Outline

• Introduction to the Institute for In Vitro Sciences, Inc.

• Pathways to *In Vitro* Methods Acceptance

• In Vitro Methods Currently Accepted at OECD Level

• Traditional Acceptance Paradigm

• Alternative Approaches

• Barriers to Routine Use of Accepted In Vitro Methods Must Be Removed
Institute for In Vitro Sciences

• Founded as a non-profit laboratory in 1997 to promote the use and acceptance of *in vitro* (non-animal) methods for toxicology

• Supported by revenues from *in vitro* testing services and contributions (mostly corporate and NGOs)

• As a non-profit we are regarded as a neutral authority when advising on non-animal testing, working with regulatory agencies, or interacting with animal protection organizations
IIVS is Unique in Combining:

- **Practical Knowledge**
  - (Science)

- **Dissemination of Information**
  - (Education)

- **Advocacy for the Methods**
  - (Outreach)

**Increased Use and Regulatory Acceptance**
Validation & Regulatory Acceptance Process

OECD Test Guideline

OECD Review

National Validation Authority

Test Method Developer

Basic Research

Development & Optimization

Prevalidation

Validation (ICCVAM, ECVAM, ???)

Regulatory Acceptance

OECD
Organization for Economic Co-Operation and Development (OECD)

- Creates international standards by making Test Guidelines (TGs) for toxicology methods

- 34 full member countries, but Brazil, China, India, Indonesia, South Africa and Russia are “observers” and potential future members

- Has published 12 *in vitro* TGs

- Member countries, i.e. their regulatory agencies, agree to accept data done to the TG standards (some caveats)
OECD In Vitro Test Guidelines

Ocular Corrosion & Irritation – TG 437 (BCOP), 438 (ICE)

Dermal Corrosivity - TGs 430 (TER), 431 (3-D skin model), 435 (Corrsitex®)

Dermal Irritation – TG 439 (3-D skin models)

Dermal Absorption – TG 428

Dermal Sensitization – TG 442C (DPRA), 442D (KeratinoSens)
OECD *In Vitro* Test Guidelines

Phototoxicity – TG 432 (3T3 NRU)

Endocrine Disruption – TG 455, 456, 457

…but not all are complete replacements or stand-alone methods!
Ocular Corrosion & Irritation
TG 437

Bovine Cornea Opacity & Permeability Assay
Ocular Corrosion & Irritation
TG 438

Isolated Chicken Eye
### BCOP & ICE Regulatory Prediction Models

#### BCOP Prediction Models per OECD TG 437 & 438

<table>
<thead>
<tr>
<th>In Vitro Score</th>
<th>UN GHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 3</td>
<td>No Category</td>
</tr>
<tr>
<td>&gt;3 ≤ 55</td>
<td>No prediction can be made</td>
</tr>
<tr>
<td>&gt; 55</td>
<td>Category 1</td>
</tr>
</tbody>
</table>

#### ICE Prediction Models

<table>
<thead>
<tr>
<th>ICE Score</th>
<th>UN GHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3XI, 2XI, 1XII</td>
<td>No Category</td>
</tr>
<tr>
<td>Other Combinations</td>
<td>No prediction can be made</td>
</tr>
</tbody>
</table>

- 3 x IV, 2 x IV, 1 x III, 2 x IV, 1 x II*, 2 x IV, 1 x I*
- Corneal opacity ≥ 3 at 30 min (in at least 2 eyes)
- Corneal opacity = 4 at any time point (in at least 2 eyes)
- Severe loosening of the epithelium (in at least 1 eye)

#### Prediction Model per US EPA AMCP Non-Animal Strategy

<table>
<thead>
<tr>
<th>In Vitro Score</th>
<th>UN GHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 75</td>
<td>Category 2</td>
</tr>
<tr>
<td>&gt; 75</td>
<td>Category 1</td>
</tr>
</tbody>
</table>
Ocular Assay Considerations

• Regulatory classification
  – OECD TG 437 & 438: cannot assign GHS Category 2 classification
  – EPA AMCP: BCOP can only assign Cat I or Cat II

• Reversibility not addressed

• Availability/source of eyes
Dermal Corrosion & Irritation
TG 431 & 439

3-D Skin Models

Stratum corneum
Granular layer
Basal layer of dividing keratinocytes
Cell culture insert

Native human skin

Courtesy of MatTek Corporation

Tissue Receipt

Tissue rinsing

Tissue treatment
### EpiSkin™ (SM)

<table>
<thead>
<tr>
<th>Viability measured after exposure time points (3, 60 and 240 minutes)</th>
<th>Prediction to be considered – UN GHS Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 35% after 3-minutes exposure</td>
<td>Corrosive: • Optional Sub-category 1A</td>
</tr>
<tr>
<td>≥ 35% after 3-minutes exposure AND &lt; 35% after 60-minutes exposure OR ≥ 35% after 60-minutes exposure AND &lt; 35% after 240-minutes exposure</td>
<td>Corrosive: • A combination of optional Sub-categories 1B and 1C</td>
</tr>
<tr>
<td>≥ 35% after 240-minutes exposure</td>
<td>Non-corrosive</td>
</tr>
</tbody>
</table>

### EpiDerm™ (EPI-200) SkinEthic™ RHE epiCS®

<table>
<thead>
<tr>
<th>Viability measured after exposure time points (3 and 60 minutes)</th>
<th>Prediction to be considered – UN GHS Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 50% after 3-minutes exposure</td>
<td>Corrosive: • Optional Sub-category 1A</td>
</tr>
<tr>
<td>≥ 50% after 3-minutes exposure AND &lt; 15% after 60-minutes exposure</td>
<td>Corrosive: • A combination of optional Sub-categories 1B and 1C</td>
</tr>
<tr>
<td>≥ 50% after 3-minutes exposure AND ≥ 15% after 60-minutes exposure</td>
<td>Non-corrosive</td>
</tr>
</tbody>
</table>
### Dermal Irritation Prediction Model

<table>
<thead>
<tr>
<th>In vitro result</th>
<th>In vivo prediction</th>
<th>UN GHS CATEGORY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean tissue viability ≤ 50%</td>
<td>Irritant (I), Possibly</td>
<td>Category 2 (Corrosive?)</td>
</tr>
<tr>
<td></td>
<td>Corrosive</td>
<td></td>
</tr>
<tr>
<td>Mean tissue viability &gt; 50%</td>
<td>Non-irritant (NI)</td>
<td>No Category</td>
</tr>
</tbody>
</table>

**Note:** GHS irritation categories are not identical to EPA hazard categories for skin irritation.
Tiered testing strategies for the assessment of skin corrosion/irritation potential

**Top-Down Strategy**

**Test material expected to be:**
Corrosive

**Assay to be used:**
*In Vitro* Corrosion Assay(s)

**Is the material predicted as corrosive?**

- **Y**

  Based on the particular *in vitro* assay(s) used:
  - Test material may be labeled as corrosive.
  - Test material may also be classified by the relevant packing group.

- **N**

  

**Actions to take:**

**Bottom-Up Strategy**

**Test material expected to be:**
Non-Corrosive

**Assay to be used:**
*In Vitro* Skin Irritation Assay(s)

**Is the material predicted as skin irritant?**

- **Y**

  The test material does not require any further skin irritation testing for submission to regulatory agencies utilizing the GHS system.

- **N**

  

**Test result:**

- **Y**

  Test material may also be classified by the relevant packing group.

- **N**

  

**Actions to take:**
1. **Seed** 96-well plates (1x10⁵ cells/mL)

2. **Dose** 2 plates with a dilution series of 8 doses. Incubate 1 hour

3. **Expose 1 plate to UVA** for 50 min (5 J/cm²). Maintain the other plate at room temperature; protected from light

4. **Decant & Rinse** (to remove test material) Add fresh medium. Incubate 24 hrs

5. **Add Neutral Red** Incubate 3 hrs

6. **Add Neutral Red Extraction Solvent**

7. **Read** plates at 550 nm (OD₅₅₀)
Photo Irritancy Factor (PIF)

Compares IC50 -UVA (dark) to the IC50 +UVA (light)

- PIF = \[ \frac{IC50 - UVA (dark)}{IC50 + UVA (light)} \]
- Example: 15.4 µg/mL
  0.73 µg/mL

PIF value = 21.1

<table>
<thead>
<tr>
<th>PIF Value</th>
<th>OECD Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIF &lt; 2</td>
<td>No Phototoxicity</td>
</tr>
<tr>
<td>2 ≥ PIF &lt; 5</td>
<td>Probable Phototoxicity</td>
</tr>
<tr>
<td>PIF ≥ 5</td>
<td>Phototoxicity</td>
</tr>
</tbody>
</table>
Mean Photo Effect (MPE)

The MPE is the product of the response effect and the dose effect.

MPE varies from a value of 0 (no difference between curves) to 1 (no toxicity in the absence of UVA, and full toxicity in the presence of UVA) within the range of doses tested.

<table>
<thead>
<tr>
<th>No Phototoxicity</th>
<th>Probable Phototoxicity</th>
<th>Phototoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPE &lt; 0.100</td>
<td>0.1 ≥ MPE &lt; 0.150</td>
<td>MPE ≥ 0.150</td>
</tr>
</tbody>
</table>

Comparing -UVA/+UVA dose response curves.
3T3 Phototoxicity Data Assessment

**PIF (Photo Irritancy Factor)**
- Compares the IC$_{50}$ of the dark plate with the IC$_{50}$ of the light plate
- Cannot be used when one or both IC$_{50}$ values are not obtained

**MPE (Mean Photo Effect)**
- Evaluates the curves (dose responses) between the light and dark plates
- Especially useful when an IC$_{50}$ value is not calculated (e.g. absence of cytotoxicity)

<table>
<thead>
<tr>
<th>Measure</th>
<th>No Phototoxicity</th>
<th>Probable Phototoxicity</th>
<th>Phototoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIF</td>
<td>PIF &lt; 2</td>
<td>2 ≥ PIF &lt; 5</td>
<td>PIF ≥ 5</td>
</tr>
<tr>
<td>MPE</td>
<td>MPE &lt; 0.100</td>
<td>0.1 ≥ MPE &lt; 0.150</td>
<td>MPE ≥ 0.150</td>
</tr>
</tbody>
</table>

OECD TG 432 Prediction
Adverse Outcome Pathways (AOPs): A New Approach To Understand *In Vitro* Relevance

Skin Sensitization
TGs 442C & 442D

- **Molecular properties**: Penetration into the viable epidermis
- **Molecular Initiating Event**: Electrophilic reactivity
- **Cellular Response**: Covalent interaction with proteins
- **Organ Response**: Expression of cell surface markers and cytokines
- **Organism Response**: Proliferation of T-cells in lymph nodes
- **Dermal inflammation (after challenge)**
In Vitro Test Strategy Along AOP

**Induction phase**

- Allergens

**Protein Reactivity**

**Keratinocyte Activation**

**LC activation**

**LC: Langerhans cells**

**T-cell proliferation**

**Lymph node**

**DPRA TG 442C**

**KeratinoSens TG 442D**

**h-CLAT**

**Chemical Properties**

**Molecular Initiating Events**

**Cellular Responses**

**Organ Response**
Direct Peptide Reactivity Assay

- *In chemico*
- The correlation of skin protein reactivity and skin sensitization is well established

Nucleophilic-electrophilic interaction:

Chemical allergen \(E\) reacts with protein to form chemical allergen and protein with modified cysteine and lysine residues.
KeratinoSens Assay

- HaCaT (immortalized keratinocyte cell line)
- Contains a reporter construct with a copy of the ARE-element of the human AKR1C2 gene upstream of a luciferase gene
- Endpoint: induction of luciferase activity by allergens
Langerhans cells (LC) play a critical role in skin sensitization.

Upon antigen capture, LC undergo maturation and migrate to the draining lymph nodes.

LC maturation is characterized by the up-regulation of CD86 and CD54 (Aiba and Katz, 1990; Ozawa et al., 1996).

Assay Procedure:
Cells (THP-1) cultured with 8 doses of chemical for 24 hours, then cell staining (CD86 and CD54), and cell viability (PI), followed by analysis by flow cytometry.
Do the Skin Sensitization Assays Provide a Full Replacement?

- Strategies for combining test results still being investigated
- The assays do not currently address skin penetration or skin metabolism
- Need for solubility is a concern
- Potency not predicted
Status of Acceptance

- List of methods and US/EU/OECD status

- For other regions it’s not as easy to find information

- OECD TG methods which provide GHS categories should be acceptable for ReACH; other TG methods likely need justification/negotiation
About ICCVAM

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is a permanent Committee of the NIEHS under the National Toxicology Program Interagency Center for the Evaluation of Toxicological Methods (NICEATM). ICCVAM is composed of representatives from 15 U.S. Federal regulatory and research agencies that require, use, generate or disseminate toxicological and safety testing information.

ICCVAM was formally established in 2000 by the ICCVAM Authorization Act (42 U.S.C. 285I-3)

"To establish, wherever feasible, guidelines, recommendations, and regulations that promote the regulatory acceptance of revised scientifically valid toxicological tests that protect human and animal health and the environment while reducing, replacing animal tests and ensuring human safety and product effectiveness."

The ICCVAM Authorization Act defines the purposes of ICCVAM as follows:

- Increase the efficiency and effectiveness of U.S. Federal agency test method review
- Eliminate unnecessary duplication of effort and share experience among U.S. Federal regulatory agencies
- Optimize utilization of scientific expertise outside the U.S. Federal government
- Ensure that new and revised test methods are validated to meet the needs of U.S. Federal agencies
- Reduce, refine, or replace the use of animals in testing where feasible

ICCVAM functions in part by:

- Facilitating interagency and international collaborations promoting the development, regulatory acceptance, and implementation of alternative test methods and testing strategies.
<table>
<thead>
<tr>
<th>No.</th>
<th>Alternative Test Method</th>
<th>ICCVAM and ICCVAM Agency Contributions</th>
<th>U.S. Regulatory Acceptance/Endorsement</th>
<th>OECD/Other Adoption</th>
<th>EU Regulatory Acceptance Endorsement</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Fixed dose procedure for acute oral toxicity</td>
<td>ICCVAM working group contributed to test guideline development</td>
<td>Accepted by U.S. via OECD TG 420</td>
<td>OECD TG 420 (2001)</td>
<td>Via OECD</td>
</tr>
<tr>
<td>5</td>
<td>Acute toxicity class method for acute oral toxicity</td>
<td>ICCVAM working group contributed to test guideline development</td>
<td>Accepted by U.S. via OECD TG 423</td>
<td>OECD TG 423 (2001)</td>
<td>Via OECD</td>
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Example of Validation and Acceptance for a Specific US EPA Use

- Anti-Microbial Cleaning Products (AMCP) Program – A special situation for the US EPA.

- By interacting throughout program with individuals who would approving the policy, as well as those who had the power to actually change policy

- Targeted a limited applicability domain

- Validation of a three method test strategy, rather than a single test method – BCOP, EpiOcular, and Cytosensor Microphysiometer,
The Challenge

• Majority of cleaning products do not have to go through a registration process before they are marketed. Companies decide how to assure safety – generally without using animals (in vitro).

• However, if “antimicrobial” claim made, then EPA regulated (animal testing for safety required).

• Both EPA and industry wanted a predictive, conservative in vitro strategy, beginning with eye irritation hazard.

• Neither group wanted this:
Cell viability measured by the continued production and secretion of hydrogen ions from glucose metabolism.
CytoSensor Validated by ECVAM Using Retrospective Review

Which led to the First Alternative Method Retrospective Weight-of-Evidence Approach to Replace the Draize Eye Test for the Identification of Non-Irritant Substances in a Defined Applicability Domain

Thomas Hartung, Leon Bruner, Rodger Curren, Chantra Eskes, Alan Goldberg, Pauline McNamee, Laurie Scott, and Valérie Zaug

1Johns Hopkins University, Bloomberg School of Public Health, Baltimore

A story which started on the cover of Science in 1989 finally became a validated method in 2009.
EpiOcular™ - A Human Epithelial Construct

Topical application

Non-keratinized tissue like cornea

Focuses on damage to the epithelium and upper stroma
Strategy For EPA Hazard Labeling

- Evaluate Components
  - Oxidizing chemistry?
    - Yes: BCOP
    - No: Expected severe or moderate?
      - Yes: Cytosensor
      - No: Water soluble?
        - Yes: EpiOcular
        - No: In vitro score
          - < 75: Category II
          - ≥ 75: Category I

  - In vitro MRD_{50} score
    - < 2 mg/ml: Category IV
    - ≥ 80 mg/ml: Category III
    - ≥ 2 but < 80 mg/ml: To distinguish Category I from II, conduct BCOP
A Success That Should Make Us All Proud

To

Models For Eye Irritation

Cytosensor Microphysiometer  3D Ocular Construct  Excised Bovine Cornea (BCOP)
Conclusions

• Many validated non-animal tests are currently available, and are accepted by regulatory authorities

• Not everyone takes advantage of this situation; barriers still need to be overcome

• Not all paths to regulatory acceptance need to go through a “validation body”, but the goal of international acceptance should generally be maintained
Questions?