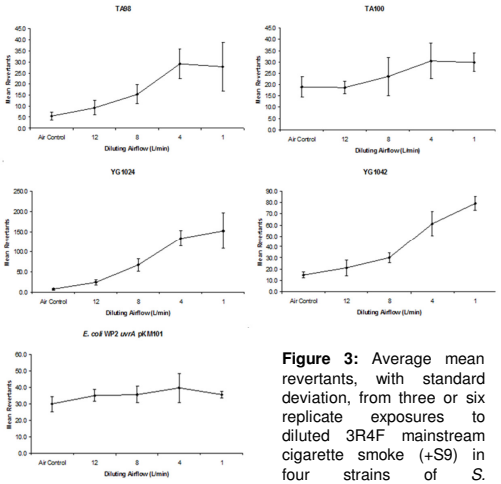


Figure 3: Average mean revertants, with standard deviation, from three or six replicate exposures to diluted 3R4F mainstream cigarette smoke (+S9) in four strains of *S. typhimurium* and one strain of *E. coli*.

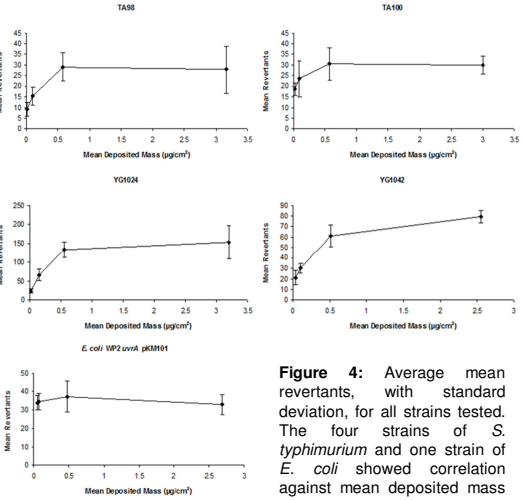


Figure 4: Average mean revertants, with standard deviation, for all strains tested. The four strains of *S. typhimurium* and one strain of *E. coli* showed correlation against mean deposited mass following 24 minute (+S9) exposure to 3R4F mainstream smoke. Deposited mass obtained real-time *in situ* of exposure.

Table 1. Average mean revertants and average fold increases (from two experiments) for all five strains tested. The four strains of *S. typhimurium* and one strain of *E. coli* all showed no response following whole smoke exposure in the absence of S9.

Table 1. -S9 Ames Data

Strain	Airflow	Average Mean revertants	Average Fold Increase
TA98	Air	2.9	1.0
	12	4.2	1.8
	8	2.5	1.1
	4	4.2	2.0
	1	4.2	1.7
T100	Air	20.9	1.0
	12	24.0	1.2
	8	21.7	1.0
	4	22.3	1.1
	1	26.3	1.3
YG1024	Air	4.0	1.0
	12	5.5	1.4
	8	5.2	1.3
	4	6.2	1.6
	1	6.8	1.7
YG1042	Air	19.8	1.0
	12	15.3	0.8
	8	18.0	0.9
	4	16.5	0.9
	1	21.0	1.1
E. coli WP2 *uvrA* pKM101	Air	34.5	1.0
	12	36.0	1.0
	8	35.3	1.0
	4	42.0	1.2
	1	43.5	1.3

Table 2. GVP Ames data

Strain	Airflow	Average Mean revertants	Average Fold Increase
TA98	Air	6.4	1.0
	12	6.3	1.1
	8	6.3	1.0
	4	7.0	1.1
	1	7.9	1.2
TA100	Air	16.2	1.0
	12	15.2	0.9
	8	15.5	1.0
	4	12.3	0.8
	1	16.5	1.0
YG1024	Air	4.8	1.0
	12	6.3	1.3
	8	4.2	0.9
	4	4.7	1.0
	1	4.0	0.8
YG1042	Air	12.5	1.0
	12	15.3	1.2
	8	16.5	1.4
	4	12.2	1.0
	1	13.0	1.0
E. coli WP2 *uvrA* pKM101	Air	23.9	1.0
	12	22.7	1.0
	8	24.2	1.0
	4	21.9	0.9
	1	24.3	1.0

Table 2. Average mean revertants and average fold increases (from two experiments) for all five strains tested. The four strains of *S. typhimurium* and one strain of *E. coli* all showed no response following 24 minute GVP exposure.

Conclusions

•Concentration-related increases in revertant numbers were observed in *S. typhimurium* strains **TA98**, **TA100**, **YG1024** and **YG1042** up to maximum mean fold increases of **5.6**, **1.7**, **24.8** and **5.5**-fold, respectively, following 24 minute exposure to diluted 3R4F mainstream cigarette smoke in the presence of S-9.

•No response to whole smoke was observed in ***E. coli* WP2 *uvrA* pKM101** in the absence or presence of S-9**.**

•Measurement of real-time deposited particulate mass using QCMs *in situ* of whole smoke exposure demonstrated that the increases in revertant numbers observed in the four *Salmonella* strains in the presence of S-9, correlated with increasing particulate deposition.

•Our results indicate that, using a 5.0 ml/min vacuum, the GVP fraction alone does not induce mutation. However, alternative vacuum rates have yet to be assessed.

•In the absence of a metabolic activation system, whole smoke failed to induce mutation, indicating that direct acting smoke constituents cannot be detected, under these conditions.

Future Directions

•We intend to develop this modified assay alongside additional strains to create a multi-strain-testing approach.

•This work will be further supplemented by assessing strains in order to identify the optimal strains for testing cigarette smoke.

•We would like to complement QCM measurements with a measure of the vapour phase dose – a technique is required for this as none currently exists.

References

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2.Perfetti, T. A., and Rodgman, A. The Complexity of Tobacco and Tobacco Smoke. Beitraege zur Tabakforschung International 2011; 24: 215-232

3.Adamson, J., Thorne, D., Dalrymple, A., Dillon, D., Meredith, C. Cigarette smoke deposition in a Vitrocell® exposure module: real-time quantification *in vitro* using quartz crystal microbalances. Chemistry Central Journal 2013; 7:15

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Related Publications

•Thorne, D., Adamson, J. **A review of cigarette smoke exposure systems.** Experimental and Toxicologic Pathology 2013; In press

- http://dx.doi.org/10.1016/j.etp.2013.06.001

•Adamson, J., Thorne, D., Dalrymple, A., Dillon, D., Meredith, C. **Cigarette smoke deposition in a Vitrocell® exposure module: real-time quantification *in vitro* using quartz crystal microbalances.** Chemistry Central Journal 2013; 7:15

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•Thorne, D., Kilford, J., Payne, R., Adamson, J., Scott, K., Dalrymple, A., Meredith, C., Dillon, D. **Characterisation of a Vitrocell® VC 10 *in vitro* smoke exposure system using dose tolls and biological analysis.** Chemistry Central Journal 2013, 7:146

- http://journal.chemistrycentral.com/content/7/1/146

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ABSTRACT

To date most toxicological testing of cigarettes has been performed on the particulate phase of cigarette smoke using standard genotoxic and cytotoxic methods, which include the AMES reverse mutation assay, neutral red uptake, mouse lymphoma and micronucleus assays. However, traditional test methods are based on a particulate test material and under submerged conditions and are not suitable for the testing of aerosols; including cigarette smoke. As a result there is a requirement for new methodologies which facilitate the testing of aerosols *in vitro*.

In this study we have modified the Ames reverse mutation assay, using a spread plate methodology, to allow exposure to a cigarette smoke aerosol at an air-agar interface (AAI). The methodology was evaluated using cigarette smoke generated from 3R4F reference cigarettes on a Vitrocell® VC 10 Smoking Robot. Four strains of *S.typhimurium* and one strain of *E. coli* were tested individually on 6 independent occasions in the presence of S-9. A dose-related increase in revertant numbers was observed in strains TA98, TA100, YG1024 and YG1042 up to mean fold increases of 5.6, 1.7, 24.8 and 5.5-fold, respectively. *E. coli* strain WP2 *uvrA* pKM101 was unresponsive at all concentrations tested. To enable us to accurately quantify dose, we measured deposited particulate mass using Quartz Crystal Microbalance technology *in situ* of exposure.

In conclusion, we have modified the traditional Ames reverse mutation assay using an aerosol-based exposure system for the assessment of cigarette smoke toxicology. Furthermore, this method is not restricted to the testing of whole smoke and could be applied to the testing of other gases, mixtures or aerosols.

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