

# Toxicity Screening of Volatile Chemicals Using a Novel Air-Liquid Interface *In Vitro* Exposure System

Alternatives for Inhalation Toxicity Testing Meeting

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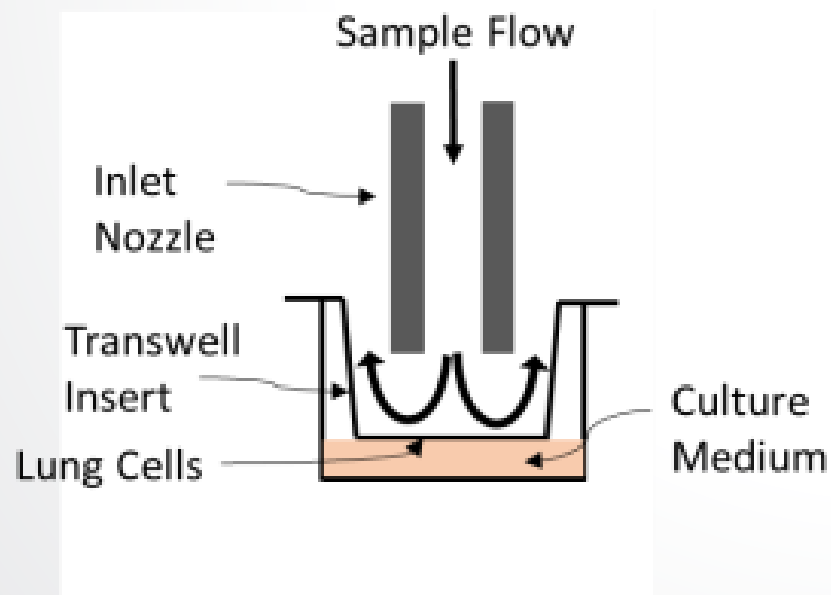




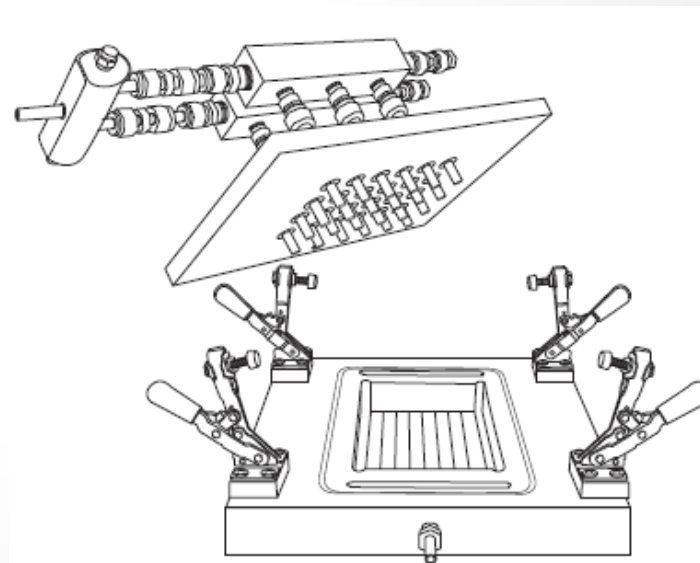
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# Main Objective

**Utilize direct exposure of volatile chemicals to cells at ALI in order to generate transcriptomic data capable of identifying concentration-dependent mechanism/mode-of-action.**



**24-Well Format**





# VOC Exposure –Study Objectives

- Develop a workflow for medium-throughput **screening of volatile chemicals** in concentration response **for bioactivity using whole transcriptome targeted RNA-Sequencing** (i.e. BioSpyder's TempO-Seq™).
- Assess the technical and biological reproducibility of transcriptomic data in an **air-liquid interface cell culture dynamic exposure model**.
- Evaluate the ability of the transcriptomic data to identify **concentration-dependent changes in mechanism/mode-of-action**.
- Evaluate the ability of the transcriptomic data from cell culture models to **group chemicals by similar bioactivity profiles** for potential grouping and read across applications.
- Evaluate the use of **ACGIH TLV as a guide for concentration test range**.



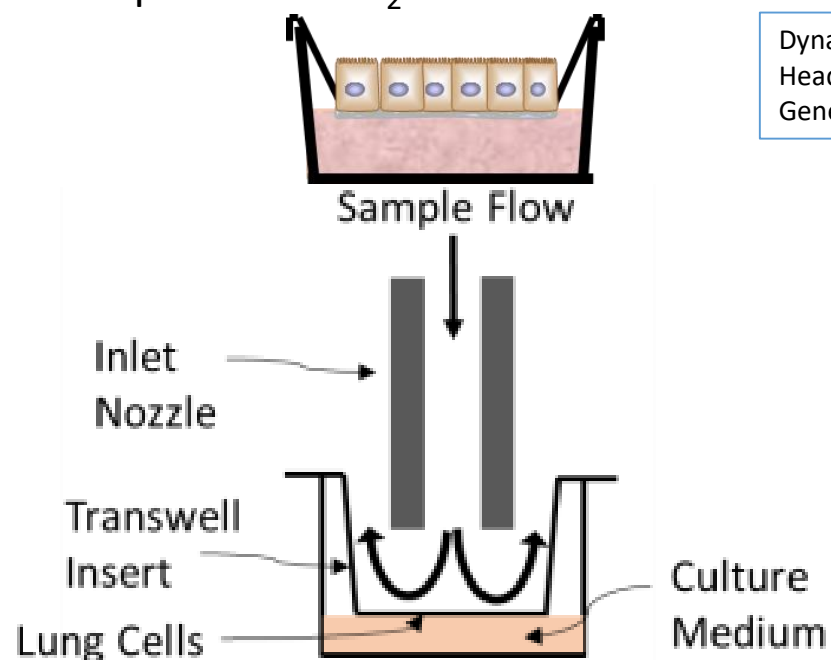
# Overview: Volatile Chemical Screening with HTTr

| Cell Types            | Primary Human Bronchial Epithelial Cells*<br>BEAS-2B cells   |   |   |
|-----------------------|--|---|---|
| Test Chemical         | 1,3-Butadiene<br>Acrolein<br>Formaldehyde  | Acetaldehyde<br>Trichloroethylene*<br>1-Bromopropane* | Carbon Tetrachloride*<br>Dichloromethane* |
| Exposure Regimen      | <ul style="list-style-type: none"><li>• 6 concentrations, sham control, incubator control</li></ul>              |   |   |
| Exposure Duration     | <ul style="list-style-type: none"><li>• 2 hours, Assays conducted 4h post exposure</li></ul>                     |   |   |
| Technical Replicates  | <ul style="list-style-type: none"><li>• TempO-Seq, n=2; Viability, n=2; Cytotoxicity, n=4</li></ul>              |   |   |
| Biological Replicates | <ul style="list-style-type: none"><li>• Exposures per cell type conducted over three days, n=3</li></ul>         |   |   |
| Assay Formats         | <ul style="list-style-type: none"><li>• TempO-Seq</li><li>• Cytotoxicity [LDH Release, Cell Titer Glo]</li></ul> |   |   |

# Exposure Overview

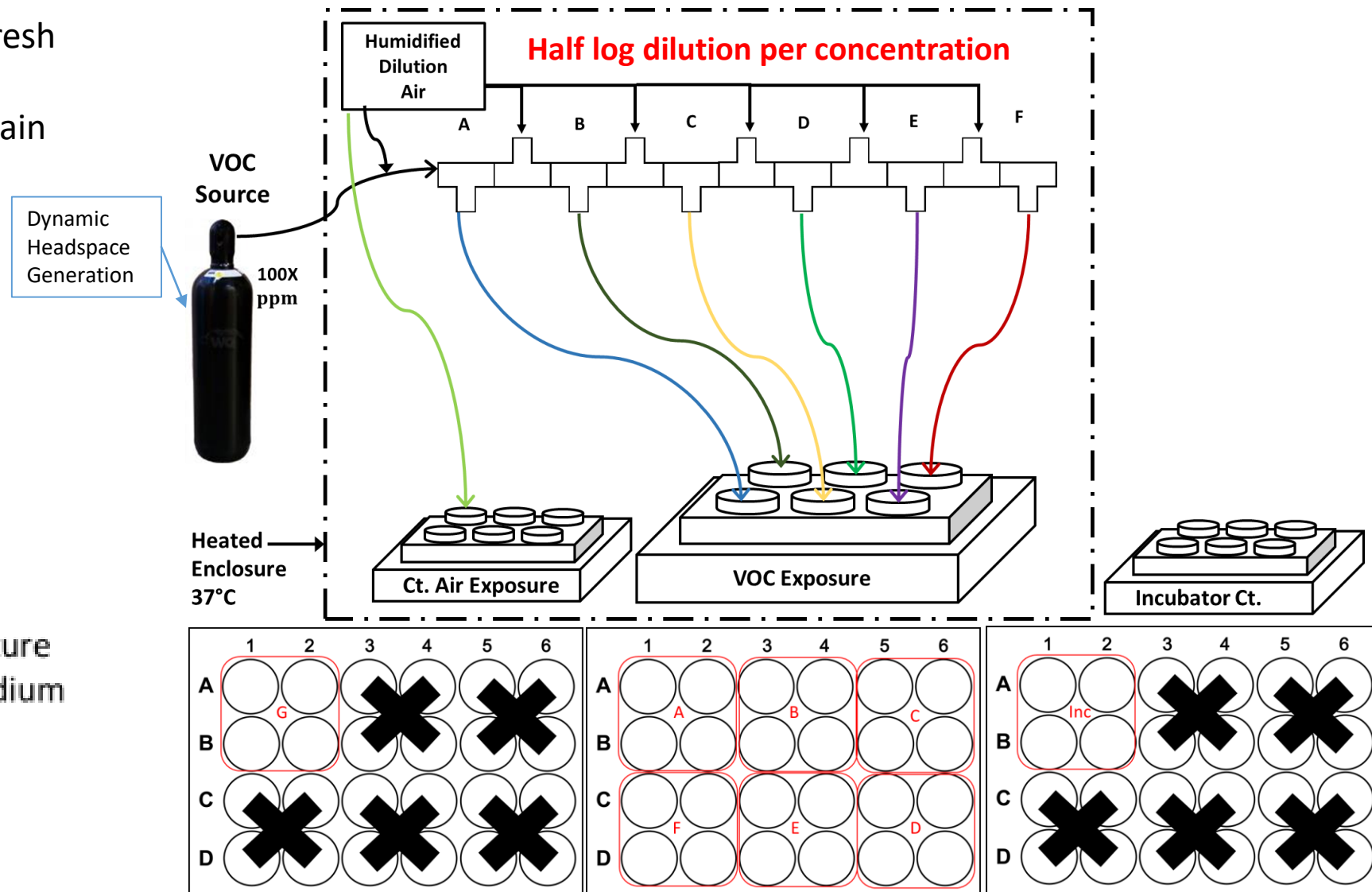
## Pre-exposure

- All cells grown at ALI
- Apical side **washed** and given fresh media 2h prior to exposure
- HEPES buffered media to maintain pH in low CO<sub>2</sub> environment



## Post-exposure

- VOC exposure for 2h
- Cells removed from CCES and samples collected 4h post-exposure

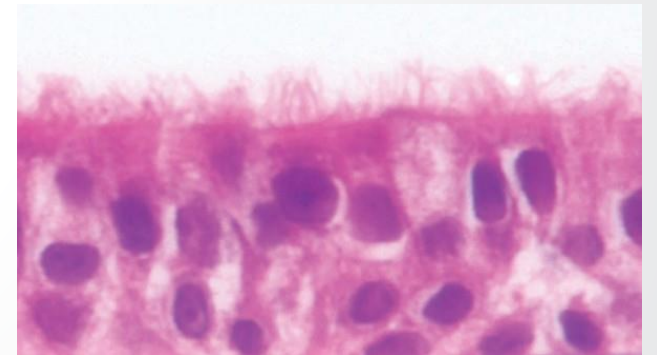
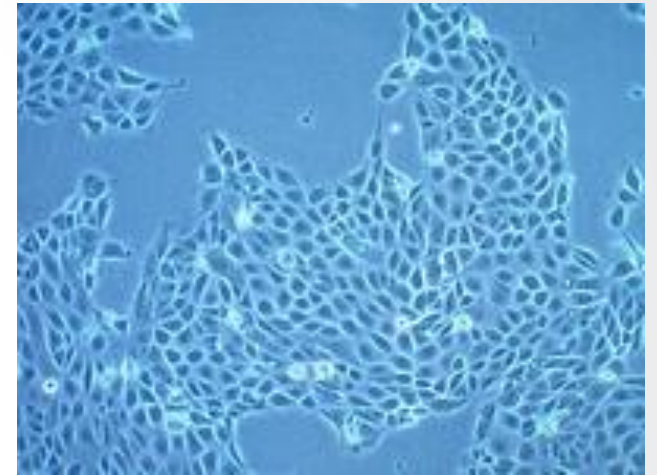


## 3 days of exposure per cell type



## Cell Models

- **BEAS-2B:** human bronchial epithelial cell line
  - 3 day expansion schedule
- **HBEC:** Human primary bronchial epithelial cells
  - Obtained from healthy volunteers at the HSF at EPA
  - Approximately 28 days to grow and fully differentiate
  - Ciliated and mucus producing cells present
  - Multiple donors





# VOCs of Interest

## Acrolein

- Chemical intermediate for acrylic acid and certain **biocide** formulations
- Combustion product, component of cigarette smoke

## 1,3-Butadiene

- Product of processing petroleum and rubber products and combustion product

## Acetaldehyde

- Synthesis of perfumes, polyesters, dyes, and combustion product
- Fruit and fish **preservative** and flavoring agent

## Formaldehyde

- Used in resins for manufacture of wood products and building materials
- Component of **fertilizers and pesticides and combustion product**

## 1-Bromopropane

- Component of **degreasing solvents**, adhesive sprays, and dry cleaning chemicals

## Dichloromethane

- Component of paint finisher, stripping agents and **pesticides**

## Trichloroethylene

- Solvent for cleaning in metal manufacturing and an intermediate for **refrigerants**

## Carbon Tetrachloride

- Component of **refrigerants** and propellants for aerosols



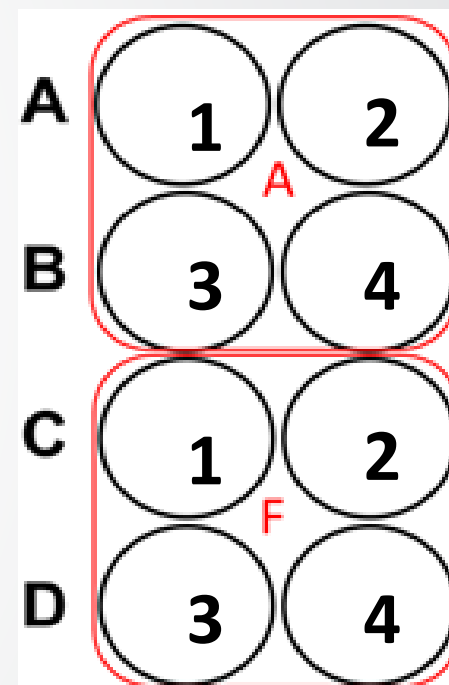




# Analytical Endpoints

## Molecular Assays

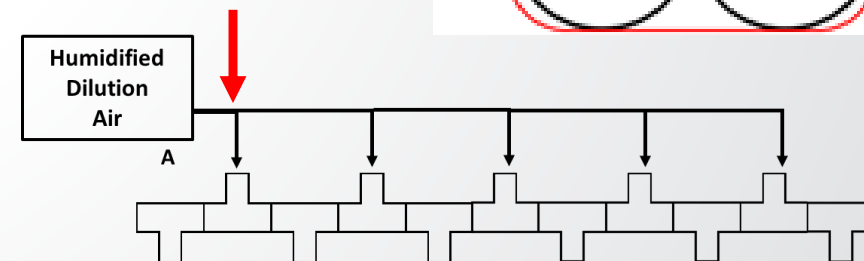
- LDH Cytotoxicity Assay ← **Sampled in all wells**
  - Measurement of cell membrane integrity by the release of LDH into the basolateral medium or apical wash (recent only)
- CellTiter-Glo Viability Assay ← **Sampled from wells 1 and 2**
  - Measures amount of ATP generated from viable cells
- Multiplexed Transcription Factor Activation Assay (BioSpyder TempO-Seq™) ← **Sampled from wells 3 and 4**
  - Whole transcriptome targeted RNA-Sequencing



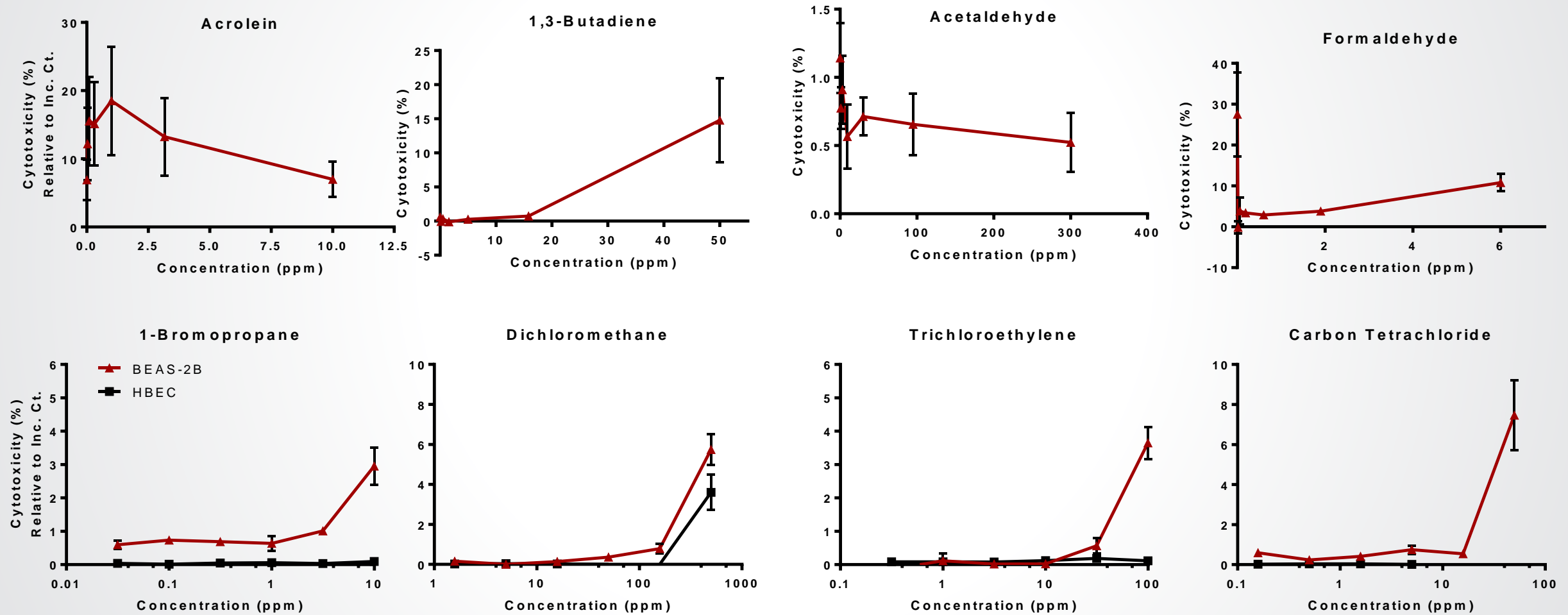
## Chemical Generation Endpoints

- Off-line GC/FID/ECD determinations of each concentration port just before entering CCES
- Analytical to Nominal ratio calculated based on total generated chemical and any losses due to transport

**Sampled after each dilution**

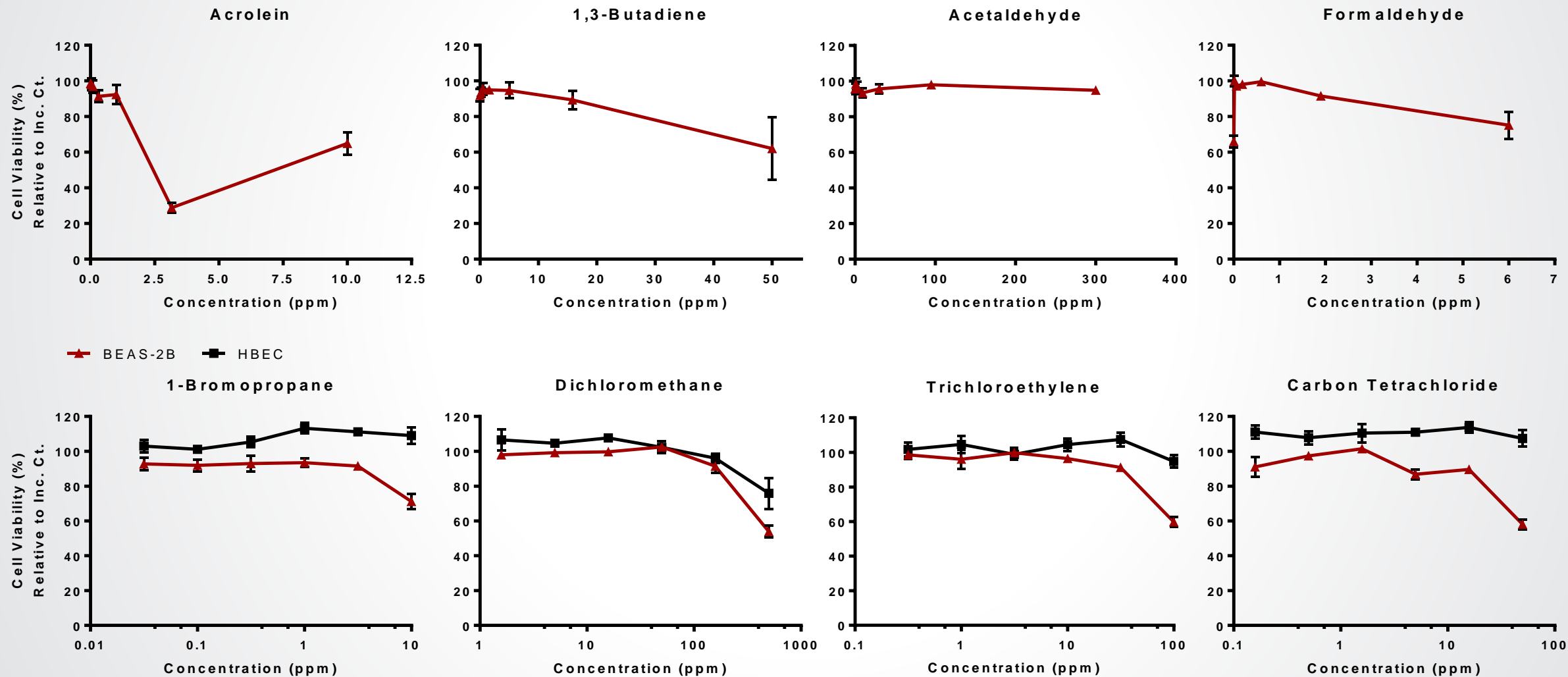


# Cytotoxicity in Tested Concentrations of Volatile Chemicals



% Change in Cytotoxicity (LDH release) from control

# Cell Viability in Tested Concentrations of Volatile Chemicals



% Change in Viability (CellTiterGlo) from control

# Benchmark Dose Modeling



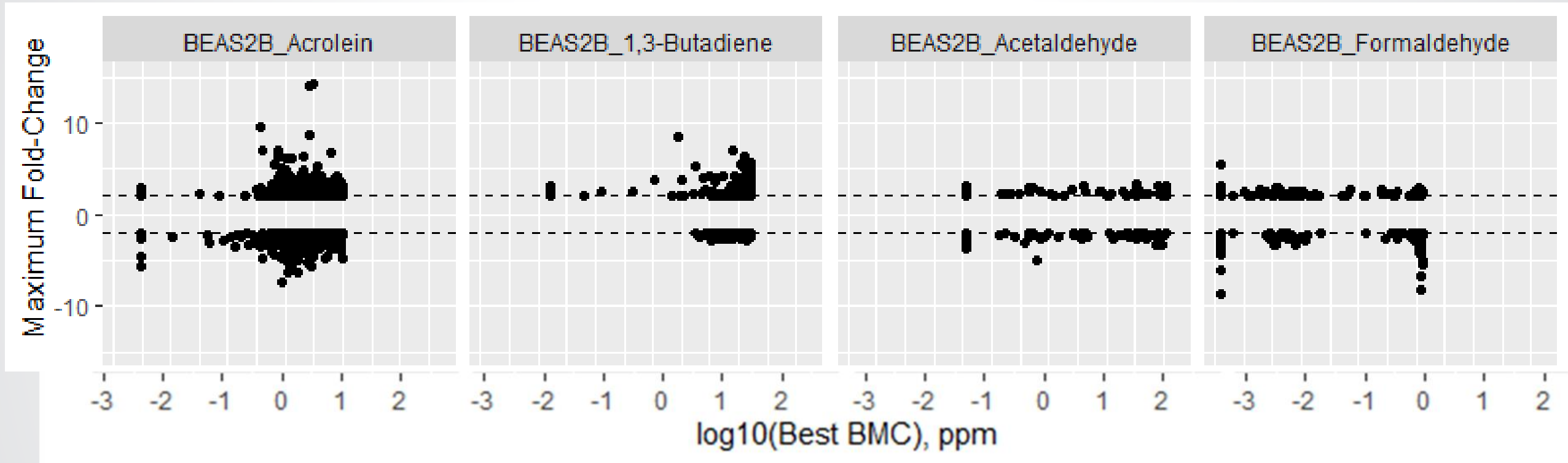
| Parameter                           | Criteria <sup>a</sup>   |
|-------------------------------------|---|
| Pre-filter:                         | ANOVA ( $p_{\text{raw}} < 0.05$ & $ FC  \geq 2$ )                           |
| Models                              | Hill, Power, Linear, Poly2, Exponential 2 3 4 5                             |
| BMR Factor:                         | BMD <sub>10</sub>   |
| Best Model Selection:               | Lowest AIC  |
| Hill Model Flagging <sup>b</sup> :  | 'k' < 1/3 Lowest Positive Dose<br>Discard Flagged Models                    |
| Pathway Analysis:                   | Genes with BMD $\leq$ Highest Dose $\geq 3$<br>$\geq 5\%$ Gene Set Coverage |
| Gene Set Collections <sup>c</sup> : | MsigDB_C2<br>MsigDB_Hallmark<br>Reactome                                    |

<sup>a</sup> Exploratory analysis – modeling criteria not finalized

<sup>c</sup> Gene Set Collections:

- **MsigDB\_C2:** Expert curated from online pathway databases and biomedical literature. (n=5501)
- **MsigDB\_Hallmark:** Well-defined biological states and coherent expression for relevance. (n=50)
- **Reactome:** Open-source, curated and peer reviewed pathway database with hierarchical pathway relationships in specific domains of biology. (n = 1764).

# BMC Modeling Results for BEAS-2B cells exposed to Volatile Chemicals (Probe Level)



Measure of potency for the most sensitive change to a single target gene meeting the BMC modeling parameters.



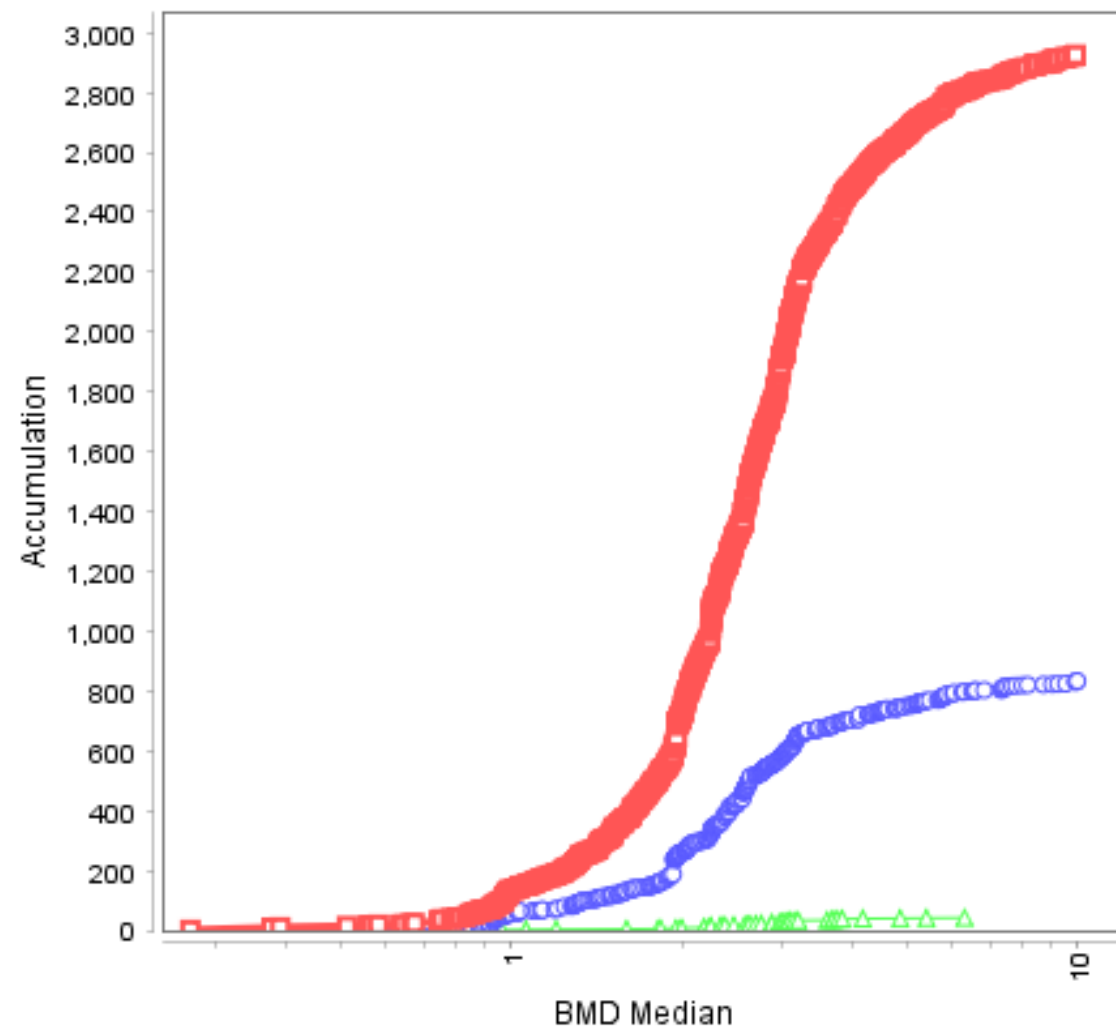
# BMC (BEAS-2B), TLV, and *In Vitro* Evidence Comparison

| Chemical Name | Gene Set Collection | BEAS-2B, BMC of most sensitive gene set (ppm) | HBEC, BMC of most sensitive gene set (ppm) | ACGIH TLV |
|---------------|---------------------|---|--|-----------|
| Acrolein      | MSigDB_C2           | 0.647371                                      | --   | 0.1ppm    |
|               | MSigDB_H            | 1.33022                                       | --   |           |
|               | Reactome            | 0.790052                                      | --   |           |
| 1,3-Butadiene | MSigDB_C2           | 10.96505                                      | --   | 10ppm     |
|               | MSigDB_H            | 38.8237                                       | --   |           |
|               | Reactome            | 32.00305                                      | --   |           |
| Acetaldehyde  | MSigDB_C2           | 245.115                                       | --   | 25ppm     |
|               | MSigDB_H            | NA  | --   |           |
|               | Reactome            | NA  | --   |           |
| Formaldehyde  | MSigDB_C2           | NA  | --   | 0.3ppm    |
|               | MSigDB_H            | NA  | --   |           |
|               | Reactome            | NA  | --   |           |

| Chemical Name | LC50  | LOAEL  | NOAEL   | LOAEL/NOAEL Conditions   |
|---------------|---|--|---|--|
| Acrolein      | 326 ppm (rats), 66 ppm (mice, 6h) <sup>13</sup> | 0.4 ppm (rats: metaplastic and inflammatory changes) <sup>13</sup>   | NR  | subchronic: 6h/d, 5 d/wk, 13 wk  |
| 1,3-Butadiene | 129000 (rats), 122000 (mice, 2h) <sup>20</sup>  | 625 ppm† (mice: alveolar epithelial hyperplasia) <sup>21</sup>       | 8000 ppm* (rat: respiratory) <sup>22</sup> , 200 ppm† (mice, respiratory) <sup>21</sup> | 6h/d, 5 d/wk, *13 wk or †40wk  |
| Acetaldehyde  | 20,555 ppm (rats) <sup>23</sup>                 | 404 ppm (rats: respiratory, 1 wk) <sup>24</sup>                      | 152 ppm (rats: respiratory, 1 wk) <sup>24</sup>   | 6h/d, 7d/wk, 1 wk  |
| Formaldehyde  | 165 ppm (rats), 325 ppm (mice) <sup>4,14</sup>  | 2 ppm <sup>#</sup> (rat: increased nasal pathology) <sup>15,16</sup> | 1 ppm* (rat: increased nasal pathology) <sup>17,18,19</sup>                             | Acute repeated <sup>#</sup> : 6h/d for 1-4 days <sup>14</sup> , or 8h/day for 3-30 days <sup>15</sup> ; subchronic: *6h/d, 5 d/wk, 13 wk |

# BMC Modeling Results for BEAS-2B cells exposed to Volatile Chemicals (Probe and Pathway Level)

## BMD Median Accumulation Plot



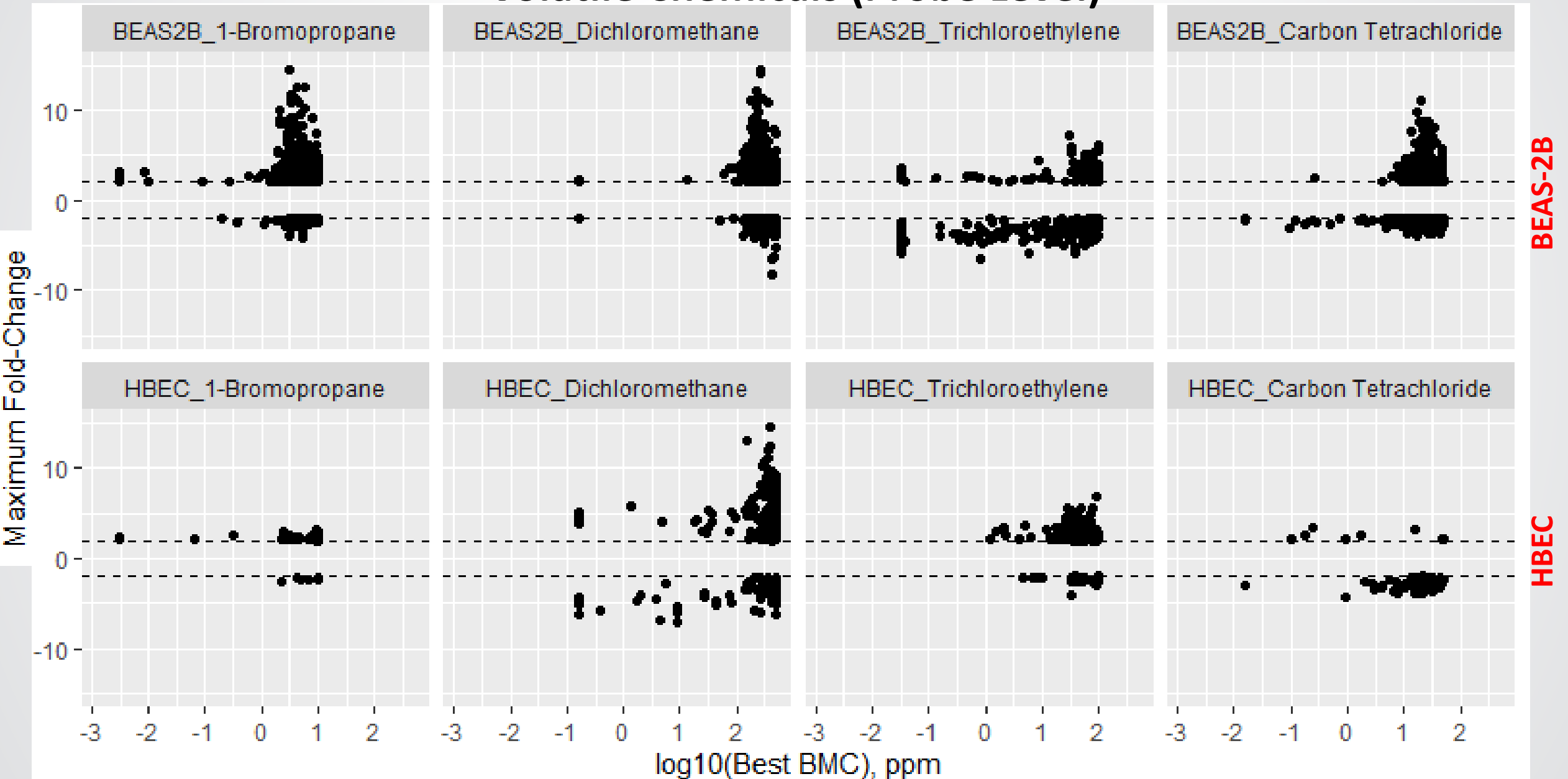
| GO/Pathway/Gene Set/Gene ID | GO/Pathway/Gene Set/Gene Name                           | BMD Median |
|-----------------------------|---|------------|
| M2053                       | ZEMBUTSU_SENSITIVITY_TO_VINBLASTINE                     | 0.271574   |
| M10762                      | IGARASHI_ATF4_TARGETS_UP                                | 0.377698   |
| M1193                       | HERNANDEZ_MITOTIC_ARREST_BY_DOCETAXEL_2_DN              | 0.377698   |
| M1244                       | WIEMANN_TELOMERE_SHORTENING_AND_CHRONIC_LIVER_DAMAGE_DN | 0.377698   |
| M19909                      | AMIT_EGF_RESPONSE_40_MCF10A                             | 0.377698   |

| GO/Pathway/Gene Set/Gene ID | GO/Pathway/Gene Set/Gene Name                  | BMD Median |
|-----------------------------|--|------------|
| R-HSA-2243919               | Reactome; Crosslinking of collagen fibrils     | 0.513108   |
| R-HSA-204626                | Reactome; Hypusine synthesis from eIF5A-lysine | 0.583292   |
| R-HSA-196783                | Reactome; Coenzyme A biosynthesis              | 0.665882   |
| R-HSA-199220                | Reactome; Vitamin B5 (pantothenate) metabolism | 0.665882   |
| R-HSA-164843                | Reactome; 2-LTR circle formation               | 0.744981   |

| GO/Pathway/Gene Set/Gene ID | GO/Pathway/Gene Set/Gene Name            | BMD Median |
|-----------------------------|--|------------|
| M5938                       | HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY | 0.962098   |
| M5913                       | HALLMARK_INTERFERON_GAMMA_RESPONSE       | 1.0616215  |
| M5936                       | HALLMARK_OXIDATIVE_PHOSPHORYLATION       | 1.2007     |
| M5934                       | HALLMARK_XENOBIOTIC_METABOLISM           | 1.59775    |
| M5902                       | HALLMARK_APOPTOSIS                       | 1.8168     |

- MsigDB\_C2
- Reactome
- MsigDB\_Hallmark

# BMC Modeling Results for BEAS-2B and Primary HBE cells exposed to Volatile Chemicals (Probe Level)



# BMC (BEAS-2B and HBEC), TLV, and *In Vitro* Evidence Comparison

| Chemical Name        | Gene Set Collection | BEAS-2B, BMC of most sensitive gene set (ppm) | HBEC, BMC of most sensitive gene set (ppm) | ACGIH TLV |
|----------------------|---------------------|---|--|-----------|
| 1-Bromopropane       | MSigDB_C2           | 1.98387                                       | 9.93639                                    | 10ppm     |
|                      | MSigDB_H            | 2.88373                                       | NA   |           |
|                      | Reactome            | 2.62083                                       | NA   |           |
| Dichloromethane      | MSigDB_C2           | 145.148                                       | 269.865                                    | 100ppm    |
|                      | MSigDB_H            | 231.7465                                      | 368.893                                    |           |
|                      | Reactome            | 145.148                                       | 338.0995                                   |           |
| Trichloroethylene    | MSigDB_C2           | 48.9539                                       | 25.9727                                    | 50ppm     |
|                      | MSigDB_H            | NA  | NA   |           |
|                      | Reactome            | 72.60365                                      | 32.0725                                    |           |
| Carbon Tetrachloride | MSigDB_C2           | 11.2604                                       | NA   | 10ppm     |
|                      | MSigDB_H            | 18.5872                                       | NA   |           |
|                      | Reactome            | 13.5783                                       | NA   |           |

| Chemical Name        | LC50  | LOAEL  | NOAEL  | LOAEL/NOAEL Conditions   |
|----------------------|---|--|--|--|
| 1-Bromopropane       | 7000 - 14374 ppm (rats) <sup>1,2</sup>          | 125 ppm (rat: nasal inflammation) <sup>3</sup>   | 250+ ppm (rat: nasal lesions, lung histopathology, death) <sup>3</sup>   | subchronic: 6h/d, 5 d/wk, 4-16 wks, often 13 wk                        |
| Dichloromethane      | 25143 ppm (rats), 14292 ppm (mice) <sup>9</sup> | 500-1000 ppm (rats: hepatic changes, 2y) <sup>10,11</sup>                              | 200 ppm (rats: hepatic changes, 2 years) <sup>12</sup> ; 4200 ppm (mice: hepatic changes, 13 wk) <sup>11</sup> | 6h/d, 5 d/wk, 13 wk or 2 y   |
| Trichloroethylene    | 12509 ppm (rats), 8456 ppm (mice) <sup>6</sup>  | 25 ppm** or 2.6 ppm*** (mice, acute: immunosuppression) <sup>7,8</sup>                 | 50 ppm** or 5.2 ppm*** (mice, acute: immunosuppression) <sup>7,8</sup>   | acute vs repeated: ** single 3h exposure, ***3h exposure, repeated 5 d |
| Carbon Tetrachloride | 23862 ppm (rats), 9513 ppm (mice) <sup>4</sup>  | 10 ppm† , ‡ (rat: body weight changes, enlarged liver with fatty changes) <sup>5</sup> | 5 ppm† or 1 ppm‡ (rat: body weight changes, enlarged liver with fatty changes) <sup>5</sup>                    | subchronic: † 7h/day, 5d/wk, 6 mo; ‡ 24hr/d, 7d/wk, 13 wks             |



## Comparison of *in vitro* to *in vivo* exposure studies

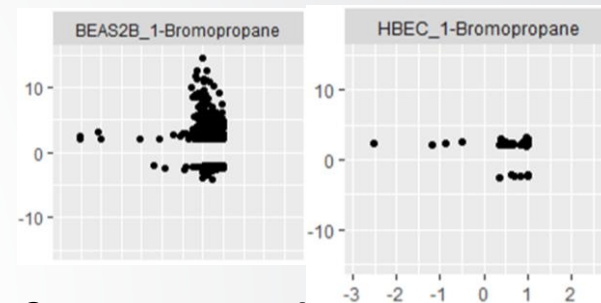
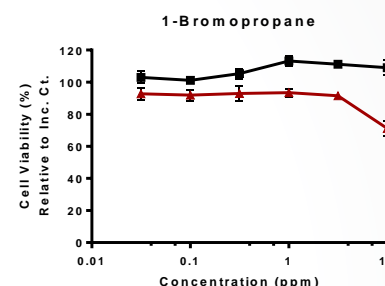
| Chemical Name        | BEAS-2B BMC<br>(MSigDB_C2), ppm | HBEC BMC (MSigDB_C2),<br>ppm | TLV (ppm) | LOAEL         | NOAEL           |
|----------------------|---------------------------------|------------------------------|-----------|---------------|-----------------|
| Acrolein             | 0.647ppm                        | --                           | 0.1ppm    | 0.4ppm        | NR              |
| Formaldehyde         | NA                              | --                           | 0.3ppm    | 404ppm        | 152ppm          |
| 1-Bromopropane       | 1.9839ppm                       | 9.936ppm                     | 10ppm     | 125ppm        | 250ppm          |
| 1,3-Butadiene        | 10.965ppm                       | --                           | 10ppm     | 625ppm        | 8000ppm, 200ppm |
| Carbon Tetrachloride | 11.2604ppm                      | NA                           | 10ppm     | 10ppm         | 5ppm, 1ppm      |
| Acetaldehyde         | 245.12ppm                       | --                           | 25ppm     | 2ppm          | 1ppm            |
| Trichloroethylene    | 48.953ppm                       | 25.973ppm                    | 50ppm     | 25ppm, 2.6ppm | 50ppm, 5.2ppm   |
| Dichloromethane      | 145.148ppm                      | 269.865ppm                   | 100ppm    | 500-1000ppm   | 200ppm          |



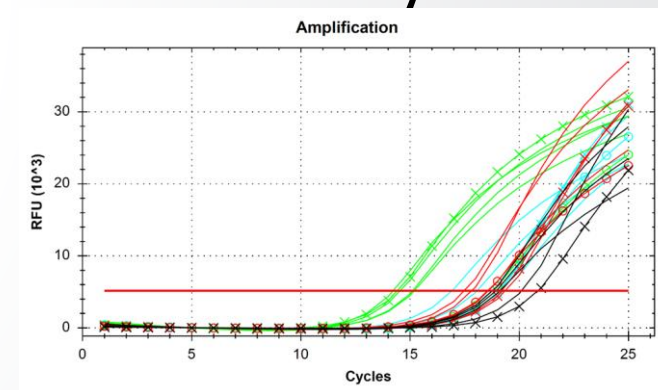


# Lessons Learned

- A higher starting target concentration may yield better curve modeling in the long run, especially for the primary HBECs.



- Immediate endpoints to determine chemical impact helps inform study design.
- Do NOT take cell lysis methodologies for granted.



- Humidity and Temperature consistency are very important.
- Timing for transcriptomic changes must be evaluated and justified.



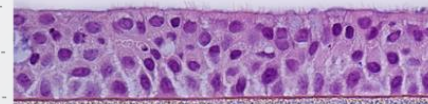
# Goals & Future Directions

- How to use in vitro data as a **predictive tool for quantitative risk assessment?**
  - Analyzing cell line vs Primary cell types differential response.
  - How do we validate acute *in vitro* exposure data with acute *in vivo* exposure data
- Evaluate the ability of the transcriptomic data to identify **concentration-dependent changes in mechanism/mode-of-action**.
- Evaluate the ability of the transcriptomic data from the cell culture model to **group chemicals by similar bioactivity profiles** for potential grouping and read across applications.
- Currently working through a schedule for medium-throughput **screening of 15 volatile or aerosolized chemicals** in order to evaluate the performance of existing human lung culture models to **identify irritants/chemicals with portal of entry effects and differentiate them from systemic toxicants**
- Currently establishing technical capabilities and developing methodology for **dosimetry of selected chemicals and/or complex mixtures**

Ciliated Apical Surface -

Mucociliary Epithelium -

Microporous Membrane -



EpiAirway

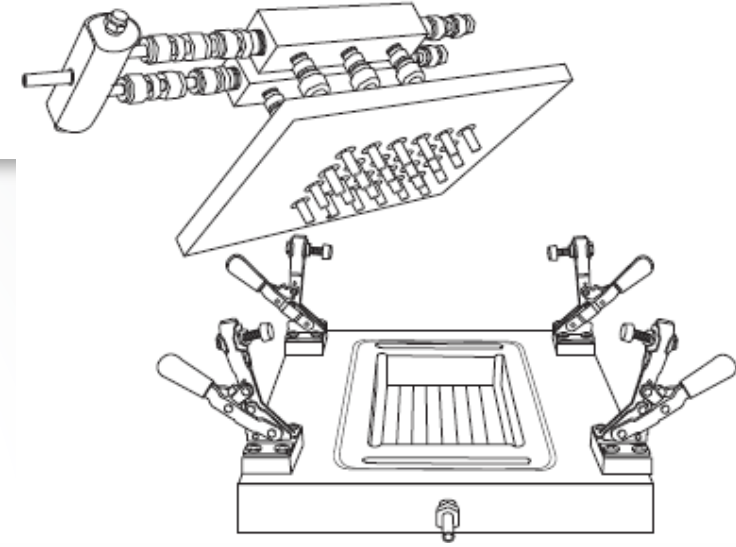


# Future Study Design for Portal vs Systemic Toxicant Case Study Design

| Cell Types            | Primary Human Bronchial Epithelial Cells<br><b>16HBE cells</b><br><b>Mattek Epi-Airway cells</b>  |
|-----------------------|---|
| Test Chemical         | Napthalene                      1,3-Dichloropropene<br>Chloropicrin                  Didecyldimethyl ammonium chloride*<br>2-phenylphenol*    *aerosol exposure |
| Exposure Regimen      | • 6 concentrations, exposure control, incubator control   |
| Exposure Duration     | • 2 hours, Assays conducted 4h post exposure  |
| Technical Replicates  | • TempO-Seq, n=2; Viability, n=2; Cytotoxicity, n=4   |
| Biological Replicates | • Exposures per cell type conducted over three days, n=3  |
| Assay Formats         | • TempO-Seq<br>• Cytotoxicity [LDH Release, Cell Titer Glo]<br>• <b>Trans Epithelial Resistance (TEER)</b><br>• <b>Inflammatory response [ELISA for IL-6 and IL-8]</b>  |



# Acknowledgements



## EPA Engineers & Biologists/Toxicologists

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