

# High Throughput Air Liquid Interface Exposure Modules: Characterization of Smoke/Aerosol Dosimetry and *in vitro* Mutagenicity and Cytotoxicity of Two Tobacco Product Types

Robert Leverette<sup>1</sup>, Brian Keyser<sup>1</sup>, Michael Hollings<sup>2</sup> and Adam Seymour<sup>2</sup>

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## Conflict of Interest

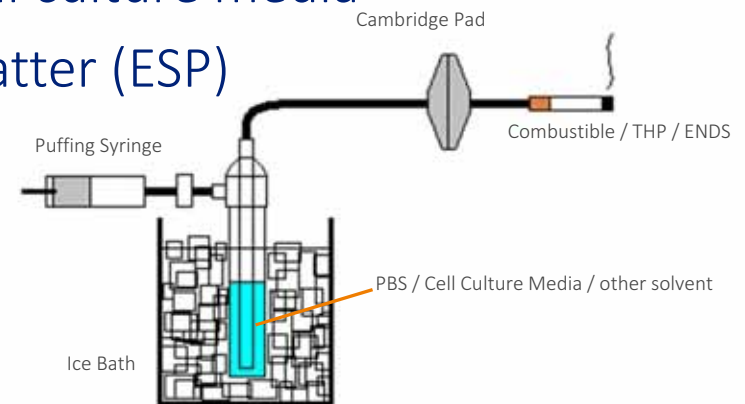
- RAI Services Company (RAISC) performs regulatory compliance services for Reynolds American Inc.'s ("RAI") subsidiary companies. RAI is an indirect, wholly owned subsidiary of British American Tobacco p.l.c.
- The data presented were generated and analyzed in studies commissioned by RAI Services Company and conducted, under contract, at Covance Laboratories Ltd.



## Introduction

Testing of smoke / aerosol generating tobacco products

- Historically, collected test samples include:
  - Pad-collected total particulate matter (TPM)
  - Liquid-trapped gas vapor phase (GVP)
  - Whole smoke bubbled / conditioned cell culture media
  - Electrostatic precipitated particulate matter (ESP)
  - Cold-trapped whole smoke condensate
- Caveats to above collection methods:
  - Smoke fractionation
  - Incomplete collection / capture





## Whole Smoke / Aerosol Exposure

Testing of smoke / aerosol generating tobacco products

- Whole smoke / aerosol exposure is the preferred method for *in vitro* testing
  - No fractionation of smoke / aerosol through separate collection of particulate and gas vapor phases
  - Direct exposure at air-liquid interface (ALI) and air-agar interface (AAI)
  - Multiple cell types and biological endpoints are adaptable to whole smoke / aerosol exposure



## Whole Smoke / Aerosol Exposure

Testing of smoke / aerosol generating tobacco products

- Challenges for ALI and AAI exposure systems
  - Complexity of exposure systems
    - Smoke / aerosol generation, dilution and delivery to cell cultures
    - Smoking / exposure atmosphere control (temperature and humidity)
    - Dose determination
  - Sample throughput requirements
    - Limited number of doses and cultures per exposure
    - Exposure times (next generation products)
  - Cell culture



## Whole Smoke / Aerosol Exposure

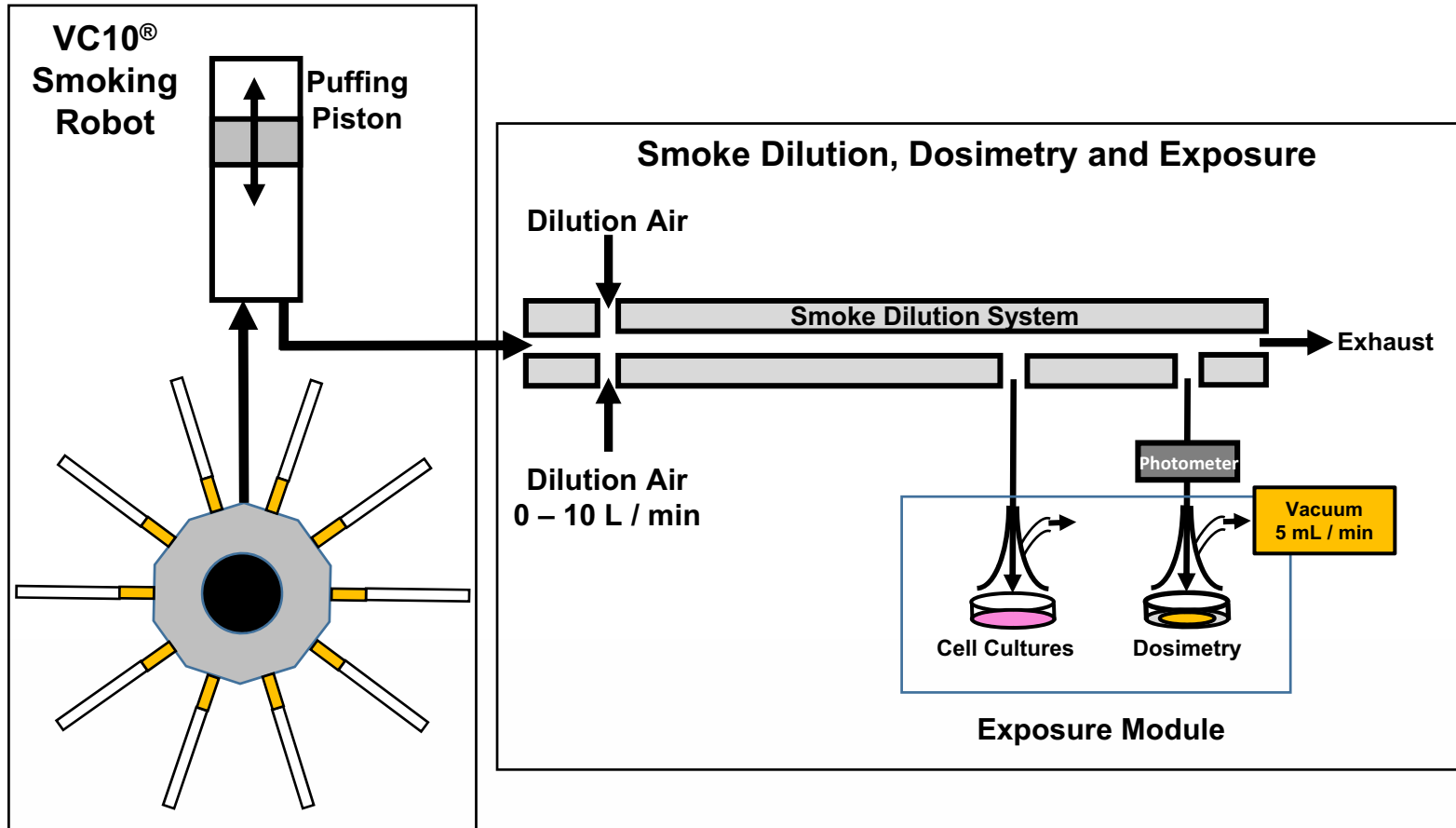
Testing of smoke / aerosol generating tobacco products

- RAISC currently utilizes Vitrocell® designed aerosol generation and exposure equipment for bacterial and mammalian cell *in vitro* exposures
  - VC10® rotary smoke machine
  - Aerosol dilution system
  - Standard exposure modules
- Current set-up limits efficient sample throughput



Standard Exposure Module  
[www.vitrocell.com](http://www.vitrocell.com)

# Vitrocell® Smoke / Aerosol Exposure System: Overview







# Whole Smoke / Aerosol High Throughput Exposure Module

## System Requirements

- Module features & capabilities:
  - Bacteria culture dishes (35mm)
  - Mammalian cell culture inserts (24mm)
  - Multi-well format
  - Serial dilution of smoke / aerosol
- Vitrocell® AMES 48 and 6/48 systems
- Characterize smoke / aerosol delivery within this system to verify if “Fit for Use” for *in vitro* testing





# Whole Smoke / Aerosol High Throughput Module

## System Characterization

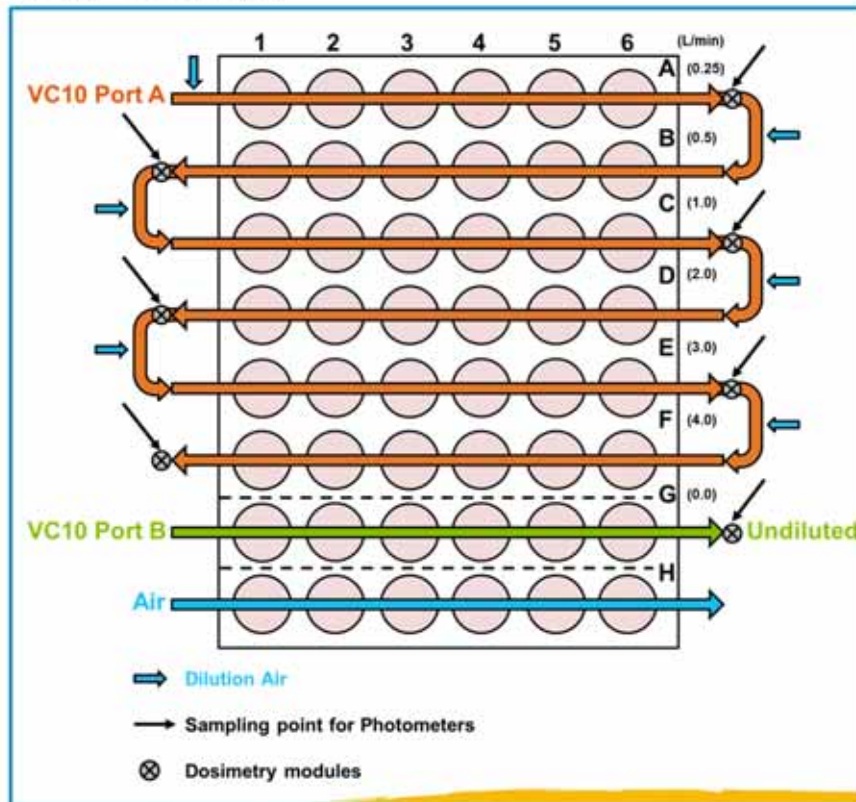
- Smoke / aerosol delivery within the module from:
  - 3R4F reference cigarette, Tobacco Heating Product (THP), Electronic Nicotine Delivery System (ENDS)
    - 3R4F: Fluorescence (Ex355/Em485) of DMSO trapped particulate matter
    - THP and ENDS: glycerol deposited in PBS traps
    - Nicotine (limited data)
- Biological endpoints: 3R4F whole smoke
  - Ames (TA98 & 100, +S9)
  - Cytotoxicity: Neutral Red Uptake (NRU; CHO cells)



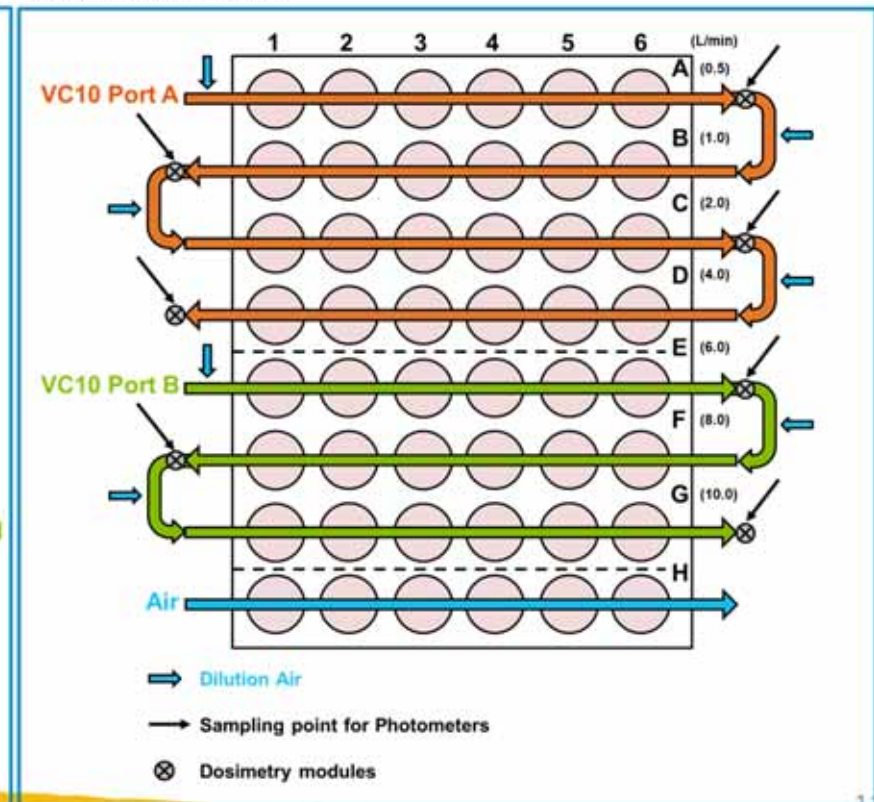
# Whole Smoke / Aerosol High Throughput Module

System Characterization: Exposure Set-up

## THP & ENDS



## Combustible





# Whole Smoke / Aerosol High Throughput Module

System Characterization

## Results





# Whole Smoke / Aerosol High Throughput Module

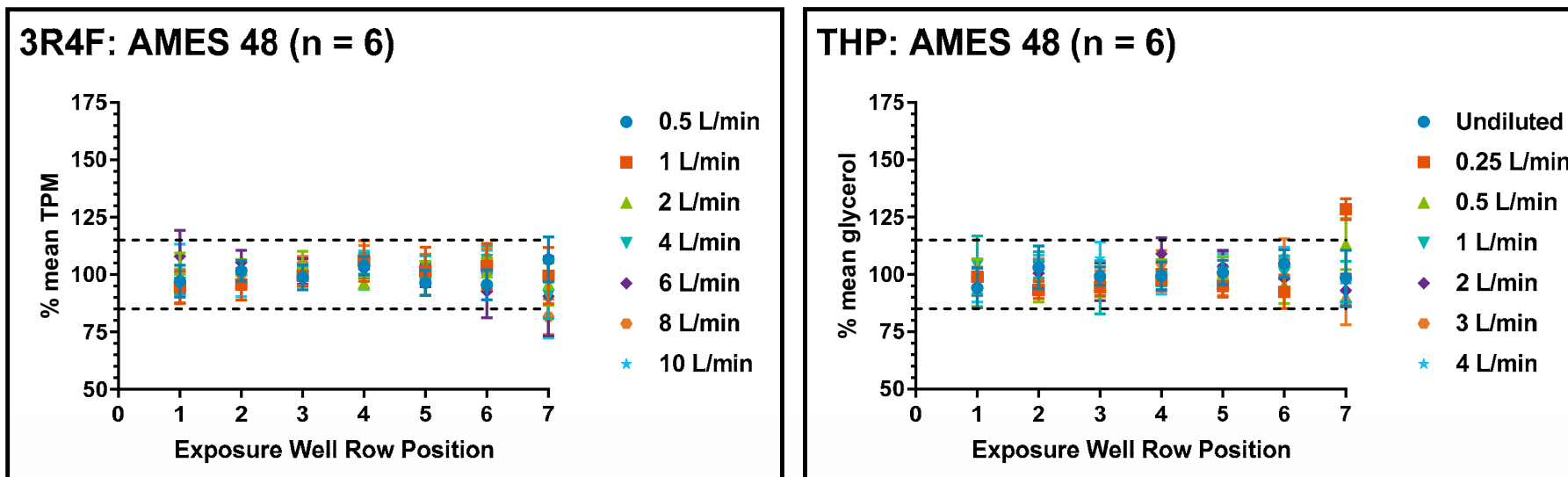
System Characterization: Deposition Results

- AMES 48 and 6/48 (mammalian) exposure modules
- Deposition
  - TPM (3R4F) and glycerol (THP, ENDS)
  - Values are presented as % of the overall mean for each dose
  - Dashed lines (--) are  $\pm 15\%$
  - Mean  $\pm$  SD from six independent experiments
- Biological Endpoints: 3R4F whole smoke
  - Mutagenicity (Ames assay)
  - Cytotoxicity (NRU)



# Whole Smoke / Aerosol High Throughput Module

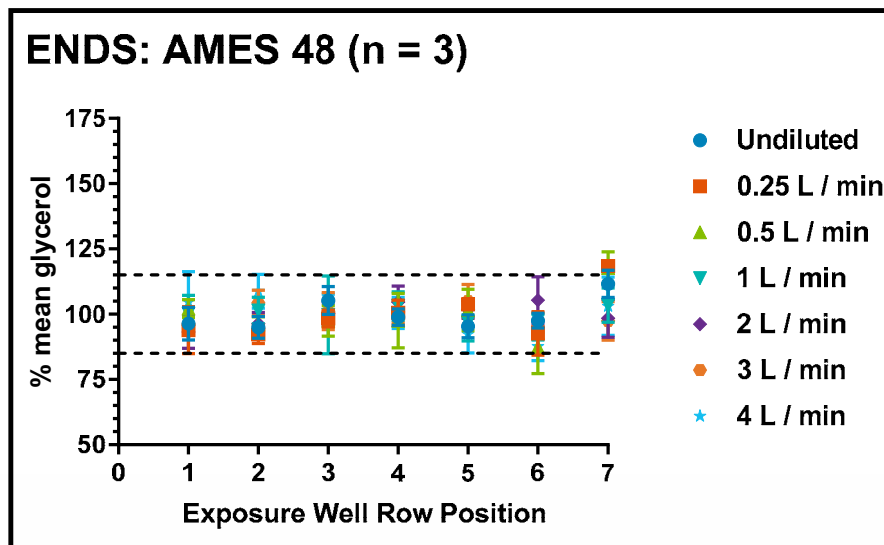
System Characterization: AMES 48; 3R4F & THP Deposition Results





## Whole Smoke / Aerosol High Throughput Module

System Characterization: AMES 48; ENDS Deposition Results



ENDS AMES 48 characterization is ongoing.  
Data shown from first 3 of 6 experiments

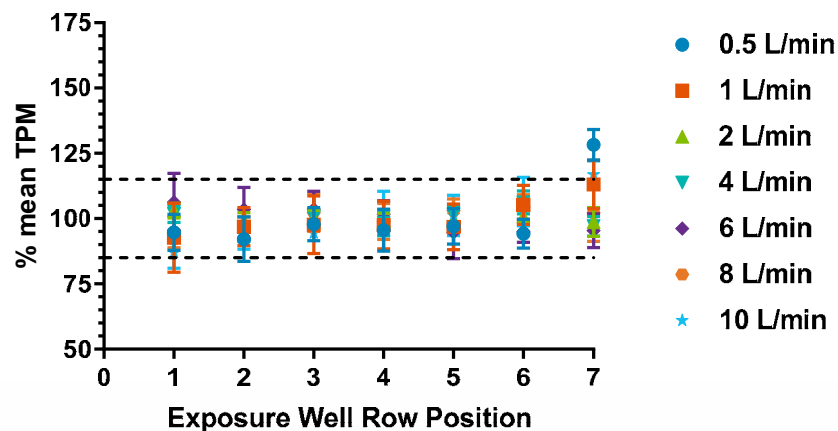
Observed consistent delivery of smoke / aerosol within the AMES 48 module at the dilutions tested.



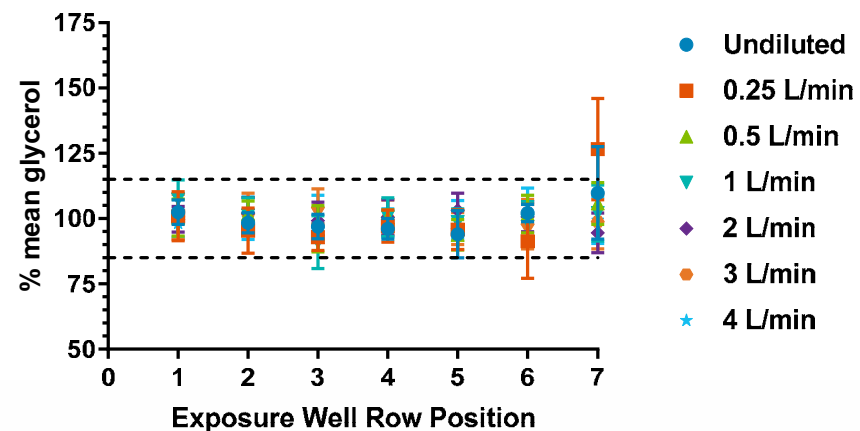
# Whole Smoke / Aerosol High Throughput Module

System Characterization: 6/48; 3R4F & THP Deposition Results

**3R4F: 6/48 "Mammalian" (n = 6)**



**THP: 6/48 "Mammalian" (n = 6)**

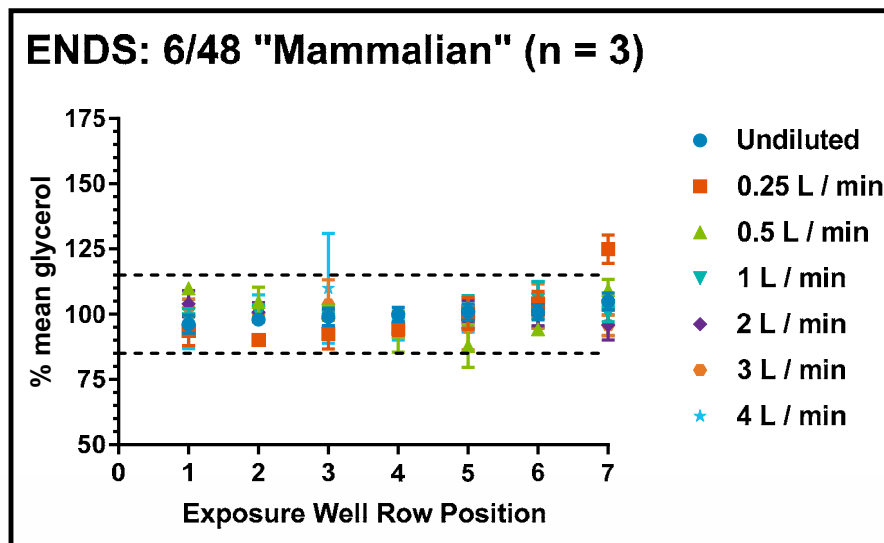






## Whole Smoke / Aerosol High Throughput Module

System Characterization: 6/48; ENDS Deposition Results



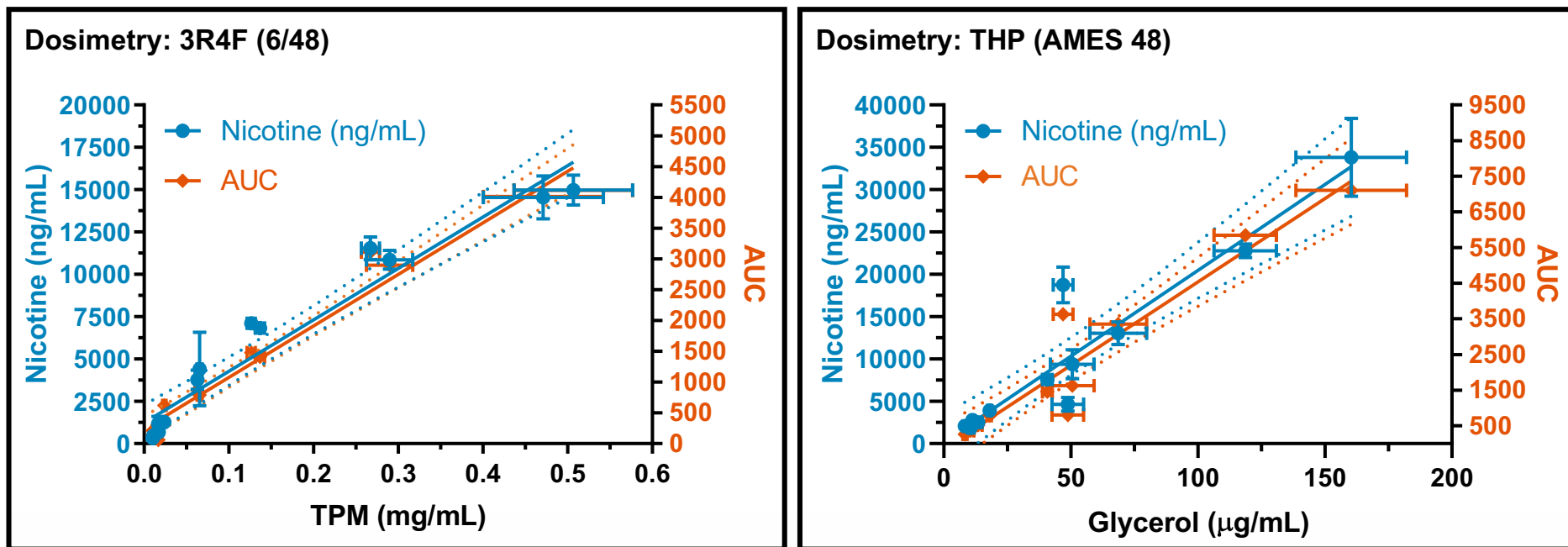
ENDS 6/48 characterization is ongoing.  
Data shown from first 3 of 6 experiments

Observed consistent delivery of smoke / aerosol within the 6/48 module at the dilutions tested.



## Whole Smoke / Aerosol High Throughput Module

System Characterization: Nicotine Delivery (ongoing analysis)



- AUC: Area Under the Curve (Volt\*sec), data from the laser photometers
- Data from representative exposures of 3R4F and THP in the 6/48 and AMES 48 modules, respectively
- Nicotine concentration correlates to smoke / aerosol deposition (TPM and glycerol)

# Whole Smoke / Aerosol High Throughput Module

System Characterization: Biological Endpoint Results

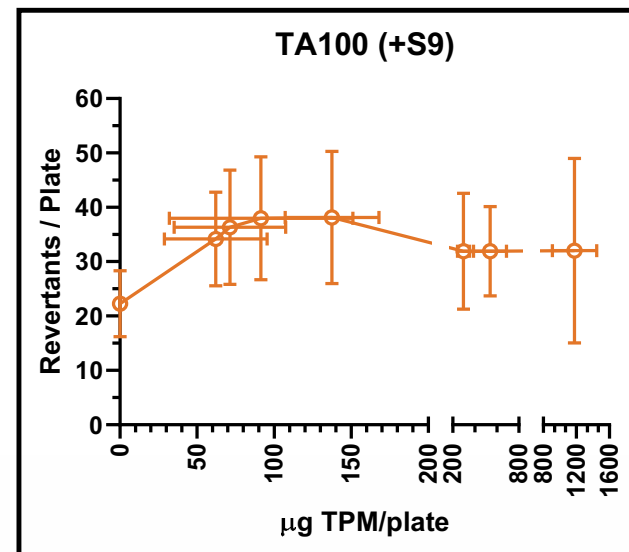
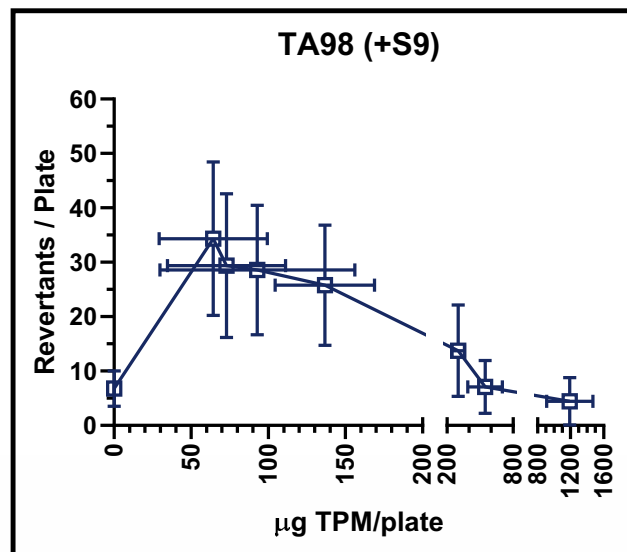
## Ames Assay



## Whole Smoke / Aerosol High Throughput Module

System Characterization: Ames Assay 3R4F Whole Smoke

- TA98 and TA100 (+S9) exposed to 3R4F whole smoke
  - HCl smoking parameters
    - 55 mL puff volume
    - 2 sec puff
    - 30 sec puff interval
  - 4 - 6 cigarettes
  - TA98: n = 16 exposures
  - TA100: n = 18 exposures



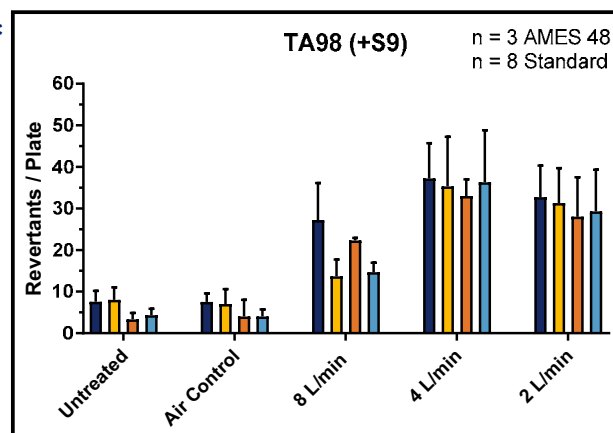
3R4F whole smoke induced an increase in revertant counts in TA98 and TA100 (+S9).  
Assay conditions for HCl generated smoke do require further optimization.



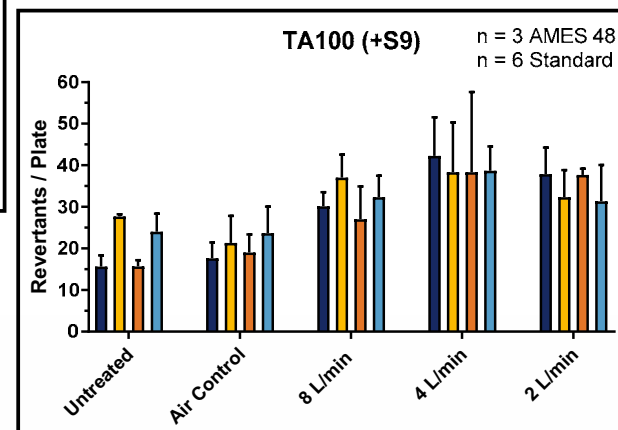
# Whole Smoke / Aerosol High Throughput Module

System Characterization: Ames Assay 3R4F Whole Smoke

- Compare to Historical Data\*
  - TA98 & TA100 (+S9)
  - ISO smoking parameters
    - 35 mL puff volume
    - 2 sec puff
    - 60 sec puff interval
  - 8 X 3R4F cigarettes
  - AMES 48 module
  - Standard AMES Module



Standard Module  
AMES 48 Ex 1  
AMES 48 Ex 2  
AMES 48 Ex 3



AMES 48 and standard exposure modules gave comparable results under similar assay conditions.

\*Fowler et al. (2018) Development, qualification, validation and application of the Ames test using a VITROCELL® VC10® smoke exposure system. *Toxicology Reports* 5, 542–551



# Whole Smoke / Aerosol High Throughput Module

System Characterization: Biological Endpoint Results

## Cytotoxicity

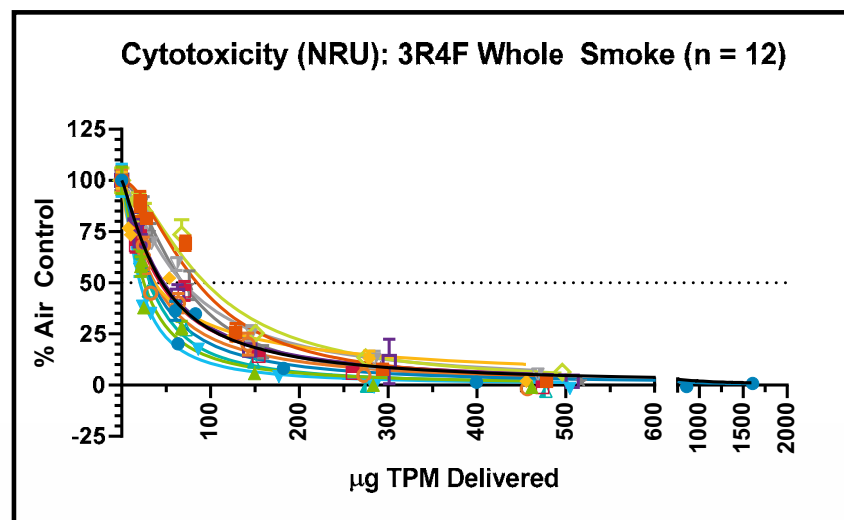




## Whole Smoke / Aerosol High Throughput Module

System Characterization: 3R4F Whole Smoke Cytotoxicity (NRU)

- CHO cells exposed to 3R4F whole smoke
  - HCI smoking parameters
  - 3 cigarettes
  - 12 independent exposures
- NRU
  - Calculate  $IC_{50}$



HCI High Throughput Exposure Module	$r^2$	$IC_{50}$ ( $\mu\text{g TPM}$ )	SE
1*	0.9625	34.8	2.7
2	0.9861	85.0	2.2
3	0.9545	25.2	0.8
4	0.9741	20.8	0.6
5	0.9715	36.4	1.4
6	0.9589	37.3	1.0
7	0.9761	47.4	1.3
8	0.9664	94.2	2.7
9	0.9634	43.4	1.4
10	0.9830	32.0	0.6
11	0.9807	69.2	1.4
12	0.9853	68.8	1.2
Global Fit	0.8929	45.6	0.7

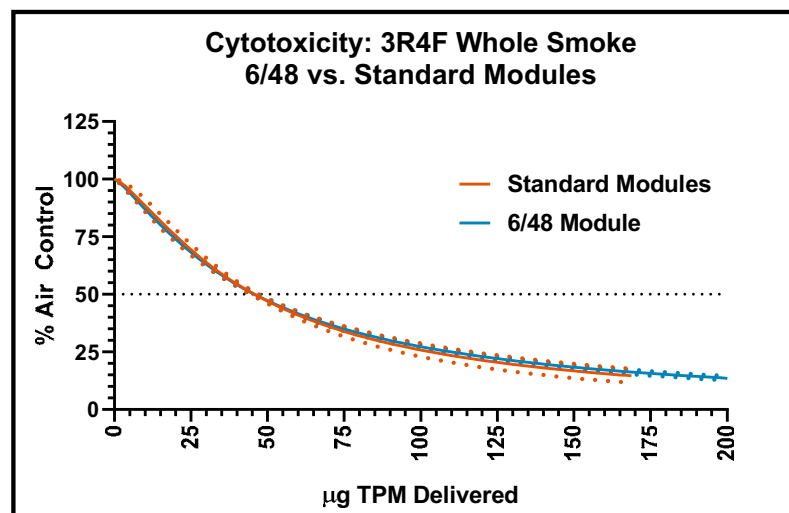
\*Exposure 1 = 8 X 3R4F cigarettes



## Whole Smoke / Aerosol High Throughput Module

System Characterization: 3R4F Whole Smoke Cytotoxicity (NRU)

- CHO cells exposed to 3R4F whole smoke
  - HCI smoking parameters
  - 3 cigarettes
  - 6/48 Module: 12 independent exposures
  - Standard Module: 3 independent exposures
- NRU
  - Global fit from all exposures
  - Calculate  $IC_{50}$



Module	$r^2$	$IC_{50}$ ( $\mu\text{g TPM}$ )	SE
6/48	0.8929	45.6	0.7
Standard	0.8544	47.7	2.6

$p = 0.4388$ ; extra sums-of-squares F test, GraphPad





## Summary and Conclusions

### Whole Smoke / Aerosol High Throughput Module

- Freshly generated whole smoke / aerosol from three different tobacco product types (3R4F, THP and ENDS) was consistently delivered within the AMES 48 and 6/48 exposure modules.
- Biological endpoints: 3R4F whole smoke induced increased revertant counts (Ames) and cytotoxicity (NRU) in the AMES 48 and 6/48 modules, respectively.
- Revertant counts (Ames) and cytotoxicity ( $IC_{50}$ ) values were comparable to those from the standard exposure modules when run under similar exposure conditions.
- Overall, the AMES 48 and 6/48 modules are deemed “Fit for Use” for the *in vitro* evaluation of different tobacco product types (combustible, THP, ENDS).



## Acknowledgements

- Vitrocell team
- Covance team
- RJRT
  - Jannell Rowe
  - Randy Weidman

