Non-Animal Testing Technologies and Strategies Used for Chemical Hazard and Risk Assessment

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Institute for In Vitro Sciences
Advancing Science & Animal Welfare Together

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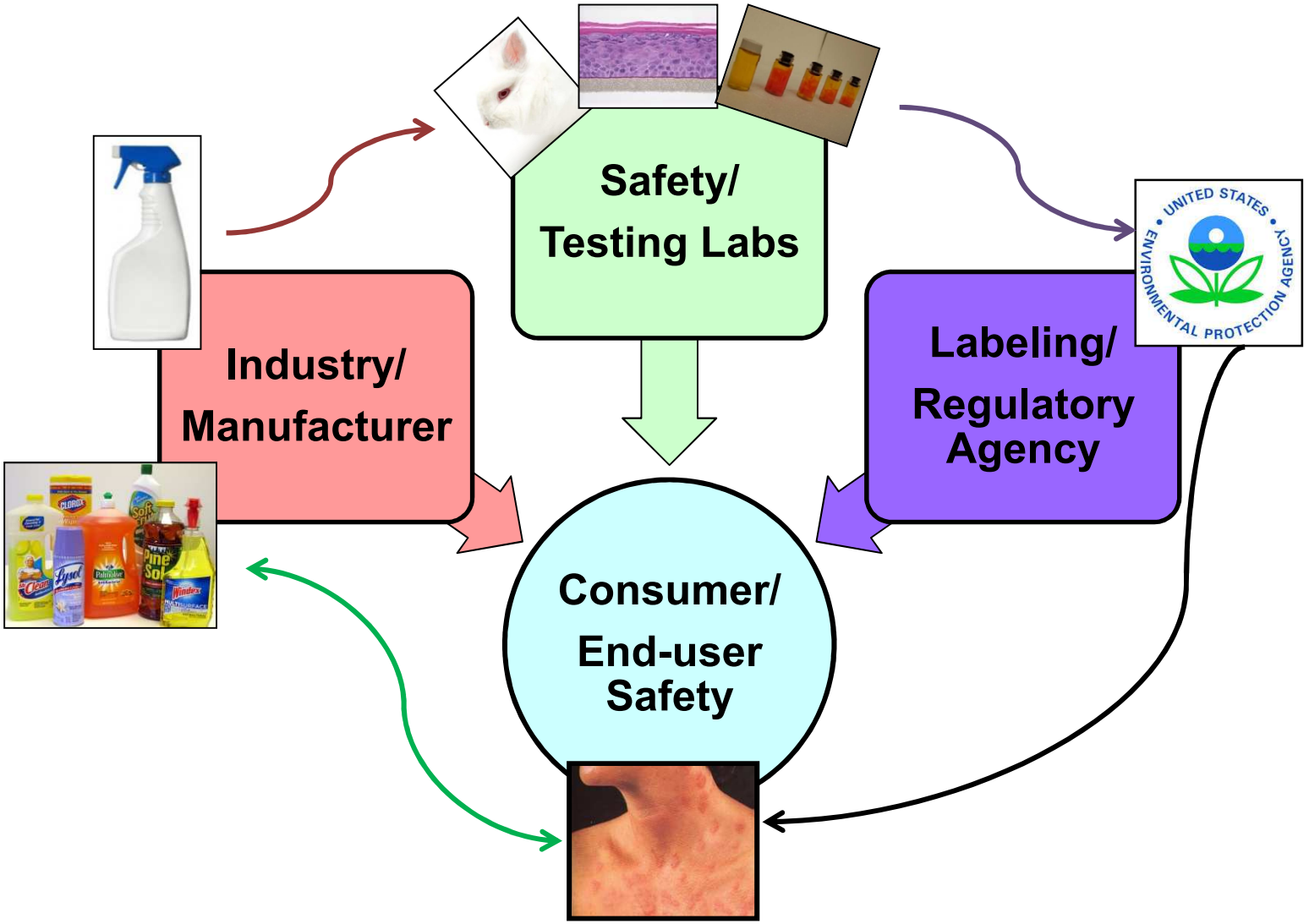
Perspectives, challenges, common goals and working together



Presentation outline

Background

Current regulatory climate – global acceptance of *in vitro* methods
Drivers of *in vitro* methods advancement
The reductionist concept of *in vitro* methods
Placing the "Safety" in the Safety Data Sheet (SDS)
Challenges with the non-animal paradigm
Major groups of non-animal test methods

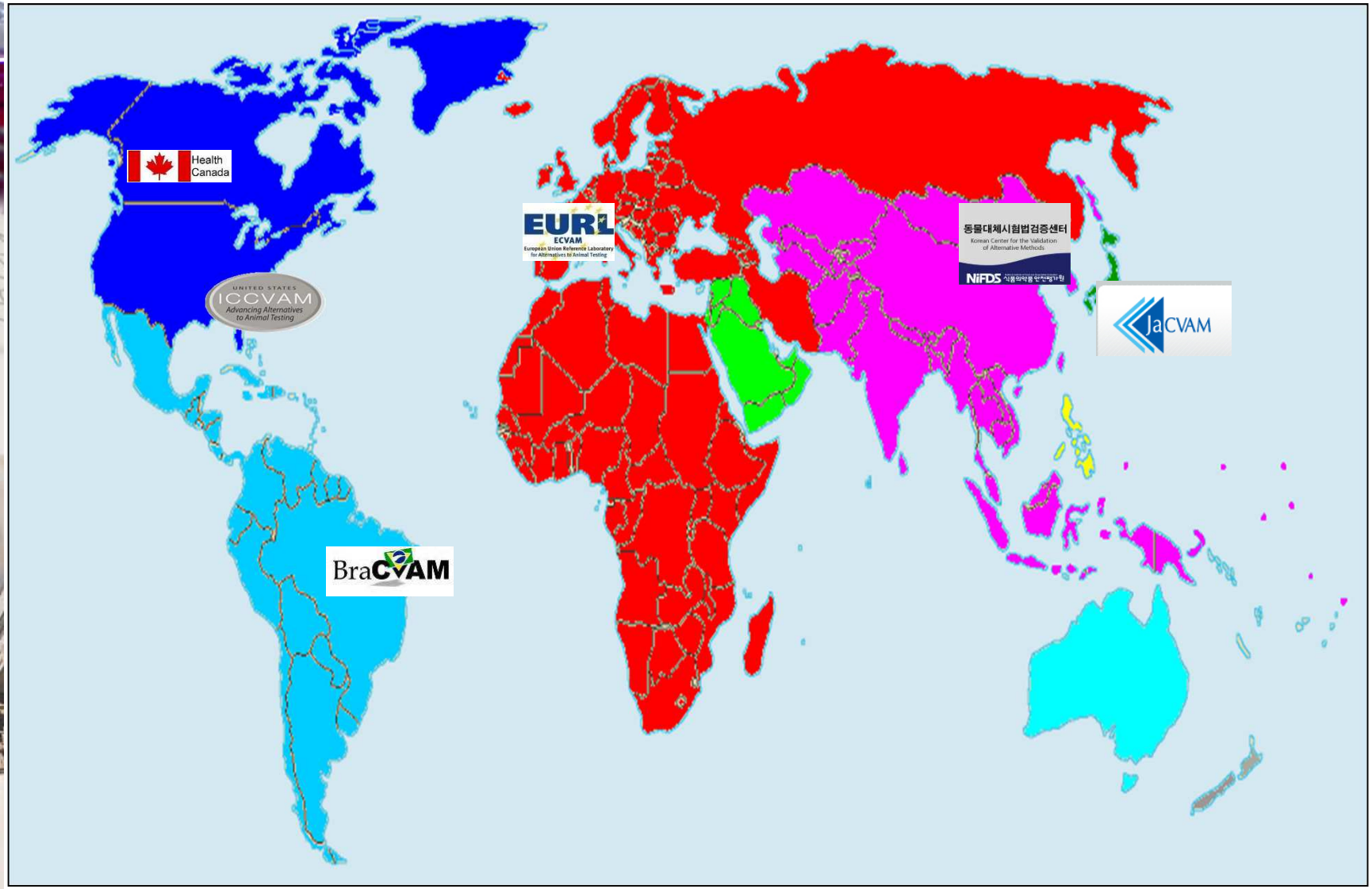
Non-animal testing technologies and strategies used for chemical hazard and risk assessment

1. ***In chemico* test systems**
2. ***In vitro* monolayer cell culture systems**
3. ***In vitro* reconstructed tissue models systems**
4. ***Ex vivo* tissues and organ systems**

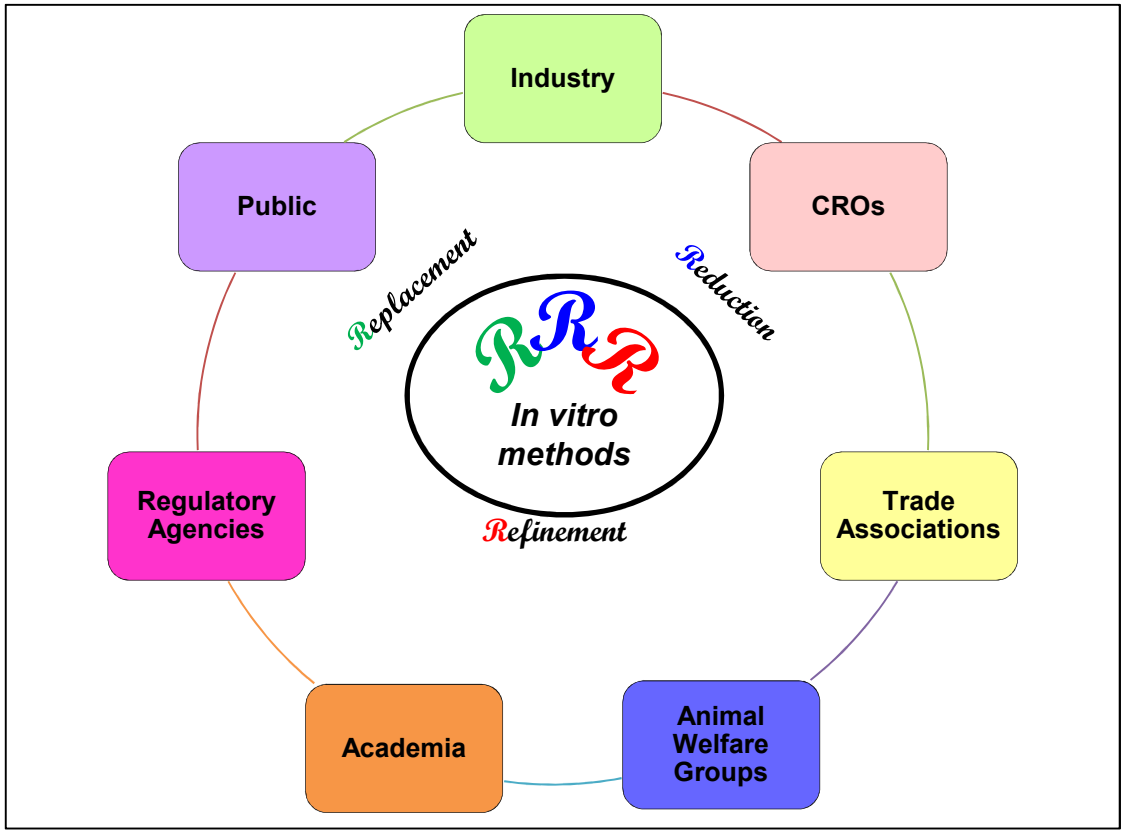
- General considerations
- Limitations
- Method overview and current regulatory status
- Typical and modified protocols
- Examples (classifications)
- Mechanisms
- Prediction models
- **A**dverse **O**utcome **P**athways
- **I**ntegrated **T**esting **S**trategies
- **I**ntegrated **A**pproaches to **T**esting and **A**ssessment
- **D**efined **A**pproaches

Key concepts – integrating information to guide testing and data analyses
Other resources

Current regulatory climate – global acceptance of *in vitro* methods



Drivers of *in vitro* methods advancement



Ongoing evolution on so many levels

- Improve scientific basis for testing using human-derived test models
- Reduce the number of animals for testing
- Increase predictivity
- Reduce time, price
- Harmonize requirements and prediction models

https://ntp.niehs.nih.gov/pubhealth/evalatm/accept-methods/index.html
http://alttox.org/mapp/table-of-validated-and-accepted-alternative-methods/

The reductionist concept of *in vitro* models

1940s

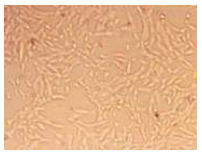
Whole animal
(Rabbit)

Organ - Eyeball
(Enucleated chicken or rabbit eye)

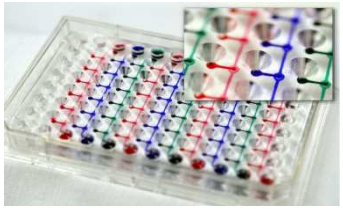
1990s

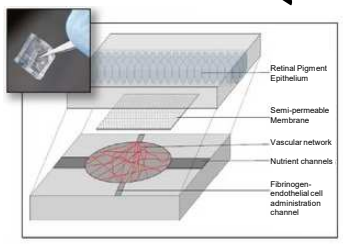
Tissue - Cornea
(Resected bovine cornea)

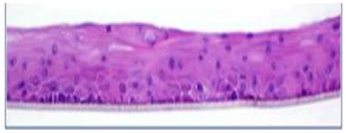
Cell culture
(Statens Seruminstitut Rabbit cornea cells)



"Less is more"







Body-on-a-chip
(Human organotypic microtissues)

Organ-on-a-chip
(Human retina)

Tissue construct
(Human EpiCorneal™ model)

Cell culture
(Normal human corneal epithelial cells)

2010s **2000s**

G.-E. Costin and H. A. Raabe. *In vitro* toxicology models. In: The Role of the Study Director in Non-clinical Studies. Pharmaceuticals, Chemicals, Medical Devices, and Pesticides. (Eds. William Brock, Barbara Mounho and Lijie Fu), John Wiley and Sons (2014)

G.-E. Costin. Advances in science: next generation of lab tools, models and testing platforms used in predictive toxicology. *Molecular Life*; 1(1), 22-28, doi: 10.26600/MolLife.1.1.3.2017. Available at: http://molecular-life.org/wp-content/uploads/2017/07/Advances-science-next-generation-lab-tools-models-testing-platforms-used-predictive-toxicology.pdf (2017)

Placing the "Safety" in the Safety Data Sheet

1. Identification
2. Hazard(s) Identification
3. Physical and Chemical Properties
4. First-aid Measures
5. Fire-fighting Measures
6. Accidental Release Measures
7. Handling and Storage
8. Exposure Controls/ Personal Protection
9. Physical and Chemical Properties
10. Stability and Reactivity
11. Toxicological Information
12. Ecological Information
13. Disposal Considerations
14. Transport Information
15. Regulatory Information
16. Other Information

Standard SDS specified by the Occupational Safety and Health administration (OSHA)

Challenges

How are classification and labeling predictions communicated to the regulatory community using the non-animal paradigm?

1. What information is acceptable?
2. Can an ingredient or a formulation be classified without testing?
3. What assays or endpoints are accepted?
4. Can they stand-alone?
5. Is there a hierarchy to follow?
6. How are data gaps addressed?

Must meet global expectations of OECD members

Mutual Acceptance of Data

Further challenges

How can the best method be selected?

How are data interpreted?

1. By target tissue (eye, skin, systemic toxicity, etc.)?
2. By endpoint? (category specific?)
3. By test system type? (*in chemico*, cellular 2D, 3D, *ex vivo*?)
4. By relevance to the test material?

   (chemicals, formulations, solubility issues)
5. By regulatory acceptance only?

   (can non-regulatory assays be used in WofE – Weight of Evidence?)

Plenty of assays to choose from

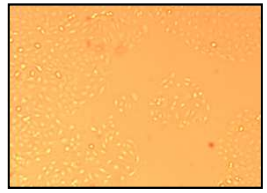
Four major groups of non-animal test methods used in research and regulatory safety testing of chemicals and products

1. *In chemico* test systems

Skin Corrosion:	**Membrane Barrier Test Method Corrositex™ (OECD TG 435)**
Eye Irritation:	"Irritection" Test (draft OECD TG)
Skin Sensitization:	Direct Peptide Reactivity Assay (DPRA) (OECD TG 442C)

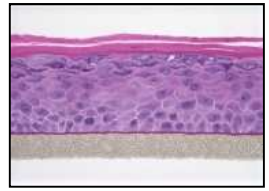
2. *In vitro* monolayer cell culture systems

Skin Phototoxicity:	Phototoxicity Test (OECD TG 432)
Ocular Irritation:	Cytosensor Microphysiometer (US EPA AMCP and draft TG)
	Short-Term Exposure (STE) Assay (OECD TG 491)
Skin Sensitization:	**KeratinoSens (OECD TG 442D)**
	hCLAT (OECD TG 442E)



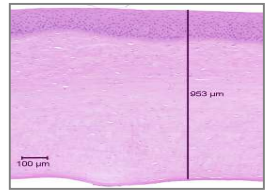
3. *In vitro* reconstructed tissue models systems

Skin Corrosion:	**Reconstructed human EpiDermis (RhE) Corrosion Assay (OECD TG 431)**
Skin Irritation:	RhE Skin Irritation Test (SIT) (OECD TG 439)
Eye Irritation:	Eye Irritation Test (EIT) (OECD TG 492)



4. *Ex vivo* tissues and organ systems

Ocular irritation:	**Bovine Corneal Opacity and Permeability Assay (OECD TG 437 and US EPA AMCP)**
	Isolated Chicken Eye Test (OECD TG 438)
Skin Absorption:	*In vitro* Skin Absorption (OECD TG 428)
Skin Corrosion:	Rat Skin Transcutaneous Electrical Resistance Test (OECD TG 430)



1. In chemico test systems

General considerations

- Do not require cell culture facility or cell culture expertise
- May be relatively inexpensive to conduct
- Standardized manufacturing or processes ensure standard testing platforms
- Some allow exposures as *in vivo*
- Some test methods may require specialized equipment (DPRA: HPLC)

Limitations

- Reliance on a limited number of manufacturers for specific commercial platforms
- Lack complex biological responses
  - *Are metabolism, inflammatory mechanisms included?*
- Assay may require further information or testing
  - *Endpoint may be simplistic*
  - *May only model chemical initiating event*

Membrane barrier test method (Corrositex®) (OECD 435)

Brief overview and current regulatory status

Test system: Artificial membrane designed to respond to corrosive substances in a manner similar to animal skin *in situ*

Assay endpoint: The time (in minutes) required for a test substance to penetrate through the Corrositex™ BioBarrier Membrane and produce a color change in the Chemical Detection System (CDS)

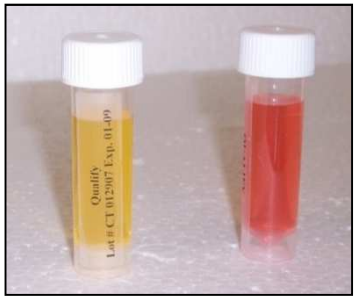
Assay controls: Negative (10% citric acid, 5% propionic acid); Positive (sodium hydroxide)

Applicability: Assigns UN Packing Group to corrosives or verifies if a test substance is non-corrosive

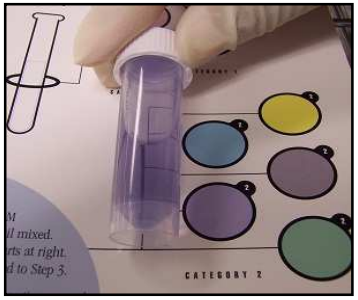
Limitations: Materials with a pH of ≥ 4.5 and ≤ 8.5 generally fail to qualify for testing based on the CDS used in the kit provided by In Vitro International

Regulatory status: OECD Test Guideline 435 (TG 435, updated 2015)

Corrositex®: typical protocol



- Test substance is added to a tube containing Chemical Detection System (CDS).
- Materials with a pH of ≥ 4.5 and ≤ 8.5 generally fail to qualify for testing.



- The test substance is added to two test tubes to determine the appropriate timetable for Packing Group Assignment.
- A Category 1 test substance will be evaluated for up to 4 hr; a Category 2 test substance will be evaluated for up to 1 hr.

Biobarrier Preparation

Biobarrier Placement

To prepare the biobarrier membranes, the biobarrier matrix powder is completely solubilized. The solubilized collagen matrix is then added to a membrane disc containing a porous cell membrane and placed onto a vial containing CDS.

Break Through Observations

Each test substance is added onto four replicate biobarrier membranes and the CDS vial is continuously monitored for the first 10 min. The vials are observed until a color change (*i.e.*, break through) occurs. When a color change occurs in each vial, the break through times are recorded.

Prediction Model

Category I

Mean Time to Produce a Change in Chemical Detection System	Packing Group
≤ 3 Minutes	I
> 3 Minutes - 1 Hour	II
> 1 - 4 Hours	III
> 4 Hours	Not Applicable

Category II

Mean Time to Produce a Change in Chemical Detection System	Packing Group
≤ 3 Minutes	I
> 3 Minutes - 30 minutes	II
> 30 - 60 minutes	III
> 60 minutes	Not Applicable

Sensitivity	Specificity	False negative rate	False positive rate	Packing Group Accuracy
89%	75%	11%	25%	96%

Classification examples:

extreme pH mixtures (alkalis)

	Solvent (% Active)	Physical Parameters		*In Vivo*	Corrositex®
		pH	Alkaline Reserve		
Product 7	20	13.7	2.83	Corrosive	Not tested
Product 8	1.5	12.95	0.91	Corrosive	Corrosive
Product 9	15	11.41	1.35	Non-corrosive	Corrosive
Product 10	0	13.5	2.36	Non-corrosive	Non-Corrosive
Product 11	32.7	12.6	0.38	Non-corrosive	Corrosive
Product 12	3	12.15	0.02	Non-corrosive	Not tested
Product 13	3	12.16	0.10	Non-corrosive	Corrosive
Product 14	10	12.76	0.91	Corrosive	Not tested
Product 15	23.8	12.15	2.51	Corrosive	Corrosive
Product 16	0	12.5	0.47	Non-Corrosive	Not tested
Product 31	27	11	1.38	Non-Corrosive	Not tested
Product 32	34.5	11	1.38	Non-Corrosive	Not tested
Product 33	15	11.9	Not recorded	Non-Corrosive	Corrosive
Product 39	0	13.2	Not recorded	Non-Corrosive	Not tested

- 3/7 products tested using the Corrositex® assay predicted the same skin classification when compared to the *in vivo* data. The remaining 4 formulas **over-predicted** the skin classification. There were no under-classifications.
- **Formulas with high levels of solvent (≥15%)** may result in a more conservative classification when using this *in vitro* assay.

Burrows-Sheppard A.M., Willmes S.S., Heitfeld F., Treichel J., Raabe H., Curren R., An evaluation of the EpiDerm Corrosivity and Corrositex assays for predicting skin corrosivity of chemical products with extreme alkaline pH, The Toxicologist, 114, 106 (2010)

2. In vitro monolayer cell culture systems

General considerations

- Generally easy to conduct – cell lines
- Quite rapid to execute
- Cost effective with batches of test materials – HTP – robotics
- Mechanistic modes of action
- Machine scored endpoints
- Identify potential hazards
- Evaluate individual chemicals (ingredients) rather than formulations

Limitations

- Dilution effects which mask toxicity of the neat material
- Buffering effects of the vehicle, and reaction of the chemical
- Solubility issues
- Pharmacokinetics poorly modeled
- No tissue barrier function modeled
- Typically lack realistic cell-cell contact: may impact cellular responses

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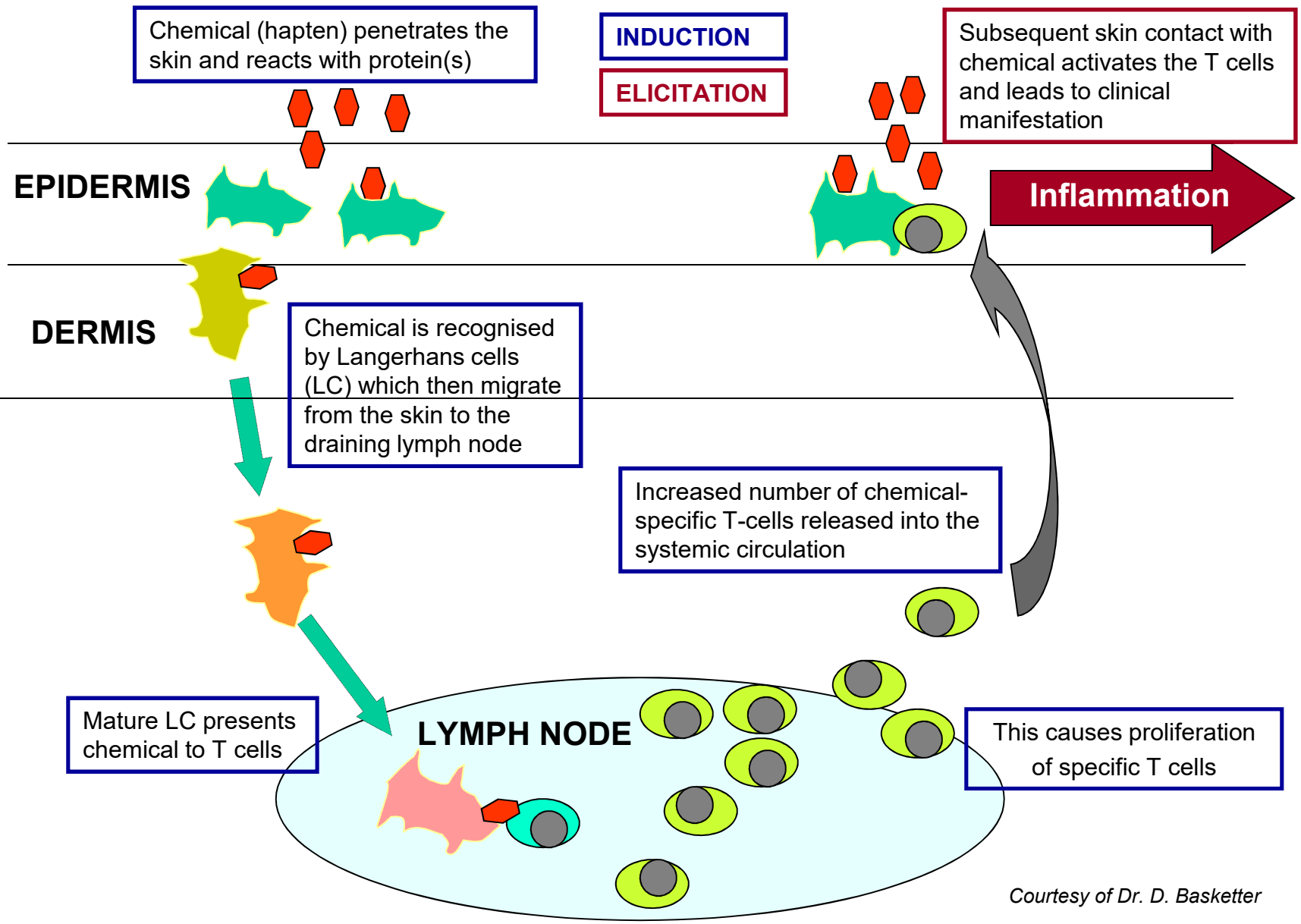
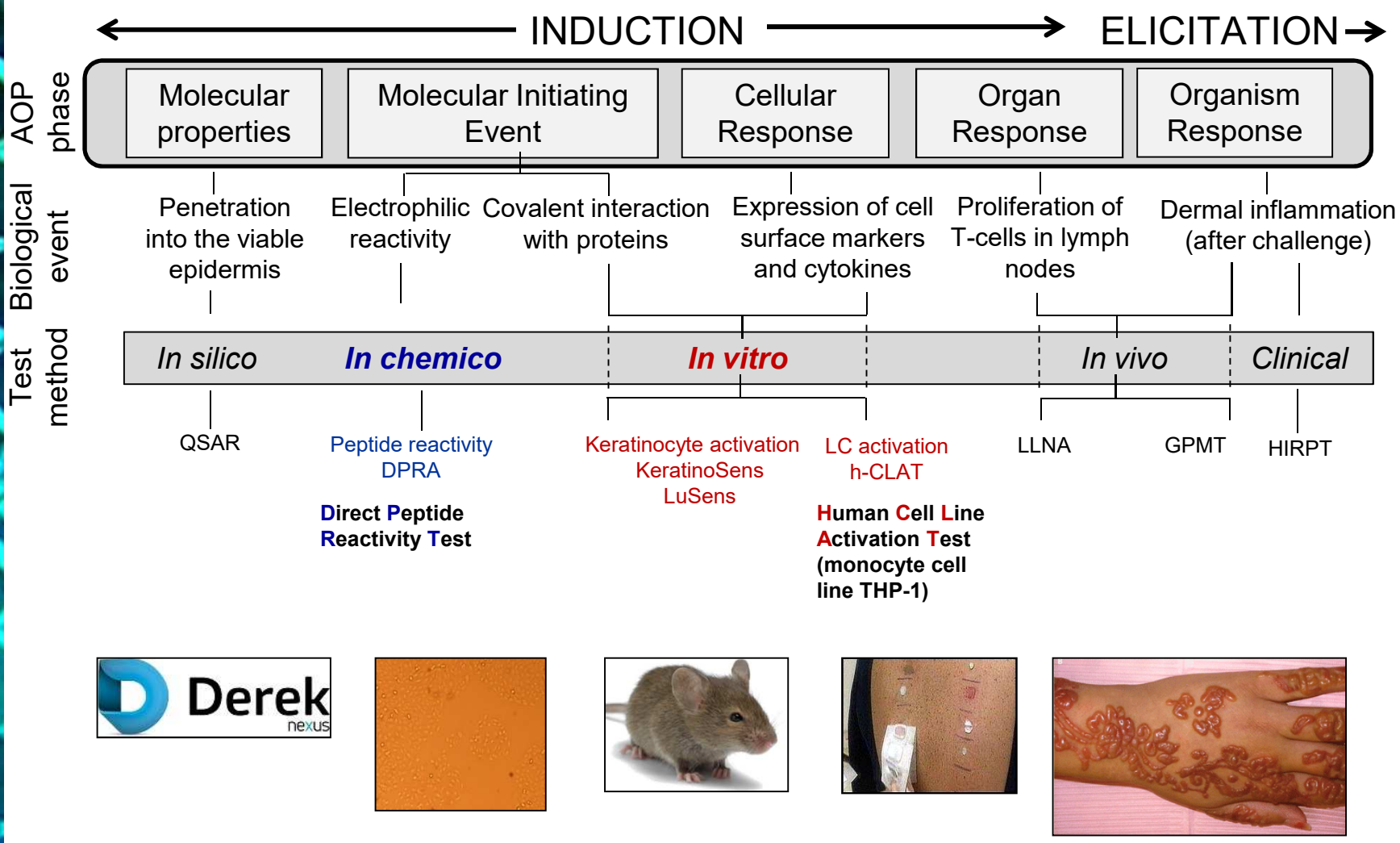
Mechanisms of skin sensitization



Courtesy of Dr. D. Basketter

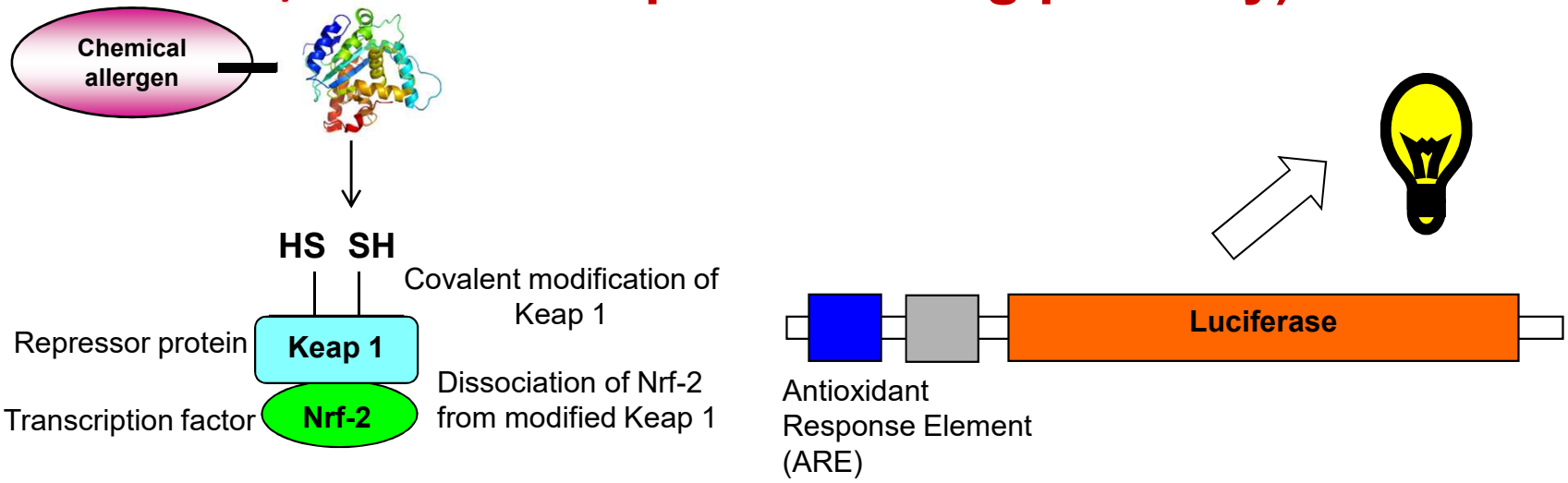
Adverse Outcome Pathway (AOP) for skin sensitization



Evans, CC and Fleming, JD. Allergic contact dermatitis from a henna tattoo. N. Engl. J. Med. 359: 627 (2008)

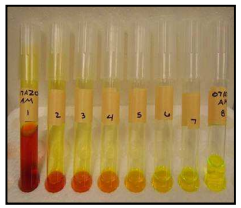
Costin G.E. Advances in science: next generation of lab tools, models and testing platforms used in predictive toxicology. Molecular Life; 1(1), 22-28, doi: 10.26600/MolLife.1.1.3.2017. Available at: http://molecular-life.org/wp-content/uploads/2017/07/Advances-science-next-generation-lab-tools-models-testing-platforms-used-predictive-toxicology.pdf (2017)

KeratinoSens assay (Nrf-2-electrophile sensing pathway)

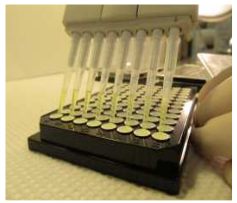


KeratinoSens: typical protocol

Pre-testing: solubility assessment

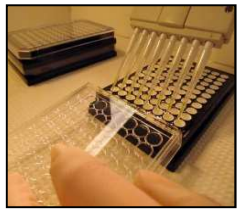


Cell dosing



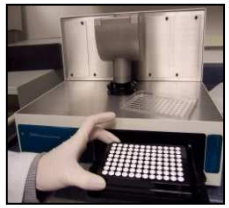
Treatment termination

Addition of luciferase



Addition of MTT

Sensitization endpoint



Cytotoxicity endpoint

KeratinoSens assay (OECD 442D)

Brief overview and current regulatory status

Test system: HaCaT cells (immortalized keratinocytes containing a reporter construct with a copy of the Antioxidant Response Element (ARE) of the human *AKRIC2* gene upstream of a luciferase gene

Assay endpoints: Induction of luciferase activity, cytotoxicity

Assay controls: Negative (Solvent: Assay Media containing 1% DMSO);
Positive (cinnamic aldehyde)

.....

Applicability : Support the discrimination between skin sensitizers and non-sensitizers for the purpose of hazard classification and labeling as part of an IATA (Integrated Approaches to Testing and Assessment)

Limitations: Since activation of the Keap1-Nrf2-ARE pathway addresses only the second key event of the skin sensitization AOP, information from test methods based on the activation of this pathway is unlikely to be sufficient when used on its own to conclude on the skin sensitization potential of chemicals.
Solubility challenges

.....

Regulatory status: OECD Test Guideline 442D (TG 442D, adopted 2015)

KeratinoSens: data interpretation

Data calculation:

EC1.5 value: test substance concentration for induction 1.5 fold time above threshold

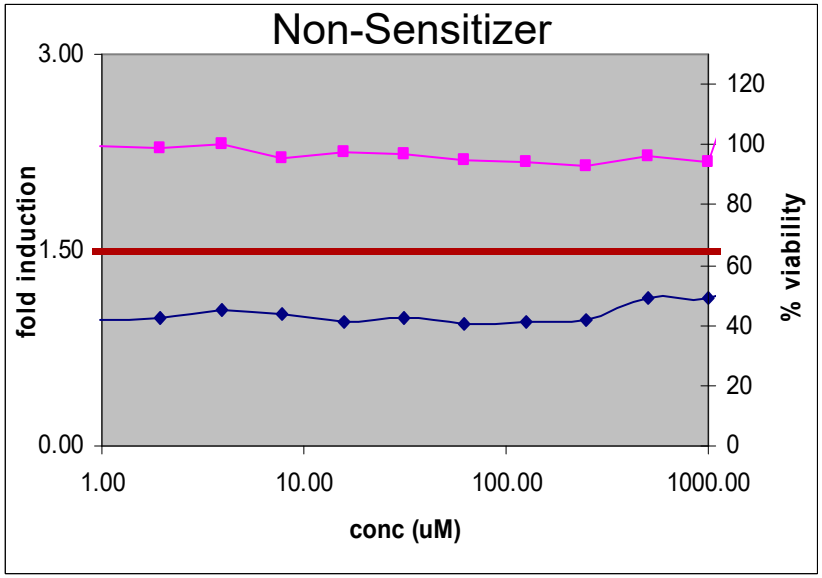
I_{max}: the largest average gene fold induction above 1.5 by the test substance

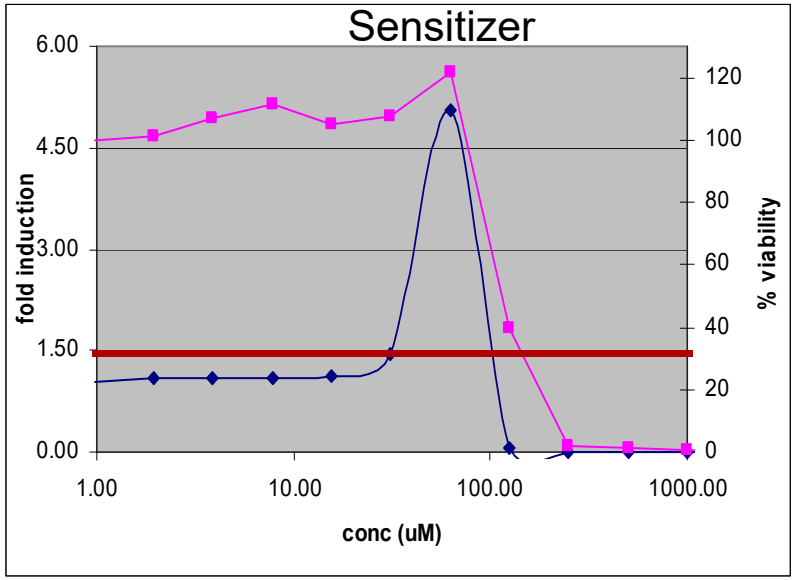
Ci_{max}: the test substance concentration at which the largest average fold induction value is achieved

Prediction Model

A test substance will be considered to have sensitization potential if:

1) The EC1.5 value falls below 1000 µM (or 200 µg/mL) in at least 2 of 3 repetitions
2) At the lowest concentration with a gene induction above 1.5, cellular viability should be greater than 70%
3) An apparent overall dose response should be similar between repetitions.





Induction- dark blue; viability- pink

Integrated Testing Strategies (ITS)

		Accuracy compared to:		
		human data	LLNA data	
		54 chemicals		145 chemicals
Assay		Bauch *et al.*, 2012		Natsch *et al.*, 2013
Individual assays	DPRA	87%	79%	82%
	ARE reporter gene assay	82%	81%	79%
2 of 3	DPRA, ARE-based assay	94%	83%	81%

The ITS is selected based on the goals of the testing:

- Screening (before animal/clinical testing)
- Stand-alone (internal)
- Submissions to regulatory agencies
- Timing and costs (sequential/parallel)
- Chemistries, risk (cosmetics/household/pharma)

Bauch C. et al. Putting the parts together: combining in vitro methods to test for skin sensitizing potential. Regul. Toxicol. Pharmacol. 63(3): 489-504 (2012)
Natsch A. et al. A dataset of 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. J. Appl. Toxicol 33(11): 1337-1352 (2013)

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Defined Approaches (DA)

Table 3. Skin sensitization potential predictivity of individual test methods and the mechanistic domains compared to both human and LLNA reference data, incl.

		Human				LLNA			
Test method	Sample size	Specificity	Sensitivity	Accuracy	Balanced accuracy	Specificity	Sensitivity	Accuracy	Balanced accuracy
LLNA	128	50.0%	85.2%	74.2%	67.6%	–	–	–	–
DPRA	124*	74.4%	72.9%	73.4%	73.6%	67.7%	66.7%	66.9%	67.2%
KeratinoSens™	128	77.5%	75.0%	75.8%	76.3%	66.7%	67.4%	67.2%	67.0%
h-CLAT	127°	52.5%	89.7%	78.0%	71.1%	51.5%	86.2%	77.2%	68.9%
U-SENS™	105#	44.7%	95.5%	77.1%	70.1%	48.0%	90.0%	80.0%	69.0%
SENS-IS	126"	47.5%	93.0%	78.6%	70.3%	50.0%	90.4%	80.2%	70.2%
Mechanistic reaction domain	122**	75.0%	86.6%	82.8%	80.8%	77.4%	81.3%	80.3%	79.4%

Table 3. Defined Approach (DA) performance in predicting human hazard (sensitizer/non-sensitizer).

Predicting Human Hazard								
Defined Approach:	BASF 2/3 (DKH)	Kao STS	Kao ITS	ICCVAM SVM (Human)	Shiseido ANN (D_hC)	Shiseido ANN (D_hC_KS)	P&G BN ITS-3	LLNA
N	127	126	120	120	126	126	119	128
Accuracy (%)*	77.2	80.2	85.0	81.7	78.6	78.6	75.6	74.2
Sensitivity (%)	79.3	97.7	93.8	86.4	95.4	100	81.3	85.2
Specificity (%)	72.5	41.0	66.7	71.8	41.0	30.8	64.1	50.0
BA (%)	75.9	69.4	80.3	79.1	68.2	65.4	72.7	67.6

DPRA; hCLAT; DEREK
Not applicable to natural products

Hoffmann S. et al. Non-animal methods to predict skin sensitization (I): the Cosmetics Europe database. Crit. Rev. Toxicol. 23: 1-15 (2018)
Kleinstreuer N.C. et al. Non-animal methods to predict skin sensitization (II): an assessment of defined approaches. Crit. Rev. Toxicol. 23: 1-16 (2018)

3. In vitro reconstructed tissue models systems

General considerations

- Higher order of complexity - Reconstructed tissues better model tissues of interest (relative to monolayer)
- Exposure to substances as *in vivo*
- Relevant mechanisms of action
- Endpoints may be machine scored
- Standardized manufacturing expected to ensure reproducibility

Limitations

- Tissue models tend to be costly
- Reliance on a small number of manufacturers
- Tissues differ slightly among manufacturers
- Still relatively simple models, and do not have support of whole body accessory functions

How might this impact the toxicity predictions?

- Care needs to be exercised not to over-interpret

(just as in the case of animal models!)

RhE test method - skin corrosion assay (OECD TG 431)

Brief overview and current regulatory status

Test system: RhE models [EpiDerm™ (EPI-200); EpiSkin™ (SM); SkinEthic™ RHE and epiCS®]

Assay endpoint: Tissue viability (%) – assessed by reduction of the vital dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by viable cells

Assay controls: Negative (sterile, deionized water or NaCl solution 9g/L);
Positive (8N KOH or glacial acetic acid – only for 4 hr exposure)

Applicability: The results can be used for regulatory purposes for distinguishing corrosive from non-corrosive test substances. The method also allows for sub-categorization, *i.e.*, 1A vs. 1B-and-1C vs. non-corrosive test substances.

Limitations: The method does not allow discriminating between skin corrosive sub-categories 1B and 1C according to the UN GHS due to a limited set of well-known *in vivo* corrosive Category 1C chemicals.

Regulatory status: OECD Test Guideline 431 (TG 431, updated 2016)

RhE - corrosion: typical protocol

Tissue Receipt

Upon receipt, tissues are incubated for at least 1 hr in standard culture conditions (37±1^{0}C in a humidified atmosphere of 5±1% CO_2 in air).

Tissue Treatment

Media is refreshed after the initial 1 hr incubation. Duplicate tissues are treated topically with control and test substances for 3 min / 1 hr (4 hr).

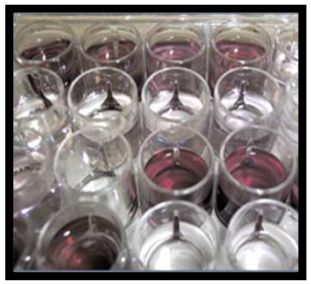
Tissue Rinsing

After exposure, tissues are rinsed to remove the control and test substances.

MTT Reduction

Individual tissues are placed into wells containing unreduced MTT solution and incubated at standard culture conditions for 3 hr.

Isopropanol Extraction



The tissues are placed in isopropanol at room temperature for 2 hr to extract the reduced MTT. Extracted MTT is thoroughly mixed and transferred to a 96-well plate.

Spectrophotometric Quantification

Optical density (OD) at 550 nm (OD_{550}) is determined using a 96-well plate reader. OD values are used to calculate relative viability values presented relative to negative control tissue values.

Prediction Models

EpiSkin™ (SM)

Viability measured after exposure time points (3, 60 and 240 minutes)	Prediction to be considered UN GHS Category
< 35% after 3-minutes exposure	Corrosive: • Optional Sub-category 1A
≥ 35% after 3-minutes exposure **AND** < 35% after 60-minutes exposure **OR** ≥ 35% after 60-minutes exposure **AND** < 35% after 240-minutes exposure	Corrosive: • A combination of optional Sub-categories 1B and 1C
≥ 35% after 240-minutes exposure	Non-corrosive

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

Viability measured after exposure time points (3- and 60-minutes)	Prediction to be considered UN GHS Category
STEP 1	
< 50% after 3-minutes exposure	Corrosive
≥ 50% after 3-minutes exposure **AND** < 15% after 60-minutes exposure	Corrosive
≥ 50% after 3-minutes exposure **AND** ≥ 15% after 60-minutes exposure	Non-corrosive
STEP 2	
<25%; **18**%; **15**% after 3-minutes exposure	Optional Sub-category 1A
≥25%; **18**%; **15**% after 3-minutes exposure	A combination of optional Sub-categories 1B-and-1C

Desprez B., Barroso J., Griesinger C., Kandarova H., Alepee N., Fuchs H.W., Two novel prediction models improve prediction of skin corrosive sub-categories by test methods of OECD, Toxicology in Vitro, 29, 2055-2080 (2015)

Classification examples: extreme pH mixtures (alkalis)

	Solvent (% Active)	Physical Parameters		*In Vivo*	Predicted by WoE*	EpiDerm™
		pH	Alkaline Reserve			
Product 7	20	13.7	2.83	Corrosive	Corrosive	Corrosive
Product 8	1.5	12.95	0.91	Corrosive	Non-corrosive	Corrosive
Product 9	15	11.41	1.35	Non-corrosive	Corrosive	Corrosive
Product 10	0	13.5	2.36	Non-corrosive	Non-corrosive	Inconclusive
Product 11	32.7	12.6	0.38	Non-corrosive	Corrosive	Inconclusive
Product 12	3	12.15	0.02	Non-corrosive	Non-corrosive	Non-Corrosive
Product 13	3	12.16	0.10	Non-corrosive	Non-corrosive	Non-corrosive
Product 14	10	12.76	0.91	Corrosive	Non-corrosive	Corrosive
Product 15	23.8	12.15	2.51	Corrosive	Corrosive	Corrosive
Product 16	0	12.5	0.47	Non-Corrosive	Non-Corrosive	Non-Corrosive
Product 31	27	11	1.38	Non-Corrosive	Non-Corrosive	Corrosive
Product 32	34.5	11	1.38	Non-Corrosive	Corrosive	Corrosive
Product 33	15	11.9	Not recorded	Non-Corrosive	Non-Corrosive	Corrosive
Product 39	0	13.2	Not recorded	Non-Corrosive	Corrosive	Non-Corrosive

- Extreme pH can be a useful predictor of irritation but may lead to over-classifications in weakly buffered systems.
- 8/12 products tested using the RhE testing system predicted the same skin classification when compared to the *in vivo* data. The remaining 4 formulas **over-predicted** the skin classification. There were no under-classifications.
- **Formulas with high levels of solvent (≥15%)** may result in a more conservative classification when using this *in vitro* assay.

Burrows-Sheppard A.M., Willmes, S.S., Heitfeld, F., Treichel, J., Raabe, H., Curren, R., An evaluation of the EpiDerm Corrosivity and Corrositex assays for predicting skin corrosivity of chemical products with extreme alkaline pH, The Toxicologist, 114 (1), 106 (2010)

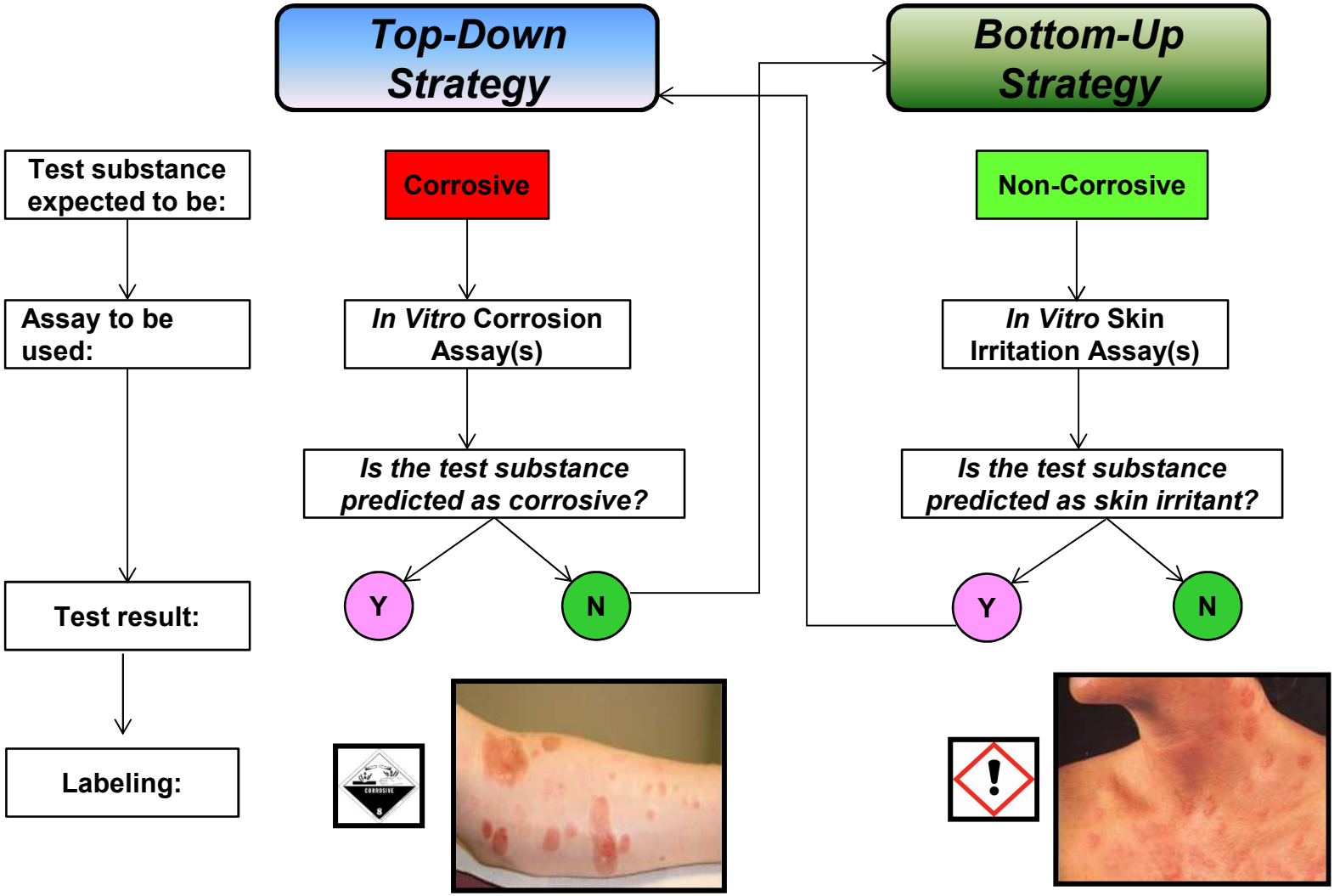
Classification examples: fatty amines

Category	Alkyl chain	CAS no.	State	Results–viability *in vitro*: Conclusion	3 min	1 h	4 h	Model	Results – *in vivo* Conclusion
PPA – Polyamine									
Propylene diamines	Coco	61791-63-7	Liquid/paste	–					Corr.1B (3 min)
	Tallow	61791-55-7	Paste	–					Corr.1B (1 h)
	HT	68603-64-5	Solid	–					Irr.Cat.2/Corr.1C
	Oleyl	7173-62-8	Liquid/paste	–					Corr.1B (3 min)
Dipropylene triamine	Coco	91771-18-5	Liquid	Non-corrosive	58%	22%		EpiDerm™	–
	Tallow	61791-57-9	Paste	Non-corrosive	98%	96%		EpiDerm™	–
	Oleyl	28872-01-7	Liquid	Non-corrosive	95%	89%		EpiDerm™	–
Tripropylene tetramine	Tallow	68911-79-5	Paste	Not possible; too sticky to remove				EpiDerm™	Corr.1C
	Oleyl	67228-83-5	Paste	Non-corrosive	91%	85%		EpiDerm™	Corr.1C
Dipropylene triamine (branched)	C_{12}	2372-82-9	Liquid	**Corr.1B/C**	**43%**	42%		EpiDerm™	Corr.1B (3 min)
	Tallow	85632-63-9	Liquid/paste	–					Corr.1B (3 min)
PPAEO – Alkylaminesethoxylated									
Alkylamines ethoxylated (2EO)	Coco-2EO	61791-31-9	Liquid	Non-corrosive	109.5%	114.8%	94.0%	EpiSkin™	Corr.1C
	C_{12-18}-2EO	71786-60-2	Liquid	Non-corrosive	106.5%	113.6%	101.2%	EpiSkin™	Corr.1C
	Tallow-2EO	61791-44-4	Paste	–					Corr.1C
	HT-2EO	90367-28-5	Solid	Non-corrosive	102.4%	105.3%	98.2%	EpiSkin™	Irr.Cat.2(*)
	Oleyl-2EO	25307-17-9	Liquid	–					Corr.1B (3 min)
AA – Amidoamine									
Alkyl amidoamine	Coco-N-DMAPA	68140-01-2	Paste	Non-corrosive	88.7%	74.8%	93.1%	EpiSkin™	Corr.1B (3 min)
	Coco-APDEA	66161-63-5	Liquid	Non-corrosive	80%	70%		EpiDerm™	Corr.1B (3 min)
ED – Etherdiamine									
Etherdiamine	iso-Tridecyl	68479-04-9	Liquid	Non-corrosive	56.3%	79.3%		EpiDerm™	Corr.1B (3 min)
QE – Quatethoxylated									
Quat ethoxylated	Coco	70750-47-9	Liquid	Non-corrosive	94%	21%		EpiDerm™	Corr.1B (1 h)

- Fatty amine derivatives are recognized for their severe irritating and corrosive effects to the skin.
- Effects are characterized by a delayed severe inflammatory reaction which may not be captured by currently validated *in vitro* assays.
- The *in vitro* RhE-based skin corrosion assay is not suitable for this category of substances (concerns with **under-predictions**).
- Authors proposed modifications of the protocol - will the data be considered by a regulatory agency?

Houthoff, E., Rugen, P., Hart, D. Predictability of in vitro dermal assays when evaluating fatty amine derivatives. Toxicol. In Vitro, 29, 1263-1267 (2015)

Integrated Approaches to Testing and Assessment (IATA)
Dermal corrosion and irritation (self-correcting)



Scott L. et al., A proposed eye irritation testing strategy to reduce and replace in vovo studies using Bottom-Up and Top-Down approaches. Toxicol. In Vitro, 24, 1-9 (2010)

Calufetti S. et al., Tiered testing strategy using validated in vitro assays for the assessment of skin and eye corrosion/irritation of pharmaceutical intermediates, The Toxicologist, 138, 268 (2014)

Wilt N. et al., A tiered in vitro irritation/corrosion testing strategy for GHS classification of pharmaceutical compounds, The Toxicologist, 144, 89 (2015)

4. Ex vivo tissues and organ systems

General Considerations

- High order of complexity
- Excised tissues directly correlate to tissues of interest
- Exposure to substances as *in vivo*
- Relevant mechanisms of action

Limitations

- Human tissues of exceptional quality are often difficult to obtain
- Tissues may differ from trial to trial
- If the tissue is non-human, is the relevance questionable?
- Excised tissues no longer have support of whole body accessory functions (inflammatory responses, metabolism, etc.)

  How might this impact the toxicity predictions?
- Care needs to be exercised not to over-interpret

  (just as in the case of animal models!)

Bovine Corneal Opacity and Permeability (BCOP) assay (OECD TG 437)

Brief overview and current regulatory status

Test system: Viable corneas maintained in culture responsive to a large variety of chemical classes and physical forms

Assay endpoint: Opacity and permeability (two relevant endpoints measured in a single, one day experiment)

Assay controls: Negative (sterile, deionized water);
Positive (imidazole – solids; ethanol - liquids)

……

Applicability: The results can be used for regulatory purposes for distinguishing eye corrosive/severe irritants (GHS Category I) from non-irritating test substances (No Category). Adopted as part of a self-correcting strategy to address the eye irritation endpoint as part of the "six pack" US EPA labeling system (antimicrobial products originally, now extended to conventional pesticides on a case by case basis).

Limitations: Cannot assign GHS Category 2 classification
Availability/source of eyeballs
Cannot address reversibility

……

Regulatory status: OECD Test Guideline 437 (TG 437, updated 2017);
US EPA OPP policy (3-2-2015)

Ocular irritation - A continuum of sensitivity

Household | **Color Cosmetics**

Cleaning Products

Industrial Chemicals

Extreme — Severe — Moderate — Mild — Very Mild

GHS 1	GHS 2	GHS Non-classified
EPA I	EPA II	EPA III / EPA IV

BCOP
ICE
IRE
HET-CAM
CM (aqueous soluble)
STE
EIT
Ocular Irritection®

Testing strategy?

GHS 2 (2A, 2B): Irritant, Reversible

GHS 1: Severe/Corrosive Irritant

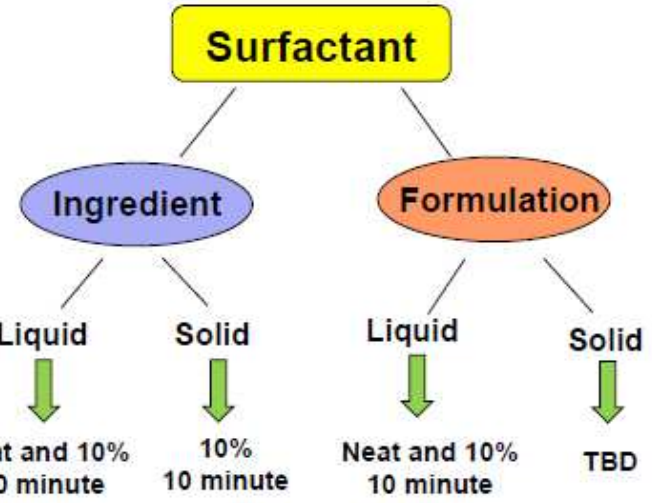
BCOP
ICE
CM (surfactants)
STE
EIT
Ocular Irritection®

Joao Barroso, Kimberly Norman, ChemWatch Webinar Series, Serious Eye Damage and Eye Irritation: http://media.simplicityweb.com/chemicalwatch/CW_serious_eye_damage_and_eye_irritation_webinar_141204.pdf

Range of protocols

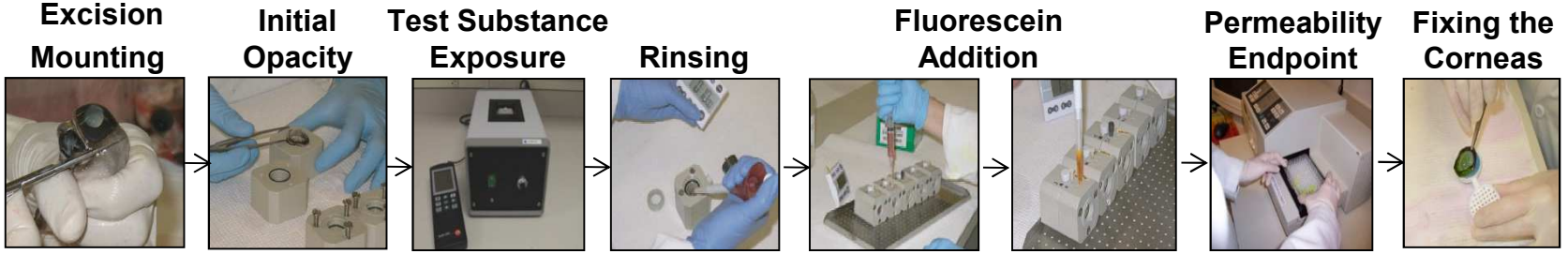
- Standard Protocols:
  - Liquid test materials: undiluted, 10-minute exposure, 120-minute post-exposure
  - Solid test materials: 20% suspension, 240-minute exposure
- Specialized Protocols:
  - Surfactant formulations: 10% solution, 60 minute exposure, 60-minute post-exposure (focus on permeability score)
  - Multiple exposures: undiluted, 3 and 10-minute exposure, 120-minute post-exposure (for organic solvent-based materials)
  - Extended post-exposure: 10-minute exposure, 4 and 20-hour post-exposures (reactive chemicals such as H_2O_2)
- Histology may be added to all protocols

Decision tree for BCOP testing approach to surfactants. ***For solid formulations, the protocol should be determined based on the formulation components.***



Bader J.E. et al. Surfactant responses in the Bovine Corneal Opacity and Permeability assay: points to condider for in vitro eye irritation testing, The Toxicologist, 132, 210 (2013)

BCOP: typical protocol



Data calculation: ***In Vitro*** **Score = Opacity + (15 x Fluor OD_{490})**

Prediction Models

Prediction Model Developed by Merck*

In Vitro Score	Predicted Irritation Potential
≤ 25	Mild
25.1 – 55	Moderate
> 55.1	Severe

Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates.* ***Fundamental and Applied Toxicology* *26:20-31.*

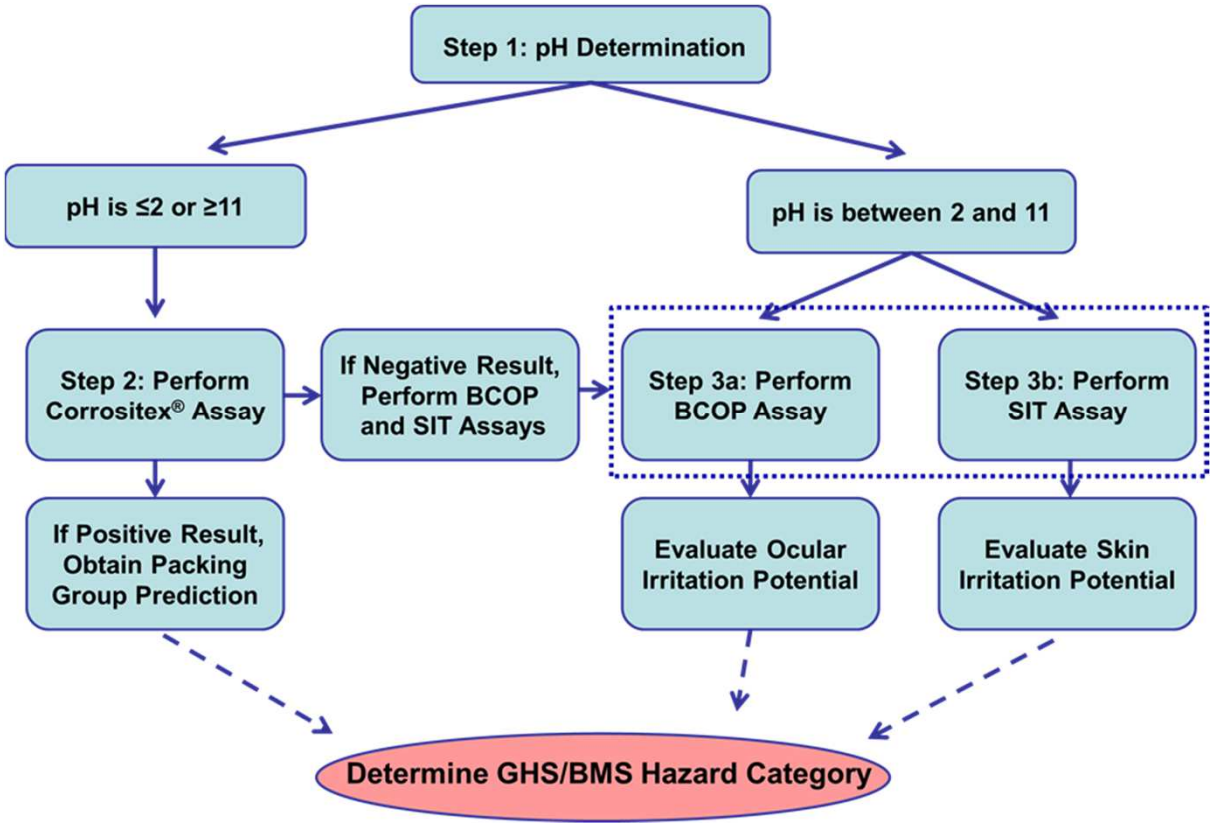
This model should be used with standard exposures & in conjunction with responses of benchmark materials; may not be appropriate for all classes of materials.

Prediction Model - OECD TG 437

In Vitro Score	UN GHS
≤ 3	No Category
>3 ≤ 55	No prediction can be made
> 55	Category 1

OECD. OECD guideline for the testing of chemicals. Bovine Corneal Opacity and Permeability Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage (OECD 437). Organisation for Economic Co-operation and Development (OECD) 2017. Available at and downloaded from: http://www.oecd-ilibrary.org/docserver/download/9713221e.pdf?expires=1513793255&id=id&accname=guest&checksum=2A6B70C3695BFF6FD957A441601B3416.

Adjusted Prediction Model and Tiered Testing Approach:
Pharmaceutical Compounds



Proposed tiered testing strategy for the assessment of ocular and dermal irritation potential of pharmaceutical compounds for the purpose of BMS worker safety

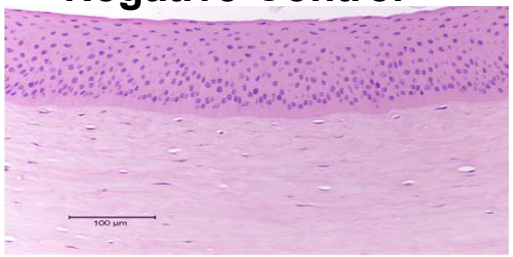
In Vitro Score	Irritation Potential
> 55	Severe Irritant
> 25 to ≤ 55	Moderate Irritant
> 3 to ≤ 25	Mild Irritant
≤ 3	Non-Irritant

Wilt N. et al., A tiered in vitro irritation/corrosion testing strategy for GHS classification of pharmaceutical compounds, The Toxicologist, 144, 89 (2015)

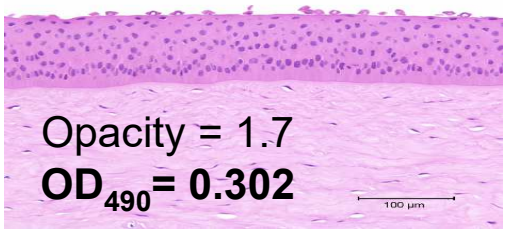
BCOP Histopathology: "classic" examples

Surfactants: membrane lysis

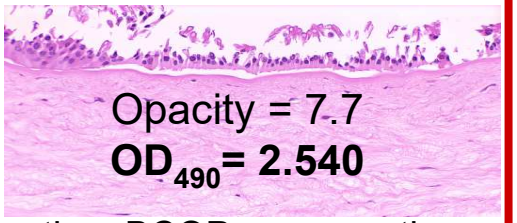
Negative Control



1.5% SLS: 10 min

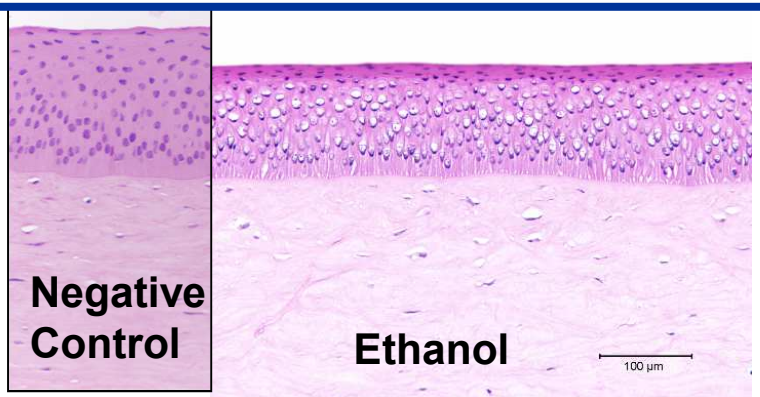


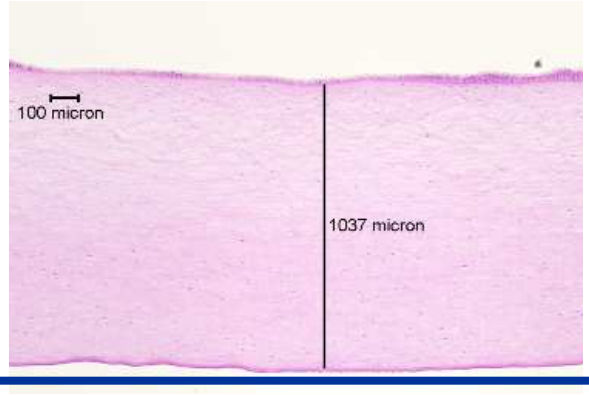
5% SLS: 30 min



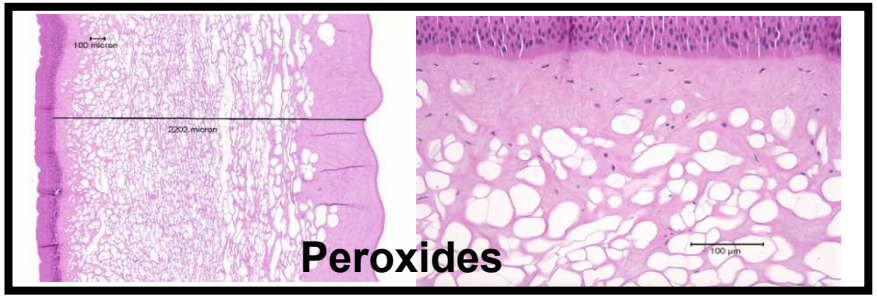
When evaluating anionic or non-ionic surfactants in the BCOP assay, the permeability endpoint should be considered independently of the opacity and In Vitro Score, because the opacity may be artificially low (potential for under-prediction).

Organic Solvents: coagulation/loss of epithelium & effects into stroma





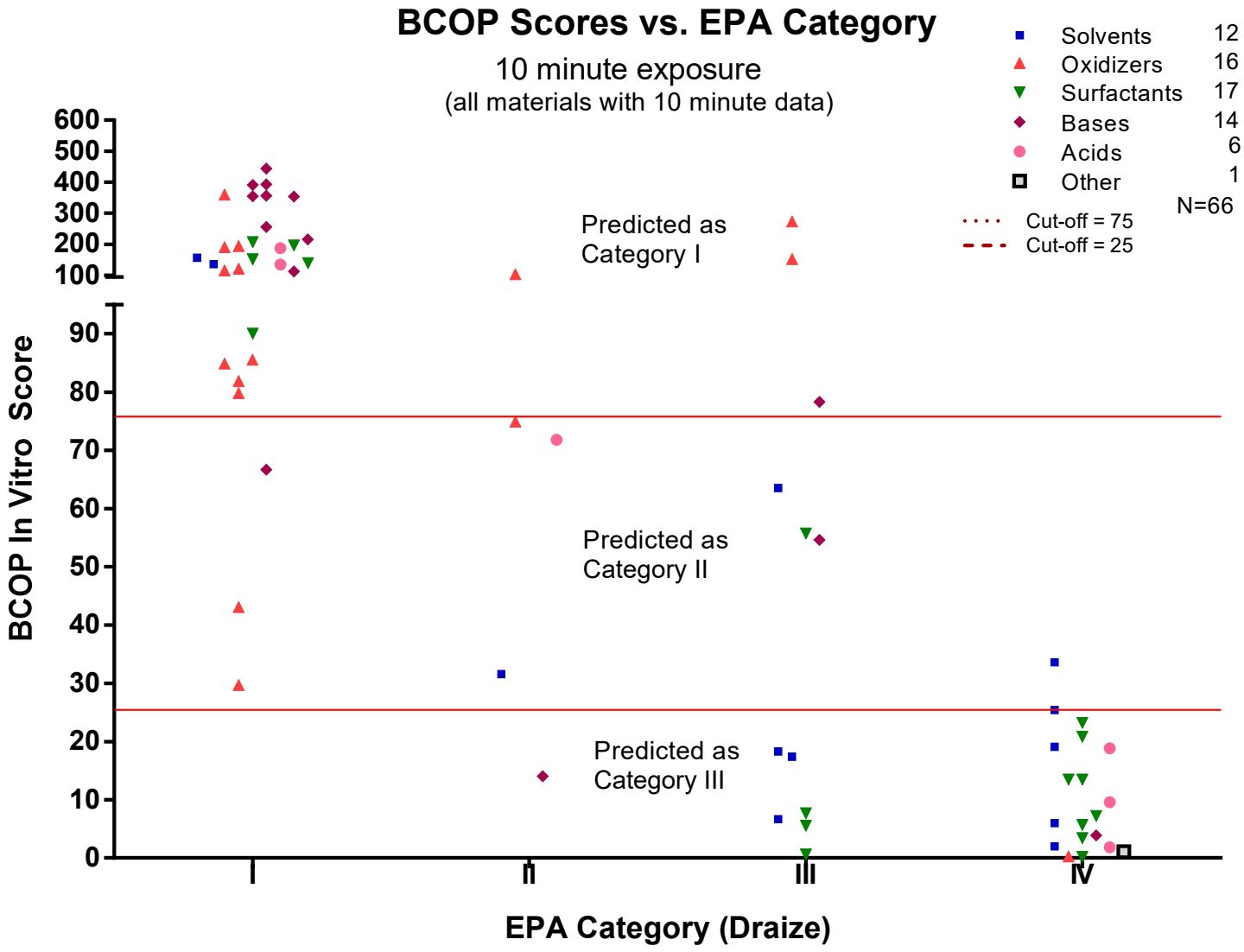
Reactive chemistries: full thickness damage



Bader J.E. et al. Surfactant responses in the Bovine Corneal Opacity and Permeability assay: points to condider for in vitro eye irritation testing, The Toxicologist, 132, 210 (2013)

Use of an alternate testing framework for classification of eye irritation potential of EPA pesticide products

*Clorox
*EcoLabs
*SC Johnson
*Colgate Palmolive
*JohnsonDiversey *(currently SealedAir)*
*The Accord Group
*Dial
*P&G
*Institute for In Vitro Sciences (IIVS)



Adjusted Prediction Model and Tiered Testing Approach: Pesticide Products Registered with US EPA

In Vitro Score	US EPA Predicted Category
< 25	Category III/IV – default to Category III and use the self-correcting strategy to discriminate between III and IV
≥ 25 <75	Category II
> 75	Category I

BCOP Assay Overall Performance

PREDICTIVITY

- Only 2 of 61 materials (8%) were under-predicted.
- All of the EPA toxicity Category IV materials are over-predicted as Category III since the BCOP does not seem to be able to differentiate between materials at this lower end of the toxicity scale.

LIMITATIONS

- If the anti-microbial cleaning product is a **High Solvent (>5 solvent) formulation**, it should be tested in the BCOP assay using a **3 minute exposure** instead of the normal 10 minute exposure.
- Testing of **ketones and alcohols** in the BCOP has been shown to result in high false positive rates for the assay, but not all ketones or alcohols are over-predicted.

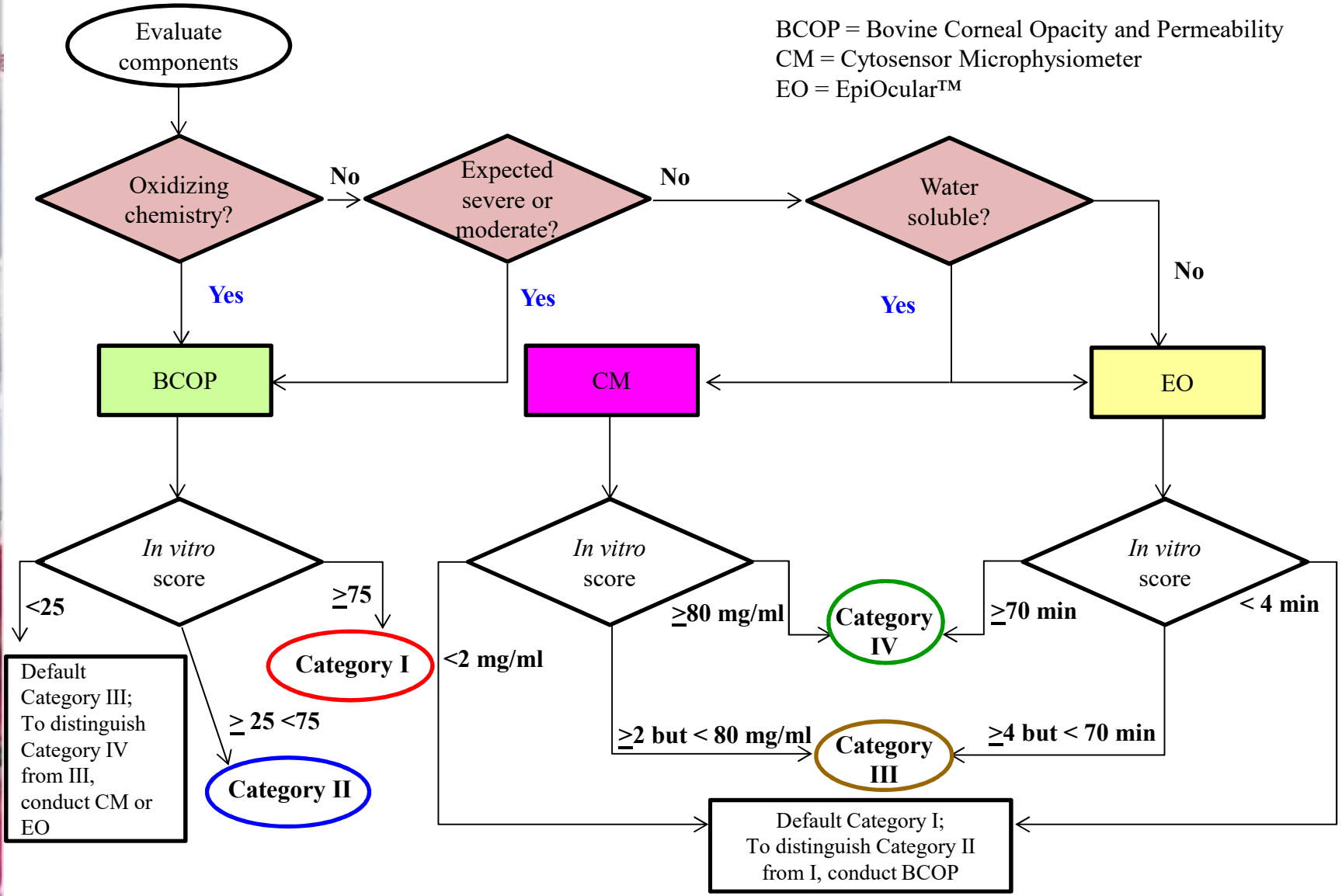
LABELING APPLICABILITY

- The BCOP assay does differentiate between EPA Category I and II materials, so it is most useful in this higher range.

US EPA OPP policy (3-2-2015): Use of an alternate testing framework for classification of eye irritation potential of EPA pesticide products - 40CFR Part 158W for AMCPs (anti-microbial cleaning products)

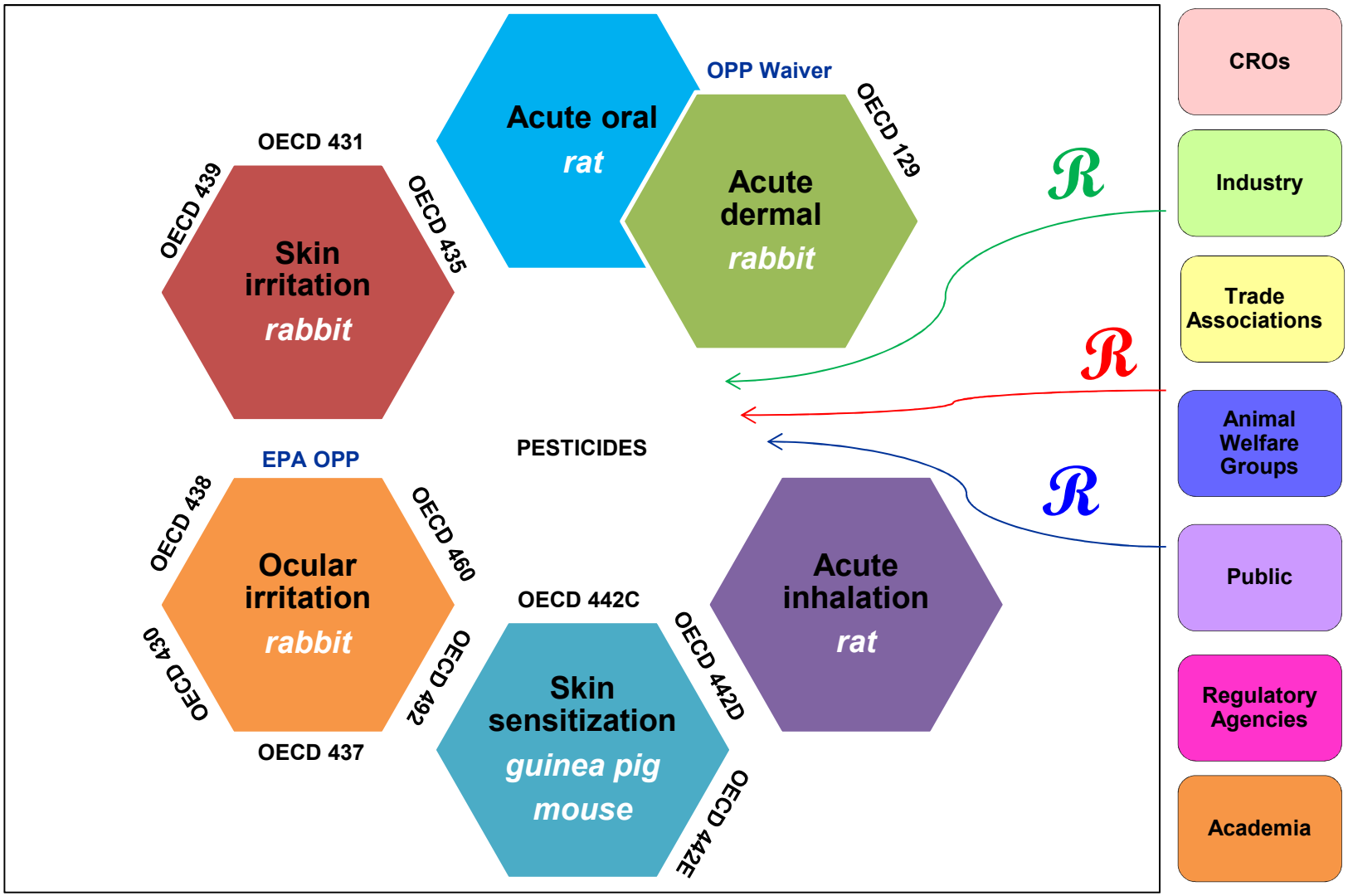
Ocular irritation - Outline of the *in vitro* testing strategy

BCOP = Bovine Corneal Opacity and Permeability
CM = Cytosensor Microphysiometer
EO = EpiOcular™



Use of an alternate testing framework for classification of eye irritation potential of EPA pesticide products. U.S. EPA (2015): https://www.epa.gov/sites/production/files/2015-05/documents/eye_policy2015update.pdf

Modernizing the "six-pack" testing strategy: influx of modern *in vitro* techniques

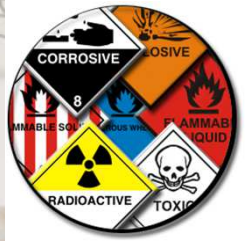


Perspectives, challenges, common goals and working together

Industry/ Manufacturer

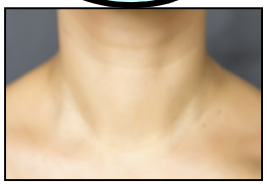
Trade Associations

Animal Welfare Groups

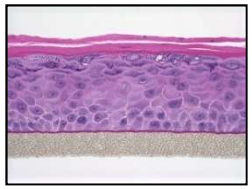


Academia

Consumer/ End-user Safety



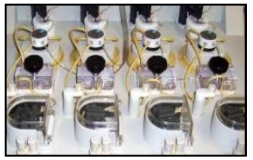
Safety/ Testing Labs



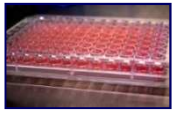
High-throughput

Sensitive

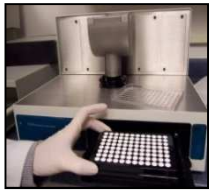
Specific



Reproducible



Reliable

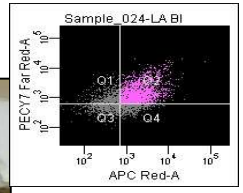


Relevant

Transferable

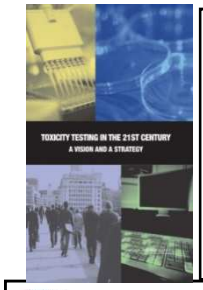
Easy to perform

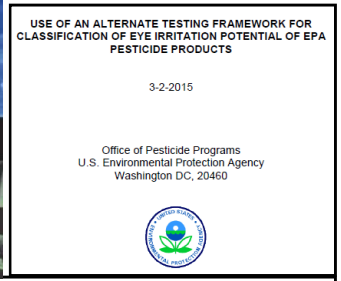
Affordable



Validated

Labeling/ Regulatory Agency

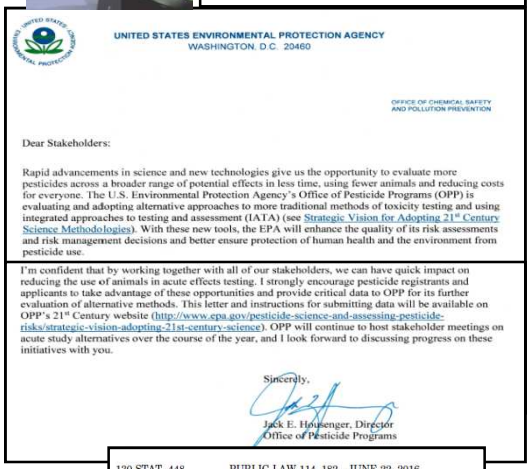




USE OF AN ALTERNATE TESTING FRAMEWORK FOR CLASSIFICATION OF EYE IRRITATION POTENTIAL OF EPA PESTICIDE PRODUCTS

3-2-2015

Office of Pesticide Programs
U.S. Environmental Protection Agency
Washington DC, 20460



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

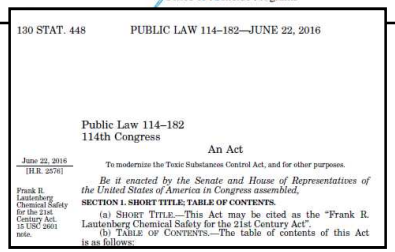
Dear Stakeholders:

Rapid advancements in science and new technologies give us the opportunity to evaluate more pesticides across a broader range of potential effects in less time, using fewer animals and reducing costs for everyone. The U.S. Environmental Protection Agency's Office of Pesticide Programs (OPP) is evaluating and adopting alternative approaches to more traditional methods of toxicity testing and using integrated approaches to testing and assessment (IATA) (see Strategic Vision for Adopting 21st Century Science Methodologies). With these new tools, the EPA will enhance the quality of its risk assessments and risk management decisions and better ensure protection of human health and the environment from pesticide use.

I'm confident that by working together with all of our stakeholders, we can have quick impact on reducing the use of animals in acute effects testing. I strongly encourage pesticide registrants and applicants to take advantage of these opportunities and provide critical data to OPP for its further evaluation of alternative methods. This letter and instructions for submitting data will be available on OPP's 21st Century website (http://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/strategic-vision-adopting-21st-century-science). OPP will continue to host stakeholder meetings on acute study alternatives over the course of the year, and I look forward to discussing progress on these initiatives with you.

Sincerely,

Jack E. Housenger, Director
Office of Pesticide Programs



130 STAT. 448 PUBLIC LAW 114–182—JUNE 22, 2016

Public Law 114–182
114th Congress

An Act

To modernize the Toxic Substances Control Act, and for other purposes.

June 22, 2016
[H.R. 2576]

Frank R. Lautenberg Chemical Safety for the 21st Century Act.
15 USC 2601 note.

Be it enacted by the Senate and House of Representatives of the United States of America in Congress assembled,

SECTION 1. SHORT TITLE; TABLE OF CONTENTS.

(a) SHORT TITLE.—This Act may be cited as the "Frank R. Lautenberg Chemical Safety for the 21st Century Act".

(b) TABLE OF CONTENTS.—The table of contents of this Act is as follows:

Integrating information to guide testing and data analyses
Key concepts

Integrated Testing Strategies (ITS)

- Designed to guide testing
- Pre-designed (**ex., US EPA AMCP eye irritation testing**)
- Series of assays, not of equal participation/importance
- Performed in a sequential manner

Integrated Approaches to Testing and Assessment

- Pragmatic, science-based approach for chemical hazard or risk assessment based on the evaluation of existing data (human – clinical or accidental; regulatory accepted *in silico*, in *vitro*, *ex vivo*, *in vivo*, physico-chemical properties)
- Methodical integration of all of the weighed data to derive predictions
- Flexible, expert opinion allowed

Adverse Outcome Pathways

- Drive endpoint development based upon mechanistic events
- Develop the IATA framework

Defined Approaches to Testing and Assessment

- Integrate information from multiple non-animal methods
- Hazard assessment and potency categorization (**ex., skin sensitization**)
- Based on a fixed set of information sources and fixed data interpretation procedure
- Fixed strategy, battery of tests all of equal importance/participation to the conclusion
- Predictions generated by these approaches are rule-based and are not influenced by expert judgment
- Usually developed by company for the chemistry domain of interest
- Loosely defined chemistry domain

Other Resources

IIVS Institute for In Vitro Sciences
http://iivs.org/newstype/webinars-videos/

ChemicalRiskManager
The hub for product safety resources

PETA INTERNATIONAL SCIENCE CONSORTIUM LTD.
https://www.piscltd.org.uk/reaching-alternatives-animal-testing/

Northern California Regional Chapter of the Society of Toxicology
http://www.toxicology.org/groups/rc/NorCal/docs/NorCal-Fall-Symposium_GECostin.pdf

National Toxicology Program
U.S. Department of Health and Human Services
https://ntp.niehs.nih.gov/pubhealth/evalatm/accept-methods/index.html
https://ntp.niehs.nih.gov/pubhealth/evalatm/accept-methods/guidance/index-2.html

European Commission
JOINT RESEARCH CENTRE
Tracking System for Alternative methods towards Regulatory acceptance (TSAR)
http://tsar.jrc.ec.europa.eu/

OECD
BETTER POLICIES FOR BETTER LIVES
http://www.oecd.org/chemicalsafety/testing/oecdguidelineapproachbyendpoints.htm

ECHA
EUROPEAN CHEMICALS AGENCY
https://echa.europa.eu/-/new-advice-on-using-non-animal-test-methods

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