EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
European Centre for the Validation of Alternative Methods (ECVAM)

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

At its 26th meeting, held on 26-27th 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)[1] 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-1α release);
2. EPISKIN (with MTT reduction and IL-1α 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 & 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-1α 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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Ispra

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)
Ms Annalaura Stammati (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 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)

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 if 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 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).

Table 1. Composition of the Management Team of the SIVS

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)
<u>Representatives of the test systems:</u>
EPISKIN (Dr Roland Roguet)
EpiDerm (Dr Manfred Liebsch)
SIFT (Dr Jon Heylings)
<u>Observers from the US:</u>
ICCVAM (Dr Karen Hamernik; *alternate*: Dr Abby Jacobs)
NICEATM (Dr William Stokes; *alternate*: Dr Ray Tice)

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 used in the previous optimisation and prevalidation phases.

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 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, 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 NCD were tested under blind conditions in the lead laboratories (EPISKIN - L'Oreal, EpiDerm - ZEBET, SIFT - Syngenta). The methods applied (with Standard Operating Procedures, SOP's) were the refined, optimised protocols developed after the ECVAM prevalidation study. For the human skin model assays this consisted in 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 or equal (≤) to 50%".

When cell viability (MTT reduction) was used as 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 sensitivity (correct prediction of R38 irritants) was 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 classifcation scheme, the MT concluded that the predictive capacity of both Epiderm and EPISKIN was sufficient to justify them to 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 re-evaluated 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 lab), Germany; Institute for *In vitro* Sciences (IIVS), USA; and BASF, Germany. The EPISKIN test was conducted in the following laboratories: L'Oréal (lead lab), France; Unilever, UK; and Sanofi-Synthélabo, 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-1α release, was added for those chemicals which did not reduce cell viability below the threshold for predicting irritancy, to determine if 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-1α 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-1α 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-1α release is less or equal (≤) 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 (MTT: based on the median classification per laboratory; MTT+IL1α: based on the classification derived from the mean viability of the independent experiments per chemical and laboratory)

	EPISKIN (MTT)	*EPISKIN (MTT+IL1-α)*	*EpiDerm (MTT)**
Sensitivity	**74.7%**	**90.7%**	57.3%
Specificity	**80.8%**	**78.8%**	83.8%
Concordance/Accuracy	**78.2%**	**83.0%**	72.4%

*The addition of IL-1α 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 4hr 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 chemicals (1) for assessment 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 collaborative project in several human reconstructed skin models, revealing cell viability reduction (MTT reduction) and IL-1α release the most promising endpoints. Because MTT reduction and IL-1α release showed a high inter-correlation, and IL-1α 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 already in phase 2 due to insufficient reproducibility, whereas the other tests [SIFT, EPISKIN and EpiDerm] showed a sufficient intra- and interlaboratory 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 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 sensitivity, 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 refinements were made using the 20 chemicals from the prevalidation study, the TF recommended to perform the SIVS in two phases: a first phase (phase 1) for the confirmation of the refinements made by the leading labs 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 proceed to a second phase (phase 2), i.e. in a blind trial involving three laboratories per test.
During 2003, the EPISKIN test was further refined by L'OREAL by extending the post incubation period of the tissues (after 15 min chemical exposure) to 42 hours which allowed significant effects to increase, and recovery from weak effects.
In May 2003, an ECVAM Stakeholder Workshop recommended to conduct a formal validation study and to 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 collaborated then 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, upon request of the ECVAM SIVS Management Team and in parallel to performing phase 1 of the SIVS, the database was further increased by testing all 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 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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