more detail. The Secretariat will consult with the US after the meeting for arrangements of a meeting preferably sometime in late February to late March 2009.

17. Hajime Kojima (JaCVAM) gave a presentation on Japanese in vitro skin irritation assays. A number of skin models like EpiDerm™, and others are readily available in Japan and several are under development or validation. The LabCyte™: epidermal skin model is under validation with the 20 reference chemicals from the ECVAM Performance Standards document, with the exemption of no 13, which is not available in Japan. The validation study is scheduled to end in December 2008 and the peer review will be coordinated by JaCVAM and will be initiated in the 2nd quarter of 2009.

REVISION OF THE DRAFT TEST GUIDELINE

18. The meeting participants were asked to go through the recent comments received from the EC, US, Sweden and Denmark and make the appropriate changes to the draft Test Guideline. Karen emphasised that before a paragraph by paragraph revision is started, a sentence should be added as a footnote to the first page of the draft TG to clarify the US Position, stating that: *"The US can not endorse a draft test guideline or annexes at this time and will need additional time to review any supporting materials and any proposals for its content, language, and utility with appropriate stakeholders"*.

19. Juan Riego Sintes (EC) explained the Commission deadlines. To be able to circulate the draft TG to the EU national coordinators for approval, he needs to circulate the draft TG within days after the meeting. The expected submission to the inter-commission services should be accomplished by mid/late November, this to meet the deadline of the re-election and closing of the EP by March 2009.

20. The meeting participants started going through the draft in a paragraph-wise manner making changes where appropriate, as suggested by the EC (for a clean version of the draft Test Guideline please see annex 3).

21. A crucial point of discussion was again the exact language in the first ten paragraphs of the draft TG regarding the proposed use of the TG and it was agreed that the text should include that the TG could be used as a stand alone *in vitro* test replacing the *in vivo* rabbit test, as a screen, or as a part of a sequential testing strategy in a weight-of-evidence approach. It was also agreed that the text should state that regulatory requirements and needs in member countries will decide the exact role (stand alsone sceen or part of a tiered testing strategy) of this TG.

22. Another issue that was extensively discussed was the classification of chemicals in accordance with UN GHS category 2. It was agreed that the TG should mirror that it only provide means for classification of skin irritation GHS sub-category 2, but there should be no restrictions for member countries to do additional testing if they require also for the classification of sub-category 3.

23. On the question of testing of constructs from a wide range of genetic backgrounds for the issue of idiosyncratic populations, the meeting agreed this was out of the scope of the TG.

24. Performance standards developed for the SIVS should be clearly reflected in the annex 2 of the draft TG. This section was considerably revised since information was missing from meeting document No. 4.4. It was evident that three chemicals will be reclassified and this will result in a less balanced set of reference chemicals from 10/10 (classified cat.2/non-classified) to 7/13. The meeting agreed that 3 new classified irritants should be added and 3 old non-classified chemicals to be taken out. This would also result in new predictive values. In addition the list of proficiency chemicals will have to be decided.

FOLLOW-UP

25. Since time did not allow the meeting to conclude the discussion on the performance standard section in the draft Test Guideline, the EC would continue the revision of the draft TG and circulate a revised new version to the expert group within days after the meeting. The major tasks were to revise the Performance Standard Section:

- Adding and removal of three chemicals in table 2 of annex 2;
- a new list of Proficiency Chemicals;
- revised Essential Test Components, and;
- a new table 3 on Predictive values.

26. The Secretariat will incorporate these technical changes into the draft Test Guideline.

27. The Secretariat thanked participants for a very productive meeting and acknowledged Horst Spielman for co-chairing the meeting and BfR as the hosting institute.

28. The meeting report will be circulated to participants for a very short turn-over time and will have a clean version of the draft Test Guideline attached as annex 3, including the technical changes from the EC and the secretariat's edits. Participants will get a short turn-over time before the draft TG will be sent to the WNT for comments.

ANNEX 1

OECD EXPERT CONSULTATION MEETING ON SKIN IRRITATION

20-21 October, 2008
Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, 12277 Berlin-Marienfelde, Germany

DRAFT AGENDA

Monday 20th October		
09h00-09h05	**Opening of the Meeting, Welcoming Note on Behalf of the Hosting Institute, BfR and of the Project lead (EC)**	
09h05-09h10	**Welcoming note on behalf of the OECD Secretariat and a brief Explanation of OECD Procedures** There will be a brief explanation of the role of the members, the status of documents and other general procedures. Listing of Meeting documents.	
09h10-09h15	**Approval of the Draft Agenda**	
09h15-09h20	**Introduction of Participants to the meeting**	*Draft list of participants*
09h20-09h50	**Presentations on the validation of the EpiDerm™ and EpiSkin™** - Valérie Zuang (EC) will present the ECVAM Skin Irritation validation Study (SIVS)	
09h50-10h30	**Presentations on the validation of the EpiDerm™ and EpiSkin™** - Manfred Liebsch (BfR) will present the EpiDerm™ optimisation study - Nathalie Alepee (L'Oreal) will present the SkinEthic™ "me-too" development study	

10h30-11h00	COFFEE/TEA BREAK	
11h00-12h00	**Discussion on the performance of the three HRE Model tests - EpiDerm™, EpiSkin™ and SkinEthic™** - Elke Genschow will present recalculated performance data	
12h00-13h00	LUNCH BREAK	
13h00-14h45	**Discussion on the performance of the three HRE Model tests - EpiDerm™, EpiSkin™ and SkinEthic™**	
14h45-15h15	COFFEE/TEA BREAK	
15h15-17h30	**Revision of the draft Test Guideline** The revised draft Test Guideline will be presented and further discussed	
17h30-18h00	**Presentation on the Japanese Validation Study on Skin Irritation** - Hajime Kojima (JaCVAM)	
18h00	**ADJOURN FOR THE DAY**	
18h00-21h00	***DINNER WILL BE ARRANGED FOR INTERESTED PARTIES***	
Tuesday 21th October		
09h00-09h10	**Opening remarks by the Secretariat**	
09h10-10h30	**Discussions on the performance of the HRE model tests, if required**	
10h30-11h00	COFFEE/TEA BREAK	
11h00-12h00	*Test Guideline revision*	
12h00-13h00	LUNCH BREAK	
13h00-14h45	*Test Guideline revision*	
14h45-15h15	*COFFEE/TEA BREAK*	

15h15-17h30	*Concluding discussions*	
17h30-18h00	*Commitments and further work (if found necessary!)*	
18h00	ADJOURN FOR THE DAY	

Meeting and Background Documents (Available at: http://webdomino1.oecd.org/comnet/env/wg-nctg.nsf/viewHtml/htm/$FILE/index.htm; username: <<toxicity>>; password: <<testing>>	
Meeting Document #1	**1.1.** Draft Test Guideline on "In Vitro Skin Irritation: Human Skin Model Test", June 2008-10-13 1.2. Revised draft Test Guideline, October 15 2008
Meeting Document #2	Compilation of comments from Czech Republic, France, Germany, Japan, Sweden, UK, BIAC and ICAPO, after the last circulation of TG433 to WNT. *Responses provided by the Commission.*
Meeting Document #3	Compilation of comments from the US after the last circulation of TG433 to WNT. *Responses provided by the Commission.*
Meeting Document #4	**SIVS Validation Documents I-VIII** are available at: [http://www.oecd.org/document/55/0,3343,en_2649_34377_2349687_1_1_1_1,00.html#Draft_TG_for_Comment] SIVS I. Validation of the EPISKIN and EpiDerm assays and of the Skin Integrity Function Test for acute Skin Irritation Testing. JRC-ECVAM Contract N° 21323-2003-10F 1ED ISP. Summary Report of the ECVAM Skin Irritation Validation Study (SIVS) SIVS II. Skin Irritation Validation Study Phase II: Analysis of the primary endpoint MTT and the secondary endpoint IL1-α Report from the study biostatistician to the management team ***SIVS III. Report from the chemical s selection sub-committee to the management team on potential reasons for the misclassifications of chemicals in the episkin and epiderm assays. Valérie Zuang, Chantra Eskes, Andrew Worth, Thomas Cole, Sebastian Hoffmann, Ana Gallegos Saliner, Tatiana Netzeva, Grace Patlewicz, Amanda Cockshott & Ingrid Gerner. BfR, Germany 2006-10-04*** SIVS IV. Performance standards for applying human skin models to in vitro skin irritation testing, 25 May 2007 ***SIVS V. Skin Irritation Validation Study: Phase I: Interim Analysis*** SIVS VI. Statement on the validity of tests for skin irritation. ESAC 26 meeting 26-27 April 2007. ***SIVS VII. The ECVAM International Validation Study on In Vitro Tests for Acute Skin Irritation: Report on the Validity of the EPISKIN and EpiDerm Assays and on the Skin Integrity Function Test.*** Horst Spielmann, Sebastian Hoffmann, Manfred Liebsch, Phil Botham, Julia H. Fentem, Chantra Eskes, Roland Roguet, José Cotovio, Thomas Cole, Andrew Worth, Jon Heylings, Penny Jones, Catherine Robles, Helena Kandárová, Armin Gamer, Marina Remmele, Rodger Curren, Hans Raabe, Amanda Cockshott, Ingrid Gerner and Valérie Zuang. ATLA 35, 559-601, 2007 SIVS VIII. The ECVAM International Validation Study on In Vitro

	Tests for Acute Skin Irritation: Selection of Test Chemicals. Chantra Eskes, Thomas Cole, Sebastian Hoffmann, Andrew Worth, Amanda Cockshott, Ingrid Gerner and Valérie Zuang. ATLA 35, 603-619, 2007.
Meeting Document #5	***CORRELATE EPIDERM OPTIMISATION DOCUMENTS (5.1-5.9)*** 5.1. Test Submission Template (TST) for ECVAM/CORRELATE submissions Submission of the Follow-up Validation Study of the Modified EpiDerm Skin Irritation Test for Hazard Identification and Labelling of Chemicals According to EU Classification System 5.2. Statistical report evaluation of the modified epiderm skin irritation test for in vitro testing of acute skin irritation potential (BfR PROJECT N° 1342-426-2) 5.3. Standard Operating procedure EpiDerm™ Skin Irritation Test Model EPI-200 5.4. Training report evaluation of the modified epiderm skin irritation test for in vitro testing of acuteskin irritation potential (BfR PROJECT N° 1342-426-2) 5.5. EpiDerm™ Skin Irritation Test: Standard Operating Procedure 5.6. Annex B: Methods Documentation Sheet (MDS) 5.7. Annex IV Relevant external data sets 5.8. Project plan evaluation of the modified epiderm skin irritation test for in vitro testing of acute skin irritation potential ***5.9. ANNEX X***
Meeting Document #6	Comparison of human skin irritation and photo irritation patch test data with cellular in vitro assays and animal in vivo data. ***Jirova, D., Liebsch, m., Basketter, D., Kandarova, H., Kejlova, K., Bendova, H., Marriot, M and Spiller, E. WC6 Proceedings***
Meeting Document #7	Skin irritation: prevalence, variability, and regulatory classification of existing in vivo data from industrial chemicals ***Sebastian Hofmann, Thomas Cole, Thomas Hartung,¤ Regulatory Toxicology and Pharmacology 41 (2005) 159–166.***
Meeting Document #8	Modelling the human epidermis in vitro: tools for basic and applied research, Review Yves Poumay, Alain Coquette, Arch Dermatol Res (2007) 298:361–369
Meeting Document #9	A simple reconstructed human epidermis: preparation of the culture model and utilization in in vitro studies. ***Poumay F. Dupont S. Marcoux M. Leclercq-Smekens M. He´ rin A. Coquette. Arch Dermatol Res (2004) 296: 203–211***
Meeting Document #10	***The Use of Reconstructed Human Epidermis for Skin Absorption Testing: Results of the Validation Study. Monika Schäfer-Korting et al ATLA 36, 161–187, 2008***
Meeting Document #11	The In Vitro Acute Skin Irritation of Chemicals: Optimisation of the EPISKIN Prediction Model within the Framework of the ECVAM

	Validation Process José Cotovio, Marie-Hélène Grandidier, Pascal Portes, Roland Roguet and G. Rubinstenn. ATLA 33, 329–349, 2005
Meeting document #12	The EpiDerm Test Protocol for the Upcoming ECVAM Validation Study on In Vitro Skin Irritation Tests — An Assessment of the Performance of the Optimised Test. Helena Kandárová, Manfred Liebsch, Ingrid Gerner, Elisabeth Schmidt, Elke Genschow, Dieter Traue and Horst Spielmann ATLA 33, 351–367, 2005
Meeting document #13	Future validated in vitro Skin Irritation Model for Full Replacement of the Draize Test? SkinEthic' Reconstructed Human Epidermis model (RHE). Nathalie Alépée, Carine Tornier, Carole Amsellem, Isabelle Goulet, Jean-Roch Meunier and Anne de Brugerolle de Fraissinette. Abstract ESTIV 2008.
Meeting document #14	Follow-up Validation of the EpiDerm Skin Irritation Test (SIT): Results of a Multi-centre Study of Twenty Reference Test Substances Manfred Liebsch, Armin Gamer, Rodger Curren, Jürgen Frank, Elke Genschow, Julian Tharmann, Marina Remmele, Britta Bauer, Hans Raabe, Nicole Barnes, Allison Hilberer, Nathan Wilt, Reza Lornejad-Schäfer, Christine Schäfer, Patrick Hayden and Helena Kandárová. Abstract ESTIV 2008.
Meeting document #15	Predictive capacity and GHS, S. Hoffman
Meeting document #16	OECD TG 404
Meeting document #17	OECD TG 431
Meeting document #18	OECD TG 435
Meeting document #19.1 & 2	In Vitro Skin Irritation Test: Increasing the Sensitivity of the EpiDerm Skin Irritation Protocol Evaluated in the ECVAM Skin Irritation Validation study. MatTek Corp. Helena Kandárová, Hisashi Torishima, Patrick Hayden, Erin Spiller, Mitch Klausner, Joseph Kubilus and John Sheasgreen
Background documents	
Background document #1	Test Submission Template (TST) for ECVAM/CORRELATE submissions
Background document #2	Skinethic™ RHE TST Submission Amendment
Background document #3	Skinethic™ Skin Irritation Test:: Determination of IL-1α concentration in conditioned media
Background document #4	SkinEthic™ Protocol Amendment
Background document #5	Skinethic™ Skin Irritation Test: Using the Reconstructed Human Epidermis (RHE) model
Background document #6	SkinEthic™ Protocol Amendment
Background document #7	SkinEthic™ Skin Irritation Raw Data

Annex 2

List of Participants

Only available to Governmental Representatives

Annex 3

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

In Vitro Skin Irritation: Reconstructed Human Epidermis (hRE)Model Test[1]

INTRODUCTION

1. Skin irritation refers to the production of reversible damage to the skin following the application of a test substance for up to 4 hours [as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS)](1). This Test Guideline provides an *in vitro* procedure that, depending on country requirements, may allow determining the skin irritancy of chemicals as a stand-alone replacement test, as a screen, or within a testing strategy in combination with a weight of evidence approach.

2. The assessment of skin irritation has typically involved the use of laboratory animals (OECD Test Guideline 404; adopted in 1981 and revised in 1992 and 2002)(2). In relation to animal welfare concerns, TG 404 was revised in 2002, allowing for the determination of skin corrosion/irritation by applying a sequential testing strategy, using validated *in vitro* and *ex vivo* methods, thus avoiding pain and suffering of animals. Three validated *in vitro* Test Guidelines, TG 430, TG 431 and TG 435 (3)(4)(5), have been adopted to be used for the corrosivity part of the sequential testing strategy of TG 404.

3. This Test Guideline is based on reconstructed human epidermis(hRE) models, which in their overall design (the use of human derived epidermis keratinocytes as cell source, representative tissue and cytoarchitecture) closely mimic the biochemical and physiological properties of the upper parts of the human skin, *i.e.*, the epidermis. The test method described under this Test Guideline allows the hazard identification of irritant substances in accordance with UN GHS category 2. This Test Guideline also includes a set of performance standards for the assessment of similar and modified hRE based test methods (6), in accordance with Guidanec Document No. 34 (7).

4. Prevalidation, optimisation and validation studies have been completed for two *in vitro* test methods (8)(9)(10)(11)(12)(13)(14)(15)(16)(17), commercially available as EpiSkin™ and EpiDerm™, using hRE models. Based on the acknowledged validity of EpiSkin™ (validated reference method 1), this validated reference method is recommended as a stand alone replacement test method for the rabbit *in vivo* test, as a screen, or as part of a sequential testing strategy in a weight of evidence approach, for classifying GHS category 2 irritant chemicals, depending on country requirements. EpiDerm™ (validated reference method 2), is only recommended as a screen test method, or as part of a sequential testing strategy in a weight of evidence approach, for classifying GHS category 2 irritant chemicals. Before a proposed *in vitro* hRE model test for skin irritation can be used for regulatory purposes, its reliability, relevance (accuracy), and limitations for its proposed use should be determined to ensure that it is comparable to that of the validated reference method 1, in accordance with the performance standards set out in this Test Guideline (Annex 2).

5. Two other *in vitro* hRE test methods, have been validated in accordance with the requirements

[1] The US can not endorse a draft Test Guideline or annexes at this time and will need additional time to review any supporting materials and any proposals for its content, language, and utility with appropriate stakeholders.

under this Test Guideline, and show similar results as the validated reference method 1 (18). These are the modified EpiDerm™ test method (modified reference method 2) and the SkinEthic RHE™ similar test method (me-too model 1).

INITIAL CONSIDERATIONS AND LIMITATIONS

6. A limitation of the validated reference method is that it only classifies compounds according to UN GHS category 2 as an irritant. It does not allow the classification of substances to the optional category 3 as defined in the UN GHS. Regulatory requirements and needs in member countries will decide if this Test Guideline will be used as a stand alone replacement test, as a screen, or as part of a testing strategy in combination with, if appropriate, a weight of evidence approach. Depending on regulatory considerations in member countries, *in vivo* testing may be needed or required to fully characterize skin irritation potential. Depending on regulatory needs and possible future inclusion of new endpoints, improvements or development of new me-too tests, this Test Guideline may have to be revised regurlarly.

7. This Test Guideline allows the hazard identification of irritant mono-, and multi-component substances (19), but it does not provide adequate information on skin corrosion. Gases and aerosols cannot be tested, while formulations and preparations have not been assessed yet in a validation study. It should also be noted that highly coloured chemicals, *i.e.*, hair dye components, may interfere with the cell viability measurements (see paragraphs 22-24).

DEFINITIONS

8. Definitions used are provided in Annex 1.

PRINCIPLE OF THE TEST

9. The test substance is applied topically to a three-dimensional hRE model, comprised of normal, human-derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo*.

10. The principle of the hRE model test is based on the premise that irritant chemicals are able to penetrate the stratum corneum by diffusion and are cytotoxic to the cells in the underlying layers. Cell viability is measured by dehydrogenase conversion of the vital dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; EINECS number 206-069-5, CAS number 298-93-1)], into a blue formazan salt that is quantitatively measured after extraction from tissues (20). Irritant substances are identified by their ability to decrease cell viability below defined threshold levels (*i.e.*, $\leq$ 50%, for UN GHS category 2 irritants). Depending on country requirements and applicability of the Test Guideline, substances that produce cell viabilities above the defined threshold level, may be considered non-irritants (*i.e.*, > 50%, no category).

11. The hRE model systems may be used to test solids, liquids, semi-solids and waxes. The liquids may be aqueous or non aqueous; solids may be soluble or insoluble in water. Whenever possible, solids should be tested as a fine powder. Since 58 carefully selected chemicals, representing a wide spectrum of chemical classes, were included in the validation of the hRE model test systems, the methods are expected to be generally applicable across chemical classes (16).

DEMONSTRATION OF PROFICIENCY

12. Prior to routine use of a validated method that adheres to this Test Guideline, laboratories may wish to demonstrate technical proficiency, using the ten chemicals recommended in Table 1. For novel similar (me-too) test methods developed under this Test Guideline that are structurally and functionally similar to the validated reference methods or for modifications of validated methods, the performance standards described in Annex 2 of this Test Guideline should be used to demonstrate comparable reliability and accuracy of the new test method prior to its use for regulatory testing.

Table 1. Proficiency Chemicals which are a subset of the Reference Chemicals listed in Annex 2[1]

Chemical	CAS Number	*In vivo* score	Physical state	GHS category
naphthalene acetic acid	86-87-3	0	S	No Cat.
isopropanol	67-63-0	0.3	L	No Cat.
methyl stearate	112-61-8	1	S	No Cat.
heptyl butyrate	5870-93-9	1.7	L	Optional Cat. 3[2]
hexyl salicylate	6259-76-3	2	L	Optional Cat. 3[2]
cyclamen aldehyde	103-95-7	2.3	L	Cat. 2
1-bromohexane	111-25-1	2.7	L	Cat. 2
butyl methacrylate	97-88-1	3	L	Cat. 2
1-methyl-3-phenyl-1-piperazine	5271-27-2	3.3	S	Cat. 2
heptanal	111-71-7	4	L	Cat. 2

[1] The proficiency chemicals is a subset of the chemicals used in the validation study.

[2] Under this Test Guideline, the UN GHS optional category 3 is considered as no category.

PROCEDURE

13. The following is a description of the components and procedures of an hRE model test for skin irritation assessment. An hRE model can be constructed, prepared or obtained commercially (*e.g.*, EpiSkin™, EpiDerm™ and SkinEthic RHE™). Sample test method protocols for EpiSkin™, EpiDerm™ and SkinEthic RHE™ can be obtained at [http://ecvam.jrc.ec.europa.eu](21)(22)(23). Testing should be performed according to the following:

hRE Model Components

General model conditions

14. Normal human keratinocytes should be used to construct the epithelium. Multiple layers of viable epithelial cells (basal layer, stratum spinosum, stratum granulosum) should be present under a functional stratum corneum. Stratum corneum should be multilayered containing the essential lipid profile to produce a functional barrier with robustness to resist rapid penetration of cytotoxic marker chemicals, *e.g.*, sodium dodecyl sulphate (SDS)] or Triton X-100. The barrier function may be assessed either by determination of the concentration at which a marker chemical reduces the viability of the tissues by 50% (IC_{50}) after a fixed exposure time, or by determination of the exposure time required to reduce cell viability by 50% (ET_{50}) upon application of the marker chemical at a specified, fixed concentration. The containment properties of the model should prevent the passage of material around the stratum corneum to the viable tissue, which would lead to poor modelling of skin exposure. The skin model should be free of contamination by bacteria, viruses, mycoplasma, or fungi.

Functional model conditions

Viability

15. The preferred assay for determining the magnitude of viability is the MTT (20). The optical density (OD) of the extracted (solubilised) dye from the tissue treated with the negative control (NC) should be at least 20 fold greater than the OD of the extraction solvent alone. It should be documented that the tissue treated with NC is stable in culture (provide similar viability measurements) for the duration of the test exposure period.

Barrier function

16. The stratum corneum and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic marker chemicals, *e.g.*, SDS or Triton X-100, as estimated by IC_{50} or ET_{50}.

Morphology

17. Histological examination of the reconstructed skin/epidermis should be performed demonstrating human skin/epidermis-like structure (including multilayered stratum corneum).

Reproducibility

18. The results of the method using a specific model should demonstrate reproducibility over time, preferably by an appropriate batch control (benchmark) substance (see Annex 1).

Quality controls (QC) of the model

19. Each batch of the epidermal model used should meet defined production release criteria, among which those for *viability* (paragraph 15) and for *barrier function* (paragraph 16) are the most relevant. An acceptability range (upper and lower limit) for the IC_{50} or the ET_{50} should be established by the skin model supplier (or investigator when using an in-house model). The barrier properties of the tissues should be verified by the laboratory after receipt of the tissues. Only results produced with qualified tissues can be accepted for reliable prediction of irritation effects. As an example, the acceptability ranges for the validated reference methods are given below.

Table 2. Examples of QC batch release criteria

	Lower acceptance limit	Mean of acceptance range	Upper acceptance limit
Validated reference method 1 (18 hours treatment with SDS)	IC_{50} = 1.0 mg/ml	IC_{50} = 2.32 mg/ml	IC_{50} = 3.0 mg/ml
Validated reference method 2 (1% Triton X100)	ET_{50} = 4.8 hr	ET_{50} = 6.7 hr	ET_{50} = 8.7 hr

Application of the Test and Control Substances

20. A sufficient number of tissue replicates should be used for each treatment and for controls (at least three replicates per run). For liquid as well as solid chemicals, sufficient amount of test substance

should be applied to uniformly cover the skin surface while avoiding an infinite dose (see Annex 1), *i.e.*, a minimum of 25 $\mu L/cm^2$ or 25 mg/cm^2 should be used. For solid substances, the epidermis surface should be moistened with deionised or distilled water before application, to ensure good contact with the skin. Whenever possible, solids should be tested as a fine powder. At the end of the exposure period, the test substance should be carefully washed from the skin surface with aqueous buffer, or 0.9% NaCl. Depending on the hRE model used, the exposure period may vary between 15 to 60 minutes, and the incubation temperature between 20 and 37°C. For details, see the Standard Operating Procedures for the three methods (21)(22)(23).

21. Concurrent NC and positive controls (PC) should be used for each study to demonstrate that viability (NC), barrier function and resulting tissue sensitivity (PC) of the tissues are within a defined historical acceptance range. The suggested PC substance is 5% aqueous SDS. The suggested NC substances are water or phosphate buffered saline (PBS).

Cell Viability Measurements

22. The most important element of the test procedure is that viability measurements are not performed immediately after the exposure to the test chemicals, but after a sufficiently long post-treatment incubation period of the rinsed tissues in fresh medium. This period allows both for recovery from weakly irritant effects and for appearance of clear cytotoxic effects. During the test optimisation phase (9)(10)(11)(12)(13), a 42 hours post-treatment incubation period proved to be optimal and was therefore used in the validation of the reference test methods.

23. The MTT conversion assay is a validated quantitative method which should be used to measure cell viability. It is compatible with use in a three-dimensional tissue construct. The skin sample is placed in MTT solution of appropriate concentration (*e.g.*, 0.3 – 1 mg/mL) for 3 hours. The precipitated blue formazan product is then extracted from the tissue using a solvent (*e.g.*, isopropanol, acidic isopropanol), and the concentration of formazan is measured by determining the OD at 570 nm using a bandpass of maximum ± 30 nm.

24. Optical properties of the test substance or its chemical action on the MTT may interfere with the assay leading to a false estimate of viability (because the test substance may prevent or reverse the colour generation as well as cause it). This may occur when a specific test substance is not completely removed from the skin by rinsing or when it penetrates the epidermis. If the test substance acts directly on the MTT, is naturally coloured, or becomes coloured during tissue treatment, additional controls should be used to detect and correct for test substance interference with the viability measurement technique. For detailed description of how to test direct MTT reduction, please consult the test method protocol for the validated reference methods (21)(22)(23). Non specific colour (NSC) due to these interferences should not exceed 30% of NC (for corrections). If NSC > 30%, the test chemical is considered as incompatible with the test.

Assay Acceptability Criteria

25. For each assay using valid batches (see paragraph 19), tissues treated with the NC should exhibit OD reflecting the quality of the tissues that followed all shipment and receipt steps and all the irritation protocol process. Control OD values should not be below historical established lower boundaries. Similarly, tissues treated with the PC, *i.e.*, 5% aqueous SDS, should reflect the sensitivity retained by tissues and their ability to respond to an irritant chemical in the conditions of each individual assay (*e.g.*, viability ≤ 40% for the validated reference method 1, and ≤ 20% for the validated reference method 2). Associated and appropriate measures of variability between tissue replicates should be defined (*e.g.*, if standard deviations

are used they should be ≤ 18%).

Interpretation of Results

26. The OD values obtained with each test sample can be used to calculate the percentage of viability compared to NC, which is set at 100%. The cut-off value of percentage cell viability distinguishing irritant from non classified test substances and the statistical procedure(s) used to evaluate the results and identify irritant substances, should be clearly defined and documented, and proven to be appropriate. The cut-off values for the prediction of irritation associated with the validated reference methods is given below:

27. The test substance is considered to be irritant to skin in accordance with UN GHS category 2 if the tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50%. Depending on country requirements, the test substance may be considered to have no category if the tissue viability after exposure and post-treatment incubation is more than (>) 50%.

DATA AND REPORTING

Data

28. For each treatment, data from individual replicate test samples (*e.g.*, OD values and calculated percentage cell viability data for each test chemical, including classification) should be reported in tabular form, including data from repeat experiments as appropriate. In addition means ± standard deviation for each trial should be reported. Observed interactions with MTT reagent and coloured test substances should be reported for each tested chemical.

Test Report

29. The test report should include the following information:

Test and Control Substances:

- Chemical name(s) such as IUPAC or CAS name and CAS number, if known;
- Purity and composition of the substance (in percentage(s) by weight);
- Physical-chemical properties relevant to the conduct of the study (*e.g.*, physical state, stability and volatility, pH,water solubility if known)
- Treatment of the test/control substances prior to testing, if applicable (*e.g.*, warming, grinding);
- Storage conditions,

Justification of the skin model and protocol used.

Test Conditions

- Cell system used;
- Calibration information for measuring device, and bandpass used for measuring cell viability (*e.g.*, spectrophotometer);
- Complete supporting information for the specific skin model used including its performance. This should include, but is not limited to:

  i) Viability

ii) Barrier function

iii) Morphology

iv) Reproducibility and predictivity

v) Quality controls (QC) of the model

- Details of the test procedure used;
- Test doses used, duration of exposure and post treatment incubation period;
- Description of any modifications of the test procedure;
- Reference to historical data of the model. This should include, but is not limited to:

  i) acceptability of the QC data with reference to historical batch data

  ii) acceptability of the positive and negative control values with reference to positive and negative control means and ranges.
- Description of evaluation criteria used including the justification for the selection of the cut-off point(s) for the prediction model

Results:

- Tabulation of data from individual test samples;
- Description of other effects observed.

Discussion of the results.

Conclusion.

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ANNEX 1

DEFINITIONS

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance. The term is often used interchangeably with "concordance" to mean the proportion of correct outcomes of a test method.

Batch control substance: Benchmark substance producing a mid-range cell viability response of the tissue.

Cell viability: Parameter measuring total activity of a cell population e.g., as ability of cellular mitochondrial dehydrogenases to reduce the vital dye MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue;), which depending on the endpoint measured and the test design used, correlates with the total number and/or vitality of living cells.

ET_{50}: Can be estimated by determination of the exposure time required to reduce cell viability by 50% upon application of the marker chemical at a specified, fixed concentration, see also IC_{50}.

False negative rate: The proportion of all positive substances falsely identified by a test method as negative. It is one indicator of test method performance.

False positive rate: The proportion of all negative (non-active) substances that are falsely identified as positive. It is one indicator of test method performance.

Infinite dose: Amount of test substance applied to the skin exceeding the amount required to completely and uniformly cover the skin surface.

GHS (Globally Harmonized System of Classification and Labelling of Chemicals): A system proposing the classification of chemicals (substances and mixtures) according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so that to convey information on their adverse effects with a view to protect people (including employers, workers, transporters, consumers and emergency responders) and the environment (1).

IC_{50}: Can be estimated by determination of the concentration at which a marker chemical reduces the viability of the tissues by 50% (IC_{50}) after a fixed exposure time, see also ET_{50}.

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are; (i) essential test method components; (ii) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals.

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability.

Sensitivity: The proportion of all positive/active substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method.

Specificity: The proportion of all negative/inactive substances that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method.

Skin irritation: The production of reversible damage to the skin following the application of a test substance for up to 4 hours. Skin irritation is a locally arising, non-immunogenic reaction, which appears shortly after stimulation (23). Its main characteristic is its reversible process involving inflammatory reactions and most of the clinical characteristic signs of irritation (erythema, oedema, itching and pain) related to an inflammatory process.