Evaluation of the EpiOral™ reconstructed human oral buccal tissue model as a testing platform for determining the oral irritation potential of tobacco products

IIVS
Institute for In Vitro Sciences
Advancing Science & Animal Welfare Together

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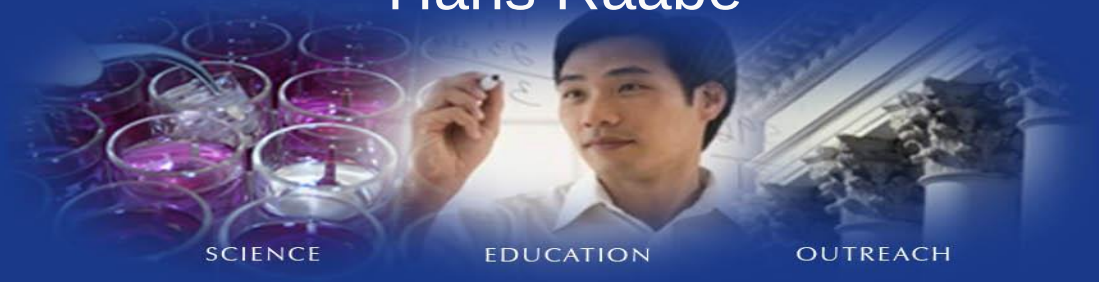
Hans Raabe



Overview

In vitro monolayer cell culture systems

applications and limitations in product development and regulatory toxicology

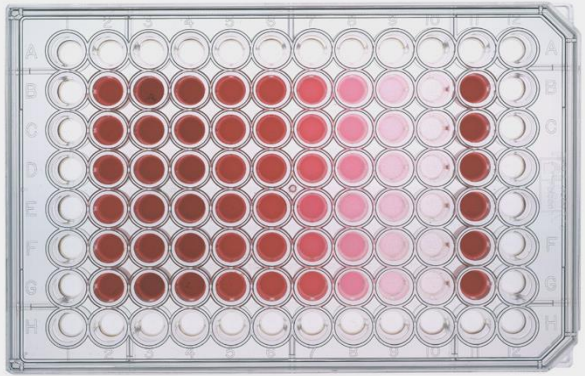
In vitro reconstructed 3D tissue models

brief intro to development and applications

Evaluation of the EpiOral™ 3D tissue model

for prediction of the oral irritation potential of tobacco products

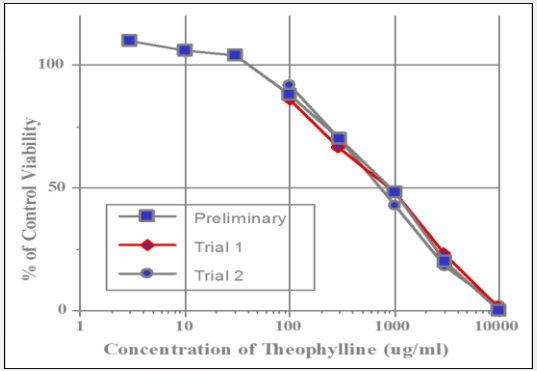
Ex. Neutral Red Uptake Viability Endpoint



low doses → high doses

Vehicle controls in columns 2 and 11

Neutral Red retention in viable cells is measured.
Optical density (550 nm)



Dose response curves are prepared;

In vitro : *in vivo* extrapolations are made

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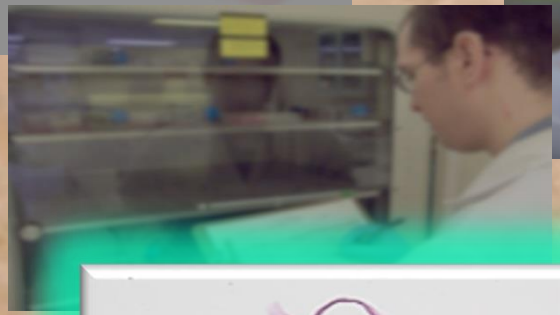
In Vitro Monolayer Cell Systems

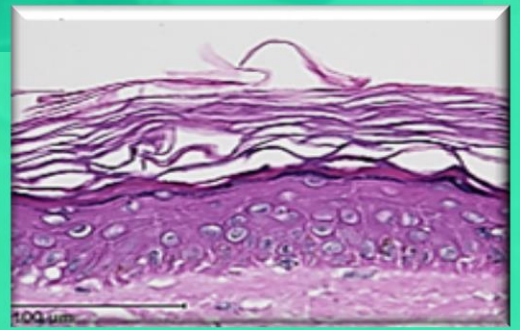
- Generally easy to conduct – cell lines
- Generally quite rapid to execute
- Evaluate individual chemicals (ingredients) rather than formulations
- Cost effective with batches of test materials – HTP – robotics
- Machine scored endpoints
- hazard oriented

Limitations of Simple Monolayer Assays

- Aqueous insoluble materials
- Dilution effects which mask toxicity of the neat material (e.g. ethanol)
- Buffering effects of the vehicle, and reaction of the chemical with medium components
- Pharmacokinetics poorly modeled
- No tissue barrier function modeled

Toxicology in Normal Human Epidermal Keratinocyte Cells





≠

Do these cells really resemble human tissues *in vivo* ?

In Vitro 3D Human Reconstructed Tissue Models

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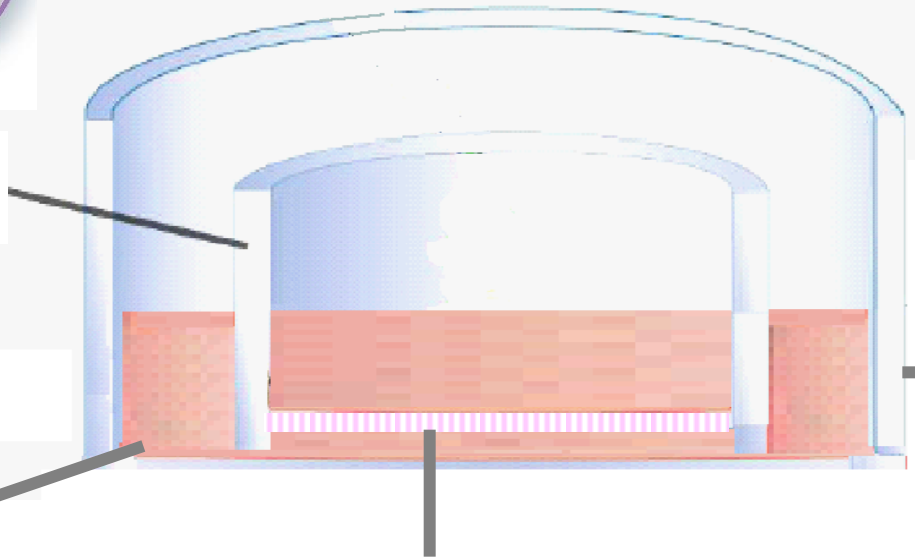
Evaluation of the EpiOral™ reconstructed human oral buccal tissue model

Technology of Tissue Reconstruction

normal human cell-based in vitro epithelial models are cultured at the Air-Liquid Interface (ALI) to reconstitute in vivo-like differentiated structure and function



courtesy Kandarova, MatTek Corporation, IIVS Practical Methods Workshop

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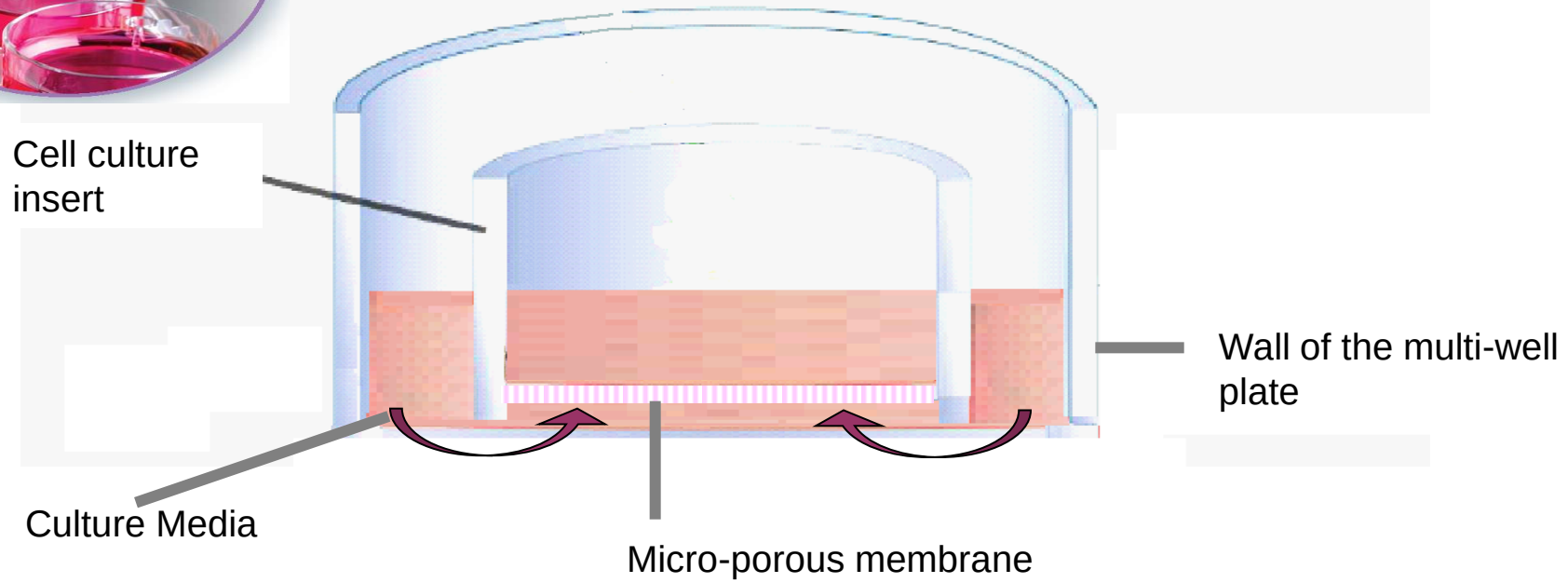

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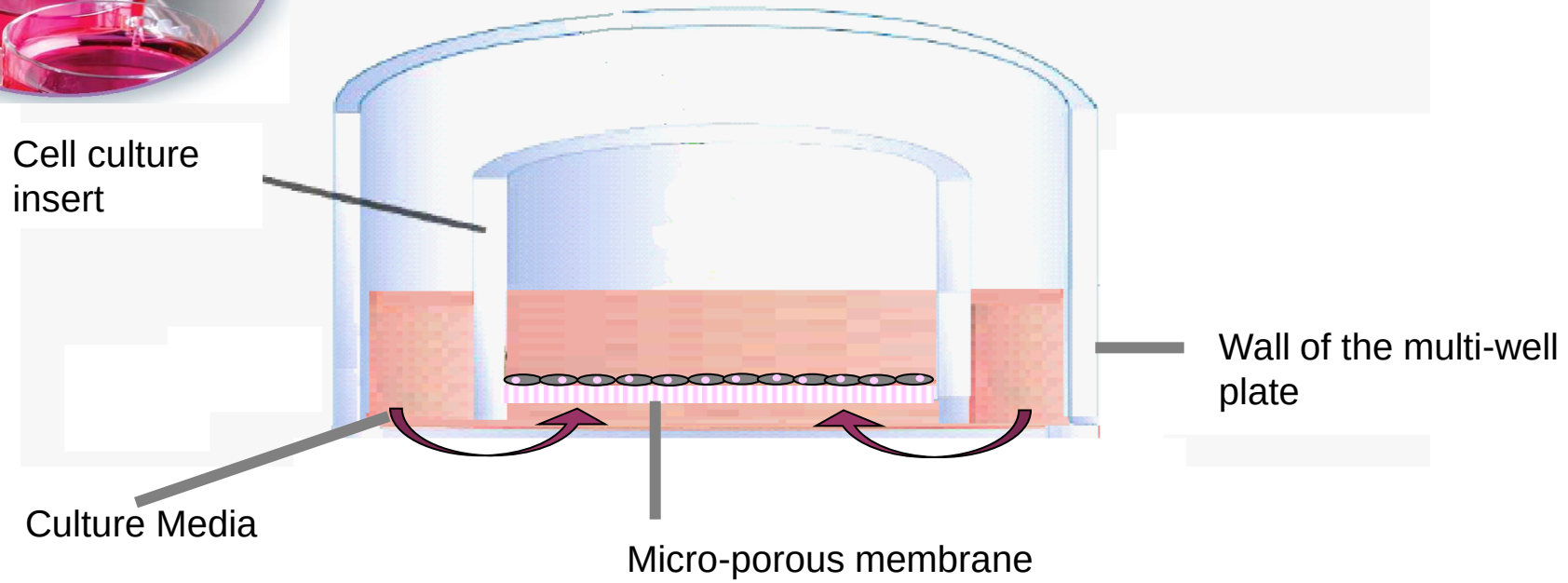

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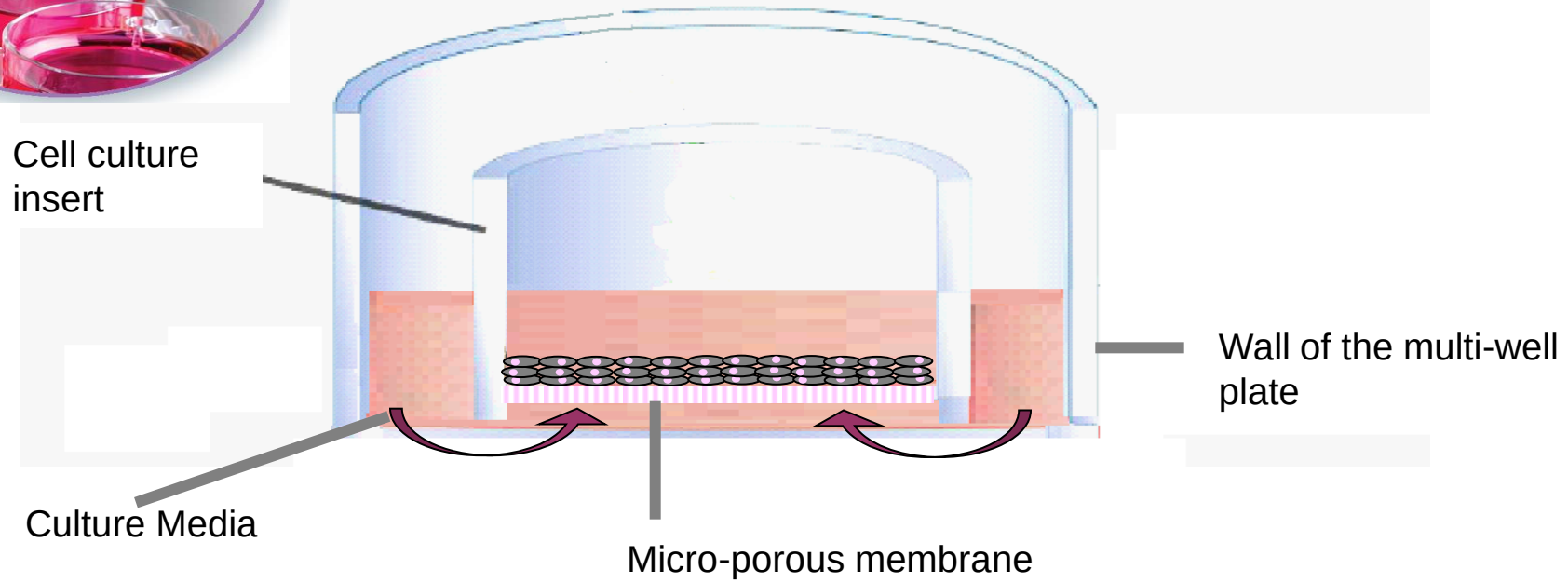

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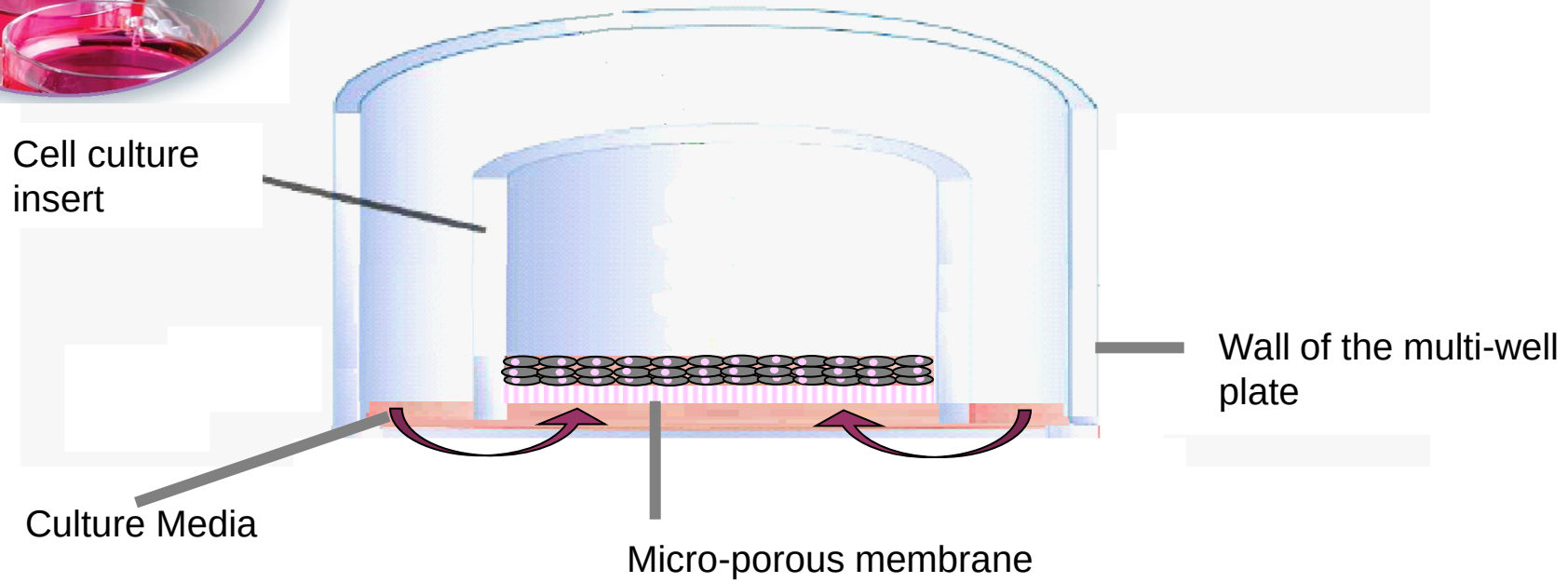

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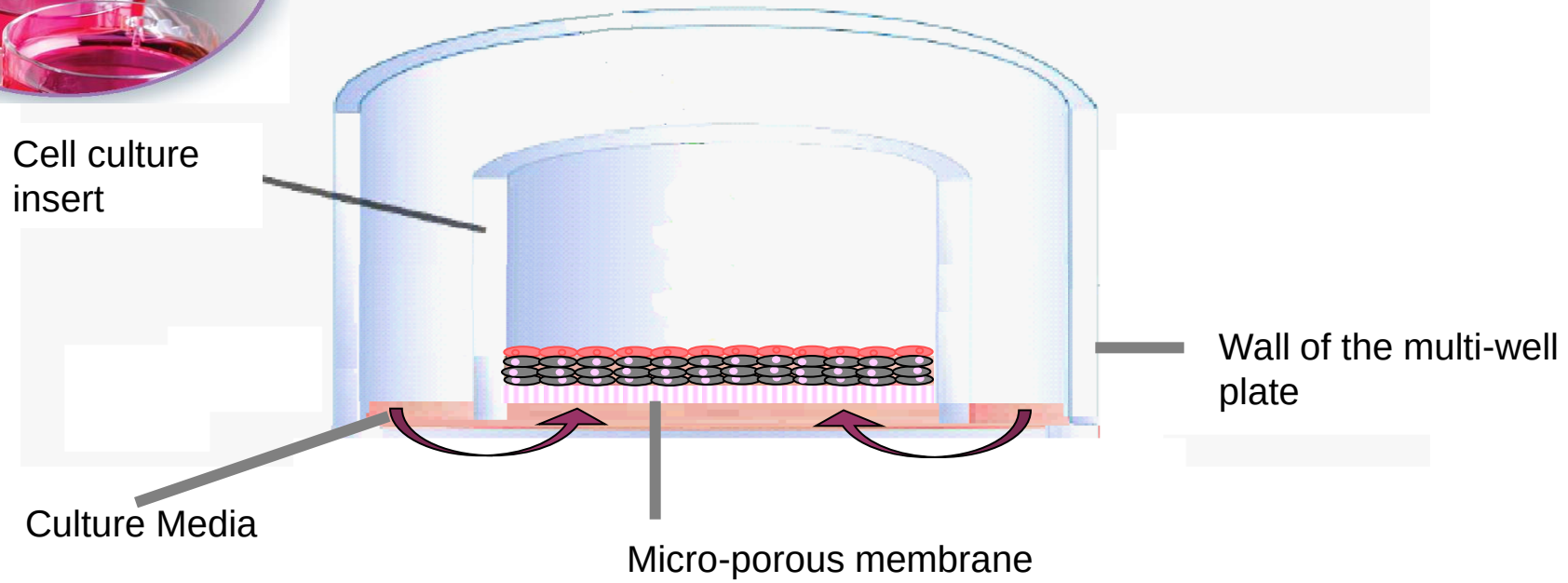

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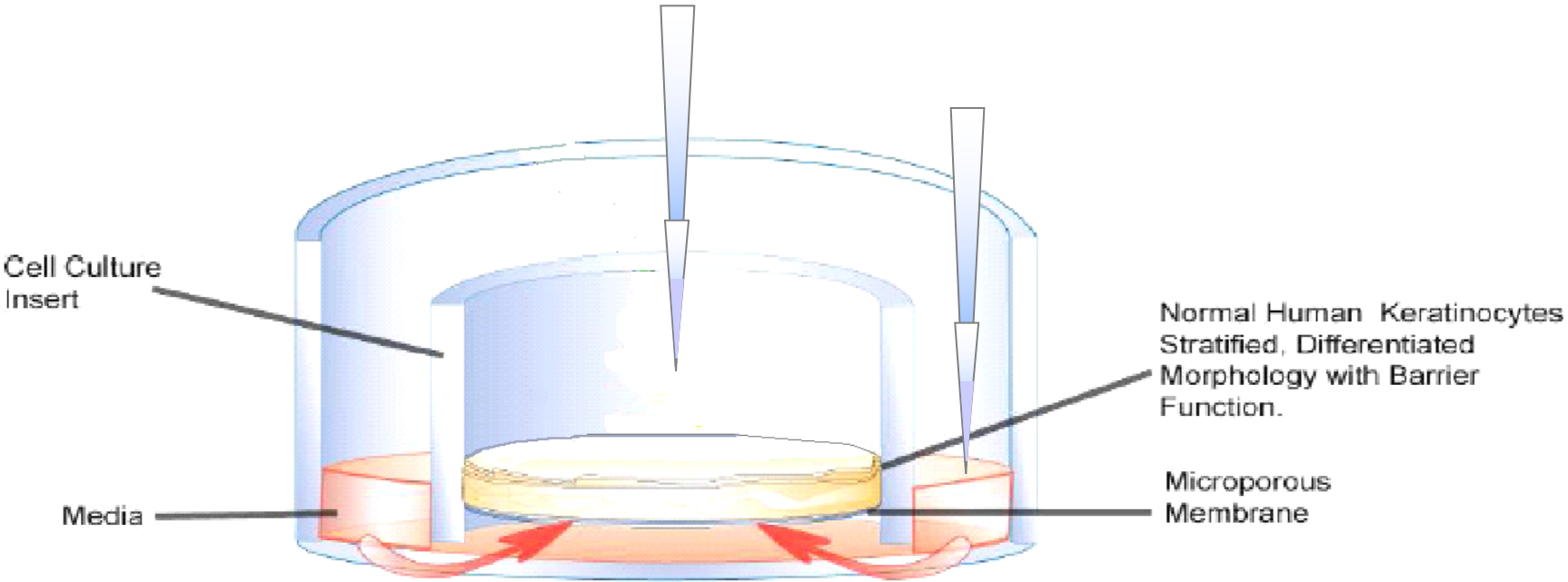
Exposure types



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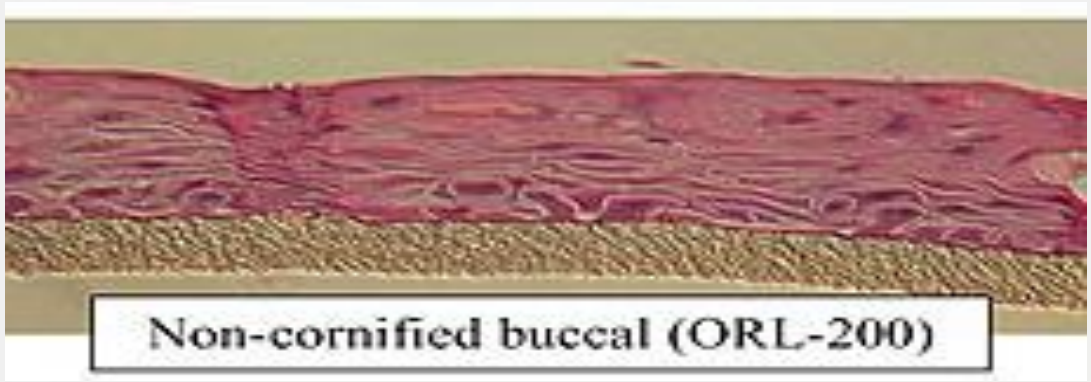
Characteristics of Reconstructed Human Oral Buccal Tissue Model

Test System: MatTek EpiOral™ Oral Buccal



Functional barrier

Basal layer of dividing buccal cells

Cell culture insert

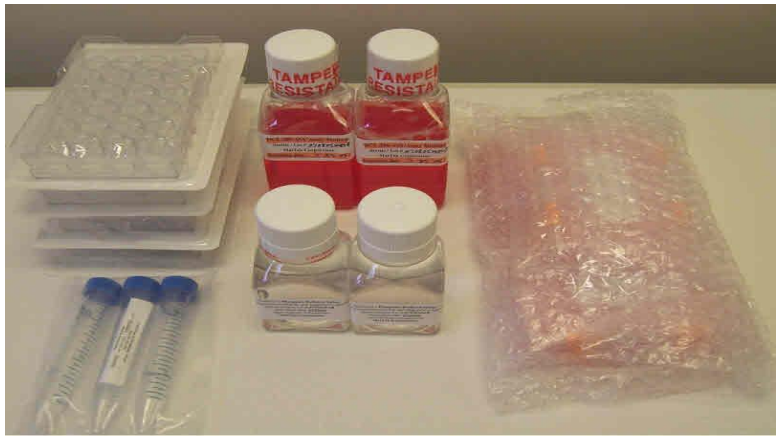
- non-cornified buccal (cheek) phenotype from non-transformed human oral epithelium

General model criteria:

- Stratified viable differentiated epithelial cells
- Tissues must be viable (MTT assay OD_{550} >1.0)
- Functional barrier

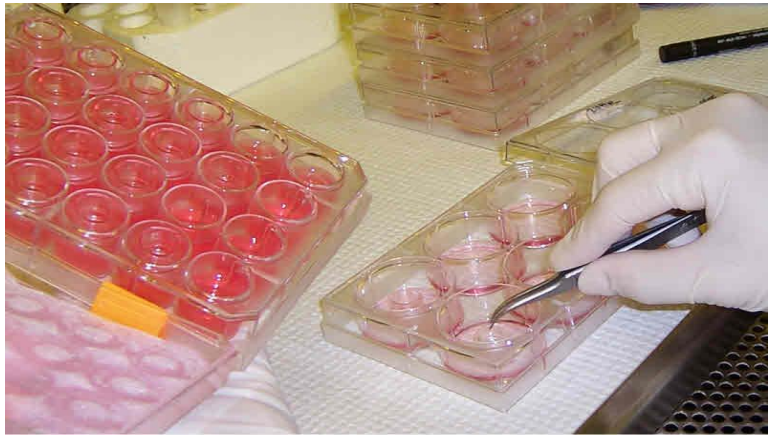
image from www.mattek.com, MatTek Corporation

Tissue Receipt and Preparation



Reconstructed human tissue models and reagents are typically shipped refrigerated and stored at 2-8°C

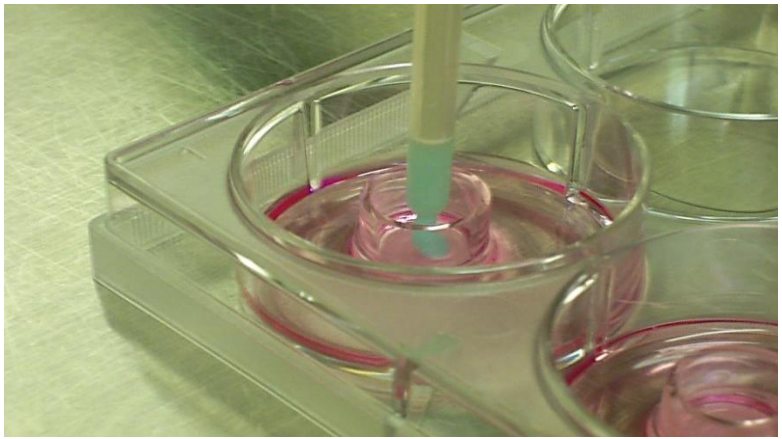
Tissues are examined for obvious defects and may be rejected based on blistering, excess fluid, air bubbles

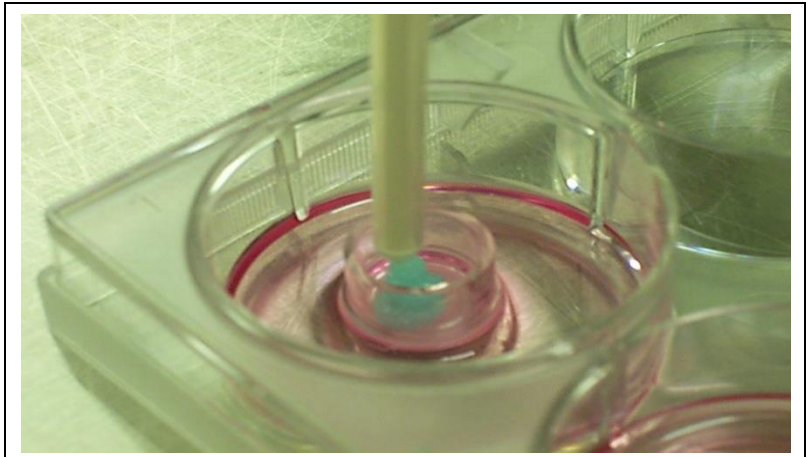


Tissues are transferred to 6-well plates that contain fresh assay medium

The tissues are initially incubated at 37°C, 5% CO_2, 90+% humidity

Dosing – Topical Exposures





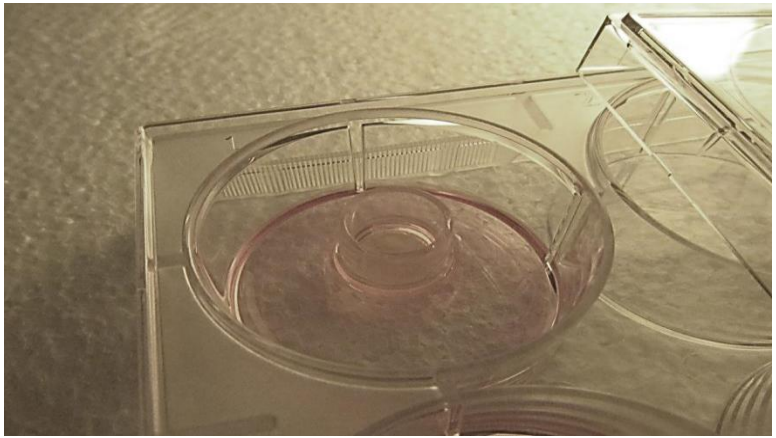
Dosing of aqueous or semi-viscous test chemicals or formulations is performed with a positive displacement pipette

Oral tobacco products and product extracts are applied directly onto the oral tissue model surfaces

The tissues are incubated at 37°C, 5% CO_2, 90+% humidity for specific exposure times

Several exposure times ranging from 1 to 24 hours may be tested

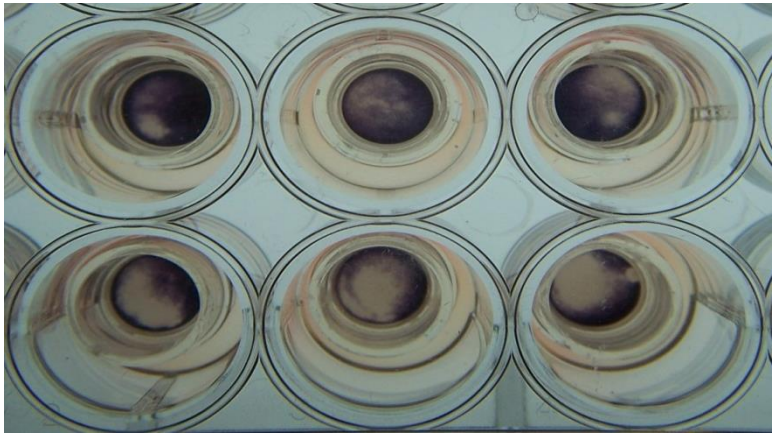
Rinsing of Treatments

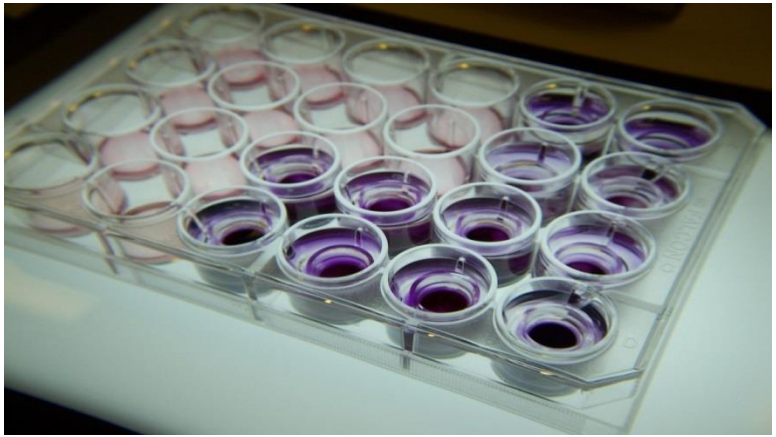


At the end of each exposure time, the test material is rinsed from the tissues with phosphate buffered saline (PBS), or Dulbecco's PBS.

Thorough removal of test material is necessary to prevent prolonged exposure and over-predictions

Viability Assessment - MTT Reduction





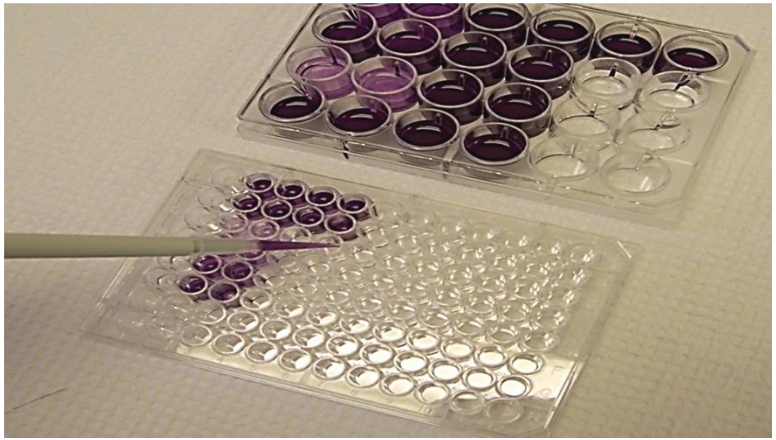
Individual tissues are placed into wells containing unreduced *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT) solution

The tissues are incubated at standard culture conditions for 3 hours

A dark blue or purple color signifies the presence of reduced MTT / viable tissue

The tissues are placed in isopropanol at room temperature for 2 hours to extract the reduced MTT

Transfer of MTT/Isopropanol and Quantification



Extracted MTT is thoroughly mixed and transferred to a 96-well plate.

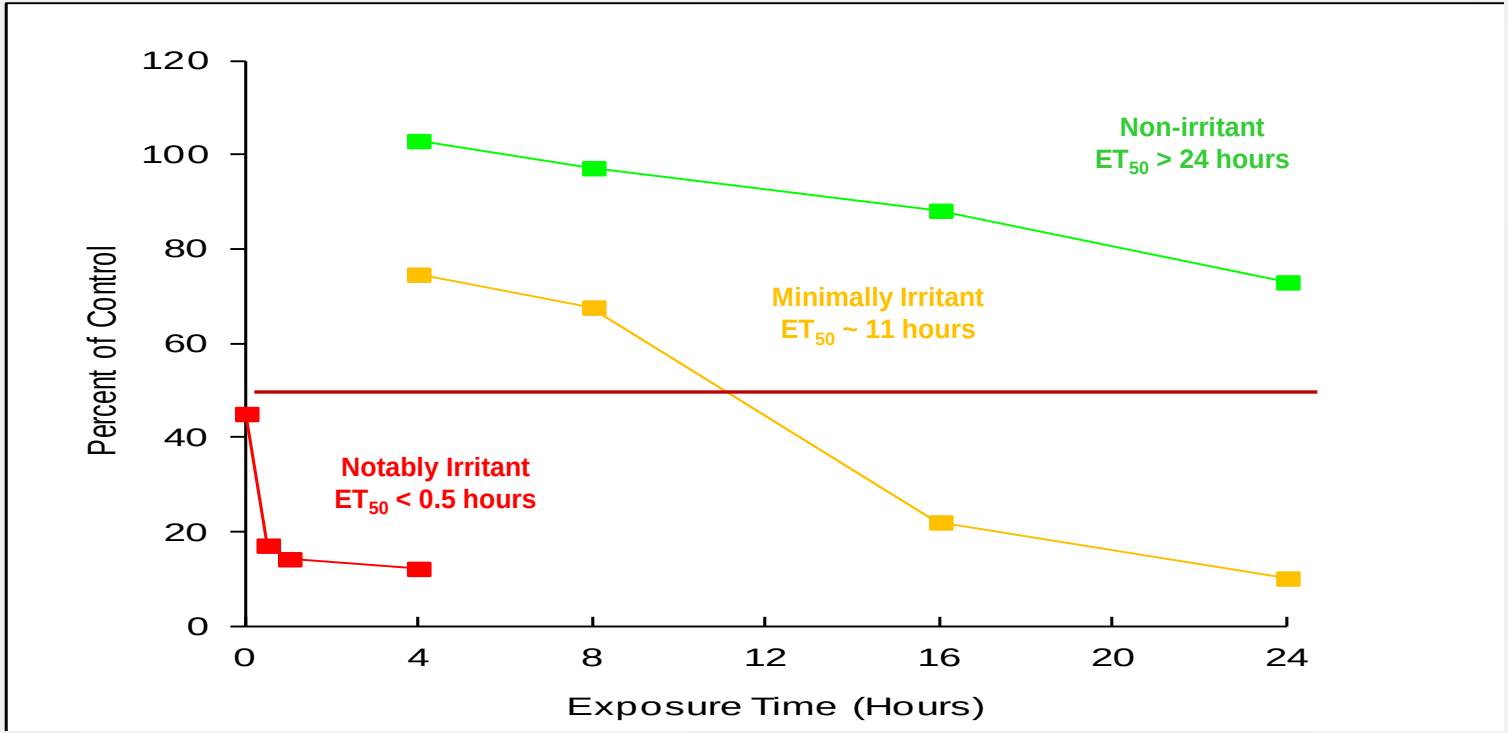
The 96-well plate/MTT-isopropanol samples are quantified using a microplate reader. Optical Density (OD) at 550 nm is measured.

OD_{550} values are used to calculate relative viability values.

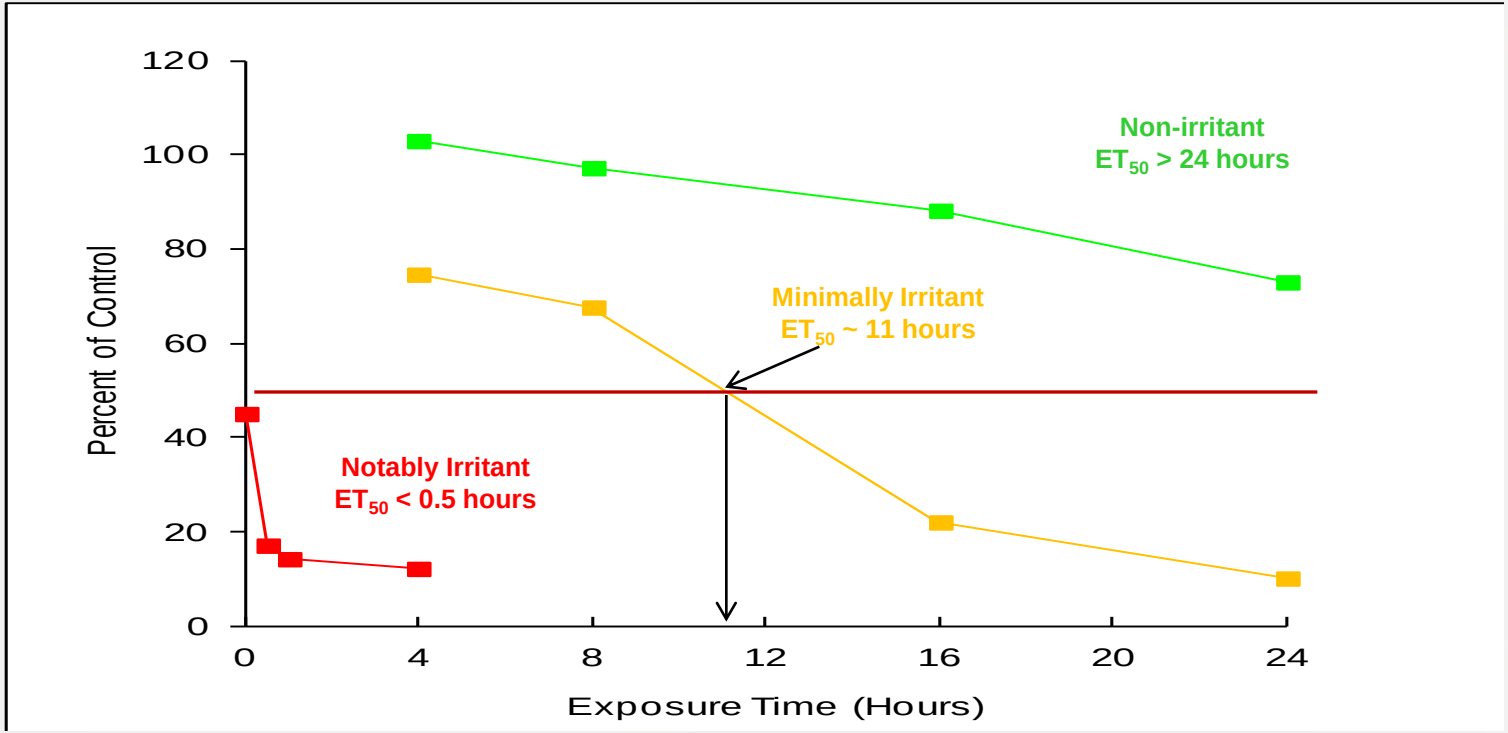
Viability is presented relative to negative control tissue values

$$\% \text{ of Control} = \frac{\text{Test Material } OD_{550}}{\text{Negative Control } OD_{550}}$$

Time-to-Toxicity Concept



Time-to-Toxicity Concept



ET_{50} is the exposure time expected to reduce viability to 50% of controls

Cytokine expression in 3D reconstructed tissues

APPLICATIONS

- Evaluate induction of inflammatory response
- Evaluate cell membrane integrity
- Generally used as a secondary endpoint
  - esp. in the absence of overt cytotoxicity
- Cytokines are inducible (secondary)
  - ex. 6-hour exposure to phorbol esters (PMA) results in 5 to 10-fold induction of IL-1α and IL-8

Based upon these concepts…

- Can we apply these *in vitro* tools to measure differences in oral irritation after exposure to oral tobacco products ?
- Can we develop and evaluate a screening program that provides accurate predictions to guide product development and product stewardship goals ?
- How might such assays be applied for other tobacco products and exposure scenarios?

Study Design – Acute Exposure

Rank order oral irritancy potential with viability and cytokine induction and release endpoints

1. **Exposure time range finding trial**
   - to identify relevant exposures for tobacco extracts (MTT viability)
   - extracts diluted in Artificial Saliva (control)

2. **Definitive trial with select exposure times**
   - rank MTT viability values
   - rank IL-1α and IL-8 expression values

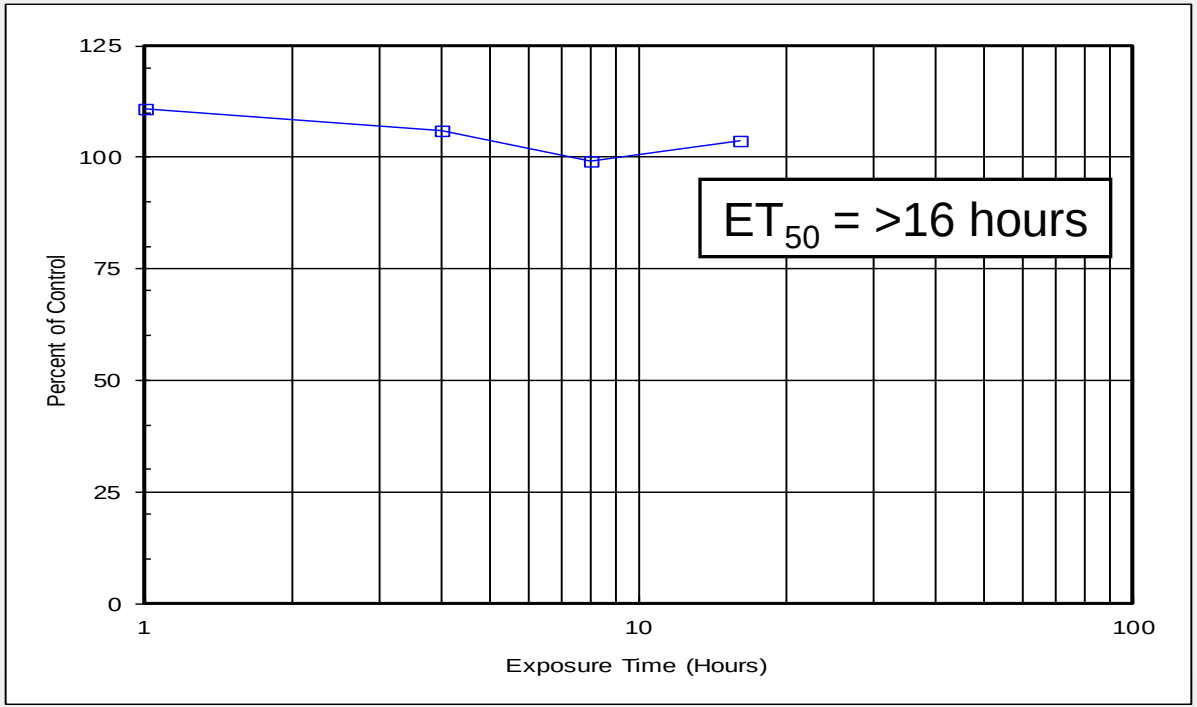
Results: Exposure Time Range Finding

Sponsor's Designation	ET_{50} (hours)[1]	pH
Tobacco Extract (100%)	8.9	discolored
Tobacco Extract (50%)	> 16	discolored
Artificial Saliva	> 16	7.0
Mouthwash	9.6	5.0
Toothpaste	2.1	5.5

[1] ET_{50} is the exposure time expected to reduce viability to 50% of controls

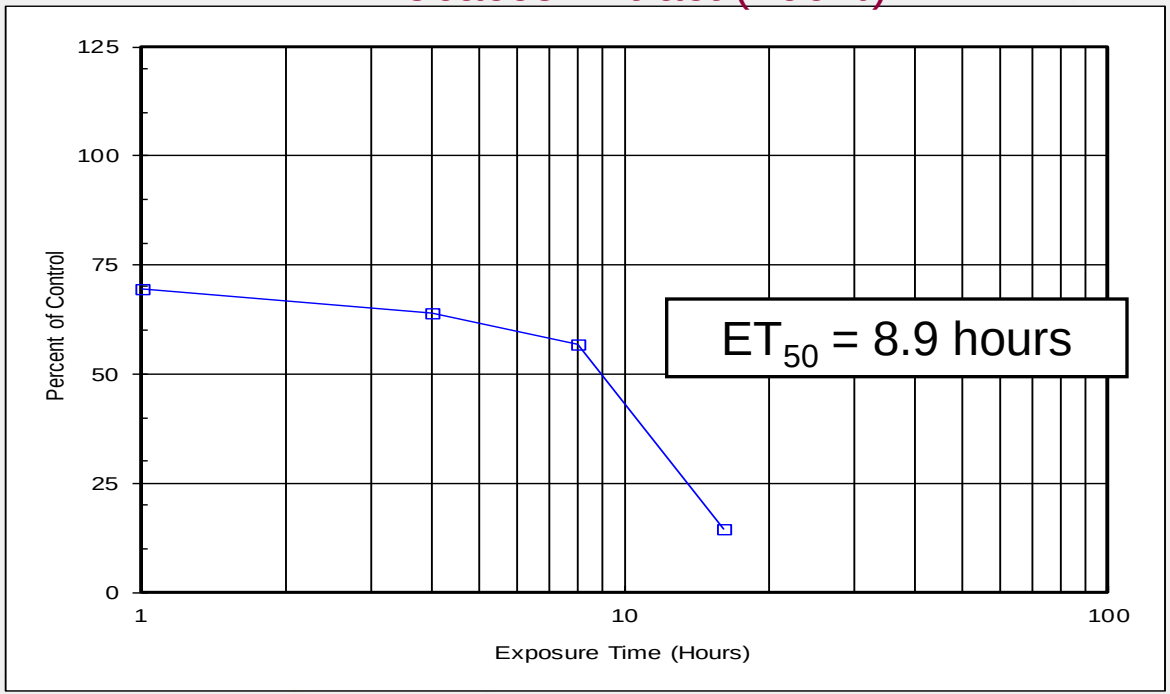
Results: Exposure Time Range Finding

MTT Viability
Artificial Saliva



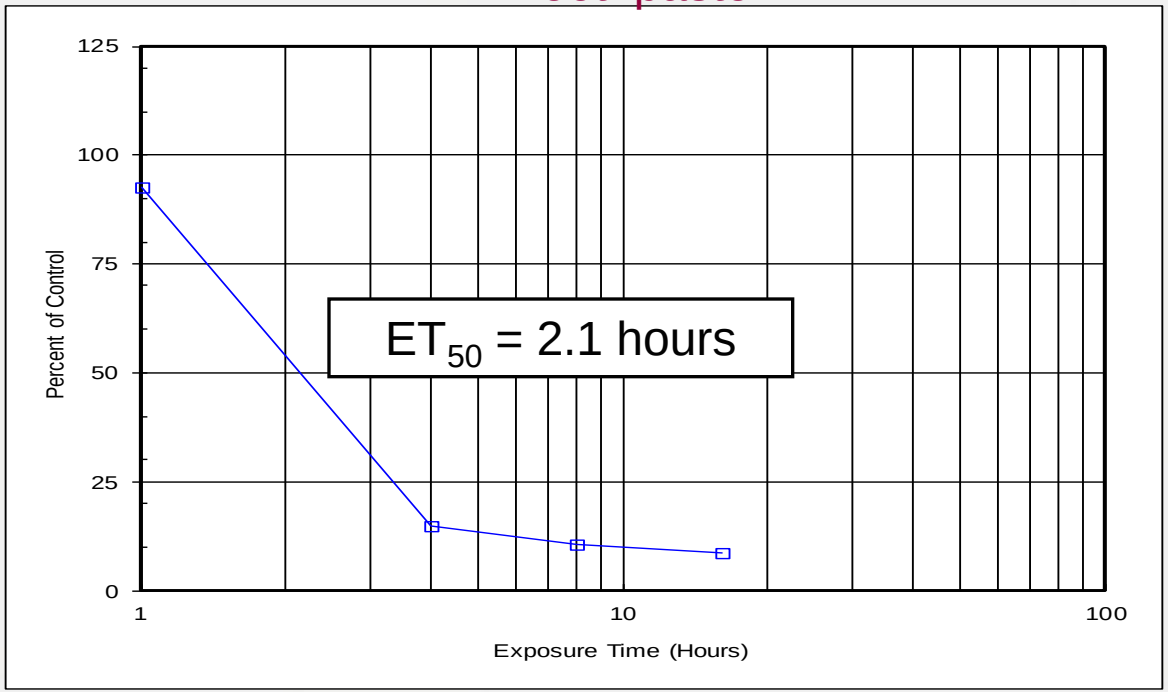
Results: Exposure Time Range Finding

MTT Viability
Tobacco Extract (100%)



Results: Exposure Time Range Finding

MTT Viability
Toothpaste



Definitive Assay Design

Tobacco Extracts

(100%, 80%, 60%, 40%, 20%, 10% v/v)

Tobacco 2S3 extracts

Whole-smoke bubbled PBS

(100%, 75%, 50%, 25% v/v)

Exposure times of 16, 8, 4, and 2 hours

Mouthwash

Exposure times of 16, 8, 4, and 2 hours

Toothpaste

Exposure times of 8, 4, 2 and 1 hours

Definitive Assay Design

Controls:

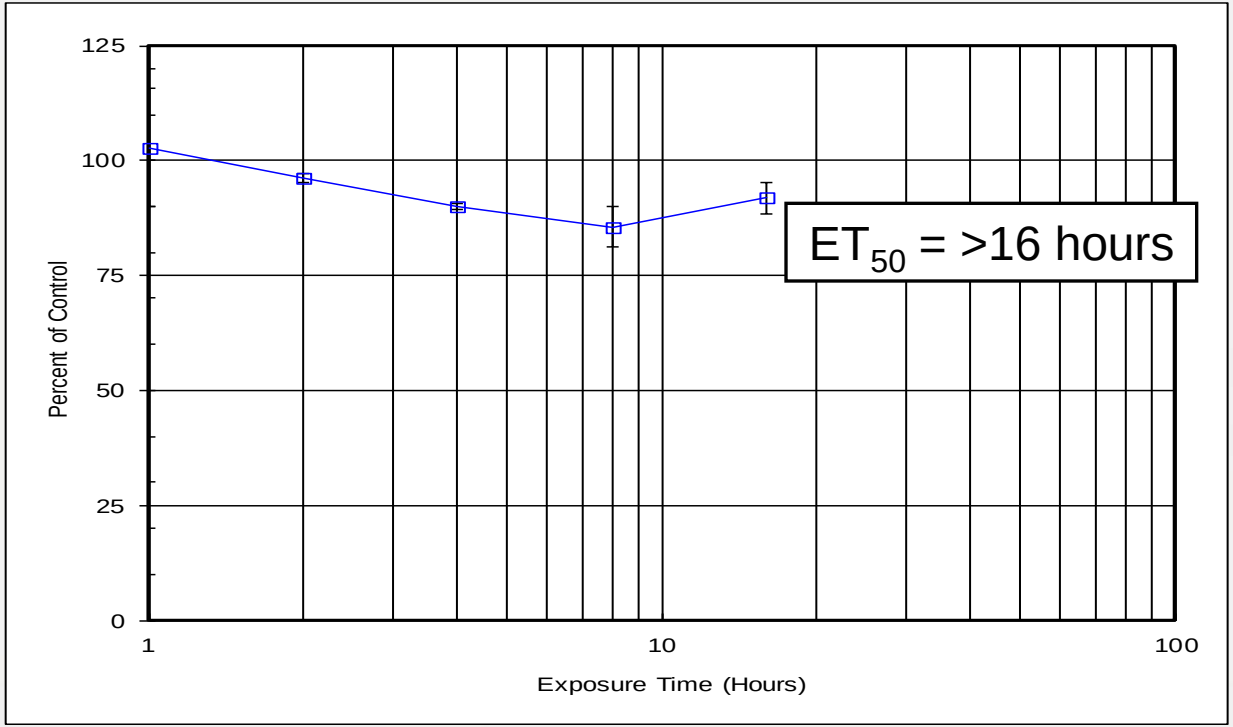
- Negative Controls
  - Deionized Water (historical)
  - Artificial Saliva (vehicle)
  - (treat at concurrent exposure times)
- Positive Control
  - 1% Triton X-100 (cytotoxicity)
  - (treat for 120, 50, and 10 minutes)
  - Phorbol ester (PMA) (inflammation)
  - (induction in medium for 6 hours)

Results: Definitive Assay

Sponsor's Designation	ET_{50} (hours)	Sponsor's Designation	ET_{50} (hours)
Artificial Saliva	> 16	Mouthwash	7.5
2S3 extract (0.22 um)	> 16	Toothpaste	2.9
2S3 extract (180 um)	> 16	Tobacco extract (100%)	8.7
Whole-smoke bubbled PBS (100%)	> 16	Tobacco extract (80%)	14.7
Whole-smoke bubbled PBS (75%)	> 16	Tobacco extract (60%)	> 16
Whole-smoke bubbled PBS (50%)	> 16	Tobacco extract (40%)	> 16
Whole-smoke bubbled PBS (25%)	> 16	Tobacco extract (20%)	> 16
1% Triton X-100	1.02	Tobacco extract (10%)	> 16

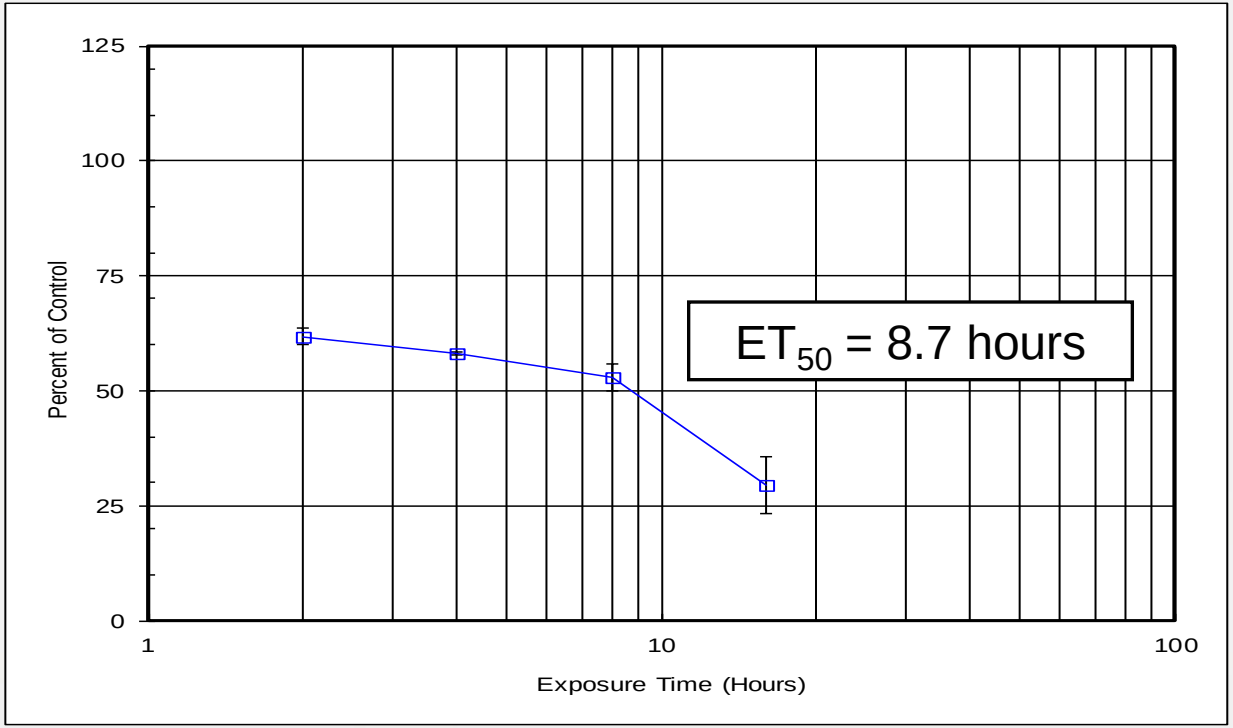
Results: Definitive Assay

MTT Viability
Artificial Saliva

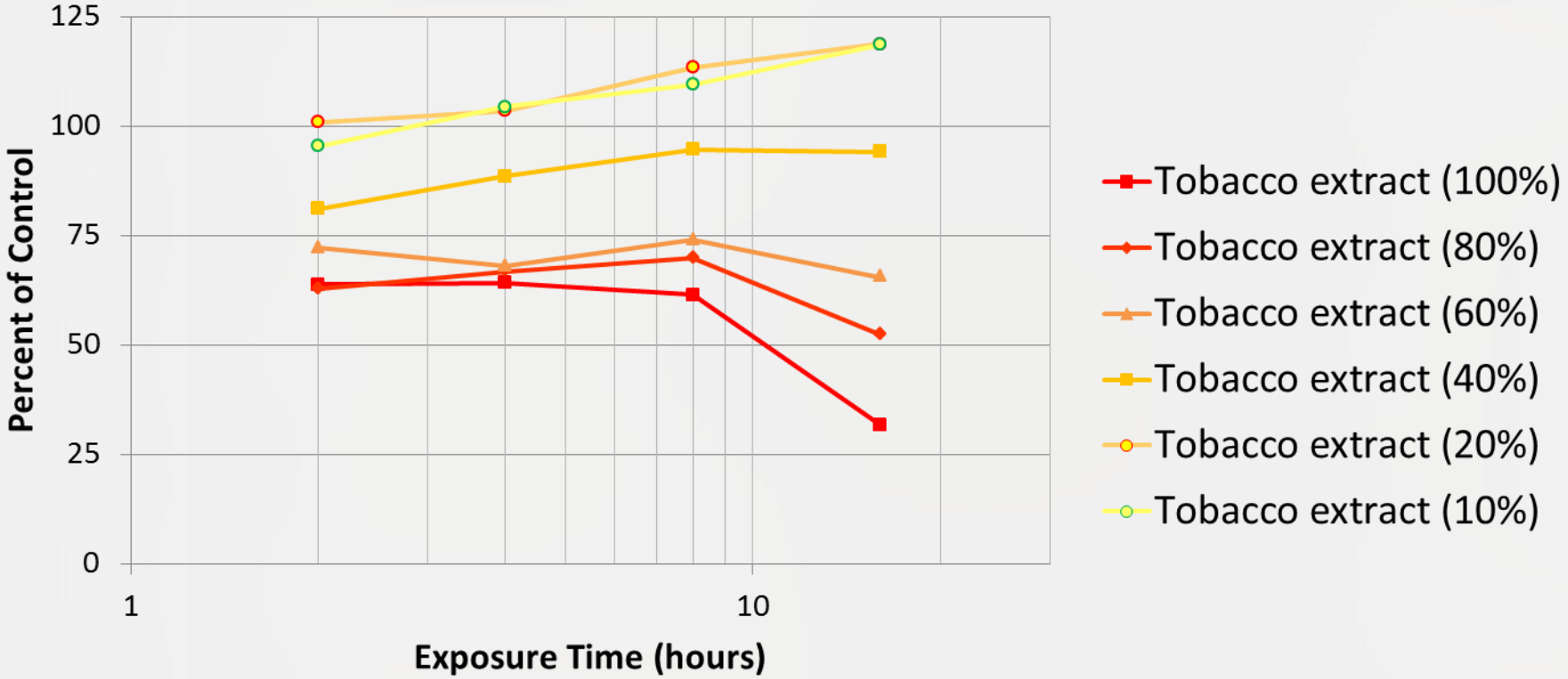


Results: Definitive Assay

MTT Viability
Tobacco extract (100%)



MTT Viability Assay
Tobacco Extracts on EpiOral™ Tissue Model



Cytokine Determinations

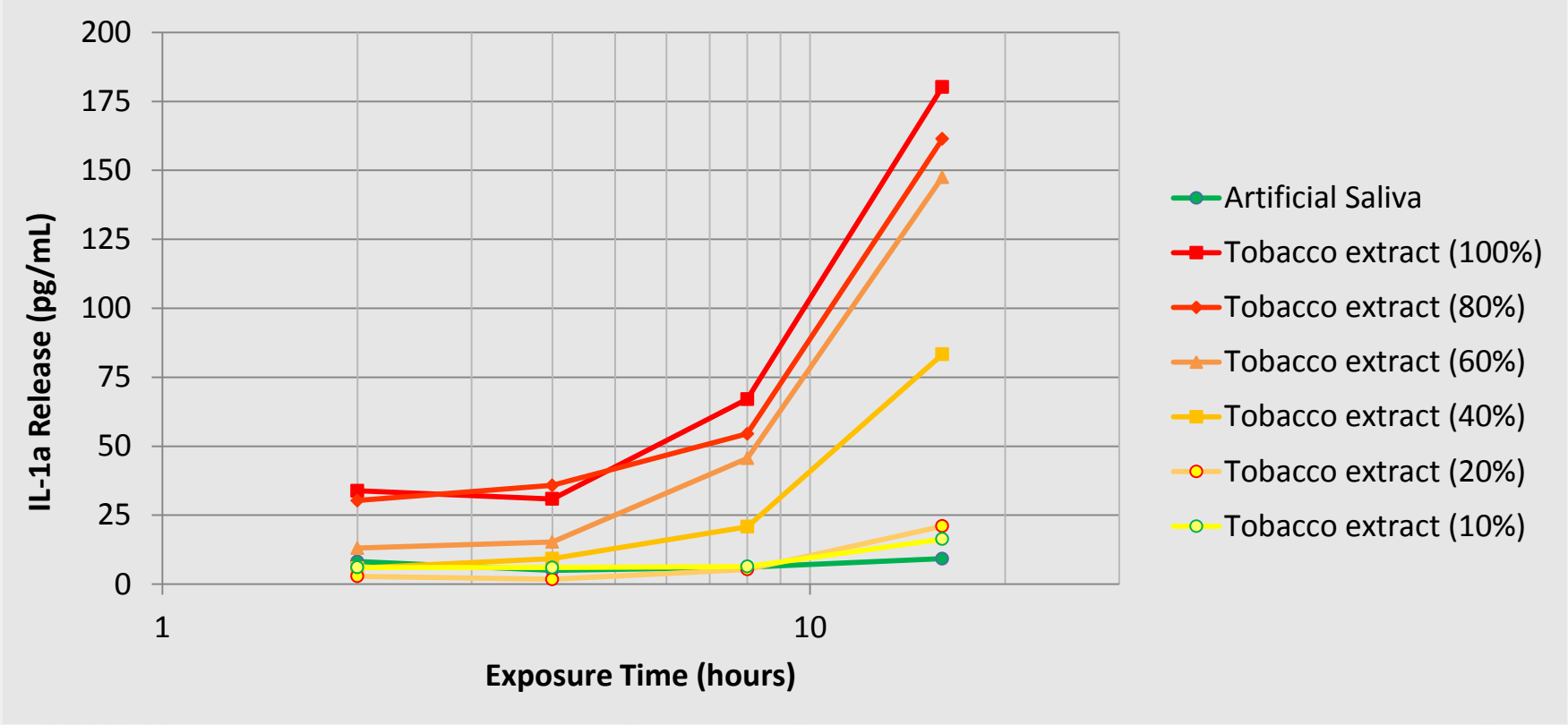
IL-1α and IL-8 expression into medium under the tissues was determined by ELISA

- for the Artificial Saliva
- Tobacco extract series
- PMA cytokine induction control
- 1% Triton X-100 cytotoxicity control

IL-1α is typically **preformed**, and **released** after cell membrane damage

IL-8 is not preformed but is **inducible** and readily **permeates intact membranes**

IL-1α Release
Tobacco Extracts on EpiOral™ Tissue Model



Cytokine Results: Definitive Assay

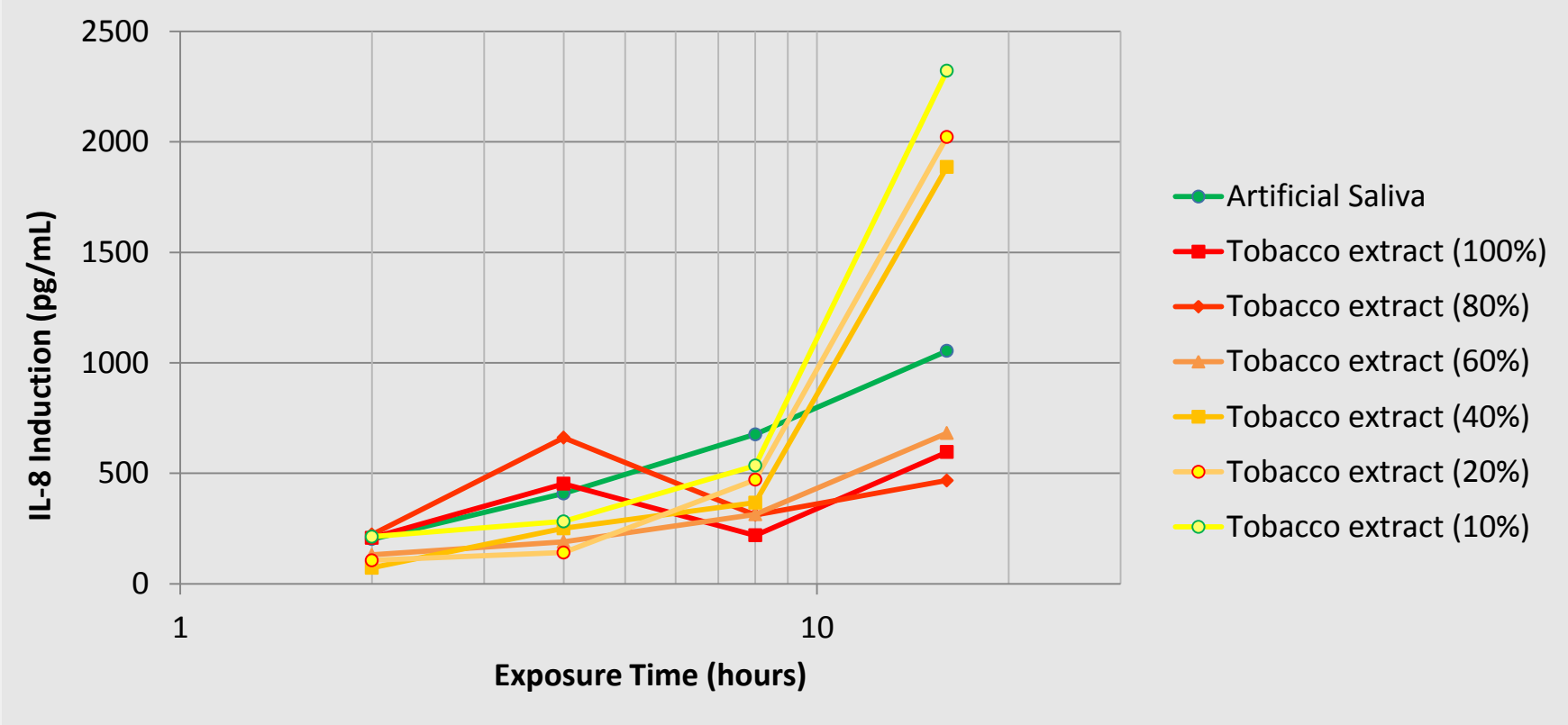
- Tobacco extract series IL-1α Release

Exposure time-related increases in IL-1α release for tobacco extract-treated tissues, relative to Artificial Saliva

The highest IL-1α release values were for the highest Tobacco extract concentrations (100% and 80%)

Shows impact of cytotoxicity on cell membrane integrity and IL-1α release

IL-8 Induction
Tobacco Extracts on EpiOral™ Tissue Model



Cytokine Results: Definitive Assay

- Tobacco extract series IL-8 Induction

Exposure time-related increases in IL-8 induction for tobacco extract-treated tissues, relative to Artificial Saliva at the **lowest** Tobacco extract concentrations (10%, 20% and 40%).

Similar to PMA induction!

Shows inflammatory activity of tobacco extracts on oral tissues, and the adverse impact of cytotoxicity on IL-8 induction

Study Conclusions

- EpiOral™ model can detect oral irritation potential of tobacco extracts by the MTT viability endpoint
- Cytokine expression profiles demonstrate the ability to respond to irritants by
  - release of the constitutively expressed primary cytokine IL-1α,
  - and the induction and synthesis of the secondary inflammatory cytokine IL-8
- Provide mechanistic evidence of the direct cytotoxic as well as inflammatory effects of tobacco extracts and oral tobacco products

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Thank You !

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Questions ??