European Commission

ESAC Opinion on the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT)

ESAC Opinion No. 2016-02
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EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM
SCIENTIFIC
ADVISORY
COMMITTEE
(ESAC)

ESAC OPINION

on the

SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT)

ESAC Opinion No.	2016-02
Relevant ESAC Request No.	2016-02
Date of Opinion	24/06/2016

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Abstract

ESAC, the EURL ECVAM Scientific Advisory Committee, advises EURL ECVAM on scientific issues. Its main role is to conduct independent peer review of validation studies of alternative test methods and to assess their scientific validity for a given purpose. The committee reviews the appropriateness of study design and management, the quality of results obtained and the plausibility of the conclusions drawn. ESAC peer reviews are formally initiated with a EURL ECVAM Request for ESAC Advice, which provides the necessary background for the peer-review and establishes its objectives, timelines and the questions to be addressed. The peer review is normally prepared by specialised ESAC Working Groups. These are typically composed of ESAC members and other external experts relevant to the test method under review. These experts may be nominated by ESAC, EURL ECVAM and partner organisations within the International Cooperation on Alternative Test Methods (ICATM). ESAC ultimately decides on the composition of these Working Groups. ESAC's advice to EURL ECVAM is formally provided as 'ESAC Opinions' and 'Working Group Reports' at the end of the peer review. ESAC may also issue Opinions on other scientific issues of relevance to the work and mission of EURL ECVAM but not directly related to a specific alternative test method.

The ESAC Opinion expressed in this report relates to the peer-review of the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT).

Ispra, 24 June 2016

ESAC Opinion

In April 2016, the EURL ECVAM Scientific Advisory Committee (ESAC) (Annex 1) received from EURL ECVAM a request for scientific advice on the L'Oréal-coordinated validation of the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT) for serious eye damage/eye irritation testing (Annex 2). ESAC established a working group (WG) (Annex 1) which delivered an ESAC WG report dated 6 June 2016 (Annex 3).

The ESAC WG Eye Irritation was established to conduct a peer review of, and provide scientific advice on:

1. A multi-laboratory trial involving 3 laboratories of the SkinEthic™ HCE Eye Irritation Test (EIT), a test method with a wide applicability domain for liquids (EITL: Eye Irritation Testing of Liquids) and solids (EITS: Eye Irritation Testing of Solids) and in particular to consider the relevance (biological/mechanistic relevance and predictive capacity; the latter in the context of an Integrated Approach to Testing and Assessment (IATA) (OECD, 2008)), and reliability (transferability; within and between laboratory reproducibility) of the test method.
2. The usefulness of HPLC/UPLC-spectrophotometry as an alternative endpoint detection system to measure MTT-formazan, in particular for highly coloured chemicals interfering with the conventional endpoint measurement by OD-photometry when used specifically with SkinEthic™ HCE EIT.

The analysis and conclusions of the ESAC WG were based primarily on the EURL ECVAM Test Submission Template (TST) and supporting documents supplied by L'Oréal, and supplementary information made available by L'Oréal during and after an 11 May 2016 teleconference.

Details of the validation study were previously published by Alépée *et al*. (2016a, b).

The pre-defined study objectives were:

- To formally validate the SkinEthic™ HCE EIT by a three laboratory ring trial study using an appropriate number and range of test chemicals and to obtain data to assess the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of the assay.
- To produce data to facilitate its international acceptance in regulatory schemes for hazard assessment of liquid and solid chemicals to discriminate chemicals (substances and chemical mixtures) not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling (Category 1 and Category 2) according to the UN GHS Classification and Labelling of Chemicals (UN GHS. UN, 2015) and as implemented by the EU CLP regulation (EU CLP. EC, 2008a).
    - N.B. The test method was not designed, intended, or evaluated to differentiate between GHS Category 1 (irreversible effects) and 2A-B (reversible effects); or to test gases or aerosols.
- To produce evidence and analysis to support the test method being incorporated into a tiered testing strategy (so-called Bottom-Up/Top-Down testing strategy,

Scott L. *et al.*, 2010). The ultimate purpose of such a tiered testing strategy being to replace the traditional *in vivo* Draize eye test [Method B.5 of EC Regulation 440/2008 (EC, 2008b) or OECD TG 405 (OECD, 2002)].

At its 42nd meeting, held on the 9th and 10th June 2016 at EURL ECVAM, Ispra, Italy, the non-Commission members of ESAC unanimously endorsed the following statement which was based on the ESAC WG report:

ESAC concludes that, subject to the specific qualifications set out below, the study design was generally appropriate and robust, the acceptance criteria appropriate at the time, and that the study findings have provided the evidence and analysis required to satisfy the study objectives.

While the SkinEthic™ HCE EIT was not developed to account for all mechanisms associated with eye irritation, the validation study demonstrated that this RhCE test method is able to correctly predict chemicals not requiring classification for serious eye damage/eye irritation independently of the types of ocular effects observed *in vivo* (i.e., corneal, iridal and conjunctival injuries). However, the available documentation does not allow an assessment of the test method's performance with mixtures.

The protocols for the testing of liquid and solid chemicals were found to be highly reproducible and to meet the predetermined acceptable performance values set by the EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study (EIVS) Validation Management Group (VMG) for sensitivity, specificity and accuracy (Barroso *et al.*, 2015; EURL ECVAM, 2016).

ESAC believes the available documentation provides sufficient evidence and reasoned arguments in favour of this RhCE test method having the potential to be at least as relevant and reliable as the currently validated *in vitro* methods for the identification of non-irritants [OECD TGs 437 (OECD, 2013a), 438 (OECD, 2013b), 491 (OECD, 2015a), and 492 (OECD, 2015b)], when combined with other alternative methods within future (tiered) testing strategies to replace the Draize eye test (Scott *et al.*, 2010).

The SkinEthic™ HCE EIT validation study was generally well designed and conducted, allowing ESAC to offer an informed opinion about the performance of the test method. ESAC believes that the overall relevance and reliability of the test method has been satisfactorily demonstrated with a view to it being considered for regulatory use in a tiered assessment strategy. The test method could be applied as a first step in Bottom-Up discrimination of 'non-irritants' (GHS No Category) or as a confirmatory last step in a Top-Down approach, where the priority is to first discriminate chemicals inducing serious eye damage (GHS Category 1). However, the method is not intended to differentiate Category 1 from Category 2 on its own.

HPLC/UPLC-spectrophotometry

The study results tend to confirm the findings of the EIVS study that suitably qualified HPLC/UPLC-spectrophotometry analytical systems can be used to quantify precipitated MTT-formazan as an alternative endpoint detection system in particular for highly coloured chemicals interfering with the conventional endpoint measurement of MTT-formazan by OD-photometry.

Table 1. Eye Irritation Validation Study (EIVS) target values and values obtained by the three laboratories participating in the ring test for within laboratory reproducibility (WLR), between laboratory reproducibility (BLR), sensitivity, specificity and accuracy. Two-sided 95 %-Confidence Intervals (CIs) are also provided.

	EIVS Target Values	EITL	EITS
WLR (Lab1)	≥ 85 %	95.0 % 95 %-CI: 86.3 % - 98.3 %	96.7 % 95 %-CI: 88.6 % - 99.1 %
WLR (Lab2)	≥ 85 %	88.3 % 95 %-CI: 77.8 % - 94.2 %	95.0 % 95 %-CI: 86.3 % - 98.3 %
WLR (Lab 3)	≥ 85 %	93.3 % 95 %-CI: 84.1 % - 97.4 %	95.0 % 95 %-CI: 86.3 % - 98.3 %
BLR (Lab 1 v Lab 2)		93.3 %	96.7 %
BLR (Lab 1 v Lab 3)		95.0 %	96.7 %
BLR (Lab 2 v Lab 3)		98.3 %	100 %
BLR (Cumulative)	≥ 80 %	93.3 % 95 %-CI: 84.1 % - 97.4 %	96.7 % 95 %-CI: 88.6 % - 99.1 %
Sensitivity (Lab 1)	≥ 90 %	100 %	92.2 %
Sensitivity (Lab 2)	≥ 90 %	97.9 %	92.2 %
Sensitivity (Lab 3)	≥ 90 %	96.9 %	92.2 %
Sensitivity (Cumulative)	≥ 90 %	98.3 %	92.2 %
Sensitivity (Bootstrap Resampling)	≥ 90 %	98.2 % 95 %-CI 93.8 % - 100 %	91.9 % 95 %-CI 90.0 % - 93.3 %
Specificity (Lab 1)	≥ 60 %	65.5 %	75.6 %
Specificity (Lab 2)	≥ 60 %	72.6 %	78.9 %
Specificity (Lab 3)	≥ 60 %	70.2 %	75.3 %
Specificity (Cumulative)	≥ 60 %	69.4 %	76.6 %
Specificity (Bootstrap Resampling)	≥ 60 %	69.4 % 95 %-CI 60.7 % - 75.0 %	76.6 % 95 %-CI 73.3 % - 80.0 %
Accuracy (Lab 1)	≥ 75 %	83.9 %	83.9 %
Accuracy (Lab 2)	≥ 75 %	86.1 %	85.6 %
Accuracy (Lab 3)	≥ 75 %	84.4 %	83.3 %
Accuracy (Cumulative)	≥ 75 %	84.8 %	84.4 %
Accuracy (Bootstrap Resampling)	≥ 75 %	84.8 % 95 %-CI 80.8 %- 88.3 %	84.3 % 95 %-CI 81.7 % - 86.7 %

References

- Alépée N, Leblanc V, Adriaens E, Grandidier MH, Lelièvre D, Meloni M, Nardelli L, Roper CS, Santirocco E, Toner F, Van Rompay A, Vinall J, Cotovio J (2016a). Multi-laboratory validation of SkinEthic HCE test method for testing serious eye damage/eye irritation using liquid chemicals. *Toxicol In Vitro* 31: 43-53.
- Alépée N, Adriaens E, Grandidier MH, Meloni M, Nardelli L, Vinall CJ, Toner F, Roper CS, Van Rompay AR, Leblanc V, Cotovio J (2016b). Multi-laboratory evaluation of SkinEthic HCE test method for testing serious eye damage/eye irritation using solid chemicals and overall performance of the test method with regard to solid and liquid chemicals testing. *Toxicol In Vitro* 34: 55-70.
- Barroso J, Alépée N, Cole T, Eskes C, Freeman SJ, Liška R, McNamee P, Pfannenbecker U, Reus AA, Rubingh CM, Schaeffer MW, Zuang V (2015). EURL ECVAM — Cosmetics Europe prospective validation study of Reconstructed human Tissue-based test methods for serious eye damage/eye irritation testing. Poster Presented at the Congress of the European Societies of Toxicology (EUROTOX), Porto, Portugal, September, 13–16, 2015. Available from:

  http://dx.doi.org/10.1016/j.toxlet.2015.08.507

  https://eurl-ecvam.jrc.ec.europa.eu/posters-1/eurl-ecvam-cosmetics-europe-prospective-validation-study-of-reconstructed-human-tissue-based-test-methods-for-serious-eye-damage-eye-irritation-testing-poster/view
- EC (2008a). Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (the 'CLP Regulation').
- EC (2008b). COUNCIL REGULATION (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).
- EURL ECVAM (2016). The EURL ECVAM - Cosmetics Europe prospective validation study of Reconstructed human Cornea-like Epithelium (RhCE)-based test methods for identifying chemicals not requiring classification and labelling for serious eye damage/eye irritation: Validation Study Report. EUR 28125 EN; doi:10.2787/41680. Available from:

  http://publications.jrc.ec.europa.eu/repository/handle/JRC100280
- OECD (2002). Acute Eye Irritation/Corrosion. OECD Guideline for the Testing of Chemicals No. 405, OECD, Paris.
- OECD (2008). OECD Environment Health and Safety Publications Series on Testing and Assessment No. 88 Workshop on Integrated Approaches to Testing and Assessment. OECD, Paris.
- OECD (2013a). 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 Guideline for the Testing of Chemicals No. 437, OECD, Paris.
- OECD (2013b). 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 Guideline for the Testing of Chemicals No. 438, OECD, Paris.
- OECD (2015a). 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 Guideline for the Testing of Chemicals No. 491, OECD, Paris.

- OECD (2015b). Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage. OECD Guideline for the Testing of Chemicals No. 492, OECD, Paris.
- Scott L, Eskes C, Hoffmann S, Adriaens E, Alépée N, Bufo M, *et al*. (2010). A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom-Up and Top-Down approaches. *Toxicol In Vitro* 24: 1-9.
- UN (2015). United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Sixth revised edition, UN New York and Geneva.

Annex 1

COMPOSITION OF ESAC AND ESAC WORKING GROUP

Composition of ESAC and ESAC Working Group

EURL ECVAM Scientific Advisory Committee (ESAC)

- Dr. Neil CARMICHAEL (ESAC Chair)
- Prof. Jürgen BORLAK
- Dr. Harvey CLEWELL
- Prof. Lucio G. COSTA
- Dr. Kristina KEJLOVÁ
- Prof. David John KIRKLAND
- Prof. Annette KOPP-SCHNEIDER
- Dr. Renate KRÄTKE
- Prof. Claus-Michael LEHR
- Dr. José Maria NAVAS
- Prof. Aldert PIERSMA
- Dr. Jonathan RICHMOND
- Dr. Erwin L. ROGGEN
- Dr. Dorothea SESARDIC

ESAC Working Group (WG)

- Dr. José Maria NAVAS (ESAC Member, WG Chair)
- Dr. Kristina KEJLOVÁ (ESAC Member)
- Prof. Annette KOPP-SCHNEIDER (ESAC Member)
- Dr. Renate KRÄTKE (ESAC Member)
- Dr. Jon RICHMOND (ESAC Member)
- Dr. Dave ALLEN (NICEATM; ICATM nomination by NICEATM/ICCVAM)
- Prof. Kyung-Min LIM (College of Pharmacy, Ewha Womans University; ICATM nomination by KoCVAM)

EURL ECVAM (Secretariat)

- Dr. João BARROSO (ESAC Coordinator)
- Dr. Thomas COLE
- Prof. Maurice WHELAN (Head of Unit)

Annex 2

EURL ECVAM REQUEST FOR ESAC ADVICE

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

ESAC Request 2016-02

EURL ECVAM Scientific Advisory Committee (ESAC)

EURL ECVAM REQUEST FOR ESAC ADVICE

on the

SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT)

Title page information	
Abbreviated title of ESAC request	SkinEthic™ HCE EIT validation
ESAC REQUEST No.	2016-02
Template used for preparing request	EP 3.02
Date of finalising request	27/05/2016
Date of submitting request to ESAC	27/05/2016
Request discussed through	Written procedure previous to ESAC 42
Opinion expected at (date)	ESAC 42 (June 2016)
File name of this request	**ER2016-02_ESAC_REQUEST_SKINETHIC_HCE_EIT.doc**

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1. TYPE OF REQUEST

Request Type	Identify request ("YES")
R1 ESAC Peer Review **of a Prevalidation Study or Validation Study**	**YES, external validation study** **(i.e. not coordinated by EURL ECVAM)**
If R1)applies please specify further:	
►Prevalidation Study	**NO**
►Prospective Validation Study	**YES** Background In December 2008, two reconstructed human eye tissue models for *in vitro* assay of eye irritation potential, EpiOcular™ Eye Irritation Test (EIT) and SkinEthic™ Human Corneal Epithelium (HCE), were sponsored for validation as alternatives to the traditional *in vivo* standard practice with rabbits (Draize test). The eye irritation validation study (EIVS) was conceived as a ring trial of comparative performance among six participant laboratories (three for each test method), testing selected chemicals to evaluate reliability (reproducibility within and between laboratories of results obtained *in vitro*) and relevance (predictive capacity of effects documented *in vivo*). Neither test method was able to comply fully with the acceptance criteria set by the validation management group (VMG). Therefore, further optimisation was recommended. With minor refinement to the EpiOcular™ EIT protocol, the method was successfully validated in 2013. The SkinEthic™ HCE protocol was subject to more comprehensive revision, followed by another validation ring trial (three laboratories) completed in 2015. In November 2015 the revised SkinEthic™ HCE test method was submitted for assessment by EURL ECVAM and formal peer-review by ESAC.
►Retrospective Validation Study	**NO**
►Validation Study based on Performance Standards	**NO**

R2 Scientific Advice on a test method submitted to EURL ECVAM for validation **(e.g. the test method's biological relevance etc.)**	**NO**
R3 Other Scientific Advice **(e.g. on test methods, their use; on technical issues such as cell culturing, stem cells, definition of performance standards etc.)**	**NO**

2. TITLE OF STUDY OR PROJECT FOR WHICH SCIENTIFIC ADVICE OF THE ESAC IS REQUESTED

L'Oréal-coordinated validation of the in vitro SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT)

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

1) Serious eye damage/eye irritation and regulatory tests

Eye irritation is the result of reversible anterior surface tissue trauma, causing degeneration of vision. Serious eye damage is not fully reversible within 21 days of exposure (UN, 2013). Traditionally, eye irritation has been determined by the *in vivo* rabbit Draize eye test (OECD Test Guideline 405, 2012).

Validated *in vitro* alternative methods include:

- organotypic assays: Bovine Corneal Opacity and Permeability test (OECD updated Test Guideline 437, 2013) and Isolated Chicken Eye test (OECD updated Test Guideline 438, 2013).
- cell-based methods: Fluorescein Leakage assay (OECD Test Guideline 460, 2012), Cytosensor Microphysiometer assay (OECD draft Test Guideline, 2012) and Short Time Exposure assay (OECD Test Guideline 491, 2015).
- reconstructed human cornea-like epithelium (RhCE) test: EpiOcular™ EIT (OECD Test Guideline 492, 2015).

2) SkinEthic™ HCE EIT purpose

At present, no single *in vitro* method can fully replace the *in vivo* Draize eye test for assessment of serious eye damage/eye irritation. However, tiered combination of alternatives (so-called Top-Down/Bottom-Up approach) can reduce/replace reliance on *in vivo* procedures (Scott *et al*., 2010; OECD draft Guidance, 2015). Top-Down differentiates chemicals inducing serious eye damage (GHS category 1) as priority, while Bottom-Up first discriminates 'non-irritants' (GHS no category).

The SkinEthic™ HCE EIT method is intended for inclusion in a Top-Down/Bottom-Up assessment strategy, particularly relevant for industrial chemicals or chemicals used in human exposure products, such as cosmetics ingredients which are banned from animal testing. The method is therefore required as an alternative, also effectively reducing the need for animal studies by their partial replacement. The SkinEthic™ HCE is the second RhCE test method that is validated following EpiOcular™ EIT. It is however important to have at least two of these methods validated and accepted by regulatory authorities in order to guarantee the widespread availability of this technology and avoid potential market monopolies.

In a tiered assessment strategy, the SkinEthic™ HCE EIT method is applicable as a first step in Bottom-Up discrimination of 'non-irritants' or as a confirmatory last step in a Top Down approach. However, the method is not intended to differentiate category 1 from 2 on its own.

3) SkinEthic™ HCE EIT principle

The test method addresses eye irritation caused by topical exposure to chemicals, manifested *in vivo* as local inflammation and/or opacity, resulting mechanistically from cell damage (cytotoxicity).

The *in vitro* test system uses immortalized human corneal epithelial cells, cultured to form a Reconstructed human Cornea-like Epithelium (RhCE), i.e. a three-dimensional tissue similar to the human corneal epithelium. The test method was developed to model *in vivo* topical exposure, with

prediction of positive or negative irritation response from cell viability assay. Tissue viability is determined quantitatively as a percentage, relative to a negative control (100% viable) by standardised MTT assay (photometric measurement of purple formazan production from enzymatic reduction of the vital dye MTT). Tissues treated with eye irritants show a decrease in viability relative to the negative control, with discrimination of positive or negative GHS classification defined by an optimised viability threshold percentage (prediction model).

The revised SkinEthic™ HCE test submission is complete with comprehensive protocols (SOPs) for eye irritation testing of liquids (EITL) and solids (EITS) as used in the validation study (Attachments 1a & 1b, respectively) and as intended for test method users (Attachments 1c & 1d, respectively).
Critical elements of the SOPs include:

- test system description (Human Corneal Epithelium tissue model, with quality control).
- TT: test treatment (application, exposure, incubation, MTT-formazan extraction).
- viability determination (MTT formazan assay: OD measurement, HPLC).
- prediction model: EITL (60% threshold) and EITS (50% threshold).

Acceptance criteria (for qualified test, qualified run, and complete test):

- NgC: negative control (PBS): $1.4 \leq OD \leq 2.5$ (mean of 2 replicate tissues).
- PC: positive control (methyl acetate): viability $\leq$ 30% (mean of 2 tissue replicates).
- viability difference between run replicates ≤ 20 (NgC, PC, TT).

4) SkinEthic™ HCE EIT optimisation

The test method was developed by L'Oréal, with prediction model optimization using 125 chemicals, including 71 liquids and 54 solids (Attachment 2).

A principal criterion for selection of test chemicals was availability of supporting complete and quality assured *in vivo* Draize eye irritation data. The selection was limited to commercially available chemicals.

The chemicals, incorporating 44/125 (35%) previously selected for the original ring trial eye irritation validation study (EIVS) provided a range of properties, including:

- Chemical class (functional group): soap/surfactant, organics (neutral, acid and base) and inorganic base.
- Several colour interfering chemicals, MTT reducers and MTT reducing coloured chemicals.
- GHS classification: 49% not classified (NC) and 51% classified (C) (divided as 53% Category 1 and 47% Category 2.

As distribution of physical state and GHS classification category, the 125 chemicals covered: 34 Category 1 (19 liquids and 15 solids), 21 Category 2A (16 liquids and 5 solids), 9 Category 2B (4 liquids and 5 solids) and 61 No Category (32 liquids and 29 solids).

The complement of chemicals used for development and optimization represents a significant and balanced set.

5) SkinEthic™ HCE EIT training and transfer

Transferability of the method was demonstrated using 18 chemicals (9 solids / 9 liquids) including strong colorants and MTT reducers known to cause interference, aiming to cover all experimental eventualities.

Two training days are required for a naïve laboratory, including practical application and data evaluation. Actual transfer of the method was arranged over two weeks, testing the 18 chemicals in replicate independent series to allow evaluation of:

- adherence to acceptance criteria.

- single and dual operator comparison.
- predictive concordance.

Results demonstrated accurate and reproducible implementation.

The training exercise has been described in full, with detailed method SOPs (Attachments 4a and 4b) and assessment reports (Attachments 5a, 5b, 5c, and 5d).
SOP implementation (transfer) by the naïve laboratories has also been reported in full (Attachments 6a and 6b) indicating the method is both robust and transferable.

6) SkinEthic™ HCE EIT validation

The ring trial validation study (EITL and EITS) for evaluation of within/between laboratory reproducibility (WLR/BLR) and predictive capacity (PC) included 120 chemicals (60 liquids, 60 solids) tested in three laboratories (L'Oréal, Charles River, Vito) with an additional 80 chemicals (45 liquids, 35 solids) tested by L'Oréal (lead laboratory).

The chemical selection (Attachment 2) again covered a range of properties:

- the full range of *in vivo* eye irritation GHS Categories (1, 2A, 2B, or No Category).
- the *in vivo* determinants of classification (cornea opacity, iritis, conjunctiva redness, chemosis, reversibility/persistence).
- wide representation of organic functional groups.
- known chemical structures.
- coloured and/or direct MTT reducers.
- availability through laboratory retail supply, at reasonable cost.

The processing and analysis of all data from the three laboratories in the ring trial was contracted to an independent consultant statistician who has compiled 2 comprehensive reports, respective of the liquid and solid protocols (Attachments 8a and 8b). The reports are clear and concise, uniformly applying the acceptance criteria and prediction model to determine within laboratory reproducibility (WLR) between laboratory reproducibility (BLR) and predictive capacity (PC).

The test submitter (L'Oréal) has also compiled all data used for method evaluation, provided as attachments in *pdf* and *xls* formats:

- Attachment 3a: Data used for relevance and reliability assessment (EITL and EITS).
- Attachment 3b: EITL: WLR assessment: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).
- Attachment 3c: EITL: WLR assessment: 45 additional chemicals, 1 lab (L'Oreal).
- Attachment 3d: EITS: WLR assessment: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).
- Attachment 3e: EITS: WLR assessment: 35 additional chemicals, 1 lab (L'Oreal).
- Attachment 7a: EITL: BLR assessment: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).
- Attachment 7b: EITS: BLR assessment: 60 ring trial chemicals, 3 labs (L'Oreal, VITO, CRL).
- Attachment 9: EIT: Predictive capacity (PC) assessment.

7) SkinEthic™ HCE EIT results

Within Laboratory Reproducibility (WLR)

WLR (concordance of predicted classification) based on the set of 120 chemicals, was reported as follows:

- CRL: 91.7% (EITL 88.3% and EITS 95.0%).
- VITO: 94.2% (EITL 93.3% and EITS 95.0%).
- L'Oreal: 95.8% (EITL 95.0% and EITS 96.7%).

WLR for the extended set of 200 chemicals (tested by L'Oreal only) was:

- 95.0% (EITL 93.3% and EITS 96.8%).

The test submission report concluded that the SkinEthic™ HCE EIT method (liquids/solids) has been shown to exceed the minimum requirement for WLR of 85% set by the validation management group (VMG) of EIVS. The WLR is also comparable to that obtained previously for a similar method, EpiOcular™ EIT.

Between Laboratory Reproducibility (BLR)

Fifty six of the 60 liquid chemicals were consistently classified (NC/C) by the three laboratories resulting in a BLR (concordance of predicted classification) of 93.3% (95% CI: 84.1% - 97.4%). BLR based on pair-wise comparison, was reported as follows:

- L'Oreal versus CRL: 93.3% (56/60 chemicals).
- L'Oreal versus VITO: 95.0% (57/60 chemicals).
- CRL versus VITO: 98.3% (59/60 chemicals).

Fifty eight of the 60 solid chemicals were consistently classified (NC/C) by the three laboratories resulting in a BLR (concordance of predicted classification) of 96.7% (95% CI: 88.6% - 99.1%). BLR based on pair-wise comparison, was reported as follows:

- L'Oreal versus CRL and L'Oreal versus VITO: 96.7% (58/60 chemicals).
- CRL versus VITO: 100%.

The test submission report concluded overall BLR for the SkinEthic™ HCE EIT method, based on the set of 120 chemicals, was 95.0% (EITL 93.3% and EITS 96.7%) exceeding the defined minimum requirement of 80% set by the VMG of EIVS.

For comparison, the test submission reported BLR from the previous ring trial validation of the similar test method EpiOcular™ EIT was 94.4% for liquids and 92.0% for solids.

Predictive Capacity (PC)

PC (ring trial) was evaluated by comparing *in vitro* viability with respect to prediction model (all runs, per laboratory and cumulatively) with documented *in vivo* classifications according to GHS.

The statistics report summarises the frequency distribution of true versus false predictions, respective of irritant classification (C) and non-irritant classification (NC). From these frequencies are calculated the sensitivity (rate of correct prediction for C, with false negatives), the specificity (rate of correct prediction for NC, with false positives) and overall accuracy (rate of correct prediction, C or NC) expressed as percentages:

Liquids protocol (EITL) predictive capacity (ring trial):

in vivo	**Cumulative**		**L'Oréal**		**CRL**		**VITO**	
	C	**NC**	**C**	**NC**	**C**	**NC**	**C**	**NC**
Classified	283	5	96	0	94	2	93	3
No Category	77	175	29	55	23	61	25	59
Total	**540**		**180**		**180**		**180**	
Sensitivity (%)	98.3		100		97.9		96.9	
False Negatives (%)	1.7		0		2.1		3.1	
Specificity (%)	69.4		65.5		72.6		70.2	
False Positives (%)	30.6		34.5		27.4		29.8	
Accuracy (%)	84.8		83.9		86.1		84.4	

From statistical bootstrap resampling (which estimates uncertainty in predictive capacity, as 95% CI) (10,000 re-samples at n=1 for the 60 chemicals) the statistics report indicates overall predictive capacity for the liquids protocol (EITL):

Parameter	Estimate	95% CI
Sensitivity (%)	98.2	93.8; 100
Specificity (%)	69.4	60.7; 75.0
Accuracy (%)	84.8	80.0; 88.3

Solids protocol (EITS) predictive capacity (ring trial):

in vivo	**Cumulative**		**L'Oréal**		**CRL**		**VITO**	
	C	**NC**	**C**	**NC**	**C**	**NC**	**C**	**NC**
Classified	249	21	83	7	83	7	83	7
No Category	63	206	22	68	19	71	22	67
Total	**539**		**180**		**180**		**179**	
Sensitivity (%)	92.2		92.2		92.2		92.2	
False Negatives (%)	7.8		7.8		7.8		7.8	
Specificity (%)	76.6		75.6		78.9		75.3	
False Positives (%)	23.4		24.4		21.1		24.7	
Accuracy (%)	84.4		83.9		85.6		83.3	

From statistical bootstrap resampling (which estimates uncertainty in predictive capacity, as 95% CI) (10,000 re-samples at n=1 for the 60 chemicals) the statistics report indicates predictive capacity for the solids protocol (EITS):

Parameter	Estimate	95% CI
Sensitivity (%)	91.9	90.0; 93.3
Specificity (%)	76.6	73.3; 80.0
Accuracy (%)	84.3	81.7; 86.7

The test submission also reports sensitivity, specificity and accuracy for the extended set of chemicals (including 45 additional liquids and 35 additional solids tested by the lead laboratory only) quoting similar figures.

8) HPLC spectrophotometry

The MTT-reduction assay for tissue viability, relevant to all *in vitro* test methods based on Reconstructed human Tissues (RhT) is limited by interference with coloured chemicals.

The test method R&D has overcome this limitation using High/Ultra High Performance Liquid Chromatography Performance (HPLC-UPLC)-spectrophotometry for endpoint detection of formazan.

The HPLC-UPLC method has been shown to be highly reproducible (BLR) between different laboratories.

Based on this, the test submission report concludes that HPLC/UPLC is relevant to all *in vitro* RhT test methods irrespective of the test system and test method and can be applied to any of the other RhT test systems within the relevant OECD Test Guidelines. Indeed, the HPLC/UPLC-spectrophotometry technique has already been implemented in OECD TGs 431 (in vitro skin corrosion based on RhE), 439 (in vitro skin irritation based on RhE) and 492 (in vitro serious eye damage/eye irritation based on RhCE).

References

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi A.J., Prieto, P., Sabbioni, E., Scott, L., Worth, A. and Zuang. V. (2004): A modular approach to the ECVAM principles on test validity. ATLA 32, 467-472.

OECD (2012), *Test No. 405: Acute Eye Irritation/Corrosion*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
DOI: http://dx.doi.org/10.1787/9789264185333-en

OECD (2012), *Test No. 460: Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
DOI: http://dx.doi.org/10.1787/9789264185401-en

OECD (2012, draft) The Cytosensor Microphysiometer Test Method: An *in vitro* Method for Identifying Ocular 3 Corrosive and Severe Irritant Chemicals as well as Chemicals not Classified as Ocular Irritants. http://www.oecd.org/env/ehs/testing/DRAFT%20Cytosensor%20TG%20(V9)%2021%20Dec%2012_clean.pdf

OECD (2013, updated), *Test No. 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 Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
DOI: http://dx.doi.org/10.1787/9789264203846-en

OECD (2013, updated), *Test No. 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 Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
DOI: http://dx.doi.org/10.1787/9789264203860-en

OECD (2015), *Test No. 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 Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
DOI: http://dx.doi.org/10.1787/9789264242432-en

OECD. (2015), *Test No. 492: Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris.
DOI: http://dx.doi.org/10.1787/9789264242548-en

OECD (2015, draft), Draft Guidance Document on an Integrated Approach to Testing and Assessment (IATA) for Serious Eye Damage and Eye Irritation: http://www.oecd.org/env/ehs/testing/draft-guidance-review-documents-monographs.htm

Scott, L., Eskes, C., Hoffmann, S., Adriaens, E., Alepée, N., Bufo, M., Clothier, R., Facchini, D., Faller, C., Guest, R., Harbell, J., Hartung, T., Kamp, H., Varlet, B.L., Meloni, M., McNamee, P., Osborne, R., Pape, W., Pfannenbecker, U., Prinsen, M., Seaman, C., Spielmann, H., Stokes, W., Trouba, K., Berghe, C.V., Goethem, F.V., Vassallo, M., Vinardell, P., Zuang, V. (2010): A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom-Up and Top-Down approaches. Toxicol In Vitro 24, 1-9.

United Nations (UN) Globally Harmonized System (GHS) for Classification and Labelling of Chemicals (2013): 5th (revised) edition.

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does EURL ECVAM require advice on the current issue?*	EURL ECVAM requests an ESAC opinion on the reliability (reproducibility within and between laboratories of results obtained *in vitro*) and relevance (predictive capacity of effects documented *in vivo*) of the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT) for prediction of eye irritation potential of chemicals. The opinion of ESAC should support EURL ECVAM with respect to the development of an EURL ECVAM recommendation on the Reconstructed human Cornea-like Epithelium (RhCE) assays for serious eye damage/eye irritation testing outlining (1) the scientific basis of the assays, (2) their overall performance (transferability, reproducibility and predictive capacity) as assessed during the validation studies and based on other (e.g. published) information, (3) their applicability and limitations, and 4) their proposed use. ESAC's advice should enable EURL ECVAM to conclude, within its EURL ECVAM Recommendation, on the potential adequacy of the SkinEthic™ HCE EIT for routine testing of serious eye damage/eye irritation for regulatory purposes.

4.2 QUESTION(S) TO BE ADDRESSED

Questions *What are the questions and issues that should be addressed in view of achieving the objective of the advice?*	The ESAC peer review of the SkinEthic™ HCE EIT should address the following aspects: (1) Scientific basis in relation to serious eye damage/eye irritation. (2) Clarity of the test definition, including: - purpose and need of the test method. - biological/mechanistic relevance in relation to the test system used and the endpoint measured. - protocol clarity and completeness. - clarity and adequacy of the prediction model and its development. (3) Clarity of the definition of the study objective(s). (4) Appropriateness of the study design and execution considering the study objective(s), including: - number and selection criteria for test chemicals (e.g., range of documented effects *in vivo*, etc.). - quality assurance of reference data (*in vivo*) for predictive capacity assessment. - number of participating laboratories. - number of replicates, number of repetitions, rules for retesting and handling of deviations. (5) Study management and conduct. (6) Results compilation and statistical analyses reporting. - appropriateness of calculation of WLR and BLR on the basis of the generated data.

	- appropriateness of calculation of Predictive Capacity on the basis of the generated data. - appropriateness of identification of limitations/applicability domain on the basis of the generated data. (7) Transferability and reproducibility (WLR/BLR). (8) Predictive capacity for distinguishing chemicals not requiring classification from chemicals requiring classification as Category 1 (serious eye damage) or Category 2 (eye irritation) and relevance to a tiered (Top-Down/Bottom-Up) testing strategy. (9) Applicability and any known limitations, assessed from the selection of the test chemicals (range of molecular class and physical properties) and analyses of possible reasons for misclassifications. (10) Possible gaps, if any, between study design and study conclusions. (11) Whether the information provided in the submission is sufficient to substantiate the proposed use of the test method within a Bottom-Up/Top-Down testing strategy. (12) Usefulness and applicability of HPLC/UPLC-spectrophotometry as an alternative endpoint detection system to standard photometry in SkinEthic™ HCE EIT. (13) What additional work, if necessary, should be undertaken in future to further characterise the test method and its proposed use. ESAC's advice should conclude on the regulatory applicability of the SkinEthic™ HCE EIT (i.e., for implementation as an EU test method and OECD Test Guideline).

4.3 TIMELINES

Timelines concerning this request *When does EURL ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	**June 2016**
	Request to be presented to ESAC by written procedure (e.g. due to urgency) prior to the next ESAC	**YES**
	Request to be presented to ESAC at ESAC plenary meeting	**NO**

5. EURL ECVAM PROPOSALS ON HOW TO ADDRESS THE REQUEST WITHIN ESAC

5.1 EURL ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request *Does the advice require an ESAC working group, an ESAC rapporteur etc.?*	**Structure(s) required**	**Required according to EURL ECVAM? (YES/NO)**
	S1 ESAC Rapporteur	**NO**
	S2 ESAC Working Group	**ESAC members** - José M. Navas (Chair) - Kristina Kejlová - Annete Kopp-Schneider - Renate Kraetke - Jon Richmond **ICATM nominations** - Dave Allen (NICEATM/ICCVAM) - Kyung-Min Lim (College of Pharmacy, Ewha Womans University; nominated by KoCVAM)
	S3 Invited Experts	**NO**
	Ad S3: If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above (S1-S3):	

5.2 DELIVERABLES AS PROPOSED BY EURL ECVAM

Deliverables *What deliverables (other than the ESAC opinion) are required for addressing the request?*	**Title of deliverable other than ESAC opinion**	**Required? (YES/NO)**
	D1 ESAC Rapporteur Report and draft opinion	**NO**
	D2 ESAC Peer Review Report and draft opinion	**YES**
	If other than above (D1-D2):	

6. LIST OF DOCUMENTS TO BE MADE AVAILABLE TO THE ESAC

Count	Description of document	Already available? (YES/NO)	File name
1	SkinEthic™ HCE test submission (TST)	YES	TST SkinEthic HCE EIT_Amended.pdf
2	EURL ECVAM Assessment Report on the SkinEthic™ HCE test submission	YES	SkinEthic_HCE_assessment_report_2016-05-09_final.pdf
3	Protocol of the SkinEthic™ HCE EITL (Liquid)	YES	Attachment 1a.pdf
4	Protocol of the SkinEthic™ HCE EITS (Solid)	YES	Attachment 1b.pdf
5	SkinEthic™ HCE EITL (Liquid) DB-ALM protocol	YES	Attachment 1c.pdf
6	SkinEthic™ HCE EITS (Solid) DB-ALM protocol	YES	Attachment 1d.pdf
7	SkinEthic™ HCE EIT - List of test items including their CAS number and basic physical/chemical properties for optimisation/transfer/WLR/BLR/Predictive capacity	YES	Attachment 2.pdf
8	SkinEthic™ HCE EIT - Data used for relevance and reliability assessment (EITL and EITS)	YES	Attachment 3a.pdf
9	SkinEthic™ HCE EITL - WLR assessment (60 chemicals – 3 labs)	YES	Attachment 3b.pdf
10	SkinEthic™ HCE EITL - WLR assessment (45 additional chemicals – 1 lab)	YES	Attachment 3c.pdf
11	SkinEthic™ HCE EITS - WLR assessment (60 chemicals – 3 labs)	YES	Attachment 3d.pdf
12	SkinEthic™ HCE EITS - WLR assessment (35 additional chemicals – 1 lab)	YES	Attachment 3e.pdf
13	Training protocol of the SkinEthic™ HCE EITL (Liquids)	YES	Attachment 4a.pdf
14	Training protocol of the SkinEthic™ HCE EITS (Solids)	YES	Attachment 4b.pdf
15	Training report of the SkinEthic™ HCE EITL (Liquids) - VITO	YES	Attachment 5a.pdf
16	Training report of the SkinEthic™ HCE EITL (Liquids) - CRL	YES	Attachment 5b.pdf
17	Training report of the SkinEthic™ HCE EITS (Solids) - VITO	YES	Attachment 5c.pdf
18	Training report of the SkinEthic™ HCE EITS (Solids) – CRL	YES	Attachment 5d.pdf
19	Transfer report of the SkinEthic™ HCE EITL (Liquids) – VITO & CRL	YES	Attachment 6a.pdf
20	Transfer report of the SkinEthic™ HCE EITS (Solids) – VITO & CRL	YES	Attachment 6b.pdf
21	SkinEthic™ HCE EITL - BLR assessment (60 chemicals – 3 labs)	YES	Attachment 7a.pdf
22	SkinEthic™ HCE EITS - BLR assessment (60 chemicals – 3 labs)	YES	Attachment 7b.pdf

23	Statistical analysis and reporting of the SkinEthic™ HCE EITL (Liquids)	YES	Attachment 8a_Revised.pdf
24	Statistical analysis and reporting of the SkinEthic™ HCE EITS (Solids)	YES	Attachment 8b.pdf
25	SkinEthic™ HCE EIT – Predictive capacity assessment	YES	Attachment 9.pdf
26	Project plan of the SkinEthic™ HCE EITL (Liquids)	YES	Attachment 10a.pdf
27	Project plan of the SkinEthic™ HCE EITS (Solids)	YES	Attachment 10b.pdf
28	SkinEthic™ HCE EIT - HPLC/UPLC-spectrophotometry (24 chemicals – 1 lab)	YES	Attachment 11.pdf
29	Publication on the validation of EITL	YES	Alépée et al. 2016 - SkinEthic HCE liquids.pdf
30	Publication on the validation of EITS	YES	Alépée et al. 2016 - SkinEthic HCE solids.pdf

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

The ESAC unanimously agreed by written procedure on the 18th of February 2016 on the composition of a new ESAC Working Group for the review of test methods in the area of serious eye damage/eye irritation.

7.2 TITLE OF THE ESAC WORKING GROUP

Full title:
ESAC Working Group on Eye Irritation Test Methods

Abbreviated title:
ESAC WG Eye Irritation

7.3 MANDATE OF THE ESAC WORKING GROUP

The ESAC WG is requested to conduct a scientific review of the l'Oréal-coordinated validation study concerning the SkinEthic™ HCE EIT. The review needs to address the questions put forward to ESAC by EURL ECVAM under section 4.2 of the current request.

The review should focus on the appropriateness of design and conduct of the study in view of the study objective and should provide an appraisal to which extent the conclusions of the test submitter are substantiated by the information generated during the study and how the information generated relates to the scientific background available.

7.4 DELIVERABLES OF THE ESAC WORKING GROUP

The ESAC WG is requested to deliver to the chair of the ESAC and the ESAC Coordinator a detailed **ESAC Working Group Report** outlining its analyses and conclusions and a **draft ESAC Opinion.** A template has been appended (Appendix 1) intended to facilitate the drafting of the WG report.

The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the report should clearly outline the differences in the appraisals and provide appropriate scientific justifications.

7.5 PROPOSED TIMELINES OF THE ESAC WORKING GROUP

Item	Proposed date/time	Action	Deliverable
1	6 May 2016	Teleconference of the Working Group	Agree procedure
2	11-13 May 2016	Working Group meeting	Draft ESAC WG report and draft ESAC opinion
3	27 May 2016	Circulation of final WG report and draft ESAC opinion to ESAC	Final draft ESAC WG report and draft ESAC opinion
4	9-10 June 2016	Endorsement of WG report and ESAC opinion at ESAC42 meeting	Final ESAC WG report and ESAC opinion

7.6 QUESTIONS WHICH SHOULD BE ADDRESSED BY THE ESAC WORKING GROUP

The review should address the **questions put forward to ESAC by EURL ECVAM** (see section 4.2) and the information requirements of the ESAC Working Group Template, where applicable. The ESAC Coordinator will provide guidance if needed.

When preparing the final ESAC WG report to address these questions, the ESAC WG is requested to use a pre-defined reporting template. This template (see appendix 1) follows EURL ECVAM's modular approach and addresses to which extent the standard information requirements have been addressed by the study. The template allows moreover for addressing the issues specific studies outlined in section 4.2. The Secretariat will provide guidance if necessary.

APPENDIX 1 REPORTING TEMPLATE

The appended ESAC WG template suggests a structure that is in close agreement with the EURL ECVAM information requirements ("modules") for scientific review following validation and allows at the same time for the description of the analysis and conclusions concerning more specific questions.

The template can be used for various types of validation studies (e.g. prospective full studies, retrospective studies, performance-based studies and prevalidation studies). Depending on the study type and the objective of the study, not all sections may be applicable.

However, for reasons of consistency and to clearly identify which information requirements have not been sufficiently addressed by a specific study, this template is uniformly used for the evaluation of validation studies.

The current template is

TEMPLATE_ESAC-WG_REPORT-v6.doc

Annex 3

ESAC WORKING GROUP PEER REVIEW CONSENSUS REPORT

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Directorate F - Health, Consumers and Reference Materials
European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM
SCIENTIFIC
ADVISORY
COMMITTEE
(ESAC)

ESAC Working Group Peer Review Consensus Report

on the

SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT)

Title page information			
File name	ESAC_WG_Report_SkinEthic_HCE_EIT.doc		
Abbreviated title of ESAC request	SkinEthic™ HCE EIT validation		
Relating to ESAC REQUEST No.	2016-02		
Request discussed through	Written procedure previous to ESAC 42		
Report to be handed over to ESAC Chair and EURL ECVAM Coordinator by	José M. Navas		
Version tracking			
Date	**Version**	**Author(s)**	**Description**
13 May 2016	V1.0	ESAC WG	First ESAC WG agreed draft
17 May 2016	V2.0	ESAC WG	Second revised draft after commenting
27 May 2016	V3.0	ESAC WG	Third revised draft after commenting
01 June 2016	V4.0	ESAC WG	Fourth revised draft after commenting
06 June 2016	V5.0	ESAC WG	Final ESAC WG approved draft sent to ESAC for endorsement

Table of Contents

ESAC Working Group

Full title: ESAC Working Group on Eye Irritation Test Methods

Abbreviated title: ESAC WG Eye irritation

The ESAC WG was established in March 2016 by written procedure to assist in the production of an ESAC Opinion by undertaking a peer review of a three laboratory ring trial of the SkinEthic™ Human Corneal Epithelium (HCE) Eye Irritation Test (EIT), a test method with a wide applicability domain for liquids (EITL: Eye Irritation Testing of Liquids) and solids (EITS: Eye Irritation Testing of Solids), developed for the prediction of the eye irritation potential of liquid and solid chemicals, specifically to distinguish chemicals requiring official classification for eye irritation or serious eye damage according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS. UN, 2015) from chemicals not requiring classification (no classification category, 'non-irritants').

Following a teleconference on 6 May 2016 the ESAC WG met at EURL-ECVAM 11-13 May 2016 to conduct its peer review.

The ESAC WG members appointed by ESAC were:

- Dr. José Maria Navas (ESAC Member, WG Chair)
- Dr. Kristina Kejlová (ESAC Member)
- Prof. Annette Kopp-Schneider (ESAC Member)
- Dr. Renate Krätke (ESAC Member)
- Dr. Jon Richmond (ESAC Member)
- Dr. Dave Allen (Nomination by NICEATM/ICCVAM)
- Prof. Kyung-Min Lim (Nomination by KoCVAM)

ESAC Coordination:

- Dr. João Barroso (ESAC Coordinator)
- Dr. Thomas Cole

Abbreviations used in the document

- **BLR** Between-laboratory reproducibility
- **CI** Confidence Interval
- **EIT** Eye Irritation Test
- **EITL** Eye Irritation Testing of Liquids
- **EITS** Eye Irritation Testing of Solids
- **EIVS** EURL ECVAM/Cosmetics Europe Eye Irritation Validation Study
- **ESAC** EURL ECVAM Scientific Advisory Committee
- **ESAC WG** ESAC Working Group
- **EU CLP** European Union Regulation on Classification, Labelling and Packaging of Substances and Mixtures
- **EURL ECVAM** European Union Reference Laboratory for Alternatives to Animal Testing
- **FDA** (US) Food and Drug Administration
- **GLP** Good Laboratory Practice
- **HCE** Human Corneal Epithelium
- **HPLC/UPLC** High Performance Liquid Chromatography/Ultra Performance Liquid Chromatography
- **IATA** Integrated Approach to Testing and Assessment
- **MTT** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- **OD** Optical density
- **OECD** Organisation for Economic Co-operation and Development
- **PC** Positive Control
- **RhCE** Reconstructed Human Cornea-like Epithelium
- **RhT** Reconstructed Human Tissue
- **SOP** Standard Operating Procedure
- **TST** Test Submission Template
- **UN GHS** United Nations Globally Harmonized System for the Classification and Labelling of Chemicals.
- **VMG** Validation Management Group
- **WLR** Within-laboratory reproducibility

1. Study objective and design

1.1 Analysis of the clarity of the study objective's definition

(a) ESAC WG summary of the study objective as outlined in the Test Submission

The TST and supporting documents provide an adequate summary of the main study objectives:

- To formally validate the SkinEthic™ HCE test method by a three laboratory ring trial study using an appropriate number and range of test chemicals and to obtain data to assess the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of the assay.
- To facilitate its international acceptance in regulatory schemes for hazard assessment of liquid and solid chemicals to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling (Category 1 and Category 2) according to the UN GHS Classification and Labelling of Chemicals (UN GHS. UN, 2015) and as implemented by the EU CLP regulation (EU CLP. EC, 2008a).
    - N.B. The test method was not designed, intended, or evaluated to differentiate between GHS Category 1 (irreversible effects) and 2A-B (reversible effects); and there is no protocol for the testing of gases and aerosols.
- To produce evidence to support the test method being incorporated into a tiered testing strategy (so-called Bottom-Up/Top-Down testing strategy, Scott L. *et al.*, 2010). The ultimate purpose of such a tiered testing strategy being to replace the traditional *in vivo* Draize eye test [Method B.5 of EC Regulation 440/2008 (EC, 2008b) or OECD TG 405 (OECD, 2002)].

(b) Appraisal of clarity of study objective as outlined in the Test Submission

The ESAC WG believes that the study objectives were sufficiently clear, and were reflected in the way the study was designed, conducted, analysed, and subsequently reported in the TST.

1.2 Quality of the background provided concerning the purpose of the test method

The TST clearly describes the intended application of the SkinEthic™ HCE test method and suitably qualified HPLC/UPLC-spectrophotometry analytical measurement systems as being for regulatory testing.

(a) Analysis of the scientific rationale provided in the Test Submission

The SkinEthic™ HCE test method validation study was designed and conducted to provide evidence of whether the test method and EITL and EITS protocols are sufficiently relevant and reliable to identify chemicals not requiring classification, i.e. chemicals that are not UN GHS/EU CLP Category 1 (serious eye damage) or Category 2 (eye irritant). The results of the study (i.e. prediction of non-classified chemicals) are to be considered for use within a testing strategy (Scott *et al.*, 2010).

The SkinEthic™ HCE test method TST describes the relevance and scientific rationale of the test method partly in terms of other validated and accepted RhT models' proven ability to measure cytotoxicity by the OD-photometric quantification of MTT reduction products (Mosmann, 1983) as a reliable surrogate measurement of a range of toxicological endpoints after the controlled exposure of an RhT air-tissue interface to solid or liquid test materials. The ESAC WG accepts that in other contexts (for example skin irritation) cytotoxicity/cell viability following chemical exposure is a recognised surrogate measure for chemically induced *in vivo* toxicity: with the *in vivo* cytotoxicity

then stimulating an inflammatory response involving the innate immune system in proportion to the degree of cytotoxicity produced by the chemical.

The TST also provides a scientific rationale and justification in the specific context of eye irritation, reasoning that RhCE test system cytotoxicity/tissue viability is a potentially plausible surrogate measure of ocular hazards capable of discriminating between UN GHS/EU CLP classified chemicals (Category 1 and Category 2) and non-irritants produced by a variety of relevant, known, *in vivo* mechanisms of ocular toxicity.

While the SkinEthic™ HCE EIT was not developed to account for all mechanisms associated with eye irritation, the validation study demonstrated that this RhCE test method is able to correctly predict chemicals not requiring classification for serious eye damage/eye irritation independently of the types of ocular effects observed *in vivo* (i.e., corneal, iridal and conjunctival injuries).

The evidence and arguments advanced in the TST in support of the test method's performance take account of the findings of previous pre-validation studies and other peer reviewed publications. The available documentation does not allow an assessment of the test method's performance with mixtures.

The TST acknowledges that, for the foreseeable future, the *in vivo* Draize rabbit eye test is unlikely to be replaced by a single *in vitro* test (Eskes *et al.*, 2005), and argues that the intended use of this RhCE test method would be within a larger testing strategy designed to replace or reduce animal testing for determining chemical-induced eye damage/irritation potential for regulatory purposes (Scott *et al.*, 2010).

The ESAC WG believes the available documentation provides sufficient evidence and reasoned arguments in favour of this RhCE test method having the potential to be at least as relevant and reliable as the currently validated *in vitro* methods for the identification of non-irritants [OECD TGs 437 (OECD, 2013a), 438 (OECD, 2013b), 491 (OECD, 2015a), and 492 (OECD, 2015b)], when combined with other alternative methods within future (tiered) testing strategies to replace the Draize eye test (Scott *et al.*, 2010).

It is proposed by Scott *et al.* (2010) that within such future testing strategies, based on the expected ocular toxicity profile of the test chemical, and using one of two tiered testing approaches (Bottom-Up or Top-Down), the systematic and sequential application of one or more *in vitro* test methods would be used to determine chemically induced eye damage/irritation potential. The full details of the appropriate integrated testing strategies and test methods have yet to be defined.

The TST discusses a known limitation of the currently used OD-photometric endpoint detection system - its inability to reliably assess the cytotoxic effects of chemicals, which interfere with the OD-photometric endpoint measurement of MTT-formazan - and provides a rationale for the use of a suitably qualified HPLC/UPLC-spectrophotometry endpoint detection system as an alternative MTT-formazan endpoint measure.

(b) Analysis of the regulatory rationale provided in the Test Submission

The TST identifies relevant regulatory requirements. In the view of the ESAC WG, the relevant legislation and regulations are appropriately referenced; and the regulatory requirements and the role of the RhCE-based methods in the context of the regulatory requirements are adequately specified.

RhCE test methods for eye damage/irritation testing such as the SkinEthic™ HCE test method could contribute to a reduction in animal testing by reliably identifying chemicals not requiring classification when used within an appropriate non-animal testing strategy (Scott *et al.*, 2010).

With respect to ocular toxicity, the ESAC WG considers that in view of the high prevalence of non-classified chemicals (Adriaens *et al.*, 2014) RhCE test methods validated for this purpose and used

within a “bottom-up approach” could significantly reduce animal testing by identifying the much larger number of chemicals not requiring classification.

1.3 Appraisal of the appropriateness of the study design

The study and data reported in the TST complies with the principles and criteria set out in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (No. 34, OECD, 2005), and described in the generally accepted Modular Approach to validation (Hartung *et al.*, 2004).

Subject to the comments below (and section 1.4 of this report), the TST and supporting documentation tends to confirm compliance with the expected standards for validation study organisation, management, and conduct, including:

- a reasoned scientific basis for the mechanistic relevance of the test method;
- suitable quality assurance systems for tissue model production and batch release;
- comprehensive SOPs for method implementation with separate protocols for the testing of liquid and solid chemicals;
- representative and balanced chemical (test item) selection;
- comprehensive training of and test method transfer to naïve laboratories;
- clear coordination and suitably defined responsibilities for the ring trial (project management, chemicals management, data management);
- appropriate use of positive and negative controls, criteria and limits for retesting, definition of qualified and non-qualified runs, data recording, calculation of WLR, BLR, specificity, sensitivity, accuracy;
- independence of ring trial testing and data statistics analysis; and
- complete documentation and transparency.

The SkinEthic^{TM} HCE test method (with separate protocols for testing liquids and solids) was assessed for its potential to identify chemicals not requiring classification for serious eye damage/eye irritation (UN GHS / EU CLP No Category; “non-irritants”) and their reliable discrimination from all classes of classified (categories 1 and 2) chemicals with a view to it forming an initial step of a Bottom-Up approach or a later stage of a Top-Down approach (Scott *et al.*, 2010).

The relevance and reliability of the test method were assessed by a three laboratory ring trial using coded test chemicals (N=120): a pre-study statistical power analysis (sample size calculation) having calculated within the EIVS study as a minimum requirement N=104 (i.e. 26 classified chemicals and 26 non-classified chemicals for each of the two protocols).

The analysis of the test method performance in the TST takes account of the study test results with these 120 chemicals, plus data from 125 chemicals used for test method development within L'Oréal, and an additional 80 chemicals tested prospectively by L'Oréal.

The performance of the SkinEthic^{TM} HCE test method (with separate protocols for testing liquids and solids) as a potential stand-alone test method for the identification of chemicals not classified for serious eye damage/eye irritation in the framework of Bottom-Up test strategies was judged against the test method performance acceptance criteria previously derived to evaluate the EIVS study.

In setting minimum acceptable values for test method performance, the EIVS VMG took into account of:

1. The background and specific objectives of the EIVS validation study;
2. The requirements of regulatory authorities and industry when testing and classifying chemicals for serious eye damage/eye irritation;

3. The within test variability inherent in the *in vivo* Draize eye test data and the manner in which these data are currently used for classifying ocular effects according to UN GHS / EU CLP;
4. The performance of other test methods already considered validated for this purpose;
5. The way in which the *in vitro* tests are to be used (as one test within a tiered testing strategy); and
6. The desired statistical power of the EIVS validation study.

The ESAC WG concludes that, subject to specific qualifications set out below (see 1.4), the study design was generally appropriate and robust, the acceptance criteria appropriate at the time, and that the study findings have provided the evidence and analysis required to satisfy the study objectives.

The ESAC WG believes the TST provides a detailed and reasoned rationale for use of the suitably qualified HPLC/UPLC-spectrophotometry analytical method (FDA, 2001; Alépée *et al.*, 2015).

1.4 Appropriateness of the statistical evaluation

The statistical and data reports annexed to the SkinEthic^{TM}HCE test method TST are detailed and informative. The method used for the calculation of the WLR is appropriate.

The method used for the calculation of predictive capacity using resampling methods (bootstrapping of single runs) is also appropriate.

However, in the view of the WG the most appropriate way of calculating the BLR would have been to apply resampling methods that better reflected how test materials would be categorised for regulatory purposes – as in practice categorization would be based on a single run not the average of three. Nevertheless, in the context of this study it would appear that had this approach been followed the reported difference in the performance of the test method would be marginal.

Non-qualifying test runs were not included in the data used to calculate final BLR or Predictive Capacity values.

No test chemical used for the ring trial was considered incompatible with the method by any of the three laboratories, with either the EITS or the EITL protocol. All chemicals were thus included in all of the statistical analyses of this test method.

The results obtained with the EITS and EITL protocols were independently assessed with respect to their reproducibility and predictive capacity.

2. Collection of existing data

2.1 Existing data used as <u>reference</u> data

The TST and annexes provide detailed information on this point. The TST references and relies on an extensive collection of information generated before and during the development and pre-validation of the test method.

The essential requirements for chemical selection for the ring trial were toxicological and physicochemical properties, and the availability of complete and quality assured supporting *in vivo* data to allow comparative evaluation of the predictive capacity of the RhCE test method as measured against *in vivo* (Draize eye test) reference method.

The chemicals selected are all commercially available chemicals and included chemicals:

- of different physical states;
- known to induce the full range of *in vivo* serious eye damage/eye irritation responses based on high quality results obtained in the reference *in vivo* rabbit eye test (Draize, 1944) and the UN GHS classification system (i.e., Categories 1, 2A, 2B, or No Category) (UN GHS. UN, 2015);
- representing the various known *in vivo* drivers of classification as reported by Adriaens *et al*. (2014) and Barroso *et al*. (2016);
- covering a good and wide representation of organic functional groups;
- having well-defined chemical structures;
- known to be coloured chemicals and/or direct MTT reducers; and
- not associated with prohibitive acquisition and/or disposal costs.

In total, 120 coded chemicals (60 liquids and 60 solids) were evaluated in three laboratories, with the test chemical set having the following qualities:

- The 120 chemicals were distributed as follows according to the UN GHS classification: 32 Category 1 (16 liquids and 16 solids), 17 Category 2A (8 liquids and 9 solids), 13 Category 2B (8 liquids and 5 solids) and 58 No Category (28 liquids and 30 solids) chemicals;
- 16 different functional groups: including organic bases, organic acids, neutral organic, inorganic bases, soap/surfactant;
- direct MTT reduction: 11 chemicals identified as MTT reducers and 43 as non MTT reducers during Eye Irritation Validation Study (2010);
- coloured chemicals (with 2 identified as coloured chemicals during EIVS) and non-coloured chemicals;
- previously used in related validation studies: 55 % (66/120) tested in EIVS.

75 of the 120 test materials had NOT been in the development or optimisation of the test method.

The Draize eye test Reference Database (DRD) published in Barroso *et al*. (2016) was consulted to identify the test chemicals used in the validation study. Such database includes data from the following sources:

1. ECETOC database of eye irritation reference chemicals (ECETOC, 1998).
2. Database from Laboratoire National de la Santé (LNS) (Gautheron *et al.*, 1992).
3. ZEBET database of eye irritation reference chemicals (Spielmann *et al.*, 1996).
4. ICCVAM (NICEATM) database of eye irritation reference chemicals.
5. EURL ECVAM database of chemicals considered for selection in EIVS, including amongst other (i) chemicals notified in the EC New Chemicals Database (NCD), (i) chemicals in the EC (DG-SANCO) Cosmetics Ingredients (CosIng) database, (iii) pesticide actives in the US EPA database

2.2 Existing data used as <u>testing</u> data

Not applicable.

2.3 Search strategy for retrieving existing data

See Section 2.1 above.

2.4 Selection criteria applied to existing data

See Section 2.1 above.

3. Quality aspects relating to data generated during the study

3.1 Quality assurance systems used when generating the data

L'Oreal's R&I laboratory developed the SkinEthic™ HCE EIT test method and was the lead laboratory for the ring trial. It is not Good Laboratory Practices (GLP) certified. However, for the purposes of the ring trial the following safeguards (Balls *et al*., 1995) were applied:

- Qualified personnel, and appropriate facilities, equipment and materials were available
- Records of the qualifications, training and experience, and a job description for each professional and technical individual, were maintained.
- For each study, an individual with appropriate qualifications, training and experience was appointed to be responsible for its overall conduct and for any report issued.
- Instruments used for the generation of experimental data were inspected regularly, cleaned, maintained and calibrated according to manufacturers' instructions. Records of these processes were kept, and made available for inspection on request.
- Reagents were labelled, as appropriate, to indicate their source, identity, concentration and stability. The labelling included the preparation and expiry dates, and specific storage conditions.
- All data generated during a study were recorded by the individual(s) responsible. These entries were attributable and dated.

The two additional laboratories involved in the ring trial were involved in the validation study namely CHARLES RIVER LABORATORIES (Edinburgh, United Kingdom) and VITO NV (Flemish Institute for Technological Research, Mol, Belgium) are GLP certified and performed the studies in accordance and in compliance with the GLP standards (OECD, 1999).

3.2 Quality check of the generated data prior to analysis

The study documents state that:

> "...*for the statistical analysis, a summary template was designed by the statistician, and the results were transferred to this template by the statistician. This summary template was compared with the summary templates received from the participating laboratories in order to check that no mistakes were made in the transfer of the results. The final conclusions for each chemical were then compared to the conclusions of the reports send by the laboratories as an additional check*".

4. Quality of data used for the purpose of the study (existing and newly generated)

4.1 Overall quality of the evaluated testing data (newly generated or existing)

The data generated and supplied are of sufficient quality to apply the predetermined acceptance criteria and prediction model.

4.2 Quality of the reference data for evaluating relevance[1]

The quality of data used is considered to be the best available Draize eye test reference data and is equivalent to that of data used in previous RhCE validation studies.

4.3 Sufficiency of the evaluated data in view of the study objective

The sample size was sufficient to produce appropriately narrow confidence intervals (CIs).

There is sufficient data for the WG to offer a reasoned opinion about the performance of the test method.

5. Test definition (Module 1)

5.1 Quality and completeness of the overall test definition

The SkinEthic™ HCE EIT test method is based on RhCE technology; the test material (solid or liquid chemicals) is applied directly to the upper surface of the air-tissue interface, and the eye damage/irritation potential of the chemical is derived from a prediction model based on percentage tissue viability (adjusted against a negative control) as estimated by OD-photometry or HPLC/UPLC-spectrophotometry of the MTT reduction product formazan.

The tissue component of the test kit is manufactured by controlled culture of immortalised human corneal epithelial cells in a chemically defined medium to produce a multi-layered epithelium similar to *in vivo* corneal epithelium with columnar basal cells, wing cells and squamous cells. The model is also characterised by the presence of relevant ultra-structural features (such as intermediate filaments, mature hemi-desmosomes and desmosomes) that characterise the corneal epithelium *in situ*. Specific cytokeratins 64kD (K.3) have also been described (Nguyen *et al.*, 2003).

The test method SOP provides separate protocols for testing liquids and solids. There are four essential differences between the two protocols:

- the length of time that the tissue interface is in contact with the test material (30 minutes exposure for liquids and 4 hours exposure for solids);
- a requirement for an 18 hours post-exposure incubation phase for solid chemicals;
- the extraction of the MTT formazan product from both the top and bottom surfaces of the tissue interface in the case of liquids, but only from the bottom surface for solids.
- the prediction models are different (see immediately below).

Following treatment with a test chemical the relative tissue viability is determined against the negative control value by the reduction of the vital dye MTT to formazan as measured by OD-photometry and/or HPLC/UPLC-spectrophotometry. For both Optical Density and HPLC/UPLC-spectrophotometry endpoints, the result is accepted if:

- The mean Optical Density (OD_{NgC}) at 570 nm (± 30nm) of the two replicate tissues treated with negative control is ≥ 1.4 with an upper acceptance limit of ≤2.5.
- The Mean Viability of the two replicate tissues (2 values from each of the two tissues) treated with positive control, expressed as percentage of the negative control, is ≤ 30 %,

[1] OECD guidance document No. 34 on validation defines relevance as follows: "Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of accuracy (concordance) of a test method."

what is much higher than the reported PC mean values (generally < 5 %), so that the established threshold (30 %) could be reduced.

- The TST indicates that when OD is chosen as endpoint the difference of viability between the two replicate tissues of a single test chemical should be ≤ 20 in the same run whatever the test item (for positive control, negative control, test substances, and all adapted controls). The WG notes that this is probably also applicable to the HPLC/UPLC-spectrophotometry measurements.

For the purposes of the Prediction Model tissues treated with chemicals inducing eye irritation or serious eye damage (UN GHS/EU CLP Category 2 or Category 1, respectively) were expected to show a decrease in tissue viability below a specified threshold in comparison to the negative control. See table below.

Table 5.1.1. Cell viability values used as threshold for the assessment of the eye irritation or serious eye damage (UN GHS/EU CLP) caused by chemicals.

Cell viability (liquids)	Cell viability (solids)	UN GHS / EU CLP classification
> 60 %	> 50 %	No Category
≤ 60 %	≤ 50 %	Categories 1 and 2

Results are expressed as mean OD, mean percentage viability and difference of viability between the two replicate tissues.

The histological features of the test system, and the use of similar endpoints and endpoint detection systems as used within other RhT systems already validated for other toxicological endpoints, make it plausible that this test RhCE system may provide insights into the likely eye damage/irritation potential of chemicals.

Although derived from cultured human corneal cells, ESAC WG notes that the cells used have been transformed and immortalised. The ESAC WG offers no opinion on whether these cells are inherently more biologically relevant in this context than other epithelial cells (e.g. eye irritation RhCE models derived from normal untransformed human keratinocytes).

5.2 Quality and completeness of the documentation concerning SOPs and prediction models

The SOPs, including the prediction model, were sufficiently detailed. See section 5.1 above.

6. Test materials

6.1 Sufficiency of the number of evaluated test items in view of the study objective

The WG believes that the number of test items was sufficient to draw meaningful conclusions with respect to the objectives of the study. See 2.1 above.

6.2 Representativeness of the test items with respect to applicability

The WG believes that the number and nature of the test items selected were adequate to provide insights into the applicability domain and the scope and limitations of the test method. See 2.1 above.

7. Within-laboratory reproducibility (WLR) (Module 2)

7.1 Assessment of repeatability and reproducibility in the same laboratory

For liquid test items

The reliability of the SkinEthic™ HCE EITL protocol was assessed in terms of concordance in predictions for three independent experiments.

The resulting WLR was 95 % (95 %-CI: 86.3 % - 98.3 %) for L'Oréal, 93.3 % (95 %-CI: 84.1 % - 97.4 %) for VITO, and 88.3 % (95 %-CI: 77.8 % - 94.2 %) for CRL. In summary, WLR ≥ 88 % between the independent runs was observed within the laboratories, exceeding the minimum acceptable value of ≥ 85 % set by the VMG.

In addition, the lead laboratory (L'Oréal) prospectively tested 45 additional liquid chemicals in three independent experiments. Twenty-two chemicals did not require classification in vivo and 23 chemicals were classified. Concordant prediction was obtained for 41 of the 45 chemicals, resulting in a WLR of 91.1 %, exceeding the minimum values set by the VMG.

For solid test items

The reliability of the SkinEthic™ HCE EITS protocol was assessed in terms of concordance in predictions for the independent valid runs. The WLR was 96.7 % (95 %-CI: 88.6 % - 99.1 %) for L'Oréal and 95.0 % (95 %-CI: 86.3 % - 98.3 %) for VITO and CRL.

In conclusion, low variation (WLR ≥ 95 %) between the independent runs was observed within the laboratories, indicating that the SkinEthic™ HCE EITS protocol is robust. This means that the WLR is higher than 95 %, exceeding the minimum value (85 %) set by the EIVS VMG (Barroso *et al*., 2015; EURL ECVAM, 2016).

The lead laboratory (L'Oréal) prospectively tested 35 additional solid chemicals in three independent runs. Twenty-three chemicals did not require classification *in vivo* and 12 chemicals were classified. A concordant prediction was obtained for 34 of the 35 chemicals, resulting in a WLR of 97.1 % exceeding the minimum values set by the VMG.

7.2 Conclusion on within-laboratory reproducibility as assessed by the study

Section 7.1 confirms that the target value set by the EIVS VMG was exceeded by all laboratories in the ring trial and further prospective testing undertaken by L'Oréal.

8. Transferability (Module 3)

8.1 Quality of design and analysis of the transfer phase

The two naïve laboratories participating in the validation of SkinEthic™ HCE received two days training by the lead laboratory (L'Oréal).

Training and proficiency testing was done with coded test chemicals (9 solids, and 9 liquids), these included colourants and direct MTT reducers.

The transfer phase included demonstrating proficiency with the SOP, and completion of the Excel spreadsheets. As confirmed by the data, both laboratories demonstrated their proficiency in performing the SkinEthic™ HCE EIT method for the Eye Irritation Testing of Liquids (EITL protocol) and Solids (EITS protocol). Only one test chemical did not produce concordant results in all cases.

Accordingly, the ESAC WG considers that the technicians from the participating laboratories demonstrated adequate proficiency in performing the SkinEthic™ HCE and readiness to enter the formal validation study.

8.2 Conclusion on transferability to a naïve laboratory / naïve laboratories as assessed by the study

The ESAC WG considers that the technicians from the participating laboratories demonstrated adequate proficiency in performing the SkinEthic™ HCE and readiness to enter the formal validation study.

9. Between-laboratory reproducibility (BLR) (Module 4)

9.1 Assessment of reproducibility in different laboratories

For each laboratory, the mean tissue viability and standard deviation over the three independent valid runs were calculated to obtain a final classification for each chemical. The evaluation of BLR was on the concordance of the final predictions Classified (C) or Not Classified (NC). BLR was reported with the Wilson 95 %-CI.

The minimum acceptable BLR value set by the EIVS VMG was ≥ 80 %.

Assessment of BLR for Liquids:

Fifty-six of the 60 liquid chemicals were consistently classified (NC/C) by the three laboratories resulting in a BLR of 93.3 % (95 %-CI: 84.1 % - 97.4 %).

The BLR for the pair-wise comparisons was 93.3 % (56/60 chemicals) for L'Oréal and CRL, 95.0 % (57/60 chemicals) for L'Oréal and VITO, and 98.3 % (59/60 chemicals) for CRL and VITO.

Assessment of BLR for Solids:

Fifty-eight of the 60 solid chemicals were consistently classified (NC/C) by the three laboratories resulting in a BLR of 96.7 % (95 %-CI: 88.6 % - 99.1 %).

The BLR for the pair-wise comparisons was 96.7 % (58/60 chemicals) for L'Oréal and CRL and for L'Oréal and VITO, a 100 % concordance was obtained between CRL and VITO.

9.2 Conclusion on between-laboratory reproducibility as assessed by the study

The overall BLR for the SkinEthic™ HCE EIT method, based on the result of the ring trial with a balanced and representative set of 120 chemicals, was 95.0 % (EITL 93.3 % and EITS 96.7 %). The BLR of the SkinEthic™ HCE EIT test method was higher than the defined minimum value of 80 % set by the EIVS VMG (Barroso *et al*., 2015; EURL ECVAM, 2016).

10. Predictive capacity and overall relevance (Module 5)

10.1 Adequacy of the assessment of the predictive capacity in view of the purpose

The predictive capacity of the assay was evaluated by comparing the prediction results based on the individual laboratory results with the existing proposed classification. Two-by-Two contingency tables (C versus NC) were constructed and sensitivity, specificity, and accuracy were calculated.

Bootstrap resampling (10,000 times with sample size = 1) was used to obtain 95 %-CIs for accuracy, sensitivity and specificity. The rationale for performing bootstrap resampling with size n=1 was the fact that in reality a chemical will be tested and classified only once.

Sensitivity, specificity, and accuracy were calculated on 10,000 simulated sets of 120 chemicals (60 liquids and 60 solids), based on observed predictions (9 predictions per chemical). Random sampling with sample size n=1 was performed per chemical (with a pool of 9 predictions, based on 3 independent runs for each chemical at each of the 3 laboratories) for the set of 60 liquids or 60 solid chemicals.

Accuracy, sensitivity and specificity were calculated for each of the 10,000 resampling sets. The mean of the bootstrap sample and 95 %-CI applying the percentile method was calculated for the three performance parameters.

Reference data were use appropriately and the resulting data sets were adequate to assess test method performance.

Assessment of predictive capacity for liquid chemicals:

Predictive capacity was calculated for each laboratory and for the cumulative results of the three laboratories using the cut-off of 60 % viability to distinguish between chemicals not requiring classification for serious eye damage/eye irritancy (No Cat) from chemicals requiring classification and labelling (Cat 1 and Cat 2) according to UN GHS (UN, 2015) (Table 10.1.1 below).

Table 10.1.1. Predictive capacity for the set of 60 liquid chemicals based on individual laboratory predictions: overall and for each laboratory.

In vivo UN GHS	Cumulative		L'Oréal		Charles River laboratories		VITO	
	I	NI	I	NI	I	NI	I	NI
Classified (n)	**283**	5	**96**	0	**94**	2	**93**	3
No Category (n)	77	**175**	29	**55**	23	**61**	25	**59**
Total (n)	540		180		180		180	
Sensitivity (%)	98.3		100		97.9		96.9	
Specificity (%)	69.4		65.5		72.6		70.2	
Accuracy (%)	84.8		83.9		86.1		84.4	

The predictive capacity was also determined for the extended dataset (60 liquid chemicals of the multicenter study and 45 additional liquid chemicals) tested by the lead laboratory. This resulted in an overall accuracy of 83.5 % with 100 % sensitivity and 65.3 % specificity for L'Oréal only.

The bootstrap resampling yielded a sensitivity of 98.2 % (95 %-CI: 93.8 % - 100 %), a specificity of 69.4 % (95 %-CI: 60.7 % - 75.0 %), and an accuracy of 84.8 % (95 %-CI: 80.8 % - 88.3 %).

Assessment of predictive capacity for solid chemicals:

The predictive capacity was calculated for each laboratory and for the cumulative results of the three laboratories using the cut-off of 50 % viability to distinguish NC (No Cat) from chemicals requiring classification and labelling (Cat 1 and Cat 2) according to UN GHS (Table 10.1.2). The calculations were based on the individual predictions derived from the qualified tests for each chemical in each laboratory. The three laboratories obtained a sensitivity of 92.2 %. The specificity varied between 75.3 % (VITO), 75.6 % (L'Oréal), and 78.9 % (CRL). An accuracy of 83.9 %, 85.6 %, and 83.3 % was obtained by L'Oréal, CRL, and VITO, respectively (See table 10.1.2 below)

Table 10.1.2. Predictive capacity for the set of 60 solid chemicals based on individual laboratory predictions: overall and for each laboratory

In vivo UN GHS	Cumulative		L'Oréal		CRL		VITO	
	C	NC	C	NC	C	NC	C	NC
Classified (n)	**249**	21	**83**	7	**83**	7	**83**	7
No Category (n)	63	**206**	22	**68**	19	**71**	22	**67**
Total (n)	539		180		180		179 [a]	
Sensitivity (%)	92.2		92.2		92.2		92.2	
Specificity (%)	76.6		75.6		78.9		75.3	
Accuracy (%)	84.4		83.9		85.6		83.3	

[a] For chemical No. 2 only two valid runs were obtained over the five runs

The predictive capacity was also determined for the extended dataset (60 solid chemicals of the multicenter study and 35 additional solid chemicals) tested by the lead laboratory. This resulted in an overall accuracy of 80.7 % with 89.7 % sensitivity and 73.6 % specificity for L'Oréal only.

The bootstrap resampling yielded a sensitivity of 91.9 % (95 %-CI: 90.0 % - 93.3 %), a specificity of 76.6 % (95 %-CI: 73.3 % - 80.0 %), and an accuracy of 84.3 % (95 %-CI: 81.7 % - 86.7 %).

All of the reported values exceeded the minimum acceptable values as set by the VMG which were a sensitivity of at least 90 %, a specificity of at least 60 %, and an accuracy of at least 75 %. None of the Category 1 chemicals were under-predicted in the majority of the runs across all laboratories. However the working group notes that false negative results were obtained in a total of 2 independent runs at the same laboratory for 1 chemical ([3-(2-Aminoethylamino)propyl]-trimethoxysilane, CASRN 1760-24-3, classified Category 1 in the Draize eye test based on persistent conjunctival and corneal effects on day 21 in the majority of the animals). Therefore this chemical was classified by this laboratory as a No Category. Having investigated this anomaly the test developer reported that due to storage problems at this laboratory there was crystal formation for this chemical during storage with the first run (viability: 25.1 %) giving the correct classification. This first run was performed in the beginning of the experimental phase whereas the second and third runs, which gave the misclassification, were performed at the end of the experimental phase (more than 60 days later). The effect of storage conditions on the stability of this chemical ([3-(2-Aminoethylamino)propyl]trimethoxysilane) was evaluated after the validation study. Indeed, confirmatory work has shown that the tissue viability increased when the container of the chemical was not closed properly. After 14 and 30 days of storage with half open or open lid, mean viability increased above 50 % (51.5 % to 66.3 %). In the two other laboratories, the independent runs for the [3-(2-Aminoethylamino)propyl]-trimethoxysilane were performed within a period of less than 30 days. The WG believes that had this chemical been properly stored then an appropriate classification would have been achieved.

In addition, another chemical (Tetraethylene glycol diacrylate, CASRN 17831-71-9, classified as Category 1 based on iritis that resulted in severe but delayed corneal opacity in the Draize eye test)

was misclassified in 1 run at 1 laboratory – but in this case the overall classification by that laboratory on the basis of three qualified run sequences was irritant in the in vitro assay.

10.2 Overall relevance (biological relevance and accuracy) of the test method in view of the purpose

See section 10.1. The WG believes that the overall relevance of the test method has been satisfactorily demonstrated with a view to it being considered for regulatory use within an integrated approach to testing and assessment (IATA) for the prediction of eye damage/irritation potential of chemicals within its applicability domain.

11. Applicability domain (Module 6)

11.1 Appropriateness of study design to conclude on applicability domain, limitations and exclusions

The validation study was undertaken with a large number of liquid and solid test materials representing a broad range of chemical classes (see 2.1) and a full range of ocular reactivity. However, there is limited information on test method performance with mixtures, and chemicals in the form of gases and aerosols were not assessed in a validation study.

11.2 Quality of the description of applicability domain, limitations, exclusions

As indicated in section 11.1, gases and aerosols were not considered in the validation study.

SkinEthic™ HCE EIT test method is not intended to differentiate between UN GHS Category 1 (serious eye damage) and UN GHS Category 2 (eye irritation). This differentiation will need to be addressed by another tier of a testing strategy (Scott *et al.*, 2010). A chemical that is identified as requiring classification for eye irritation/serious eye damage with SkinEthic™ HCE EIT will thus require additional testing (in vitro and/or in vivo) to establish a definitive classification, using e.g., OECD TG 437 (OECD, 2013a), 438 (OECD, 2013b), 460 (OECD, 2012) or 491 (OECD, 2015a).

12. Performance standards (Module 7)

Performance standards were not addressed by TST but are already available at OECD based on the similar method EpiOcular™ EIT (OECD, 2015c).

12.1 Adequacy of the proposed Essential Test Method Components

Not applicable – see immediately above.

12.2 Adequacy of the Reference Chemicals

Not applicable – see immediately above.

12.3 Adequacy of proposed performance target values

Not applicable – see immediately above.

13. Readiness for standardised use

13.1 Assessment of the readiness for regulatory purposes

The WG believes that the overall relevance and reliability of the test method has been satisfactorily demonstrated with a view to it being considered for regulatory use in a tiered assessment strategy. The test could be applied as a first step in Bottom-Up discrimination of 'non-irritants' (GHS No Category) or as a confirmatory last step in a Top-Down approach, where the priority is to first discriminate chemicals inducing serious eye damage (GHS Category 1). However, the method is not intended to differentiate Category 1 from Category 2 on its own.

13.2 Assessment of the readiness for other uses

The study findings justify the SkinEthic™ HCE EIT method being considered as a means of identifying chemicals do not cause eye damage/irritation in non-regulatory contexts.

13.3 Critical aspects impacting on standardised use

The TST indicates that two training days are required for a naïve laboratory.

All HPLC/UPLC-spectrophotometry systems have to be suitably qualified (FDA, 2001; Alépée *et al.*, 2015).

13.4 Gap analysis

1. The SkinEthic™ HCE EIT test method liquid and solid chemical protocols could form components of future integrated approaches to testing and assessment (IATA) for determining the eye damage/irritation potential of chemicals (Scott *et al.*, 2010). The other components of such a testing strategy and the precise role of these RhCE test methods have yet to be formally defined.
2. As mentioned above the ESAC WG notes that there is limited information on the performance of this test method with chemical mixtures and no information about gases and aerosols.
3. The WG acknowledges that including a wide range of chemical mixtures in validation studies currently raises several problems, e.g. availability of *in vivo* data, selection of test mixtures, and continuity of supply. However, most of the chemicals which have to be classified are mixtures and there is a need to confirm that *in vitro* methods can be used for the classification of chemical mixtures. The ESAC WG recommends the inclusion of a broader range of chemical mixtures in future validation studies, and proposes consideration of the use of reference data available for the classification of mixtures, using the additivity approach recommended by the UN GHS (UN, 2015) as well as the CLP (EC, 2008a), and/or the use of mixtures already assessed and identified as Cat.1, Cat.2, or No Cat.
4. While the SkinEthic™ HCE EIT was not developed to account for all mechanisms associated with eye irritation, the validation study demonstrated that this RhCE test method is able to correctly predict chemicals not requiring classification for serious eye damage/eye irritation independently of the types of ocular effects observed in vivo (i.e., corneal, iridal and conjunctival injuries).
5. The available documentation does not allow an assessment of the test method's performance with mixtures.

6. For RhCE test methods that use cytotoxicity as a surrogate measure of eye damage/irritation potential it is plausible that related test methods could be developed to allow histological and other evaluation of the tissue damage to provide additional insights into the mechanism of injury.
7. There is a lack of information in the TST about inter-laboratory reproducibility of HPLC/UPLC-spectrophotometry analytical method. However, this has been demonstrated for the HPLC/UPLC-spectrophotometry measurement of formazan in isopropanol extracts obtained from other RhTs (ESAC, 2014; Alépée *et al.*, 2015).

14. Other considerations

The ESAC WG notes that due to the variability of individual animal responses within the *in vivo* Draize eye test there is ≥ 12 % probability, if chemicals are retested, of chemicals currently classified as UN GHS Category 2 by the *in vivo* test being identified as UN GHS No Category (Adriaens *et al.*, 2014). As *in vivo* Draize eye test data served as reference data for chemical selection and Predictive Capacity within validation studies, the reported performance of the *in vivo* test should be borne in mind when evaluating the reported performance, and validity, of alternative methods and testing strategies for detecting chemically induced eye damage/irritation.

The reported PC mean values (generally < 5 %) are significantly lower than the 30 % acceptance threshold set out in the SOP. The PC was also generally reproducible with maximum viabilities unequivocally acceptable. The WG suggests that consideration be given to reducing the PC acceptance criterion threshold.

15. Conclusions on the study

15.1 ESAC WG summary of the results and conclusions of the study

1. The SkinEthic™ HCE EIT test method study was generally well designed and conducted, and the TST provides analysis and insights that allows the WG to offer an informed opinion about the performance of the test method.
2. The ESAC WG considers that the SkinEthic™ HCE EIT test method liquid and solid chemical protocols are scientifically valid (reproducible and accurate) in the context of identifying chemicals not requiring classification for serious eye damage/eye irritation under UN GHS.
3. The ESAC WG considers that although a wide range of chemical types, chemical classes, molecular weights, LogP, chemical structures, etc., were tested, no clear additional limitations regarding applicability could be identified beyond those identified in the TST.
4. The ESAC WG has noted that currently there is only a limited range of chemical mixtures available for use as test chemicals within eye damage/irritation validation studies, and would like to see more data presented with respect to the test method performance in the case of chemical mixtures requiring the classification for eye damage/irritation potential.
5. For coloured chemicals interfering too strongly with the MTT-reduction assay an alternative endpoint detection system should be used (e.g., suitably qualified HPLC/UPLC-spectrophotometry). The ESAC WG agrees, and believes that qualified HPLC/UPLC-spectrophotometry endpoint measurement system described in the TST is suitable for this purpose.

15.2 Extent to which study conclusions are justified by the study results alone

All of the ESAC WG conclusions given above can be derived and argued or justified from the TST and supporting documents.

15.3 Extent to which conclusions are plausible in the context of existing information

In reaching its conclusions the ESAC WG has also taken account of the larger body of information and knowledge set out in the technical annexes supplied and the references cited in the study documents. It is on consideration of both the study findings, and that larger body of knowledge of information, that the ESAC WG established and confirmed the plausibility of the conclusions set out above.

16. Recommendations

16.1 General recommendations

The ESAC WG believes that the overall relevance and reliability of the SkinEthic™ HCE EIT has been satisfactorily demonstrated with a view to it being considered for regulatory use in a tiered assessment strategy. The test method could be applied as a first step in Bottom-Up discrimination of 'non-irritants' (GHS No Category) or as a confirmatory last step in a Top-Down approach, where the priority is to first discriminate chemicals inducing serious eye damage (GHS Category 1). However, the method is not intended to differentiate Category 1 from Category 2 on its own.

The validation study results tend to confirm the findings of the EIVS study that suitably qualified HPLC/UPLC-spectrophotometry analytical systems can be used to quantify precipitated MTT-formazan as an alternative endpoint detection system in particular for highly coloured chemicals interfering with the conventional endpoint measurement of MTT-formazan by OD-photometry.

16.2 Specific recommendations (e.g. concerning improvement of SOPs)

The ESAC WG offers the following additional recommendations for consideration.

While the SkinEthic™ HCE EIT was not developed to account for all mechanisms associated with eye irritation, the validation study demonstrated that this RhCE test method is able to correctly predict chemicals not requiring classification for serious eye damage/eye irritation independently of the types of ocular effects observed in vivo (i.e., corneal, iridal and conjunctival injuries).

There may be an option to harmonise the protocols for liquid and solid chemicals in relation to the extraction procedures of formazan, if categorisation of chemicals based on extraction from only under-surface of the tissue interface meets the performance of the different extraction procedures given in the SOP for liquid and solid chemicals.

The ESAC WG recommends insights into test performance with chemical mixtures be obtained by testing mixtures based on their current classification, or based on the known properties of their components.

The ESAC WG believes that potential problems with the reliability of historical Draize eye test data must also be taken into account when evaluating the predictive capacity of alternative test methods and testing strategies.

17. References

- Adriaens E, Barroso J, Eskes C, Hoffmann S, McNamee P, Alépée N, Bessou-Touya S, De Smedt A, De Wever B, Pfannenbecker U, Tailhardat M, Zuang V (2014). Retrospective analysis of the Draize test for serious eye damage/eye irritation: importance of understanding the in vivo endpoints under UN GHS/EU CLP for the development and evaluation of in vitro test methods. *Arch Toxicol* 88: 701-23.
- Alépée N, Barroso J, De Smedt A, De Wever B, Hibatallah J, Klaric M, Mewes KR, Millet M, Pfannenbecker U, Tailhardat M, Templier M, McNamee P (2015). Use of HPLC/UPLC-Spectrophotometry for Detection of MTT Formazan in In Vitro Reconstructed Human Tissue (RhT)-Based Test Methods Employing the MTT Assay to Expand their Applicability to Strongly Coloured Test Chemicals. *Toxicol In Vitro* 29: 741-761.
- Barroso J, Alépée N, Cole T, Eskes C, Freeman SJ, Liška R, McNamee P, Pfannenbecker U, Reus AA, Rubingh CM, Schaeffer MW, Zuang V (2015). EURL ECVAM — Cosmetics Europe prospective validation study of Reconstructed human Tissue-based test methods for serious eye damage/eye irritation testing. Poster Presented at the Congress of the European Societies of Toxicology (EUROTOX), Porto, Portugal, September, 13–16, 2015. Available from:

  http://dx.doi.org/10.1016/j.toxlet.2015.08.507

  https://eurl-ecvam.jrc.ec.europa.eu/posters-1/eurl-ecvam-cosmetics-europe-prospective-validation-study-of-reconstructed-human-tissue-based-test-methods-for-serious-eye-damage-eye-irritation-testing-poster/view
- Barroso J, Pfannenbecker U, Adriaens E, Alépée N, Cluzel M, De Smedt A, Hibatallah J, Klaric M, Mewes KR, Millet M, Templier M, McNamee P (2016). Cosmetics Europe compilation of historical serious eye damage/eye irritation in vivo data analysed by drivers of classification to support the selection of chemicals for development and evaluation of alternative methods/strategies: the Draize eye test Reference Database (DRD). *Arch Toxicol.* In press. doi: 10.1007/s00204-016-1679-x.
- Balls M, Blaauboer BJ, Fentem JH, *et al*. (1995). Practical aspects of the validation of toxicity test procedures. The report and recommendations of the ECVAM Workshop 5. *ATLA* 23: 129-147.
- Bland JM, Altman DG (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **i**: 307-310.
- Draize, J.H., Woodard, G., and Calvery, H.O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exper Therap.* 82: 377-390.
- EC (2008a). Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures (the 'CLP Regulation').
- EC (2008b). COUNCIL REGULATION (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).
- ECETOC (1998). Eye irritation reference chemicals data bank. Technical report n. 48 (2), Brussels, Belgium.
- ESAC (2014). ESAC Opinion No. 2014-03 from 17 November 2014, based on the ESAC Scientific Peer Review of the ECVAM Eye Irritation Validation Study (EIVS) and a related Cosmetics Europe study on HPLC/UPLC-photometry as an alternative endpoint detection system for formazan reaction product.
- Eskes C, Bessou S, Bruner L, *et al*. (2005). Eye irritation. *ATLA* 33: Suppl. 1, 47-81.
- EURL ECVAM (2016). The EURL ECVAM - Cosmetics Europe prospective validation study of Reconstructed human Cornea-like Epithelium (RhCE)-based test methods for identifying chemicals not requiring classification and labelling for serious eye damage/eye irritation: Validation Study Report. EUR 28125 EN; doi:10.2787/41680. Available from:

  http://publications.jrc.ec.europa.eu/repository/handle/JRC100280

- Food and Drug Administration (2001) Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research.
- Gautheron P, Dukic M, Alix D, Sina JF. (1992). Bovine corneal opacity and permeability test: An in vitro assay of ocular irritancy. *Fundam Appl Toxicol* 18: 442-449.
- Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortaner S, Gribaldo L, Halder M, Hoffmann S, Roi AJ, Prieto P, Sabbioni E, Scott L, Worth A, Zuang V (2004). A modular approach to the ECVAM principles on test validity. *ATLA* 32: 467-472.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival:application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63.
- Nguyen DH, Beuerman RW, DeWever B, Rosdy M (2003) Three-dimensional construct of the human corneal epithelium for in vitro toxicology. In: Alternative toxicological methods, 1st ed., chapter 14, pp.147-159, Ed. Salem H and Katz SA, CRC, New York.
- OECD (1999) OECD Series of Good Laboratory Pactice and Compliance Monitoring No. 5. Compliance of Laboratory Suppliers with GLP principles. Paris, France: Organisation for Economic Cooperation and Development. Available at: [http://www.oecd.org/env/testguidelines].
- OECD (2002). Acute Eye Irritation/Corrosion. OECD Guideline for the Testing of Chemicals No. 405, OECD, Paris.
- OECD (2005). Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment, Series on Testing and Assessment, No. 34. OECD, Paris.
- OECD (2008). OECD Environment Health and Safety Publications Series on Testing and Assessment No. 88 WORKSHOP ON INTEGRATED APPROACHES TO TESTING AND ASSESSMENT. OECD, Paris.
- OECD (2012). Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants. OECD Guideline for the Testing of Chemicals No. 460, OECD, Paris.
- OECD (2013a). 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 Guideline for the Testing of Chemicals No. 437, OECD, Paris.
- OECD (2013b). 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 Guideline for the Testing of Chemicals No. 438, OECD, Paris.
- OECD (2015a). 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 Guideline for the Testing of Chemicals No. 491, OECD, Paris.
- OECD (2015b). Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labelling for eye irritation or serious eye damage. OECD Guideline for the Testing of Chemicals No. 492, OECD, Paris.
- OECD (2015c). Performance standards for the assessment of proposed similar or modified *in vitro* reconstructed human cornea-like epithelium (RhCE) test methods for eye hazard. OECD Series on Testing & Assessment No. 216, OECD, Paris.
- Scott L, Eskes C, Hoffmann S, Adriaens E, Alépée N, Bufo M, *et al*. (2010). A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom-Up and Top-Down approaches. *Toxicol In Vitro* 24: 1-9.
- Spielmann H, Liebsch M, Kalweit S, Moldenhauer F, Wirnsberger T, Holzhütter H-G, Schneider B, Glaser S, Gerner I, Pape WJW, Kreiling R, Krauser K, Miltenburger HG, Steiling W, Luepke NP, Müller N, Kreuzer H, Mürmann P, Spengler J, Bertram-Neis E, Siegemund B, Wiebel FJ. (1996). Results of a validation study in Germany on two in vitro alternatives to the Draize eye irritation test, HET-CAM test and the 3T3 NRU cytotoxicity test. *ATLA* 24: 741–858.

- UN (2015). United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Sixth revised edition, UN New York and Geneva.

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