

**ESAC STATEMENT ON THE CONCLUSIONS OF THE ICCVAM RETROSPECTIVE STUDY ON ORGANOTYPIC *IN VITRO* ASSAYS AS SCREENING TESTS TO IDENTIFY POTENTIAL OCULAR CORROSIVES AND SEVERE IRRITANTS AS DETERMINED BY US EPA, EU(R41) AND UN GHS CLASSIFICATIONS IN A TIERED TESTING STRATEGY, AS PART OF A WEIGHT OF EVIDENCE APPROACH**

At its 26th Meeting, held on 26–27 April 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)<sup>1</sup> unanimously endorsed the following statement:

1. With regard to the results and conclusions from the ICCVAM retrospective study<sup>2,3</sup> on:

“Organotypic *in vitro* assays as screening tests to identify potential ocular corrosives and severe irritants as determined by US EPA, EU(R41) and UN GHS classifications in a tiered testing strategy, as part of a weight of evidence approach”

the ESAC endorses the following conclusion:

There are sufficient data to support the use of the Bovine Corneal Opacity and Permeability (BCOP) test method, and the Isolated Chicken Eye (ICE) test method in appropriate circumstances and with certain limitations,<sup>3</sup> as screening tests to identify substances as ocular corrosives and severe irritants in a tiered-testing strategy, as part of a weight-of-evidence approach.

2. With regard to the Isolated Rabbit Eye assay (IRE) and the Hen’s Egg Test–Chorio-Allantoic Membrane assay (HET–CAM), the ESAC recommends that further work should be performed before a statement on their validity can be made.

It should be noted, however, that European authorities previously stated that while all four tests were not yet validated, positive outcomes from these tests could be used as the basis for classifying and labelling substances as severe eye irritants (R41).<sup>4</sup>

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27 April 2007

1. The ESAC was established by the European Commission, and is composed of nominees from the EU Members States, industry, academia and animal welfare, together with representatives of the relevant Commission services.

This statement was endorsed by the following Members of the ESAC:

Ms Sonja Beken (Belgium)  
Ms Dagmar Jírová (Czech Republic)  
Mr Tõnu Püssa (Estonia)  
Mr Lionel Larue (France)  
Mr Manfred Liebsch (Germany)  
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Mr Robert Combes (ESTIV)  
Mr Hasso Seibert (European Science Foundation)

The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself:

Mr Thomas Hartung (ECVAM; chairman)  
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Ms Susanna Louhimies (DG Environment)  
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 Mr Barry Philips (Eurogroup for Animal Welfare)  
 Mr William Stokes (NICEATM, USA)

[ivocutox/ocu\\_brd.htm](http://ivocutox/ocu_brd.htm)

2. "Background Review Documents on *in vitro* test methods for detecting ocular corrosives and severe irritants":
  - Bovine Corneal Opacity and Permeability test;
  - Isolated Rabbit Eye test;
  - Hen's Egg Test-Chorio-Allantoic Membrane test;
  - Isolated Chicken Eye test.
 Website: <http://iccvam.niehs.nih.gov/methods/ocutox/>
3. The "ICCVAM Test Method Evaluation Report on *in vitro* test methods for detecting ocular corrosives and severe irritants". Website: [http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu\\_tmer.htm](http://iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_tmer.htm)
4. EC (2004). *Manual of Decisions for Implementation of the 6th and 7th Amendments to Directive 67/548/EEC on Dangerous Substances. Updated version of July 2004*, 189pp. Ispra, Italy: European Chemicals Bureau, European Commission JRC. Website: [http://ecb.jrc.it/DOCUMENTS/New-Chemicals/Manual\\_of\\_decisions.pdf](http://ecb.jrc.it/DOCUMENTS/New-Chemicals/Manual_of_decisions.pdf)

### ESAC STATEMENT ON THE REDUCED LOCAL LYMPH NODE ASSAY (rLLNA)

At its 26th Meeting, held on 26–27 April 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)<sup>1</sup> unanimously endorsed the following statement:

Skin sensitisation is an important toxicological endpoint with respect to human safety. Having reviewed the final report of the independent peer review evaluation co-ordinated by ICCVAM and NICEATM,<sup>2</sup> the report by the EMEA,<sup>3</sup> the pre-report of the SCCNFP,<sup>4</sup> and evidence made available since the original submissions to ICCVAM in March 2000, the 14th meeting of ESAC stated:

"Following a review of the scientific report and publications on the local lymph node assay (LLNA), it is concluded that the LLNA is a scientifically validated test which can be used to assess the skin sensitisation potential of chemicals. The LLNA should be the preferred method, as it uses fewer animals and causes less pain and distress than the conventional guinea-pig methods. In some instances and for scientific reasons, the conventional methods can be used."

Since its acceptance for regulatory purposes, the LLNA has proved suitable for the purposes of satisfying a range of EU and other regulatory requirements.<sup>5</sup> The developers of the LLNA have now undertaken a retrospective analysis of published data obtained with the LLNA.<sup>6</sup> They conclude that, within a tiered testing strategy in the context of REACH, a "reduced" version of the LLNA (rLLNA), using only a negative control group and the equivalent of the high-dose group from the full LLNA, can be used as a screening test to distinguish between sensitisers and non-sensitisers.

The ESAC established a peer review panel to evaluate whether there was the potential to minimise animal use, by employing the rLLNA as a screening test as part of a tiered-testing strategy for chemicals. Mindful that with the rLLNA:

- When compared with the full LLNA, the rLLNA cannot and will not result in additional false-positives.
- When compared with the full LLNA, the rLLNA may produce a few false-negatives (3:169 in the reference document, reducing to 2:169 when negative results obtained with concentrations of <10% are considered invalid).
- The test results provided by the rLLNA do not allow the determination of the potency of a sensitising chemical.

The ESAC states that the peer reviewed and published information is of a quality and nature to support the use of the rLLNA within tiered-testing strategies, to reliably distinguish between chemicals that are skin sensitisers and non-sensitisers, and that animal use can be minimised, providing that:

- The concentration used to evaluate sensitisation potential is the maximum consistent with solubility and the need to avoid local and other systemic adverse effects, and that this principle, rather than strict adherence to the specific recommended absolute concentrations as in OECD TG 429, should be used.
- Negative test results associated with testing using concentrations of less than 10%, should undergo further evaluation.
- Positive and negative (vehicle) control groups are used, as appropriate, as per OECD TG 429.

— The full LLNA should be performed, when it is known that an assessment of sensitisation potency is required.

The ESAC recommends that further work should be undertaken to determine whether the 10% concentration threshold referenced above is optimal.

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27 April 2007

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## ESAC STATEMENT ON THE VALIDITY OF *IN VITRO* TESTS FOR SKIN IRRITATION

At its 26th meeting, held on 26–27 April 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)<sup>1</sup> unanimously endorsed the following statement:

After a review of scientific reports and peer reviewed publications on the following range of *in vitro* tests, which had been subjected to a full validation study:

1. EpiDerm (with MTT reduction and IL-1 $\alpha$  release); and
2. EPISKIN (with MTT reduction and IL-1 $\alpha$  release);

of these, the EPISKIN method showed evidence of being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction, and for being used as a replacement (*based on the performance of the assay as specified in the annex*) for the Draize Skin Irritation Test (OECD TG 404 and Method B.4 of Annex V to *Directive 67/548/EEC*) for the purposes of distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances. At the present time, the IL-1 $\alpha$  endpoint should be regarded as a useful adjunct to the MTT assay, as it has the potential to increase the sensitivity of the test, without reducing its specificity. This endpoint could be used to confirm negatives obtained with the MTT endpoint.

At this time, due to its high specificity, the EpiDerm model reliably identifies skin irritants, but negative results may require further testing (e.g. according to the tiered strategy, as described in the OECD TG 404). Improvement of the EpiDerm protocol should be made to increase the level of sensitivity. This endorsement takes account of the dossiers prepared for peer review; the views of independent experts who evaluated the dossiers against defined validation criteria; supplementary submissions made by the Management Team; and the considered view of the Peer Review Panel appointed to oversee the process.

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Mr William Stokes (NICEATM, USA)

## Annex

### General information on the ECVAM skin irritation validation study

After extensive optimisation and prevalidation activities (see background to the SIVS here below), ECVAM launched a formal validation study on three *in vitro* test systems in 2003. Two of the assays employed reconstituted human epidermis models (EPISKIN, EpiDerm) and one, the skin integrity function test (SIFT), employed *ex vivo* mouse skin. The aim of the study was to replace the regulatory Draize skin irritation test (EU B.4 method; OECD TG 404) currently performed on albino rabbits by assessing the relevance (predictive capacity) and reliability (reproducibility within and between laboratories) of these test systems with a set of 58 coded test chemicals. The goal of the study was to evaluate whether the *in vitro* tests would predict *in vivo* classification according to the EU classification system, using the risk phrase R38 for skin irritants and no classification for non-irritants. In addition, the chemical selection was representative for the three categories (strong [category 2], mild [category 3] and non-irritants [no category]) of the Globally Harmonised Classification System (GHS) for permitting a *post-hoc* evaluation of the results according to the GHS. The validation study was conducted according to the principles and criteria documented in the OECD *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (No. 34). Furthermore, to ensure a high quality of the commercially produced human skin models, the facilities of the producers of the human skin models EPISKIN and EpiDerm were evaluated by independent auditors at the beginning of the ECVAM Skin Irritation Validation Study (SIVS). The study was sponsored by ECVAM, coordinated by a main contractor (ZEBET-BfR, Germany) and managed by a Management Team (MT; see Table 1 for the composition of MT).

A Chemicals Selection Sub-Committee (CSSC) was appointed to identify test chemicals to be used in the SIVS, having high quality existing *in vivo* data with which to correlate the *in vitro* measurements. Since chemicals from the European Centre for the Ecotoxicology and Toxicology of Chemicals (ECETOC) database of reference chemicals for skin irritation/skin corrosion had been extensively used in the preceding studies, the CSSC was requested to make use of novel sources for potential test chemicals. For this purpose, chemicals were selected from the New Chemicals Database (NCD), which is the central archive within the EU notification scheme for new commercial chemicals. In addition, existing

chemicals readily available from major manufacturing and/or distribution sources were selected from alternative databases such as the Toxic Substance Control Act (TSCA) database maintained by the US Environmental Protection Agency (EPA) and the ECETOC database, excluding those chemicals which had been used in the previous optimisation and prevalidation phases.

**Table 1: Composition of the Management Team of the SIVS**

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Chair (Dr Phil Botham)  
 Co-chair (Dr Julia Fentem)  
 Sponsor representative (Dr Valérie Zuang, *alternate*: Dr Chantra Eskes)  
 Independent biostatistician (Dr Sebastian Hoffmann)  
 Representative of the main contractor (Dr Horst Spielmann)  
 Representative of the CSSC (Dr Andrew Worth)  
 ECB customer (Dr Thomas Cole)

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*Representatives of the test systems:*  
 EPISKIN (Dr Roland Roguet)  
 EpiDerm (Dr Manfred Liebsch)  
 SIFT (Dr Jon Heylings)

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*Observers from the USA:*  
 ICCVAM (Dr Karen Hamernik, *alternate*: Dr Abby Jacobs)  
 NICEATM (Dr William Stokes; *alternate*: Dr Ray Tice)

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A total set of 58 chemicals, comprising a set of 25 existing chemicals and 33 chemicals from the NCD, were selected and tested in the SIVS. The selected chemicals: a) represented statistically justified sample sizes for distinguishing R38 from non-classified chemicals, b) provided a balanced representation of the three GHS categories, to allow for *post-hoc* evaluation of the performance of the assays for that classification system, and c) accounted as far as possible for the large prevalence known to exist for chemicals which have oedema and erythema scores of 0. These chemicals were independently coded and distributed to the participating laboratories. The selected chemicals presented a variety of molecular structures, functional chemical groups, and effect and use categories, as well as a wide range of physical-chemical properties. They represented a challenging set of chemicals relevant to current industrial commerce for the alternative methods being validated.

In phase 1 of the ECVAM SIVS, 20 chemicals (9 irritant, 11 non-irritant) from the NCD were tested under blind conditions in the lead laboratories (EPISKIN — L'Oréal; EpiDerm — ZEBET; SIFT — Syngenta). The methods applied (with Standard

Operating Procedures [SOPs]) were the refined, optimised protocols developed after the ECVAM prevalidation study. For the human skin model assays, this consisted of applying the test chemicals to the surface of the skin for 15 minutes, followed by a post-treatment incubation period of 42 hours, and the subsequent assessment of their effects on cell viability by using the MTT assay.

The prediction model related to MTT used in Phases 1 and 2 was the following:

“The test substance is considered to be irritating to skin (R38), if the tissue viability after exposure and post incubation is less than or equal ( $\leq$ ) to 50%.”

When cell viability (MTT reduction) was used as the endpoint, the two skin models met the acceptance criteria set by the MT of the study. While the specificity (correct prediction of non-irritants) of both the EPISKIN and EpiDerm assays was 91%, their sensitivities (correct prediction of R38 irritants) were 67% and 56%, respectively. However, since almost all of the misclassified chemicals were lying at the threshold between irritant and non-irritant chemicals according to the EU classification scheme, the MT concluded that the predictive capacity of both Epiderm and EPISKIN was sufficient to justify that they proceed to Phase 2. On the other hand, the predictive capacity of the SIFT method was considered inadequate. For SIFT, it was suggested that the lead laboratory should re-evaluate the test protocol and prediction model, particularly in relation to the manner in which solids and non-surfactant materials are handled.

In Phase 2, all 58 chemicals were assessed in three different laboratories for each of the two reconstituted human skin methods. The EpiDerm test was conducted in the following laboratories: ZEBET (lead laboratory), Germany; Institute for *In Vitro* Sciences (IIVS), USA; and BASF, Germany. The EPISKIN test was conducted in the following laboratories: L'Oréal (lead laboratory), France; Unilever, UK; and Sanofi-Synthelabo, France. Chemicals were re-coded from Phase 1 to ensure blind testing. The main endpoint measured for both Epiderm and EPISKIN was cell viability, measured by MTT reduction, as used in all previous testing. However, a second endpoint, interleukin-1 $\alpha$  release, was added for those chemicals which did not reduce cell viability below the threshold for predicting irritancy, to determine whether it could be used to improve the sensitivity of the assays. This second endpoint was used in all three laboratories assessing EPISKIN and by the lead laboratory for Epiderm. The prediction model related to the combined use of MTT and IL-1 $\alpha$  release in Phase 2, was the following:

“The test substance is considered to be *irritant* to skin, if the viability after 15 minutes of exposure

and 42 hours of post-treatment incubation is more (>) than 50%, and the amount of IL-1 $\alpha$  release is more (>) than 60pg/ml.”

“The test substance is considered to be non-irritant to skin, if the viability after 15 minutes of exposure and 42 hours of post-treatment incubation is more (>) than 50%, and the amount of IL-1 $\alpha$  release is less than or equal ( $\leq$ ) to 60pg/ml.”

The predictive capacities of the assays in this second phase are shown in Table 2. The within-laboratory reproducibility of classifications over three independent experiments meeting the acceptance criteria was 93.9% for EPISKIN (MTT) and 96.0% for EpiDerm (MTT). The between-laboratory reproducibility, measured as the proportion of identical median classifications between laboratories, was 89.5% for EPISKIN (MTT) and 88.5% for EpiDerm.

**Table 2: Predictive capacities of EPISKIN and EpiDerm**

	EPISKIN (MTT)	EPISKIN (MTT+IL-1 $\alpha$ )	EpiDerm (MTT)*
Sensitivity	74.7%	90.7%	57.3%
Specificity	80.8%	78.8%	83.8%
Concordance/ Accuracy	78.2%	83.0%	72.4%

*MTT: based on the median classification per laboratory; MTT+IL-1 $\alpha$ : based on the classification derived from the mean viability of the independent experiments per chemical and laboratory. \*The addition of IL-1 $\alpha$  to the EpiDerm protocol gave no improvement to the outcome.*

The study was forwarded to the ECVAM Scientific Advisory Committee (ESAC), with a proposal that EPISKIN could be considered as a replacement for the rabbit skin irritation method, and Epiderm as a constituent of a testing strategy.

### Background to the ECVAM skin irritation validation study

In 1998, the ECVAM Skin Irritation Task Force published a report on the actual status of *in vitro* skin irritation testing, and proposed 10 “challenge chemicals” for which promising, concordant *in vivo* data from the rabbit test, *in vivo* data from the 4-hour human patch test, and *in vitro* data from the human skin model, EpiDerm, were available. Proponents of new *in vitro* test systems were encouraged to submit data obtained with new *in vitro* skin irritation test protocols for these chemi-

cals (1), for assessment as to whether these tests could be considered in an ECVAM prevalidation study. At the same time, the suitability of various endpoints for prediction of human skin irritation was evaluated in an EU 4th Framework Programme collaborative project on several human reconstructed skin models, revealing cell viability reduction (MTT reduction) and IL-1 $\alpha$  release to be the most promising endpoints. Because MTT reduction and IL-1 $\alpha$  release showed a high inter-correlation, and IL-1 $\alpha$  release was more variable, MTT reduction was proposed to be the best endpoint for human skin models (2).

Of the test systems for which data were submitted to the ECVAM TF, five tests (perfused pig-ear, Prediskin, SIFT, EPISKIN, EpiDerm) had been considered promising for participation in the ECVAM prevalidation study. However, during the prevalidation study, two tests failed in phase 2 due to insufficient reproducibility, whereas the other tests (SIFT, EPISKIN and EpiDerm) showed a sufficient intra-laboratory and inter-laboratory reproducibility, but failed in their ability to correctly predict the skin irritation potential of 20 chemicals that were tested in phase 3 of the ECVAM prevalidation study (3). The ECVAM Management Team of the study therefore proposed the refinement and optimisation of these three tests before considering them for formal validation.

In 2001, the ECVAM Skin Irritation Task Force and the laboratories responsible for the refinement of the tests met again, and discussed ways forward to approach formal validation. In addition, since a *post-hoc* analysis of prevalidation data for MTT reduction for EPISKIN and EpiDerm revealed similar sensitivities, it was recommended to develop a common test protocol for both skin models before the start of a formal validation study (4).

In November 2002, the ECVAM Skin Irritation Task Force (TF) discussed the refinements of the SIFT (5) and the skin model tests (6), and came to the conclusion that processing the tests to formal validation could be recommended. However, because all the refinements were made by using the 20 chemicals from the prevalidation study, the TF recommended that the SIVS be performed in two phases: a first phase (phase 1) for the confirmation of the refinements made by the lead laboratories (Syngenta [SIFT], L'Oréal [EPISKIN], and ZEBET [EpiDerm]), by testing new chemicals in a controlled way under blind conditions. If the outcome of phase 1 were still promising, the tests would then proceed to a second phase (phase 2), i.e. to a blind trial involving three laboratories per test.

During 2003, the EPISKIN test was further refined by L'Oréal, by extending the post-incubation period of the tissues (after a 15-minute chemical exposure) to 42 hours, which allowed significant effects to increase, and recovery from weak effects.

In May 2003, an ECVAM Stakeholder Workshop recommended that a formal validation study be conducted, and that work should concentrate on the predictions of the EU classification system (R38 vs. no label), because the tests were developed and optimised for this classification scheme. L'Oréal and ZEBET then collaborated in developing a common test protocol to be used in the ECVAM SIVS, and evaluated it first with the 20 "challenge" chemicals of the ECVAM prevalidation study. In 2004, at the request of the ECVAM SIVS Management Team and in parallel with performing phase 1 of the SIVS, the database was further increased by testing all the non-corrosive chemicals recommended in the ECETOC reference data base (ECETOC Report No. 66). The data obtained in both skin models with the optimised common protocol were very promising, and were published back-to-back in 2005 (7, 8). The BfR was contracted in November 2003, further to the publication of a call for tender for the ECVAM SIVS by the European Commission in June 2003. The study started formally with the 1st Meeting of the SIVS Management Team (MT), on 17–18 November 2003.

Manuscripts on the outcome of the skin irritation validation study, and on the chemicals selection, are currently being finalised for publication.

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## ESAC INTERIM STATEMENT ON THE RETox ASSAY FOR EMBRYOTOXICITY TESTING

At its 26th Meeting, held on 26–27 April 2007 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the non-Commission members of the ECVAM Scientific Advisory Committee (ESAC)<sup>1</sup> unanimously endorsed the following statement:

On the basis of the available data submitted for peer review, the ESAC has not endorsed the scientific validity of the RETox assay.

Thomas Hartung  
Head of Unit  
ECVAM  
Institute for Health & Consumer Protection  
Joint Research Centre  
European Commission  
Ispra

27 April 2007

The ESAC was established by the European Commission, and is composed of nominees from the EU Members States, industry, academia and animal welfare, together with representatives of the relevant Commission services.

This statement was endorsed by the following members of the ESAC:

Ms Sonja Beken (Belgium)  
Ms Dagmar Jírová (Czech Republic)  
Mr Tõnu Püssa (Estonia)  
Mr Lionel Larue (France)  
Mr Manfred Liebsch (Germany)  
Ms Annalaura Stamatì (Italy)  
Mr Jan van der Valk (The Netherlands)  
Mr Constantin Mircioiu (Romania)  
Mr Albert Breier (Slovakia)  
Ms Argelia Castaño (Spain)  
Mr Patric Amcoff (Sweden)  
Mr Jon Richmond (UK)

Mr Carl Westmoreland (COLIPA)  
Ms Vera Rogiers (ECOPA)  
Ms Nathalie Alépée (EFPIA)  
Mr Robert Combes (ESTIV)  
Mr Hasso Seibert (European Science Foundation)

The following Commission Services and Observer Organisations were involved in the consultation process, but not in the endorsement process itself:

Mr Thomas Hartung (ECVAM; chairman)  
Mr Jens Linge (ECVAM; ESAC secretary)  
Ms Elke Anklam (Director of IHCP)  
Ms Susanna Louhimies (DG Environment)  
Ms Barbara Mentré (DG ENTR)  
Ms Grace Patlewicz (ECB, DG JRC)  
Mr Christian Wimmer (DG Research)  
Mr Hajime Kojima (JACVAM)  
Ms Laurence Musset (OECD)  
Mr Barry Philips (Eurogroup for Animal Welfare)  
Mr William Stokes (NICEATM, USA)