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The assessment of eye irritation potential of chemicals, cosmetics, and household products using reconstructed human cornea – like model, EpiOcular

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Introduction

Eye irritation tests using in vitro test methods

- reconstructed 3D cornea-like model - EpiOcular™ model
- *in vitro* eye irritation assays for testing chemicals, cosmetics and household products using reconstructed cornea-like tissue model:
    - EpiOcular™ EIT – OECD TG 492
    - EpiOcular™ time-to-toxicity (ET-50) assays:
        - neat method
        - dilution method
        - sub-Draize mildness testing
- CON4EI project

Conclusion

A piece of history behind the *in vivo* eye irritation test:

Fig. 1. To depict the need for governmental regulations over cosmetics, the U.S. Food and Drug Administration prepared this poster in 1933 from photographs of a 38-year-old Ohio woman. The patient's first photo was taken an hour before eyelash dyeing, and the other was obtained during the following month showing bilateral staphylococcal corneal ulcers that resulted in vision of light perception. (Reprinted from Lamb RdeF[109] with permission of Farrar and Reinhart.)

Fig. 3. John H. Draize, PhD, the FDA pharmacologist who developed methods for assessing the ocular and dermal toxicity of drugs and cosmetics (Courtesy of the U.S. Food and Drug Administration History Office).

To protect us, he selected the most sensitive animal!

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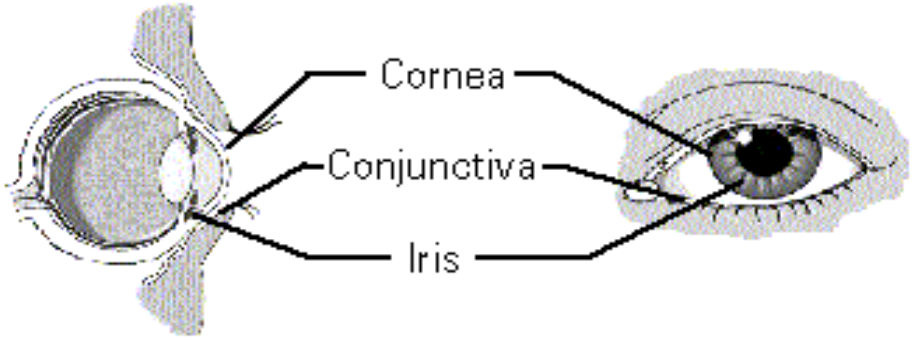
Draize rabbit eye test (OECD TG 405)

A substance to be tested is applied in a single dose (0.1 mL) to one of the eyes of the experimental animal; the untreated eye serves as the control. **Exposure time and washing conditions are not strictly determined** !

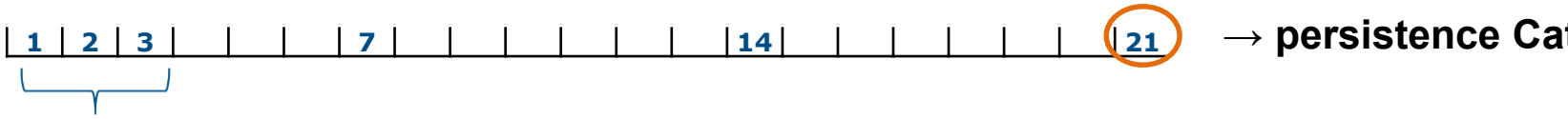
- Corneal opacity (CO: score 0 to 4)
- Iris lesions (IR: score 0 to 2)
- Conjunctiva redness (CR: score 0 to 3)
- Conjunctiva chemosis (CC: score 0 to 4)



Human eye

The eyes should be examined at 1, 24, 48, and 72 hours after test substance application.
The observations can be extended up to 2 weeks.

Tissue observation for up to 21 days

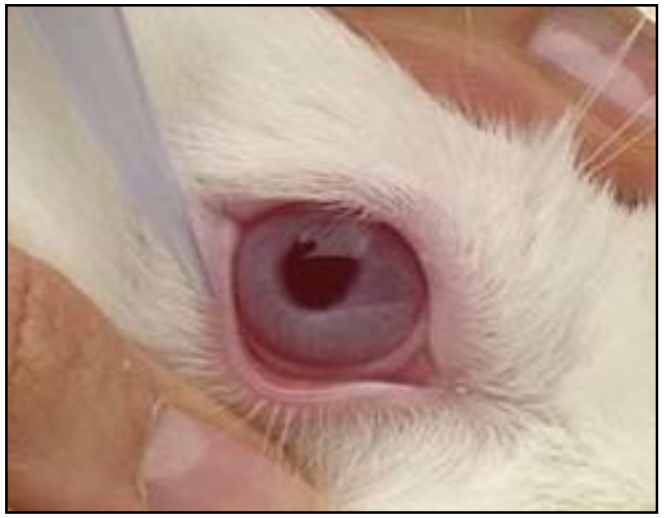


→ **persistence Cat 1**

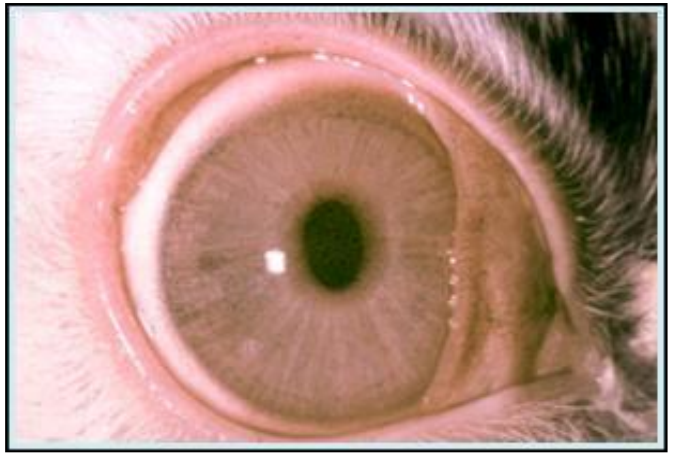
Calculate for each rabbit mean CO, IR, CR, and CC values over days 1 to 3

Draize rabbit eye test (OECD TG 405)

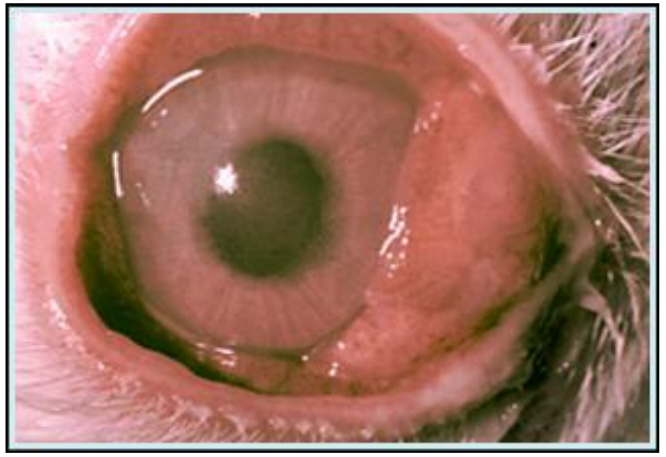
The degree of eye irritation/corrosion is evaluated by **subjective scoring of a trained personnel.** Lesions of conjunctiva, cornea, and iris are recorded at specific intervals.



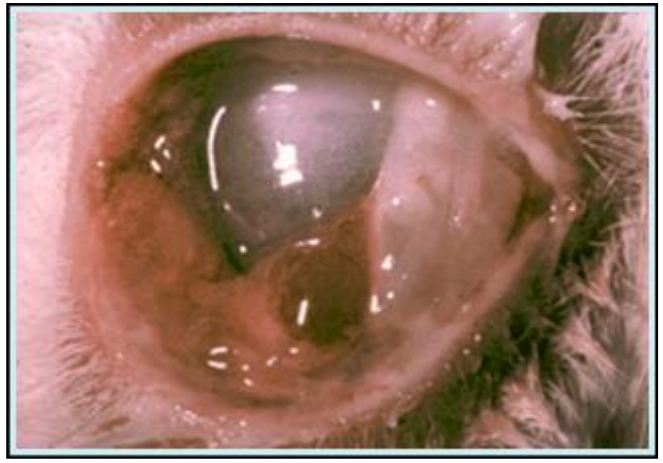
Application



No effect



Irritation = H319 (R36), GHS 2

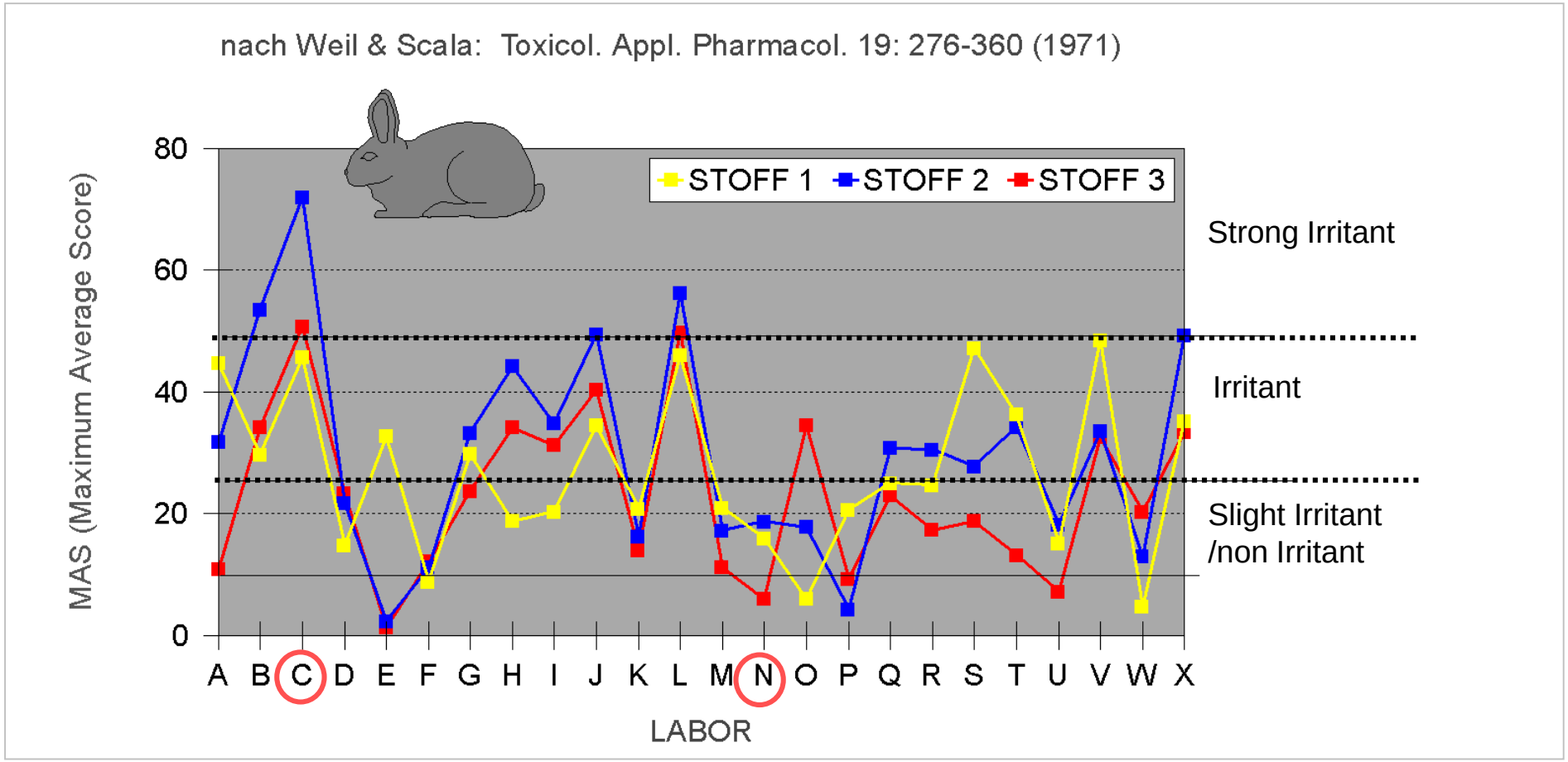


Corrosion = H318 (R41), GHS 1

Pictures provided by ZEBET, at the BfR

Inter-laboratory comparison of the observed effects *in vivo*:

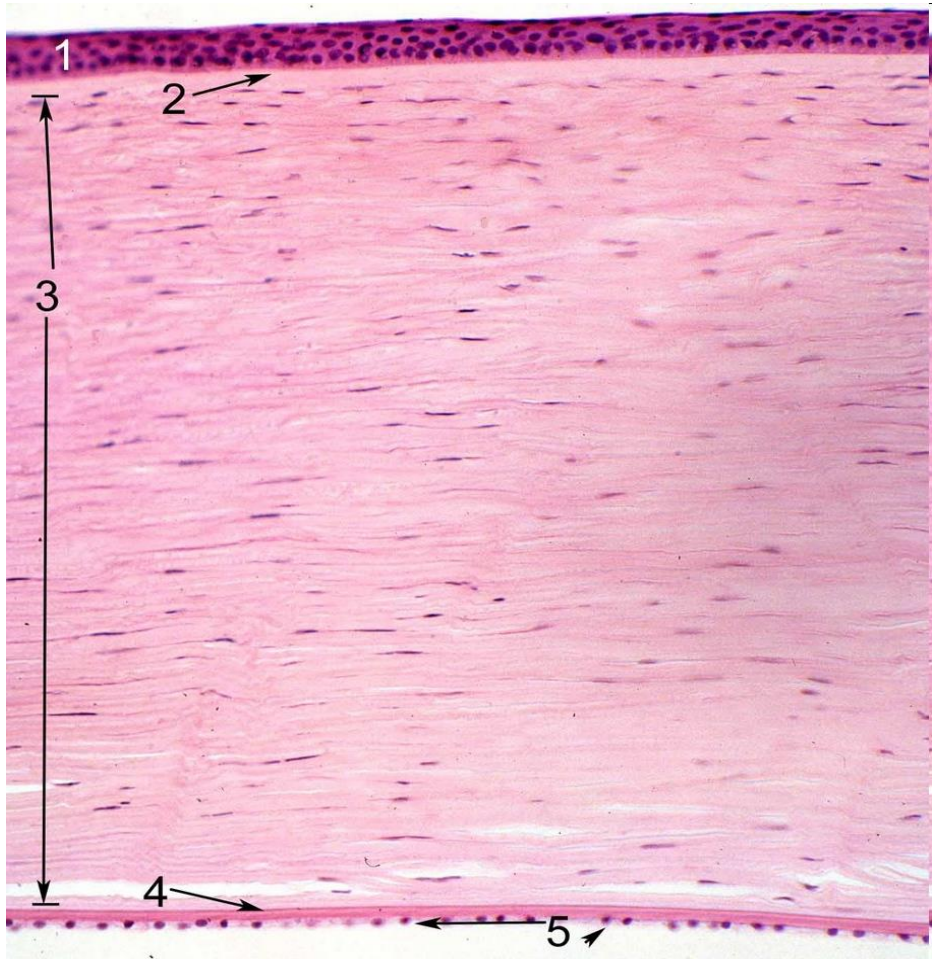
3 substances in 24 laboratories



Picture provided by ZEBET, at the BfR

Note: MMAS/MAS score are not used anymore for classification in most of the countries. Individual ocular tissue scores for cornea, iris and conjunctiva used for C&L purposes. Persistence is also taken into the account.

What we try to replace? Light micrograph of the human cornea



1. The outer surface (top) is lined by **non-keratinizing stratified squamous epithelium** about five cells thick (total thickness of about 50 µm).

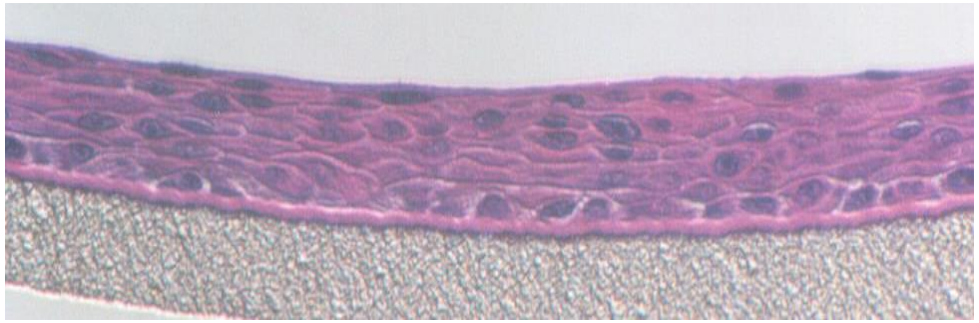
2. It is supported by a specialised basement membrane called **Bowman's membrane**.

3.The bulk of the cornea (substantia propria) consists of a regular form of dense collagenous **connective tissue**.

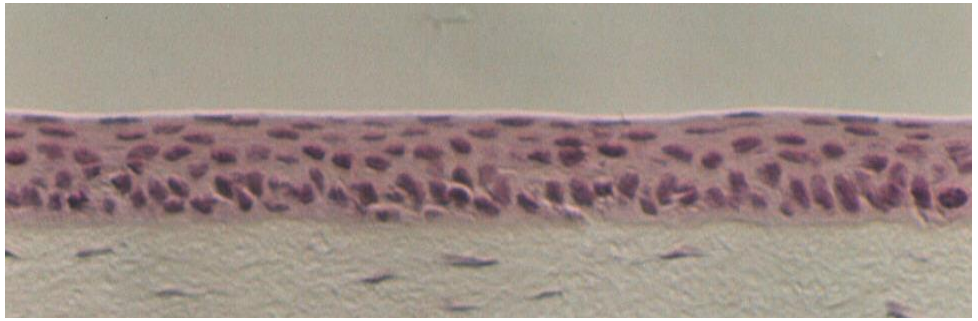
4. The inner surface (bottom) is lined by a layer of flattened endothelial cells, which are supported by an elastic basement membrane called **5. Descemet's membrane**

Reconstructed 3D cornea-like model EpiOcular™

Reconstructed human Cornea-like Epithelium - EpiOcular™



A) *in vitro* EpiOcular™ tissue (OCL-200)



B) rabbit corneal epithelium.

- **Test system**: non-keratinized multi-layered epithelium reconstructed from primary human epidermal keratinocytes.
- **H&E** stained cross-section of the EpiOcular tissue (NHEK) reveals **highly organized basal and supra-basal cell layers** followed by cells which flatten out as the apical surface is approached, similar to *in vivo* corneal tissue.
- **Endpoints usually measured**: cytotoxicity (MTT assay), permeation, release of inflammatory mediators
- **Applicability and limitations:** Applicable to all types of chemicals and also to intensely coloured chemicals (with use of HPLC/UPLC-spectrophotometry)

Quality control (QC) and reproducibility

QC testing for the EpiOcular consists of topically dosing tissues (n=2) with 100 µl of 0,3 % Triton X-100, for various times - ET 50 values are then determined for every manufactured batch.

Summary of yearly average from quality control (QC) testing of EpiOcular (OCL-200) tissue from 1996 to 2016. ET-50 values are obtained after the exposure of RhCE tissues to 100 microliters of 0.3% Triton X-100. The QC acceptance range for EpiOcular tissues of 12.2 – 37.5 min was established in 1996.

Calendar Year	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
EpiOcular ET-50 (min)	24.6	23.0	25.2	22.0	22.9	23.3	22.4	24.6	22.2	24.8	27.3	24.4	25.0	23.2	23,8	24,6	23,9	25,3	25,1	29,5	29,9	25,0	20,9	24,6	19,2	18,1
Avg CV* (%)	5.1	5.5	5.5	5.6	5.5	4.7	5.0	5.9	6.7	5.8	6.4	5.7	4.9	5.3	4,9	5,8	5,1	5,8	5,5	5,6	5,1	6,7	6,2	6,6	5,7	6,3

** Intermediate point was added to the assay in 2004 in order to increase precision of the ET-50 assay. This caused the average CV to increase slightly.*

Parameter	QC Acceptance Criteria
ET-50 (minutes)	12.2 > ET-50 > 37.5
Negative control (OD)	> 1.00
Histological evaluation	Organised stratified non-keratinising squamous epithelium, 4–6 cell layers present
Sterility	No signs of contamination by bacteria or fungi following storage for 14 days

Detailed information in:

Development of the EpiOcular™ Eye Irritation Test for Hazard Identification and Labelling of Eye Irritating Chemicals in Response to the Requirements of the EU Cosmetics Directive and REACH Legislation.

Kaluzhny, Y.,Kandárová,H. Hayden P. et al, (2011). ATLA **39**, 339–364

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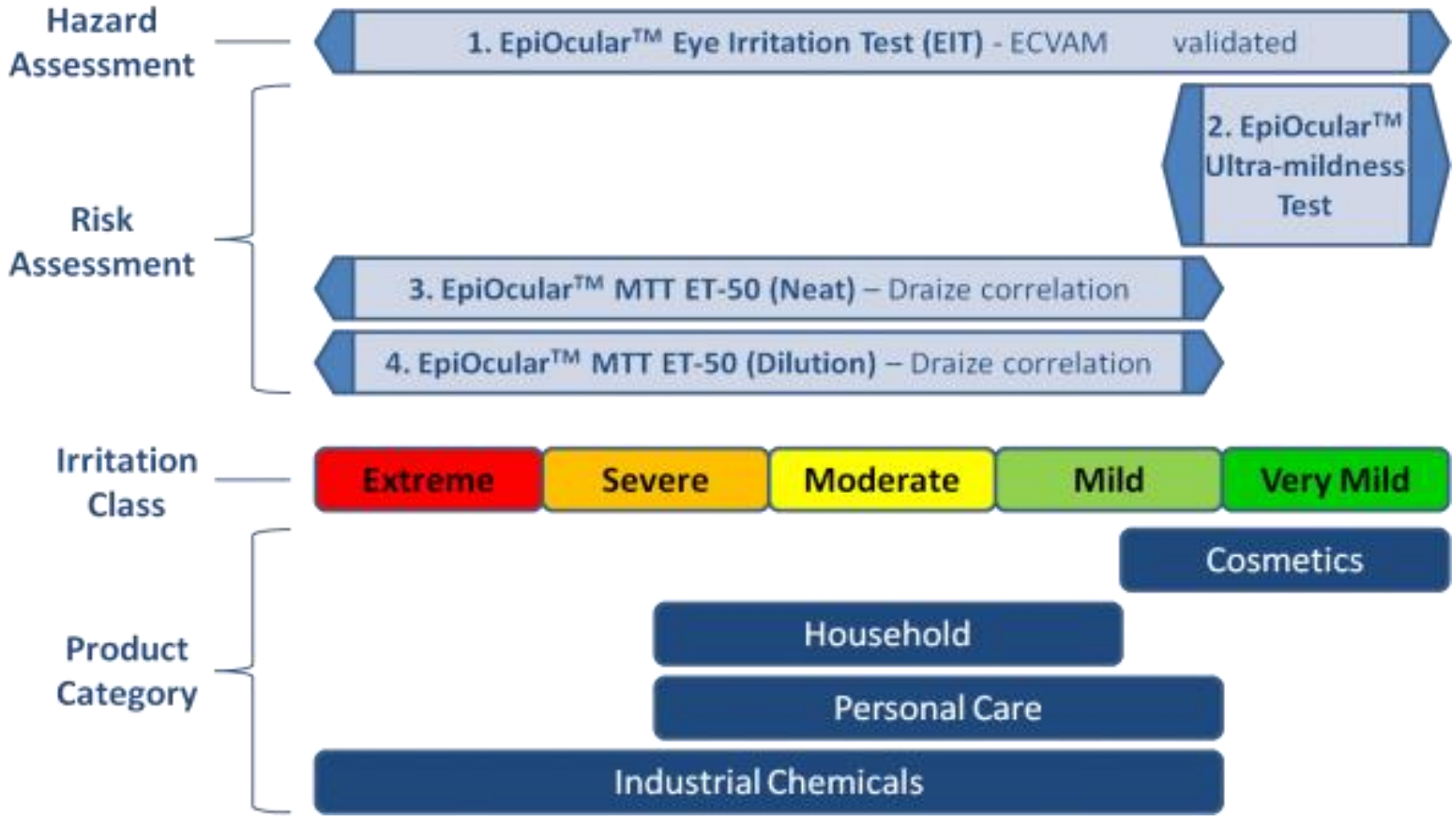
EpiOcular™ test methods

- several ocular irritation assays based on the EpiOcular tissue model:



In vitro assays for testing chemicals, formulations and cosmetics – EpiOcular™ EIT

EpiOcular™ Eye Irritation Test (EIT) – OECD TG 492

- EpiOcular EIT Protocol was developed by MatTek Corporation in 2007, as a reaction on ECVAM's request for a simple and straightforward assay that could be used in the framework of REACH and 7th Amendment to the Cosmetic Legislation (on demand protocol).

- EIT was developed for classification and labeling substances for regulatory purposes of chemicals including raw cosmetic ingredients**.**

- The protocol and its prediction model was designed to discriminate between ocular irritant / corrosive materials (GHS Categories 1 and 2 combined) and those that require no labeling (GHS No Category).

- In total **112 chemicals** were tested during the development of the protocol by MatTek.

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THE EUROPEAN COSMETICS ASSOCIATION
Now Cosmetics Europe

=

Cosmetics Europe
the personal care association

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OECD/OCDE 492

Adopted:
28 July 2015

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage

INTRODUCTION

1. *Serious eye damage* refers to the production of tissue damage in the eye, or serious physical decay of vision, following application of a test chemical to the anterior surface of the eye, which is not fully reversible within 21 days of application, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1). Also according to UN GHS, *eye irritation* refers to the production of changes in the eye following the application of a test chemical to the anterior surface of the eye, which are fully reversible within 21 days of application. Test chemicals inducing serious eye damage are classified as UN GHS Category 1, while those inducing eye irritation are classified as UN GHS Category 2. Test chemicals not classified for eye irritation or serious eye damage are defined as those that do not meet the requirements for classification as UN GHS Category 1 or 2 (2A or 2B) i.e., they are referred to as UN GHS No Category.

2. The assessment of serious eye damage/eye irritation has typically involved the use of laboratory animals (OECD Test Guideline (TG) 405; adopted in 1981 and revised in 1987, 2002 and 2012) (2). In relation to animal welfare concerns, TG 405 recommends the use of a sequential testing strategy for the determination of the serious eye damage/eye irritation potential of chemicals. This testing strategy is described in a Supplement to the Guideline and includes the use of validated, scientifically valid and accepted *in vitro* test methods, thus decreasing or avoiding pain and suffering of animals (2).

3. This Test Guideline describes an *in vitro* procedure allowing the identification of chemicals (substances and mixtures) not requiring classification and labelling for eye irritation or serious eye damage in accordance with UN GHS. It makes use of reconstructed human cornea-like epithelium (RhCE) which closely mimics the histological, morphological, biochemical and physiological properties of the human corneal epithelium. Four other *in vitro* test methods have been validated, considered scientifically valid and adopted as OECD Test Guidelines (TGs) 437 (3), 438 (4), 460 (5) and 491 (32) to address the human health endpoint serious eye damage/eye irritation.

2015 4. The only *in vitro* test method currently covered by this Test Guideline is the EpiOcular™ Eye Irritation Test (EIT), which makes use of a commercially available RhCE tissue construct as test system.

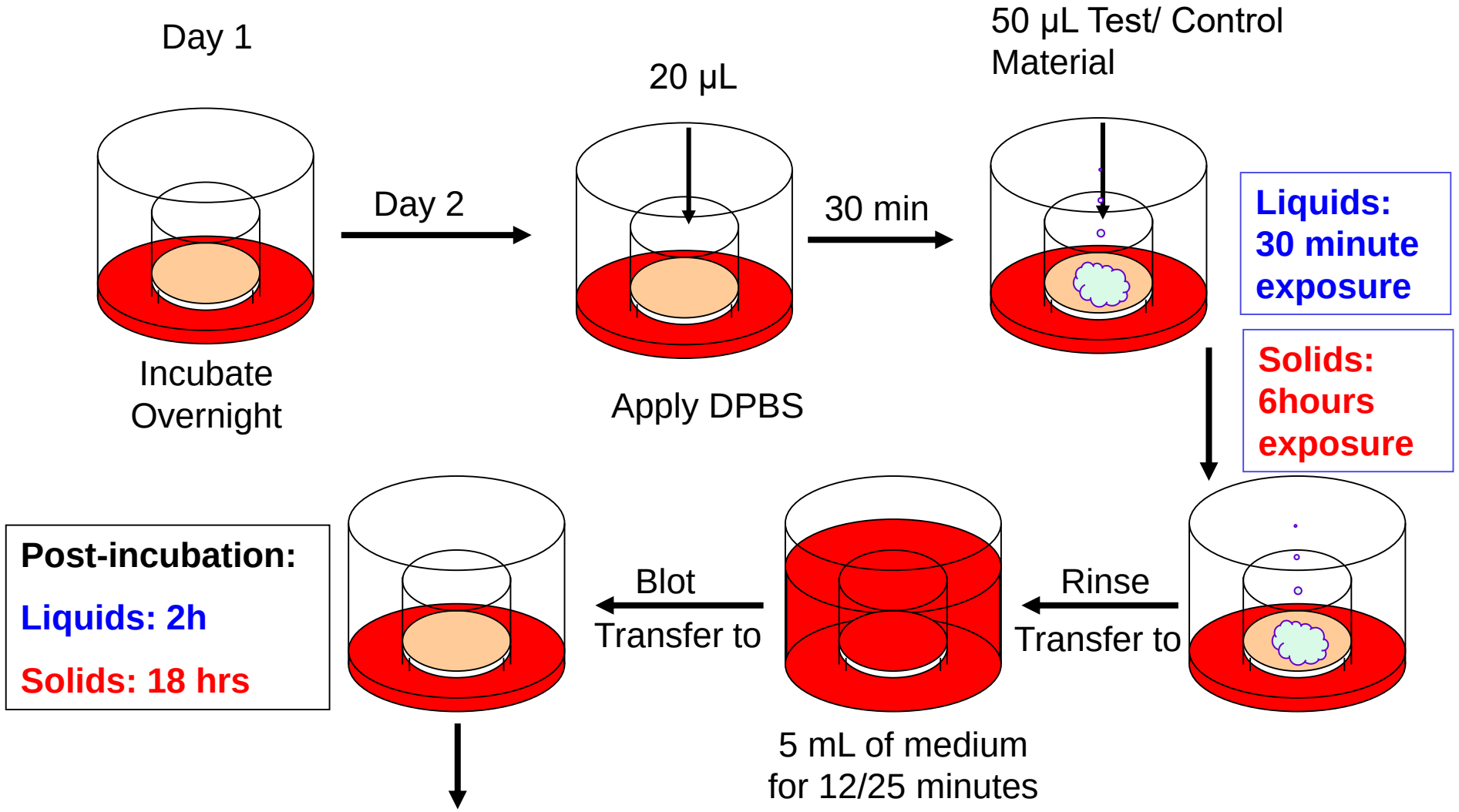
2015 – **EpiOcular EIT** was covered by OECD TG 492 – adopted on July 28, 2015

2016 – **SkinEthic HCE** included into the updated draft OECD TG 492 – adopted on October 09, 2017

2018 – **LabCyte Cornea-Model 24** EIT included into the updated draft OECD TG 492 – adopted on June 25, 2018

2019 – **MCTT HCE™** EIT included into the updated draft OECD TG 492 – adopted on June 14, 2019

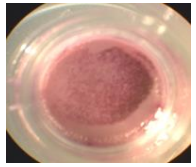
EpiOcular™ EIT protocol



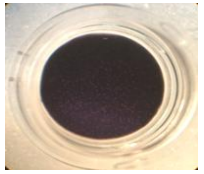
EpiOcular™ EIT protocol

MTT assay (3hours)

Dead tissue



Viable tissue



Prediction model:

Eye Irritating chemicals are cytotoxic in specific test conditions of the in vitro eye irritation test:

If viability > 60%, Non Irritant (No Category)

If viability ≤ 60 %, Irritant ("No prediction can be made" within the interpretation of the OECD TG 492)

OECD TG 492 can be utilized as part of a tiered testing strategy to identify chemicals that do not require classification and labeling.

All compounds predicted as irritants are undergoing further testing to identify the irritation potency/severity or to exclude false positive prediction.

Methods to identify the irritation potency/severity

OECD TG 437 - 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 TG 438 - Isolated Chicken Eye Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

OECD TG 460 - Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants

OECD TG 491 - Short Time Exposure In Vitro Test Method for Identifying i) Chemicals Inducing Serious Eye Damage and ii) Chemicals Not Requiring Classification for Eye Irritation or Serious Eye Damage

OECD TG 405 - Acute Eye Irritation/Corrosion - used as a last option

Testing strategies

It is generally accepted that **no single in vitro eye irritation test** will be able to fully replace the OECD 405 acute eye irritation/corrosion test in vivo (also known as the Draize eye test) across the full range of irritation for different chemical classes.

Combinations of several alternative test methods may be able to replace the Draize eye test in the future.

Testing strategies such as the top-down or bottom-up approaches provide a means of incorporating existing information, QSAR predictions, and in vitro test results.

Proposed testing strategy

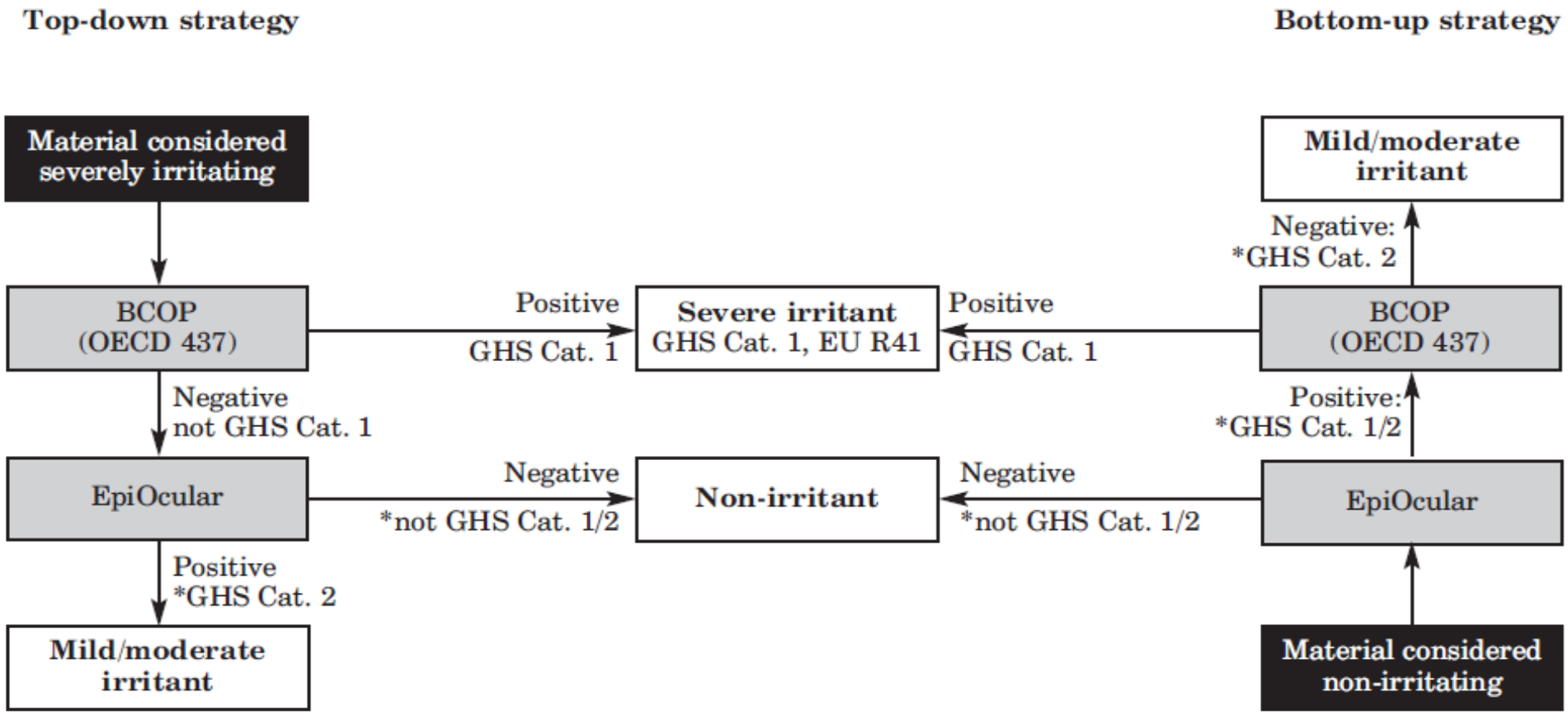


Figure 5 in Kolle et al *

Detailed information in:

In-house Validation of the EpiOcular™ Eye Irritation Test and its Combination with the Bovine Corneal Opacity and Permeability Test for the Assessment of Ocular Irritation. * Kolle, S.N., Kandárová, H., Wareing,B. van Ravenzwaay,B., Landsiedel, R.(2011). ATLA **39**, 365–387 (60 chemicals).

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In vitro assays for testing chemicals, formulations and cosmetics – ET-50 protocols

EpiOcular™ time-to-toxicity (ET-50) protocols

- Protocols exist for more than 25 years
- Suitable for the assessment of the irritation potency, tolerance and mildness of a cosmetic formulations – AVON and Mary Kay use the EpiOcular assay for the mildness testing of their products for more than 20 years!
- Specific protocols were developed for surfactants and surfactant based formulations. Validation was conducted by IIVS and Colgate in 1998. Data from the validation were submitted to ECVAM in 2000 and resubmitted in 2004. Applicability domain increase was requested. New studies initiated within CON4EI project (2015-2017).
- Accepted by EPA for the assessment of Antimicrobial cleaning products. Level of irritation is assessed as tissue viability in MTT assay, determination of ET-50 value.
- Different test design and PM than EpiOcular EIT – more time-points required, but more information can be obtained compared to the EIT.

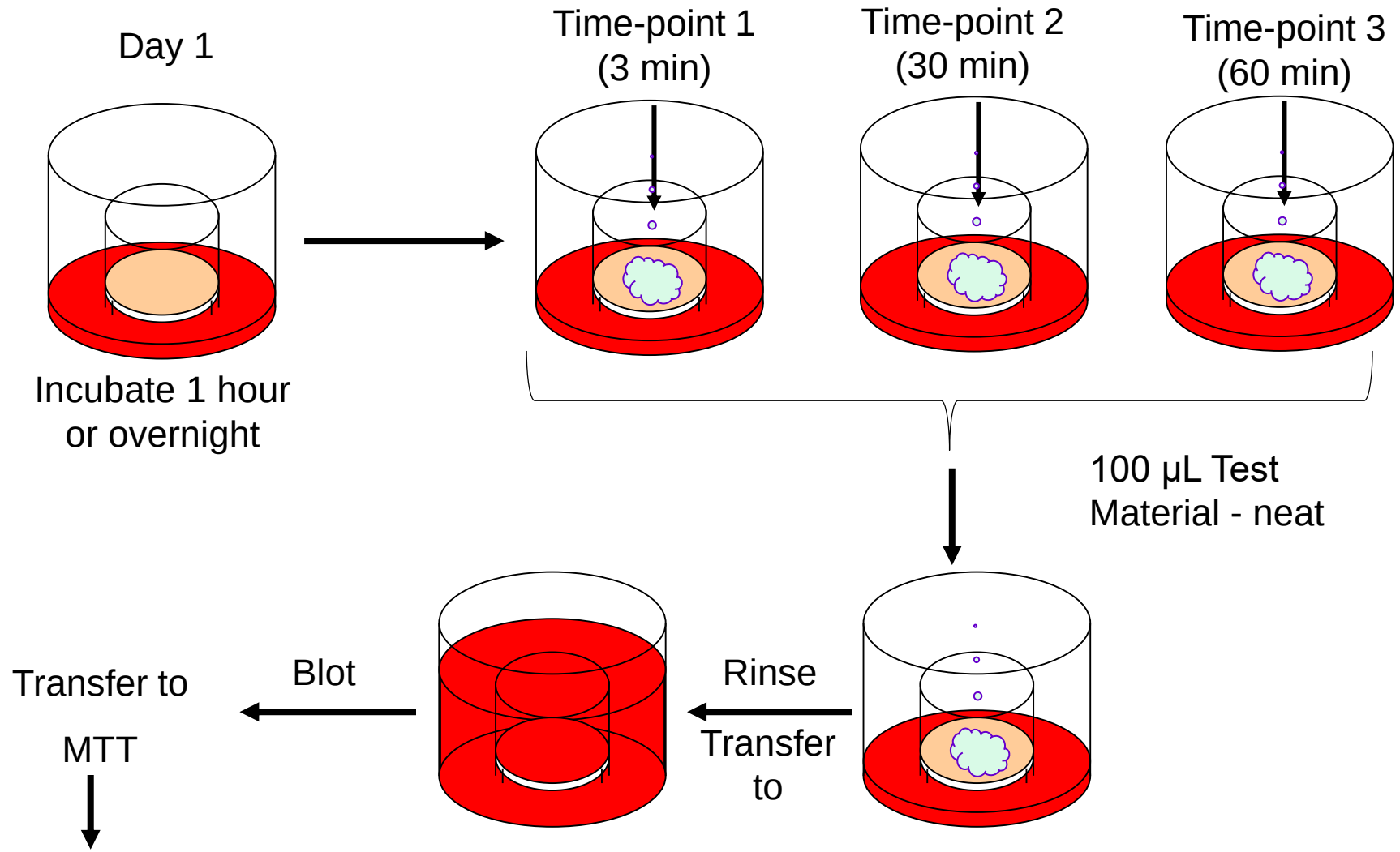
EpiOcular™ time-to-toxicity (ET-50) protocols

- **Neat Method:** used for non-water soluble materials or for exposing test articles undiluted.

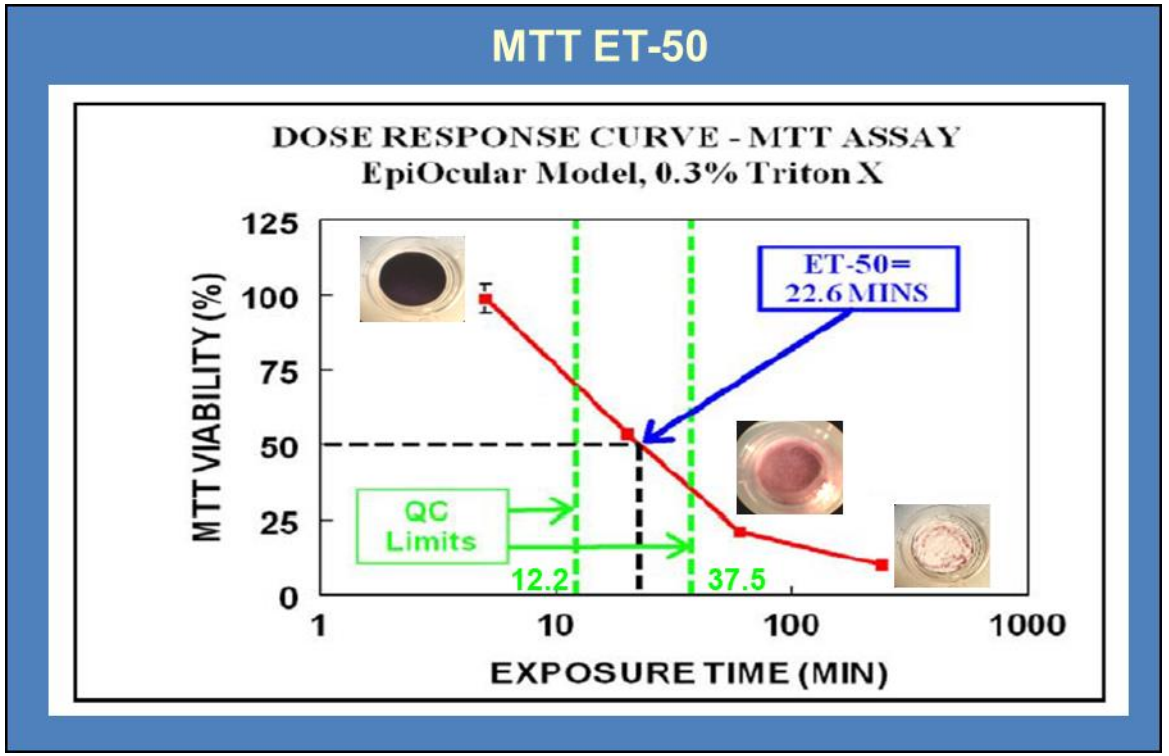
- **Dilution Method:** applicable to water-soluble materials with a specific gravity of ≥ 0.95 and requires an initial dilution of the test article to 20 % in water. Recommended for surfactants and rinse-off cosmetics.

- **Sub-Draize Mildness Testing:** involves applying neat test articles to the EpiOcular tissue model and is used to differentiate between materials for which standard Rabbit Eye Draize testing is insensitive (eye care cosmetics).

Example of the ET-50 Protocol: Neat Method



Prediction model – Neat method:

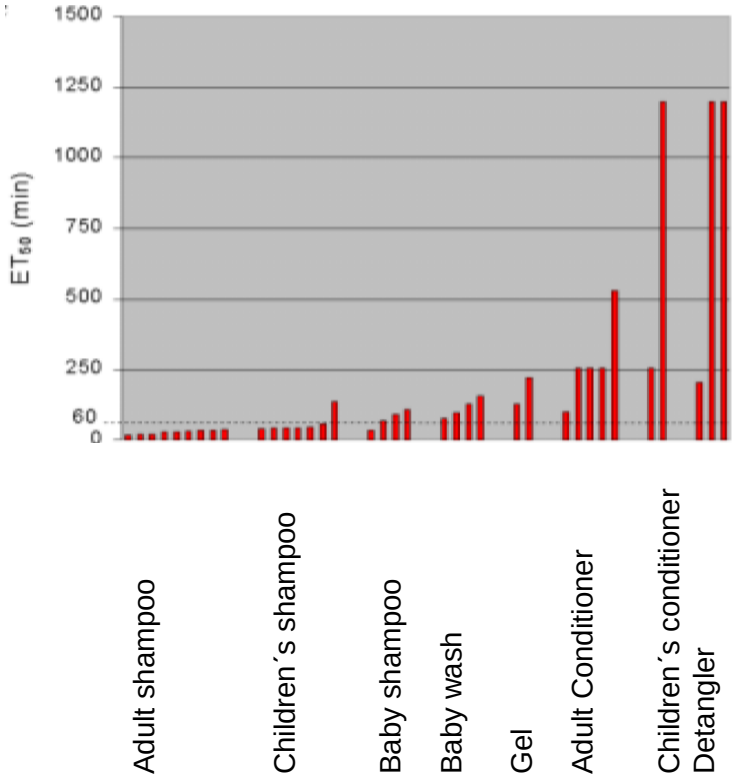


Draize Score	Irritancy Classification	Example	EpiOcular ET-50 (min)
0-15	Non-irritating, Minimal	PEG-75 Lanolin, Tween 20	>60
15.1-25	Mild	Pareth 25-12	30-60
25.1-50	Moderate	1% Triton X-100	3-29.99
50.1-110	Severe, Extreme	5% Benzalkonium Chloride	<3

Example of use: Assessment of Ocular Irritation Ranges of Market-Leading Cosmetic and Personal-Care Products Using an *In Vitro* Tissue Equivalent – SOT 2002 – The Toxicologist 66, 243, 2002

NE McCain, RR Binetti, SD Gettings, and BC Jones, **Avon Products**, Inc., Suffern, NY, USA

Formulation Category	Number of Samples	Mean ET50 (min)	Median ET50 (min)	Range of ET50 Scores (min)
Surfactant Formulas				
Adult Shampoo	9	27.0	28.8	17.1-35.1
Children's Shampoo	7	58.2	45.4	37.9-135.4
Baby Shampoo	4	74.9	80.3	31.9-107.1
Baby Wash	4	114.5	112.6	76.9-155.9
Eye Area Cosmetics				
Liquid Eyeliner	15	532.2	127.0	64-1440
Eye Cream	11	581.8	454.0	102-1200
Mascara	20	655.1	626.5	199-1080
Pencil Eyeliner	3	1440.0	1440.0	1440
Creams/Lotions				
Adult Lotion/Cream	17	511.4	440.8	234-1440
Sunscreen	15	673.6	486.0	252-1440
Baby Lotion	2	1200.0	not applicable	1200
Non-Surfactant Haircare				
Children's Styling Gel	2	172.7	not applicable	126-219.3
Adult Conditioner	5	279.6	256.0	100-530
Children's Conditioner	2	728.0	not applicable	256-1200
Children's Hair Detangler	3	868.4	1200.0	205.1-1200



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CON4EI project (2015 – 2017)

CON4EI aims into **development of tiered testing strategies for** eye irritation assessment for all eye irritation drivers of classifications.

8 test methods to identify irritancy potency

The irritancy potency of a set of 80 chemicals was identified based on existing *in vivo* data.
Eight test methods were included in this project:

- BCOP (Bovine Corneal Opacity and Permeability),
- ICE (Isolated Chicken Eye),
- STE (Short Term Exposure),
- EpiOcular EIT (EpiOcular Eye Irritation Test) *,
- EpiOcular ET-50 tests (neat and dilution protocols) *,
- SkinEthic HCE (Human Corneal Epithelial) EIT,
- SMI (Slug Mucosal Irritation),
- BCOP-LLBO (BCOP-laser light-based opacitometer).

Detailed information in:
CON4EI: Development of Testing Strategies for Hazard Identification and Labelling of Eye Irritating Chemicals. *
E. Adriaens, S. Verstraelen, N. Alépée, H. Kandarova, A. Drzewiecka, K. Gruszka, R. Guest, J.A. Sr. Willoughby, A.R. Van Rompay. . (2018) Toxicology in Vitro 49, 99 -115.
* Are published in a Special Issue of TIV 2018 with other papers describing the single methodologies and their performance

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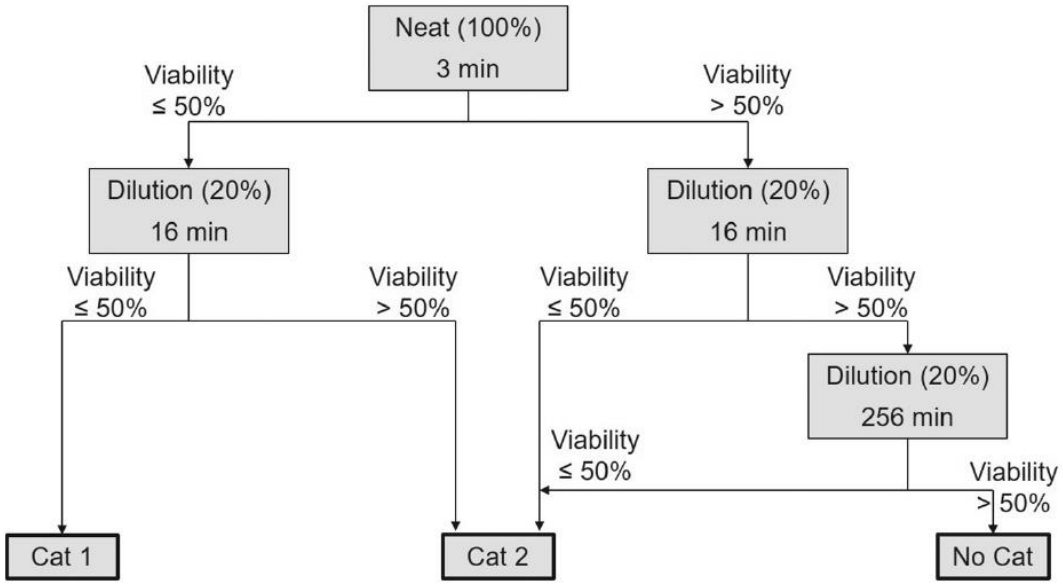
EpiOcular ET-50 methods – summary of results

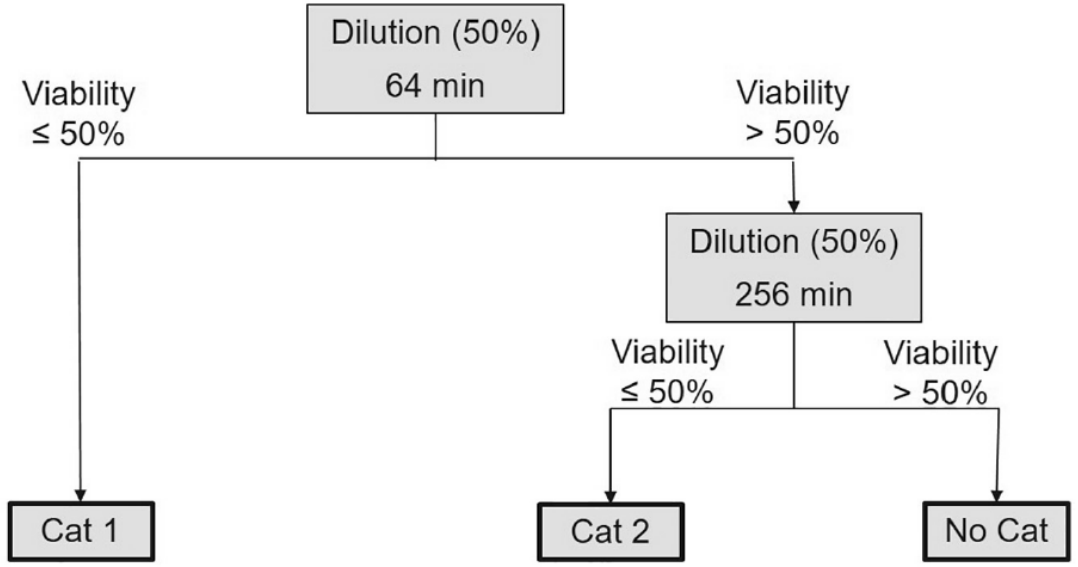
UN GHS	Overall - ET-50 (min)		
	Cat 1	Cat 2	No Cat
Cat 1	73.3%	26.7%	0%
Cat 2	28.3%	64.2%	7.5%
No Cat	0%	3.4%	96.6%

UN GHS	Liquids - ET-50 (min)			Solids - ET-50 (min)		
	Cat 1	Cat 2	No Cat	Cat 1	Cat 2	No Cat
Cat 1	78.8%	21.2%	0%	69%	31%	0%
Cat 2	28%	68%	4%	28.6%	60.7%	10.7%
No Cat	0%	6.7%	93.3%	0%	0%	100%

CON4EI proposed PM for liquids and solids



Prediction model for liquids



Prediction model for solids

Detailed information in:

CON4EI: CONsortium for in vitro Eye Irritation testing strategy – EpiOcular™ time-to-toxicity (EpiOcular ET-50) protocols for hazard identification and labelling of eye irritating chemicals. *
H. Kandarova, S. Letasiova, E. Adriaens, R. Guest , J.A. Sr. Willoughby, A. Drzewiecka, K. Gruszka, N. Alépée, S. Verstraelen, A.R. Van Rompay. (2018) Toxicology in Vitro 49, 39-52.

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ALT4EI project - Summary of results

UN GHS	Overall - ET-50 (min)		
	Cat 1	Cat 2	No Cat
Cat 1	63.6 %	31.8 %	4.5 %
Cat 2	33.3%	56.6 %	10.0 %
No Cat	4.7 %	18.8 %	76.6 %

Conclusion

- **EpiOcular EIT test protocol has been adopted on July 28, 2015** as the new **OECD TG 492**. This test enables reliable identification of substances that do not require labelling.
- If a chemical classifies as NI in the EIT, no further testing is required.
- In case of positive results from the EIT it is necessary to conduct further *in vitro* testing to exclude corrosive nature of the test article or false positive prediction. Such a test could be BCOP, ICE or STE if the applicability domain is not limited.
- AVON, Mary Kay and some other **major cosmetic companies have established a reference database** of *in vitro* ocular irritation using the **EpiOcular ET-50 method** for a cross-section of marketed cosmetic and personal care products.
- The *in vitro* EpiOcular tissue model used in the ET-50 test design is able to distinguish differences between moderate "**mild" and "ultra-mild" formulations**.
- **EpiOcular time-to-toxicity assay** – protocols for liquids and solids developed in CON4EI project seems to be promising in an integrated testing strategy (ITS) for eye irritation assessment.


Thank you very much for your attention!
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