Validation of the VITROCELL VC10® Smoke Exposure System for *In Vitro* Assays

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Abstract

The VITROCELL VC10® smoke exposure system offers multiple platforms and air liquid interface (ALI) exposure to mimic *in vivo*-like conditions for assessing the toxicological impact of smoke in *in vitro* assays. The validation plan to ensure the system and corresponding assays were fit-for-purpose consisted of equipment qualification, pre-validation method development and method validation for neutral red uptake (NRU), sister chromatid exchange (SCE) and Ames assay (TA98, TA100). Parameters assessed for establishing the experimental model consisted of 1) optimization of mammalian and bacterial cell growth at ALI in static and flowing air conditions; 2) investigation of pH changes during exposure; and 3) determination of smoke concentrations and appropriate positive controls for all assay types. In each validation protocol, at least six experiments were performed. Smoke airflows of 12, 10, 8, 6, 4 or 2L/min and air-control were used for evaluating the assays, and acceptance criteria were established accordingly. NRU acceptance criteria were established to be 1) coefficient of variance is < 15% in optical density (540nm;OD_{540}) values between chamber replicates; 2) the positive control elicits > 50% decrease in NRU relative to the air control; 3) mean OD_{540} of all air control replicates is > 0.2 and 4) viability values above and below the IC_{50} are required. Acceptance criteria for the Ames assays were set as follows: 1) ALI control counts fall within specified ranges for TA98 and TA100) and 2) positive controls induce ≥ 2.0-fold increases in revertant numbers over the ALI control. SCE assay acceptance criteria were set as follows: 1) the average number of SCE/cell in the incubator and air controls is within a specified range of 3.7 to 7.2; 2) at least one positive control treatment exhibits ≥ doubling in the average number of SCE/cell over the incubator and air controls; 3) ≥ 15 cells must be scored for at least two chamber replicates and 4) data must be generated from at least three non-zero concentrations. Through critical function and assay assessments via the installation, operational, performance and validation protocols, the VC10 was deemed fit-for-purpose and has been shown to induce exposure-related changes in cell survival, sister chromatid exchange and bacterial mutagenesis. Collectively, these criteria support the validity of the use of the VC10 system with several *in vitro* genetic toxicological assays.

Results

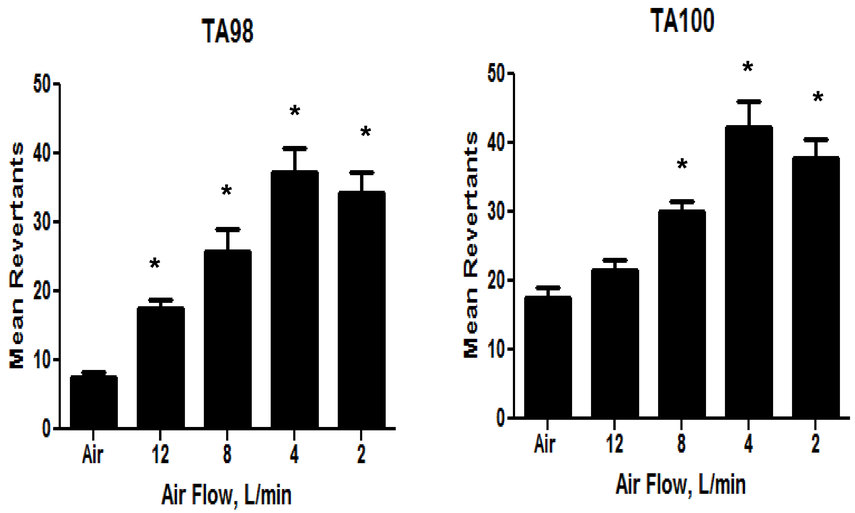

Figure 2. Ames Assay +S9: TA98 and TA100 bacterial strains were exposed to whole smoke at airflows of 12, 8, 4 and 2 L/min for 64 minutes, incubated for 3 days post-exposure and assessed for revertant colonies. Data represent mean response ± SE from seven (TA98) and six (TA100) independent experiments. Asterisk denotes statistically significant (p<0.05; Dunnett's Multiple Comparison Test) difference as compared to ALI.

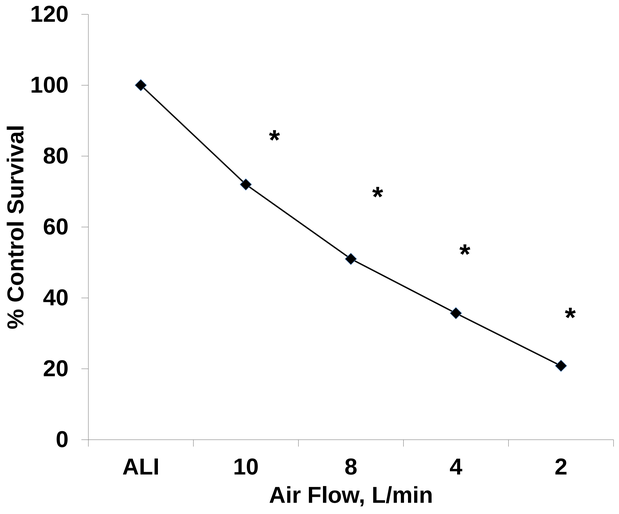

Figure 1. NRU Assay: CHO cells were exposed to whole smoke at air flows of 10, 8, 6 and 2L/min for 64 minutes, and cytotoxicity was assessed 24 hours post-exposure with the NRU assay. Data represent mean response ± SE from six independent experiments. Asterisk denotes statistically significant (p<0.05; Dunnett's Multiple Comparison Test) difference as compared to ALI.

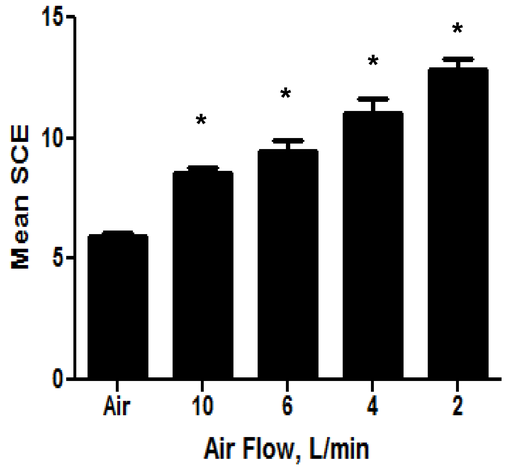

Figure 3. SCE Assay: CHO cells were exposed to whole smoke at air flows of 10, 6, 4 and 2L/min for 64 minutes, and exchange of sister chromatids was assessed at least 24 hours post-exposure with a harvest delay as noted in the Materials and Methods. Data represent mean response ± SE from thirteen independent experiments. Asterisk denotes statistically significant (p<0.05; Dunnett's Multiple Comparison Test) difference as compared to ALI.

Materials and Methods

VC10 Equipment Qualification: Qualification procedures included factory and site acceptance testing, installation, operational and performance qualifications (IQ, OQ and PQ).

Cell Maintenance: Chinese Hamster Ovary (CHO) cells were maintained in McCoy's 5A medium (supplied containing 25 mM HEPES buffer) with 10% (v/v) heat-inactivated fetal bovine serum and 0.52% penicillin-streptomycin added (McCoy's 5A complete). Cell were not used past passage 15. For experiments, cell seeding, exposures and post-exposures were also performed with McCoy's 5A complete.

Cigarette Smoke Generation and Exposures: Whole smoke from Kentucky Reference 3R4F cigarette was generated with a VITROCELL VC10® Smoking Robot using ISO smoking regime. 64-minute exposures (8 cigarettes, 8 puffs/cigarette) at the air liquid interface (ALI) were performed for all endpoints using four smoke concentrations expressed in terms of diluting airflow. Smoke concentrations consisted of airflows of 12, 10, 8, 6, 4 or 2 L/min with 5 mL/minute vacuum. For each endpoint, at least six replicate experiments were performed using the same four smoke concentrations. ALI controls (airflow of 0.2 L/min), untreated controls and positive controls were included for all experiments.

Neutral Red Uptake Assay: CHO cells were seeded 24 hours prior to exposure in 12 mm Transwells™; cell confluence at time of exposure was ~ 50%. Transwells™ were incubated 24 hours post-exposure prior to NRU analysis. Blank Transwells™ (set up at beginning of assay) were used to correct for uptake of NRU dye by the Transwell™ itself. IC_{50} and R^2 values were estimated by fitting an exponential decay model to the data.

Ames Assay: *Salmonella typhimurium* strains TA98 and TA100 were cultured in nutrient broth containing ampicillin at 37°C for 8 hours and subsequently plated on 35 mm plates in the presence of 10% Aroclor-induced rat liver S9. Following whole smoke exposure, plates were incubated at 37°C for up to three days and revertant colonies counted. The slope was determined from the linear portion of the concentration-response curve by fitting a generalized linear model with Poisson distribution and identity link function with a separate parameter 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).

SCE Assay: CHO cells were seeded 24 hours prior to exposure in 24 mm Transwells™; cell confluence at time of exposure was 50-70%. Airflows were treated in separate exposures to allow 12 Transwells™ to be exposed per airflow (3 chambers x 4 dilution bars) to ensure a sufficient number of cells were treated to complete the SCE endpoint. Transwells™ were incubated post-exposure in media containing 5-bromo-2'-deoxyuridine (BrdU) for two cell cycles, and then treated with colchicine for 1.5 hours to accumulate cells in metaphase. Cells were harvested by trypsinization. The three chambers per dilution bar were pooled at harvest. A delayed harvest approach was adopted (based on visual evaluation of cells post exposure) to ensure sufficient cells had reached second division. Chromosome preparations were stained using the fluorescence-plus-Geimsa technique and scored blind for exchanges. Slope values were determined by fitting a linear regression to mean SCE counts with a separate parameter fitted for the top dose; an iterative point-rejection approach was taken, by which the top dose was excluded until the linear portion of the curve was identified.

Summary

- Installation and operational qualification demonstrated that the VC10 smoking robot was supplied as specified, was installed in an environment equipped and suitable for the operation of the equipment and demonstrated that the equipment was suitable for its intended use. Performance qualification demonstrated that all aspects of the VC10 robot perform as intended, met predetermined acceptance criteria and operated to the user specific requirements.
- Critical assay parameters such as cell growth at ALI and stability of pH during exposures were established during development of the NRU, Ames and SCE assays for use with the VC10 instrument, and ICCVAM and OECD guidelines (1 -3) were used in establishing acceptance criteria for the respective assays.
- NRU Assay: Cell survival was impacted across the exposure range, and a mean IC_{50} value of 5.71L/min was observed. Positive control, sodium dodecyl sulphate (2,000µg/ml), caused >50% reduction in survival relative to the ALI control. Consistency was observed across six independent NRU whole smoke experiments, and all acceptance criteria were met.
- Ames Assay: Statistically significant increases in mean revertants were observed in each 3R4F whole smoke experiment. For TA98, >2-fold increase was observed at 12L/min exposure and revertants increased to > 4-fold over the exposure range. TA100 reached a maximum revertant response of 2 to 3-fold increase between 2 and 4L/min exposures. Positive control response for B[A]P (0.8ug/plate) for TA98 and 2-aminoanthracene (0.4ug/plate) for TA100 were > 8-fold and > 10-fold, respectively, and all acceptance criteria were met.
- SCE Assay: Generally, a maximal 2-fold increase in SCEs was achieved in response to whole smoke. However, high variability in the slopes was observed across experiments, and optimization studies were conducted. The positive control, mitomycin C (0.15 and 0.20 µg/ml), yielded > 2 fold-increase in SCEs, and all acceptance criteria were met.
- VC10 was deemed fit-for-purpose and has been shown to induce exposure-related changes in cell survival, bacterial mutagenicity and sister chromatid exchange with several *in vitro* genetic toxicological assays.

References

1) ICCVAM: *In vitro* Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Testing. Publication Date: Nov 2006.
2) OECD Test No. 471: Bacterial Reverse Mutation Test. Publication Date: 21 July 1997 Pages: 11.
3) OECD Test No. 479: Genetic Toxicology: In vitro Sister Chromatid Exchange Assay in Mammalian Cells; Publication Date : 23 Oct 1986 Pages: 5.

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