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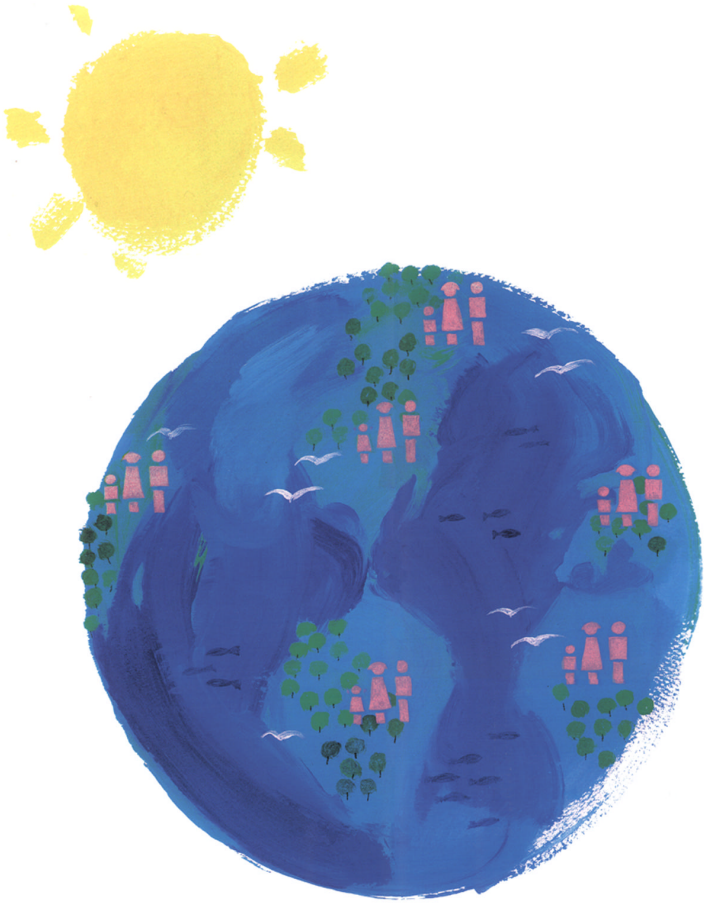

ICCVAM Evaluation of EPISKIN™, EpiDerm™ (EPI-200), and the Rat Skin Transcutaneous Electrical Resistance (TER) Assay:

In Vitro Test Methods for Assessing Dermal Corrosivity Potential of Chemicals

Interagency Coordinating Committee on the Validation of Alternative Methods
(ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

THE INTERAGENCY COORDINATING COMMITTEE
ON THE VALIDATION OF ALTERNATIVE METHODS
AND
THE NTP INTERAGENCY CENTER FOR THE
EVALUATION OF ALTERNATIVE TOXICOLOGICAL METHODS

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 1997 by the Director of the National Institute of Environmental Health Sciences (NIEHS) to implement NIEHS directives in Public Law 103-43. P.L. 103-43 directed NIEHS to develop and validate new test methods, and to establish criteria and processes for the validation and regulatory acceptance of toxicological testing methods. P. L. 106-545, the ICCVAM Authorization Act of 2000, establishes ICCVAM as a permanent committee. The Committee is composed of representatives from 15 Federal regulatory and research agencies that generate, use, or provide information from toxicity test methods for risk assessment purposes. P.L. 106-545 directs ICCVAM to coordinate technical reviews and evaluations of new, revised, and alternative test methods of interagency interest. ICCVAM also coordinates cross-agency issues relating to development, validation, acceptance, and national/international harmonization of toxicological test methods.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) was established in 1998 to provide operational support for the ICCVAM and to collaborate with the ICCVAM to carry out committee-related activities such as test method peer reviews and workshops. NICEATM and ICCVAM coordinate the scientific review of the validation status of proposed methods and provide recommendations regarding their usefulness to appropriate agencies. NICEATM and ICCVAM seek to promote the validation and regulatory acceptance of toxicological test methods that will enhance agencies' abilities to assess risks and make decisions, and that will refine, reduce, and replace animal use. The ultimate goal is to improve public health by gaining the regulatory acceptance of new scientifically valid test methods that are more predictive of human and ecological effects than currently available methods.

Additional Information

Additional information can be found at the ICCVAM/Center Website: http://iccvam.niehs.nih.gov and the publication: ***Validation and Regulatory Acceptance of Toxicological Test Methods, a Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods*** (NIH Publication No. 97-3981), or you may contact the Center at telephone 919-541-3398, or by e-mail at iccvam@niehs.nih.gov. Specific questions about ICCVAM and the Center can be directed to the Director of NICEATM:

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Environmental Protection Agency
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National Institute of Environmental Health Sciences
National Institutes of Health, Office of the Director
National Institute of Occupational Safety and Health
National Library of Medicine
Occupational Safety and Health Administration

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Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

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Table of Contents

List of Tables ..... **v**
List of Abbreviations ..... **vii**
Acknowledgements ..... **x**
Preface ..... **xiii**
Executive Summary ..... **xv**

1.0 ICCVAM Evaluation and Test Recommendations **1**
1.1 Introduction 1
1.2 Background 2
1.3 ICCVAM Test Method Recommendations 5

2.0 EPISKIN™ **7**
2.1 Summary Report of the EPISKIN™ Assay 9
2.2 EPISKIN™ Test Method Protocol 23

3.0 EpiDerm™ (EPI-200) **41**
3.1 Summary Report of the EpiDerm™ Assay 43
3.2 EpiDerm™ Test Method Protocol 57

4.0 Rat Skin Transcutaneous Electrical Resistance (TER) Assay **77**
4.1 Summary Report of the Rat Skin TER Assay 79
4.2 Rat Skin TER Test Method Protocol 93

5.0 References **111**

6.0 Appendices

A. Federal Regulations and Guidelines for Corrosivity **A-1**
A-1 Summary Table of Guidelines and Regulations for Dermal Corrosivity A-3
A-2 OPPTS 870.2500 Acute Dermal Irritation / U.S. EPA Health Effects Test Guidelines A-5
A-3 Chapter 8 (Precautionary Labeling) of U.S. EPA Office of Pesticide Programs Label Review Manual A-13

B. European Commission Evaluations and Statements **B-1**
B-1 Statements by the ECVAM Scientific Advisory Committee (ESAC)
· Statement on Rat Skin TER and EPISKIN™ B-3
· Statement on EpiDerm™ B-13
B-2 · SCCNFP's Opinion on *In Vitro* Skin Corrosivity Methods B-15
· SCCNFP's Committee Members B-19
B-3 European Commission Directive for Rat Skin TER and a Human Skin Model Assay B-21

Table of Contents
(continued)

C. OECD Related Test Guidelines and Documents..... C-1

D. *Federal Register* Notice (Vol. 66 (189): 49685-6; September 28, 2001) D-1

E. Public Comments in Response to the *Federal Register* Request for Comments E-1

List of Tables

2.0 EPISKIN™

Table 2.1 Performance of the EPISKIN™ Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Fentem et al., 1998) 14

Table 2.2 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays 16

Table 2.3 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays Based on a Weight-of-Evidence Approach by Chemical using Data from the ECVAM and other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000) 17

Table 2.4 General comparison of the Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) assays from independent test results in the ECVAM validation studies (Fentem et al., 1998; Liebsch et al., 2000) 18

Table 2.5 Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000) 19

3.0 EpiDerm™ (EPI-200)

Table 3.1 General Protocol Comparison between EPISKIN™ and EpiDermä (EPI-200) 46

Table 3.2 Summary of Results for EpiDerm™ (EPI-200) and EPISKIN™ Compared to *In Vivo* Rabbit Results 48

Table 3.3 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays 50

Table 3.4 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays Based on a Weight-of-Evidence Approach by Chemical using Data from the ECVAM and other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000 51

Table 3.5 General Comparison of the Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays from Independent Test Results in the ECVAM Validation Studies (Fentem et al., 1998; Liebsch et al., 2000) 52

List of Tables
(continued)

Table 3.6 Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000) 53

4.0 Rat Skin Transcutaneous Electrical Resistance (TER) Assay

Table 4.1 Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Overall) 83

Table 4.2 Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Fentem et al., 1998) 84

Table 4.3 Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Botham et al., 1992; 1995) 85

Table 4.4 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assay 87

Table 4.5 General Comparison of the Rat Skin TER Assay, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays Based on a Weight-of-Evidence Approach by Chemical using Data from the ECVAM and Other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000 88

Table 4.6 General Comparison of the Rat Skin TER, EPISKIN™ and EpiDerm™ (EPI-200), Assays from Independent Test Results in the ECVAM Validation Studies (Fentem et al., 1998; Liebsch et al., 2000 89

Table 4.7 Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000) 90

List of Abbreviations

ACLAM	American College of Laboratory Animal Medicine
Anon.	Anonymous
ANOVA	Analysis of Variance Software
BRD	Background Review Document
C	corrosive
°C	Degrees Centigrade
CAAT	Center for Alternatives to Animal Testing
CFR	Code of Federal Regulations
cm	Centimeter
CPSC	Consumer Product Safety Commission
CWG	Corrosivity Working Group (ICCVAM)
DABT	Dipolomate, American Board of Toxicology
EC	European Commission
ECB	European Chemicals Bureau
ECVAM	European Centre for the Validation of Alternative Methods
ELECTRO	Electrophile
EPI-200	EpiDerm™ (MatTek Corp., Ashland, MA, USA.)ä
ERGATT	European Research Group for Alternatives in Toxicity Testing
ESAC	ECVAM Scientific Advisory Committee
EU	European Union
FDA	Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FR	*Federal Register*
g	gram
GHS	Globally Harmonized Classification System
GLP	Good Laboratory Practice
GPO	Government Printing Office
HIV	Human immunodeficiency virus
hr	hour
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Standards Organization
kg	kilogram
l	liter
MDS	Methods Documentation Sheet
mg	milligrams
ml	milliliter
mM	millimolar
MSHA	Mine Safety and Health Administration
MT	Management Team

List of Abbreviations
(continued)

MTT	Mitochchondrial tetrazolium salt assay
N/A	Not applicable
NC	Noncorrosive
NHEERL	National Health and Environmental Effects Research Laboratory/U.S. EPA
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIOSH	National Institute of Occupational Safety and Health
nM	nanomolar
NM	Not measured
NQ	Non-qualified
NTP	National Toxicology Program
OD	Optical densities
OECD	Organisation for Economic Co-operation and Development
OPP	Office of Pesticide Programs/U.S. EPA
OPPT	Office of Pollution Prevention and Toxics/U.S. EPA
OPPTS	Office of Prevention, Pesticides, and Toxic Substances/U.S. EPA
ORD	Office of Research and Development/U.S. EPA
OSHA	Occupational Safety and Hazards Administration
PBS	Phosphate buffered saline
PDF	Portable document format
PFTE	Polytetrafluoroethylene
pH	A measure of the negative logarithm of the H^+ ion concentration
P.L.	Public Law
PM	Prediction model
PPE	Personal protective equipment
PR	Pesticide registration
psi	pounds per square inch (pressure)
QC	Quality control
REDs	Registration Eligibility Documents
RPD	Respiratory protection device
SAR	Structure activity relationships
SAS	Statistical Analysis System – (SAS Institute, Inc., Cary, NC, USA)
SCBA	Self-contained breathing apparatus
SCCNFP	Scientific Committee for Cosmetic Products and Non-Food Products (European Commission)
S.D.	Standard deviation
SDS	Sodium dodecylsulphate
SOP	Standard Operating Procedure
TC	Toxicity category
TER	Transcutaneous electrical resistance assay

List of Abbreviations
(continued)

TG	Test Guideline
TG 404	Test Guideline 404 (Acute Dermal Irritation/Corrosion) [OECD]
TG 405	Test Guideline 405 (Acute Eye Irritation/Corrosion) [OECD]
TG 430	Test Guideline 430 (*In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance Test (TER)) [OECD]
TG 431	Test Guideline 431 (*In Vitro* Skin Corrosion: Human Skin Model Test) [OECD]
UN	United Nations
U.S. DOT	U.S. Department of Transportation
U.S. EPA	U.S. Environmental Protection Agency
v/v	volume to volume ratio
WPS	Worker Protection Standard
w/v	weight to volume ratio
ZEBET	German Center for Documentation and Evaluation of Alternative Methods to Animal Experiments
µg	microgram
µL	microliter
â	Registered Trademark
TM	Trademark
KΩ	Change in transcutaneous electrical resistance
R34	UN chemical classification for Packing Groups II and III
R35	UN chemical classification for Packing Group I
3Rs	Refinement, Reduction, and Replacement (of animal use)

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Preface

Corrosive substances are defined as chemicals that cause visible destruction or irreversible alterations in living tissue by chemical action at the site of contact (29CFR 1917.28). Dermal corrosivity testing is conducted to identify corrosive chemicals that may cause burns and permanent scarring to the skin. Test results are used to classify and label corrosive chemicals so that consumers and workers can take appropriate precautions to prevent injury. Test results are also used to determine appropriate packaging that will minimize hazardous spills during transport. While corrosive chemicals and products have typically been identified with an *in vivo* procedure involving application of test substances to the intact skin of a rabbit, animal welfare concerns have led to the recent development and validation of *in vitro* testing methods for assessing skin corrosivity.

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) coordinated the independent peer review evaluation of Corrositex® (In Vitro International, Inc., Irvine, CA), an *in vitro* corrosivity testing method. ICCVAM recommendations for using Corositex® to assess dermal corrosivity were forwarded to and subsequently accepted by U.S. regulatory agencies. In 2000, a second *in vitro* method for corrosivity testing, EpiDermä (EPI-200) (MatTek Inc, Ashland, MA), was submitted to ICCVAM for consideration. ICCVAM was also notified that EpiDermä and two other *in vitro* corrosivity test methods, EPISKINä (EPISKIN SNC, Lyon, France) and the Rat Skin Transcutaneous Electrical Resistance (TER) assay, had been reviewed and endorsed by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (ESAC). The ICCVAM agreed that it should evaluate all three proposed test methods.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) subsequently prepared a background review document (BRD) summarizing available data, prior ECVAM validation studies, and the ESAC reviews for the three test methods. An ICCVAM Corrosivity Working Group (CWG) composed of Federal employees reviewed the BRD and concluded, based on the information provided and the outcomes of the previous reviews, that further evaluation by an independent scientific peer review panel did not appear necessary. The CWG therefore recommended that these methods undergo ICCVAM evaluation using a newly created expedited review process, and ICCVAM agreed to proceed with an expedited review. This evaluation process involved the development of a draft ICCVAM position (proposed ICCVAM test recommendations) and publication of the position in the *Federal Register* (Vol. 66, No. 189, pp.49685-6; Sept. 28, 2001) for public comment. Public comments were considered by the CWG and ICCVAM, after which the test recommendations were finalized.

ICCVAM recommends that EpiDermä, EPISKINä, and the Rat Skin TER assay can be used to assess the dermal corrosion potential of chemicals and chemical mixtures in a weight-of-evidence approach

using an integrated testing strategy for dermal irritation/corrosion. In this approach, positive *in vitro* corrosivity responses will not generally require further testing and results can be used for classification and labeling without the need for animal testing. Accordingly, these methods provide for the replacement of animal use when positive results are obtained.

As required by the ICCVAM Authorization Act of 2000 (P. L. 106-545), these ICCVAM test recommendations will be forwarded to Federal agencies for their consideration and appropriate action. Agency responses to ICCVAM test recommendations will be made available on the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov). This publication and supporting documents are also available on this website.

An added benefit realized from this review was the further development and application of the new ICCVAM expedited review process. The experience gained during this review will facilitate future ICCVAM consideration of ECVAM-validated and ESAC-endorsed methods. This process enhances the likelihood of international harmonization and provides an opportunity to develop concordant recommendations between the United States and the European Union where feasible. It also minimizes or avoids duplication of effort and avoids needless delays in achieving mutual endorsement and acceptance of scientifically valid methods.

These test method evaluations required the efforts of many individuals. We especially acknowledge the ECVAM staff who designed, managed, and analyzed the results of the independent validation studies and the efforts of the participating laboratories that conducted the validation studies. The ESAC is recognized for their careful review of the study results. Special thanks go to the NICEATM staff for preparing the Background Review Document on the test methods and for editing and publishing this final report. We appreciate the efforts of the CWG and the ICCVAM for conducting a diligent and thorough review of these three methods. Finally, we appreciate and acknowledge the reviews and comments by members of the public.

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In Vitro Skin Corrosivity Methods: EPISKIN™, EpiDerm™ (EPI-200), and the Rat Skin Transcutaneous Electrical Resistance (TER) Assay

Executive Summary

Corrosive substances are defined as chemicals causing visible destruction or irreversible alterations in living tissue by chemical action at the site of contact (29 CFR 1917.28). Dermal corrosivity testing is conducted to identify chemicals that potentially pose this hazard to humans. U.S. Federal regulations and guidelines include test methods for assessing dermal corrosivity. Testing data is used to determine appropriate hazard classification and labeling, appropriate transport and/or storage methods, and appropriate precautions for workers in industrial settings. For regulatory purposes, corrosive chemicals and chemical mixtures have typically been identified using an *in vivo* test method involving the application of chemicals or chemical mixtures to the intact skin of a rabbit. The skin is visually evaluated for corrosion after exposures of three minutes, one hour, and four hours. Animal welfare considerations have led to efforts to develop *in vitro* alternative test methods. One such method, Corrositex®, (In Vitro International, Inc., Irvine, CA) was submitted to ICCVAM for consideration. Following independent scientific peer review (ICCVAM, 1999), ICCVAM recommended that Corrositex® could be used to assess the dermal corrosion potential of chemicals as part of a tiered testing strategy.

Three other alternative *in vitro* test methods – EpiDerm™ (EPI-200), EPISKIN™, and the Rat Skin Transcutaneous Electrical Resistance (TER) assay – have subsequently undergone validation studies by the European Centre for the Validation of Alternative Methods (ECVAM), and have been accepted for corrosivity testing in the European Union (EU, 2000). ICCVAM subsequently implemented an expedited test method review process to consider methods which have been evaluated by the ECVAM (ICCVAM, 2001). This process will accelerate interagency consideration of these test methods, thereby avoiding duplication of effort and unnecessary delays in recommending useful test methods to Federal agencies in accordance with Public Law 106-545. This report describes the information and data considered by ICCVAM during its expedited review of the three methods, and provides the ICCVAM test recommendations for these methods.

Validation and Regulatory Acceptance Status of EPISKIN™, EpiDerm™ (EPI-200), and the Rat Skin TER Assay

Independent validation studies on these three *in vitro* assays were conducted by ECVAM (Barratt et al., 1998; Fentem et al., 1998; Liebsch et al., 2000). The ECVAM Validation Management Team concluded that the EpiDerm™ (EPI-200), Rat Skin TER, and EPISKIN™ methods were able to distinguish between corrosive and non-corrosive chemicals for all of the chemical classes considered.

A review of these validation studies and the analyses conducted by NICEATM are presented in Sections 2.0, 3.0, and 4.0 of this report.

The validation status of the three methods was reviewed by the ECVAM Scientific Advisory Committee (ESAC) (Balls and Corcelle, 1998; Balls and Hellsten, 2000). The ESAC

concluded that the Rat Skin TER, Episkinä, and EpiDerm™ (EPI-200) tests can be used to distinguish between corrosive and non-corrosive chemicals within the context of the draft EU and Organisation for Economic Co-operation and Development (OECD) test guidelines on skin corrosion (Balls and Corcelle, 1998; Balls and Hellsten, 2000). EPISKINä and Rat Skin TER were also reviewed by the European Commission's Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) which concluded that the methods were considered applicable to the safety evaluation of cosmetic ingredients or mixtures of ingredients (SCCNFP, 1999).

EPISKIN™

The EPISKINä human skin model is commercially available from EPISKIN SNC, Lyon, France, a wholly owned subsidiary of L'OREAL. EPISKIN™ is a three-dimensional human skin model composed of a human collagen (Types III and I) matrix, representing the dermis, covered with a film of Type IV human collagen and stratified differentiated epidermis derived from human keratinocytes. Test materials can be applied directly to the stratum corneum. The model utilizes cell viability as the measured endpoint. The topical mode of application of test material mimics the route of human exposure. For use in corrosivity testing, the test material (liquids: 50 µL; solids: 20 mg) is applied to an epidermis unit for 3, 60, and 240 minutes. Cell viability is assessed by measuring mitochondrial activity using the MTT (a tetrazolium salt) assay as compared to concurrent negative controls. A 35% decrease in cell viability is used to indicate a potential for human corrosivity.

ECVAM conducted an independent validation study on the EPISKIN™ method as an *in vitro* replacement assay for *in vivo* corrosivity testing (Fentem et al., 1998). Sixty chemicals were evaluated in duplicate in three different laboratories; chemical selection and *in vivo* reference data were described by Barratt et al. (1998). The ECVAM validation chemical test set included:

- organic acids
- organic bases
- neutral organics
- phenols
- inorganic acids
- inorganic bases
- inorganic salts
- electrophiles
- soaps/surfactants

The database used in the EPISKIN™ evaluation consisted of data from the ECVAM validation study only; other data were not located. An analysis of the results of the database of 60 chemicals and chemical mixtures evaluated in the validation study had the following performance:

- accuracy: 83% (50/60 chemicals or chemical mixtures)
- sensitivity: 82% (23/28 chemicals or chemical mixtures)
- specificity: 84% (27/32 chemicals or chemical mixtures)
- false positive rate: 16% (5/32)
- false negative rate: 18% (5/28)

Furthermore, EPISKIN™ was able to distinguish between known R35/I and R34/II & III chemicals.[1]

Inter- and intra-laboratory reproducibility of EPISKIN™ was also evaluated by Fentem et al. (1998). In each laboratory, each chemical was tested three times using three different batches of EPISKIN™. Of the 60 chemicals tested, 42 gave the same corrosivity classification in all three tests in all three laboratories. Discordant results for the remaining chemicals were as follows: one of nine tests for six chemicals, two to three of nine tests for seven chemicals, and four to five of nine tests for the remaining five chemicals. The study concluded that EPISKIN™ had acceptable intra- and inter-laboratory reproducibility (Fentem et al., 1998).

EpiDerm™ (EPI-200)

EpiDerm™ (EPI-200) is commercially available from MatTek Corporation, Ashland, MA, USA. The EpiDerm™ (EPI-200) skin model is mechanistically and functionally related to EPISKIN™. The assay consists of normal human epidermal keratinocytes which have been cultured in chemically defined medium to produce a stratified, highly differentiated, organotypic tissue model of the human epidermis. The EpiDerm™ (EPI-200) tissue consists of metabolically and mitotically active cells which are organized into basal, spinous, and granular layers along a multi-layered stratum corneum (MatTek Corporation, 2000). Like EPISKIN™, the EpiDerm™ (EPI-200) tissue approximates the barrier of normal human skin, and the topical mode of application of the test material in EpiDerm™ (EPI-200) mimics the route of human exposure. For use in corrosivity testing, the test material (liquids and semi-solids: 50 µL; solids: 25 mg plus 25 µl of H2O) is applied to a tissue for three and 60 minutes. For each test substance, duplicate plates are analyzed at each test period. As with EPISKIN™, cell viability is assessed by measuring mitochondrial activity using the MTT assay. A test chemical is classified as corrosive if it induces ≥50% decrease in relative cell viability at 3 minutes or ≥85% decrease in relative cell viability at 60 minutes.

ECVAM conducted an independent validation study on EpiDerm™ (EPI-200) as an *in vitro* replacement assay for *in vivo* corrosivity testing (Liebsch et al., 2000). Twenty-four chemicals representative of the 60 chemicals tested in the Fentem et al. (1998) ECVAM validation study for the EPISKIN™ assay were tested. The 24 chemicals selected included 12 corrosive and 12 noncorrosive chemicals composed of; organic acids and bases, neutral organic bases, phenols, inorganic acids and bases, electrophiles, and surfactants.

The database used in the evaluation of EpiDerm™ (EPI-200) consisted of data from the ECVAM pre-validation/validation study only (Liebsch et al., 2000); other data were not located. (see Section 2.0) Based on an analysis of the results of 24 chemicals and chemical mixtures evaluated in the validation study, EpiDerm™ (EPI-200) had the following performance:

[1]UN packing group classifications I, II, and III are assigned based on the capacity of a chemical, when tested on the intact skin of rabbits, to produce skin corrosion following exposure intervals of 3 minutes, 1 hour, or 4 hours, respectively (Fentem et al., 1998). EU regulations require classification of chemicals according to certain risk phases, such as those assigned based on whether the chemical causes corrosion following a 3-minute application (R35 – "causes severe burns"; analogous to packing group I) or 4 hours (R34 – "causes burns"; analogous to packing groups II and III) (Barratt et al., 1998; Fentem et al., 1998).

- accuracy: 92% (22/24 chemicals or chemical mixtures)
- sensitivity: 92% (11/12 chemicals or chemical mixtures)
- specificity: 83% (10/12 chemicals or chemical mixtures).
- false positive rate: 17% (2/12)
- false negative rate: 8% (1/12)

Unlike EPISKIN™, EpiDerm™ (EPI-200) was not able to distinguish between known R35/I and R34/II & III chemicals.

Intra- and inter-laboratory reliability was evaluated by testing each chemical twice, using different tissue lots, in each of three laboratories. Of the 24 chemicals tested, 19 gave the same corrosivity classification in the two replicates in all three laboratories (six tests). Discordant results for the remaining chemicals were as follows: one of six tests for three chemicals and two of six tests for two chemicals. Based on the results obtained, the study concluded that EpiDerm™ (EPI-200) provided excellent reliability (Liebsch et al., 2000).

Rat Skin TER

Transcutaneous electrical resistance is measured using an AIM electronic databridge 401 or 6401, which is commercially available from H. Tinsley and Co., New Addington, Croydon, Surrey, UK. In the Rat Skin TER assay, test materials (liquids: 150 µL; solids 100 mg plus 150 µL of water) are applied for two and 24 hours to the epidermal surfaces of skin discs obtained from the skin of humanely killed young rats. Nine to 15 discs can be prepared from one rat pelt which can be used to test up to five chemicals. Corrosive materials produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction of the inherent transcutaneous electrical resistance below a predetermined threshold level of 5 kΩ.

A prevalidation study of the Rat Skin TER assay was conducted during 1993 and 1994 (Botham et al., 1995) to evaluate the relative performance and interlaboratory variability of the method. Subsequently, in 1997, the Rat Skin TER method was also evaluated in an ECVAM validation study as an alternative for traditional *in vivo* testing using the same 60 chemicals and chemical mixtures as EPISKIN™ (Fentem et al., 1998).

The database used in the TER evaluation consisted of data from three published sources (Botham et al., 1992; Botham et al., 1995; Fentem et al., 1998). Based on a database of 122 chemical and chemical mixtures, TER had the following performance:

- accuracy: 81% (99/122 chemicals or chemical mixtures)
- sensitivity: 94% (51/54 chemicals or chemical mixtures)
- specificity: of 71% (48/68 chemicals or chemical mixtures)
- false positive rate: 29% (20/68)
- false negative rate: 6% (3/54)

These performance characteristics were not different when the Botham et al. (1992) and (1995) studies were evaluated independently of the ECVAM validation study (Fentem et al., 1998). The Rat Skin TER assay was not capable of classifying chemicals or chemical mixtures by UN corrosivity packing group.

In the ECVAM validation study (Fentem et al., 1998), the intra- and inter- laboratory reliability was evaluated. Inter- and intra-laboratory reproducibility were approximately equivalent, with no evidence of systematic differences between experiments within a laboratory. Of the 60 chemicals tested, 37 gave the same corrosivity classification in both experiments in all three laboratories (six

tests). Discordant results for the remaining chemicals were as follows: one of six tests for 11 chemicals and two to three of six tests for 12 chemicals. ECVAM concluded the TER assay had acceptable reproducibility.

ICCVAM Recommendations

Draft proposed test recommendations were developed by the ICCVAM Corrosivity Working Group (CWG), which was composed of Federal Agency scientists who have experience and/or expertise with corrosivity testing. These proposed recommendations were endorsed by ICCVAM and made available with background review materials for a 45-day public comment period as announced in a September 28, 2001, *Federal Register* notice (Appendix D).

Twenty-one public comments were received and considered by the CWG, which then drafted final test recommendations that were forwarded with the public comments for consideration by ICCVAM. The ICCVAM revised and approved the final test recommendations in May, 2002.

Based on an evaluation of the ECVAM validation studies and all other available data, ICCVAM concludes that there are sufficient data to substantiate the use of these assays for assessing the dermal corrosion potential of chemicals in a weight-of-evidence approach in an integrated testing scheme (OECD, 2001b; OECD, 2001d). EPISKIN™, EpiDerm™ (EPI-200), and Rat Skin TER are not appropriate methods for assessing irritation. The integrated testing schemes for dermal irritation/corrosion allow for the use of validated and accepted *in vitro* methods. In this approach, positive *in vitro* corrosivity responses do not generally require further testing and can be used for classification and labeling. Negative *in vitro* corrosivity responses would be followed by *in vivo* dermal irritation/corrosion testing. (Note: The first animal used in the irritation/corrosivity assessment would be expected to identify any chemical corrosives that were false negatives in the *in vitro* test). Furthermore, as is appropriate for any *in vitro* assay, there is the opportunity for confirmatory testing if false positive results are indicated based on a weight-of-evidence evaluation of supplemental information, such as pH, structure-activity relationships (SAR), and other chemical and testing information.

ICCVAM concludes also that each of the three *in vitro* corrosivity methods sufficiently consider and incorporate, where scientifically feasible and applicable, the 3Rs of animal use alternatives (refinement, reduction, and replacement). When EpiDerm™ (EPI-200) and EPISKIN™ are used as part of the integrated testing strategy for corrosivity/irritation, there is a reduction in the number of animals required because positive results usually eliminate the need for animal testing, and when further testing in animals is determined to be necessary, only one animal could be required to identify a corrosive chemical (one animal is used if the *in vitro* test is negative). Compared to the rabbit corrosivity test, the Rat Skin TER method reduces the number of animals used because skin from only one rat may be used to test up to five chemicals. Similar to EpiDerm™ (EPI-200) and EPISKIN™, use of the Rat Skin TER assay as part of the integrated testing strategy for corrosivity/irritation reduces and refines the use of animals when negative *in vitro* results are obtained.

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ICCVAM Evaluation of *In Vitro* Methods for Assessing the Dermal Corrosivity Potential of Chemicals: EPISKIN™, EpiDerm™ (EPI-200 Model), and Rat Skin Transcutaneous Electrical Resistance (TER)

Public Law 106-545 directs the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate new, revised and alternative test methods, and to develop and forward test recommendations to appropriate Federal agencies. ICCVAM recently evaluated and developed test recommendations for three *in vitro* methods for assessing the dermal corrosivity potential of chemicals. The methods are:

- EPISKIN™
- EpiDerm™ (EPI-200)
- Rat Skin Transcutaneous Electrical Resistance (TER) Assay

Draft proposed test recommendations were developed by the ICCVAM Corrosivity Working Group (CWG), which is composed of Federal Agency scientists who have experience and/or expertise with corrosivity testing. These proposed recommendations were endorsed by ICCVAM and made available with background review materials for a 45-day public comment period as announced in a September 28, 2001 *Federal Register* notice (NIEHS 2001, Appendix D). Written public comments were received from 15 individuals and six organizations; these comments are provided in Appendix E. The comments were considered by the CWG, which then drafted final test recommendations that were forwarded to and approved by ICCVAM in May 2002.

1.1 Introduction

ICCVAM has developed test recommendations for the use of three *in vitro* test methods to assess the dermal corrosivity potential of chemicals and chemical mixtures: EpiDerm™ (EPI-200), EPISKIN™, and the Rat Skin TER assay. Validation studies for these methods were conducted by the European Centre for the Validation of Alternative Methods (ECVAM) (Baratt et al., 1998; Fentem et al., 1998; Liebsch et al., 2000). The validation status of these three methods has been evaluated by the ECVAM Scientific Advisory Committee (ESAC) (Balls and Corcelle, 1998; Balls and Hellsten, 2000), and EPISKIN™ and Rat Skin TER have also been evaluated by the European Commission's Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) (SCCNFP, 1998). The three methods have been adopted for regulatory use within the European Union (EU) by the European Commission (EU, 2000). The EPISKIN™ human skin model is commercially available from EPISKIN SNC, Lyon, France, a wholly owned subsidiary of L'OREAL. EpiDerm™ (EPI-200) is commercially available from MatTek Corporation, Ashland, MA, USA. In the TER assay, transcutaneous electrical resistance is measured using an AIM electronic databridge 401 or 6401, which is commercially available from H. Tinsley and Co., New Addington, Croydon, Surrey, UK.

ICCVAM Expedited Review Process

ICCVAM used an expedited test method review process to consider these three methods because they had already been evaluated by ECVAM (ICCVAM, 2001). The ICCVAM CWG considered background review documents prepared by the NTP

Interagency Center for the Evaluation of Alternative Methods (NICEATM) for each of the three corrosivity methods. Based on the information provided and previous reviews, a formal independent scientific peer review panel evaluation was not considered necessary. In accordance with the expedited review process procedures, the CWG developed proposed test recommendations which were reviewed and endorsed by ICCVAM. A *Federal Register* notice (September 28, 2001, Vol. 66, No. 189, pp.49685-6) announced the availability and requested public comment on the proposed recommendations and the test method background review documents (Appendix D). These public comments are discussed below in Section 1.2.3 of this document and are provided in Appendix E. Following receipt and consideration of public comments, ICCVAM prepared final recommendations on these methods. In accordance with Public Law 106-545, these ICCVAM recommendations will be forwarded to U.S. agencies for their consideration and acceptance where appropriate.

1.2 Background

1.2.1 ECVAM Evaluation

Validation studies on these three *in vitro* assays were conducted by ECVAM (Barratt et al., 1998; Fentem et al., 1998; Liebsch et al., 2000). Based on the results, which met pre-study acceptance criteria of no more than 20% false negatives and no more than 20% false positives, the ECVAM Study Management Team concluded that EpiDerm™ (EPI-200), Rat Skin TER, and EPISKIN™ were scientifically valid for use as replacements for the animal test currently used to distinguish between corrosive and non-corrosive chemicals for all chemical classes (Fentem et al., 1998; Liebsch et al., 2000). Of the three test methods, only EPISKIN™ was able to distinguish between chemicals in the EU skin corrosion hazard classes (R35 and R34) and for two of the three United Nations (UN) packing group classifications (I and II/III) (Fentem et al., 1998).[1] A detailed review of these validation studies is described in this final report (ICCVAM, 2002).

1.2.2 Relevant Comments from an OECD Expert Consultation Meeting

In 1999, the Organisation for Economic Co-operation and Development (OECD) proposed a draft test guideline (TG) describing the Rat Skin TER assay and a generic *in vitro* skin model assay (OECD, 1999). A generic skin model assay procedure was proposed rather than the specific EPISKIN™ and EpiDerm™ (EPI-200) test method protocols because of OECD's policy not to adopt TGs for tests that require equipment or material that can only be obtained from unique sources. OECD requested review of the draft TG by member countries in 2000. Extensive comments were received, and an Extended Expert Consultation Meeting was convened in Berlin, Germany on November 1-2, 2001 to address these comments and other technical issues.

[1] UN packing group classifications I, II, and III are assigned based on the capacity of a chemical, when tested on the intact skin of rabbits, to produce skin corrosion following exposure intervals of 3 minutes, 1 hour, or 4 hours, respectively (Fentem et al., 1998). Current EU regulations require classification of chemicals according to certain risk phrases, such as those assigned based on whether the chemical causes corrosion following a 3-minute application (R35 – "causes severe burns"; analogous to packing group I) or 4 hours (R34 – "causes burns"; analogous to packing groups II and III) (Barratt et al., 1998; Fentem et al., 1998). Internationally harmonized classification schemes for corrosivity, which include the UN packing group classifications, have recently been adopted (OECD, 2001a).

Section 2.0

EPISKIN™

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The meeting experts agreed to prepare two separate test guidelines, one for the TER, and one for the human skin test model. With regard to use of these methods, the Expert Meeting participants agreed that, in the majority of all applications, the *in vitro* skin corrosion tests would be applied as one of the initial steps of a tiered approach. Consequently, false negative predictions are likely to be detected when the test chemical is tested on the first rabbit for skin irritation (OECD, 2002c). The deliberations at the meeting did not change the general procedures for the generic human skin model assay; however, the following revisions were proposed for the TER assay:

Rat Skin TER Assay

- Substances with a resistance value greater than 5 kΩ are considered non-corrosive. Most test substances typically have produced resistance values in two ranges, <3 kΩ (positive) and >10 kΩ (negative). It was recommended that if the resistance value for a test substance is close to the 5 kΩ decision criteria, a judgment of whether to classify the substance as positive or negative should consider a weight-of-evidence strategy or assume the more conservative approach, based on regulatory needs. If classified as positive, the standard positive confirmatory dye-binding test to demonstrate physical destruction of the stratum corneum should be conducted to avoid a false positive classification.
- Several critical aspects of the test system were defined, including the surface area of skin used, the use of magnesium sulfate ($MgSO_4$) as the electrochemical solution for measuring resistance, and the age of the animals.

Two revised draft test guidelines were subsequently circulated for comment in March 2002, and further revised for consideration at the Test Guideline Program National Coordinators Meeting in June, 2002. Both guidelines were accepted pending further revisions agreed on at the meeting (personal communication, June 2002, Angela Auletta, U.S. EPA, Washington, D.C.).

1.2.3 Public Comments

Twenty-one public comments were received in response to the September 28, 2001 *Federal Register* notice. Three of the 21 responses provided general comments about the Background Review Document (BRD), stating that it was well organized, comprehensive and clearly written. The remaining comments addressed specific aspects of the proposed test recommendations as discussed below.

Integrated testing scheme vs. stand-alone

Seventeen of the 21 public responses disagreed with or stated opposition to the proposed ICCVAM recommendation that these three *in vitro* methods should be used in the context of a weight-of-evidence approach in an integrated scheme, where negative *in vitro* corrosivity responses would be followed by *in vivo* dermal irritation/ corrosion testing. Three of the 21 comments stated that the three *in vitro* tests should be used as stand-alone tests, such that negative results would be classified as non-corrosives without further confirmatory testing.

ICCVAM recognizes that it would be highly desirable to completely replace animals for corrosivity testing. However, the current performance characteristics resulting from validation studies of these *in vitro* assays do not adequately support their use as stand-alone assays for hazard classification. Specifically, the results of the ECVAM validation studies indicate that significant false negative results may occur with these

assays (12% for TER; 13% for EpiDerm™ (EPI-200); 17% for EPISKIN™) (Fentem et al., 1998; Liebsch et al., 2000). In such instances, a false negative result for a corrosive chemical would result in erroneous classification as a non-corrosive. Accordingly, the corrosive chemical would not be labeled with appropriate hazard warnings of corrosivity. Serious and irreversible damage can result from human exposure to corrosive substances, including dermal ulceration and scarring. Given that results of dermal corrosion are often used by regulators to identify corrosives to the eye, false negative responses in the *in vitro* dermal corrosion tests will fail to identify potential serious effects to the eye for 12-17% of true dermal corrosives. Therefore, this level of error was not considered by ICCVAM to provide adequate protection for public health and safety. ICCVAM is also cognizant of the fact that nearly all regulatory authorities that require corrosive testing also require a determination of dermal irritation potential if substances are not found to be corrosive. Current international guidance and test guidelines for dermal irritation/corrosion call for sequential testing, so that if a corrosive substance is erroneously identified in the *in vitro* test as non-corrosive, it will be detected as corrosive in an *in vivo* irritancy test (EPA, 1998; OECD 2001a, OECD 2001b; Worth, et al. 1998). *In vitro* tests for irritancy are being developed and may be coupled with *in vitro* corrosion tests. Such test strategies will need to be evaluated for their ability to correctly identify corrosive and irritant chemicals that produce false negative results in such *in vitro* tests. Thus, as outlined in Section 1.3, ICCVAM concludes that the false negative rates obtained in these three *in vitro* assays preclude their use as stand-alone assays. Instead, these assays should be considered as screens, where positive results are classified as corrosives and negative results require further testing for corrosive potential.

General test method guideline vs. specific validated test method protocols

One comment suggested using a general "skin model corrosivity test" description rather than the specific test method protocols for EpiDerm™ (EPI-200) and EPISKIN™. The basis for this suggestion was: 1) the 2 assays are similar with regard to test material exposure, endpoints, prediction models, and predictive power; and 2) this would better allow the future use of other skin models that are similar with regard to structure and function and that perform comparably to these previously validated skin models. The respondent also acknowledged that this would require the development of structural and performance criteria, including a set of reference chemicals, to evaluate such new skin models.

While ICCVAM recognizes the increased flexibility of general test method descriptions, it also recognizes the critical importance of determining the acceptability of validated specific protocols for which the reliability and performance characteristics have been carefully determined. The use of protocols that adhere to a general test method description but have not been adequately validated could lead to erroneous results. Therefore, ICCVAM is only recommending validated, specific test method protocols. However, ICCVAM appreciates that similar test methods could be found to be acceptable if adequate performance and reliability are demonstrated for a standardized test method protocol in appropriate validation studies. The provision of a list of reference chemicals and minimum performance criteria would

certainly be helpful to those interested in validating such models in the future.

Concern about limited availability

Another comment suggested revising the BRD and related documents to remove any reference to EPISKIN™, or alternatively, to include a qualifying statement regarding the current commercial unavailability of this human skin model. The basis for the comment was to avoid recommending a test method that is not otherwise commercially available. ICCVAM has added a statement regarding the current availability of each assay.

1.3 ICCVAM Test Method Recommendations

EPISKIN™, EpiDerm™ (EPI-200), and Rat Skin Transcutaneous Electrical Resistance (TER)

Based on evaluation of the ECVAM validation studies and other available data, ICCVAM concludes that there are sufficient data to substantiate the use of these three *in vitro* assays for assessing the dermal corrosion potential of chemicals in a weight-of-evidence approach in an integrated testing scheme (EPA, 1996; OECD, 2001c; OECD, 2001d; OECD, 2001e; OECD, 2001f; Worth, et al. 1998). EPISKIN™, EpiDerm™ (EPI-200), and Rat Skin TER are not appropriate methods for assessing irritation. Integrated testing schemes for dermal irritation/corrosion allow for the use of validated and accepted *in vitro* methods. In this approach, positive *in vitro* corrosivity responses do not generally require further testing and can be used for classification and labeling. Negative *in vitro* corrosivity responses shall be followed by *in vivo* dermal irritation/corrosion testing. (Animals used in the irritation/corrosivity assessment would be expected to identify any chemical corrosives that were false negatives in the *in vitro* test). Furthermore, as is appropriate for any test system, there is the opportunity for confirmatory testing if false positive results are indicated based on a weight-of-evidence evaluation of supplemental information, such as pH, structure-activity relationships (SAR), and other chemical and testing information.

ICCVAM previously evaluated another *in vitro* method for determining corrosivity, Corrositex® (ICCVAM, 1999), and recommended that it could be used in a similar manner as recommended for EPISKIN™, EpiDerm™ (EPI-200), and Rat Skin TER. Corrositex® is also approved by the U.S. Department of Transportation for identifying the three United Nations packing group classifications for certain chemical classes (ICCVAM, 1999; U.S. DOT, 2000). The ICCVAM report on Corrositex® is available at http://iccvam.niehs.nih.gov/docs/reports/corprrep.pdf.

Animal Welfare Considerations

ICCVAM concludes that each of the three *in vitro* corrosivity methods sufficiently incorporates, where scientifically feasible and applicable, the 3Rs of animal use alternatives (refinement, reduction, and replacement). When EpiDerm™ (EPI-200) and EPISKIN™ are used as part of an integrated testing strategy for irritation/corrosion, there is replacement of animals because positive *in vitro* results usually eliminate the need for animal testing. There is a reduction in animal use with negative *in vitro* results because only one positive animal may be needed to identify an *in vitro* false negative as a corrosive chemical. Compared to the rabbit corrosivity test, the Rat Skin TER assay reduces the number of animals used because skin from one rat may be used to test up to five chemicals. Similar to EpiDerm™ (EPI-200) and EPISKIN™, use of the Rat Skin TER assay as part of the

integrated testing strategy for irritation/ corrosion reduces and refines the use of animals when negative *in vitro* results are obtained.

Summary Report of the EPISKIN™ *In Vitro* Assay for Assessing Dermal Corrosivity

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PURPOSE

This report focuses on the performance of EPISKIN™ to determine the usefulness and limitations of the assay for the identification of potential human corrosive chemicals. This report also discusses how the EPISKIN™ assay compares to the *in vivo* rabbit skin corrosivity test and to other *in vitro* corrosivity tests (Rat Skin Transcutaneous Electrical Resistance [TER], EpiDerm™ [EPI-200], and Corrositex®). The data and assessments in the European Centre for the Validation of Alternative Methods (ECVAM) formal validation study on EPISKIN™ (Barratt et al., 1998; Fentem et al., 1998) were reviewed. Additionally, an independent analysis of the performance data, based on the information provided in Fentem et al. (1998), was conducted.

EVALUATION OF REGULATORY AND SCIENTIFIC RATIONALE

EPISKIN™ is one of several *in vitro* corrosivity assays evaluated as alternatives to the *in vivo* rabbit corrosivity test by ECVAM in a formal validation study (Fentem et al., 1998). EPISKIN™ is a three-dimensional human skin model that measures cell viability. Because it is a human skin model, it may be more relevant to assessing human skin corrosivity potential than a test based on skin from another species. Also, the mode of application (topical) of the test material mimics the route of human exposure.

EPISKIN™ has been endorsed by the ECVAM Scientific Advisory Committee for use in corrosivity testing in Europe (Balls and Corcelle, 1998) and EPISKIN™ has also been evaluated and endorsed for its intended use by the European Commission Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) (Anon., 1999). This method has been adopted for regulatory use within the European Union (EU) by the European Commission (EU, 2000).

EVALUATION OF THE TEST METHOD

A standard kit contains media, reagents, and 12 epidermis units. The epidermis units provided in the test kit are comprised of a reconstructed epidermis and a functional stratum corneum. For use in corrosivity testing, the test material (liquids: 50 µL; solids: 20 mg) is topically applied to an epidermis unit for 3, 60, and 240 minutes. Per test compound, one epidermis unit is needed for each of the three test periods. Cell viability is assessed by measuring mitochondrial activity using the MTT (a tetrazolium salt) assay. A 35% decrease in cell viability is used to indicate a potential for human corrosivity. The scientific and mechanistic basis of the test and the rationale for using a 35% decrease in cell viability as the criterion for identifying potential human corrosivity were not discussed by Fentem et al. (1998). However, mechanistically, corrosivity is associated with cell death.

EVALUATION OF TEST METHOD DATA QUALITY

Only limited validation test data are available on EPISKIN™. In the single published validation study by Fentem et al. (1998), ECVAM evaluated 60 chemicals. The chemical selection procedure was described in sufficient detail by Barratt et al. (1998). The main criterion for including chemicals in the study was that their corrosivity classification (C= corrosive; NC = noncorrosive) was based on unequivocal animal data (Barratt et al., 1998). The ECVAM validation chemical test set

included organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1 NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC), and soaps/surfactants (3NC). Despite the small numbers of chemicals in some categories, ECVAM concluded that the set of test chemicals represented the best possible group for evaluating the performance characteristics of the *in vitro* assays, given the limited availability of unequivocal animal data (Barratt et al., 1998).

Each chemical was tested three times by each of three different laboratories. The tests were stated to have been conducted in the "spirit" of GLP (Fentem et al., 1998). A formal audit of the ECVAM data by a Quality Assurance Unit was not conducted; however, it was stated that all data submitted by the participating laboratories were verified against the original data sheets by ECVAM staff on at least three separate occasions.

EVALUATION OF TEST METHOD PERFORMANCE

For this summary report, an analysis was conducted, similar to the performance analysis conducted for the ICCVAM Peer Review of Corrositex®; the current analysis evaluated the performance characteristics of the EPISKIN™ assay against the corresponding *in vivo* rabbit corrosivity data. The database used in the EPISKIN™ evaluation consisted of data from the ECVAM validation study only; other data were not located. For ease of comparison, chemicals evaluated in the EPISKIN™ assay were classified into the same chemical and product class designations used in the Corrositex® evaluation. A weight-of-evidence approach was used for classifying discordant results within or between laboratories; in instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls), the chemical was eliminated from inclusion in the performance calculations.

Based on the database of 60 chemicals and chemical mixtures used in the validation study (**Table 2.1**), EPISKIN™ had an accuracy of 83% (50/60 chemicals or chemical mixtures), a sensitivity of 82% (23/28 chemicals or chemical mixtures), a specificity of 84% (27/32 chemicals or chemical mixtures), a false positive rate of 16% (5/32 chemicals or chemical mixtures), and a false negative rate of 18% (5/28 chemicals or chemical mixtures). Furthermore, EPISKIN™ was able to distinguish between known R35/I and R34/II & III chemicals[1]. Based on these data, which met pre-study acceptance criteria of no more than 20% false negatives and 20% false positives, the ECVAM study Management Team concluded that EPISKIN™ was valid for use as a replacement for the *in vivo* rabbit skin test for distinguishing between corrosive and noncorrosive chemicals for all of the chemical classes studied (Fentem et al., 1998; Balls and Corcelle, 1998). Because of the relatively small numbers of chemicals evaluated in some chemical classes (i.e., cleaners and detergents), definitive conclusions as to the adequacy of

[1]UN packing group classifications I, II, and III are assigned based on the capacity of a chemical, when tested on the intact skin of rabbits, to produce skin corrosion following exposure intervals of 3 minutes, 1 hour, or 4 hours, respectively (Fentem et al., 1998). EU regulations require classification of chemicals according to certain risk phases, such as those assigned based on whether the chemical causes corrosion following a 3-minute application (R35 – “causes severe burns”; analogous to packing group I) or 4 hours (R34 – “causes burns”; analogous to packing groups II and III) (Barratt et al., 1998; Fentem et al., 1998).

EPISKIN™ for some classes of chemicals were difficult to make with a high degree of confidence. Additionally, no assessment could be made with respect to mixtures. However, it was stated that taking into account the relative simplicity of the mechanism of action of corrosives, this method would be generally applicable across all chemical classes (Fentem et al., 1998).

EVALUATION OF TEST METHOD RELIABILITY (REPEATABILITY/ REPRODUCIBILITY)

The inter- and intra-laboratory reliability of EPISKIN™ was evaluated in the ECVAM validation study (Fentem et al., 1998). In each laboratory, each chemical was tested three times using three different batches of EPISKIN™. Intra- and inter-laboratory reliability was evaluated using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors), scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Of the 60 chemicals tested, 42 gave the same corrosivity classification in all three experiments in all three laboratories. In seven cases, the median results for the three laboratories gave identical predictions. In only three cases did one laboratory give results that were consistently in a different classification category than those from the other laboratories. In an additional three cases, the median result from one laboratory was in a different category than those from the other laboratories, and in five cases, chemicals gave results that crossed the classification boundaries in more than one laboratory. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that EPISKIN™ met the criteria agreed by the Management Team concerning acceptable intra- and inter-laboratory reproducibility (Fentem et al., 1998). Due to the lack of quantitative data, by experiment and laboratory, for individual chemicals in the published studies, no independent evaluation of repeatability or reproducibility for EPISKIN™ could be conducted. However, after reviewing the intra- and inter-laboratory evaluations conducted by ECVAM, it was concluded by NICEATM that the analyses were appropriate and that the conclusions were accurate.

Table 2.1 Performance of the EPISKIN™ Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Fentem et al., 1998)

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	**60**	**83**	**(50/60)**	**82**	**(23/28)**	**84**	**(27/32)**
Organic and Inorganic Acids and Bases[1]	**41**	**78**	**(32/41)**	**81**	**(21/26)**	**73**	**(11/15)**
Organic and Inorganic Bases and Base Mixtures[2]	**14**	**64**	**(9/14)**	**60**	**(6/10)**	**75**	**(3/4)**
Organic and Inorganic Acids and Acid Mixtures	**20**	**85**	**(17/20)**	**100**	**(11/11)**	**67**	**(6/9)**
Amines	**10**	**60**	**(6/10)**	**57**	**(4/7)**	**67**	**(2/3)**
Inorganic Bases and Base Mixtures	**4**	**75**	**(3/4)**	**67**	**(2/3)**	**100**	**(1/1)**
Acid Derivatives	**7**	**86**	**(6/7)**	**80**	**(4/5)**	**100**	**(2/2)**
Surfactants	**5**	**80**	**(4/5)**	**NA**	**(0/0)**	**80**	**(4/5)**
Industrial Chemicals	**10**	**100**	**(10/10)**	**100**	**(1/1)**	**100**	**(9/9)**
Cleaners and Detergents	**1**	**100**	**(1/1)**	**NA**	**(0/0)**	**100**	**(1/1)**

[1] This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives

[2] This chemical class includes amines, inorganic bases, and base mixtures.

OTHER SCIENTIFIC REVIEWS

In March 1999, a search of the open literature was conducted to locate additional EPISKIN™ studies. Six databases (Medline, Toxline, Embase, Biosis, Caba, and LifeSci) were searched using the key terms "Episkin", and "Epi" within one word of "skin". The search found no additional relevant studies conducted with EPISKIN™. In May 2001, another search was conducted to locate additional EPISKIN™ studies. Four databases (PubMed, Web of Science, Toxline, and Current Contents Connect) were searched using the same search strategy and no additional relevant studies were found.

OTHER CONSIDERATIONS

The EPISKIN™ kit contains all of the necessary materials to conduct the test and does not require additional preparation. No animals are used in this test. ECVAM concluded that, compared to the *in vivo* test method, EPISKIN™ costs less to perform (Fentem et al., 1998). The cost for conducting EPISKIN™ is reported by L'OREAL Recherche (e-mail communication from Odile de Silva, L'OREAL Recherche) to be approximately $450 per kit (**Table 2.2**). When compared to other *in vitro* corrosivity test methods, the cost of EPISKIN™ is stated to be greater than that of the Corrositexâ and EpiDerm™ (EPI-200) assays and somewhat less than the Rat Skin TER (Fentem et al., 1998).). The EPISKIN™ human skin model is commercially available from EPISKIN SNC, Lyon, France, a wholly owned subsidiary of L'OREAL. The time needed to conduct the EPISKIN™ assay is greater than the Corrositex® assay, comparable to the EpiDerm™ (EPI-200) assay, and less than the Rat Skin TER assay.

RELATED ISSUES

Refinement, Reduction, and Replacement

Since the method is designed as a replacement for animals, EPISKIN™ would clearly reduce the requirement for animal testing for corrosivity. Therefore, it has the potential to eliminate the use of animals for the determination of corrosivity. If used in an integrated approach, EPISKIN™ provides for reduction and refinement of animal use.

Comparison to Other *In Vitro* Assays

General comparative information on the TER, EPISKIN™, and Corrositexâ assays is provided in **Tables 2.2** through **2.5**.

Table 2.2 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays

	Rat Skin TER	EPISKIN™ (prediction model B)	EpiDerm™ (EPI-200) (prediction model 2)	Corrositex®
Test Method Description	Acceptable	Acceptable	Acceptable	Acceptable
Adequacy/Completeness of Protocol	Acceptable	Acceptable	Acceptable	Acceptable
Usefulness for Assessing Corrosivity/Non-corrosivity	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Usefulness for Determining Packing Groups	Not Acceptable (Fentem et al., 1998)	Can group as UN packing group II/III or I (Fentem et al., 1998)[a]	Not Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Repeatability and Reproducibility	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (Fentem et al., 1998; ICCVAM, 1999)
Animal Use Refinement, Reduction, and Replacement Considerations	Refines and reduces animal use when used as a stand-alone test or in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.	Refines and reduces animal use when used in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.
Cost	~$500-850/test	~$450/test kit[b]	~$200/test chemical	~$300/test chemical
Study Duration	2 work-days	1 work-day	1 work-day	≤ 4 hr/chemical

[a] Since the performance of EPISKINä was not assessed for distinguishing between UN packing groups II and III, all R34 classifications would be conservatively classified as UN packing group II.

[b] One to three chemicals may be tested per test kit; however, it is recommended by the supplier that each test chemical be assayed using 3 different skin batches/kits which equates to a total cost of ~$430/ test chemical.

Table 2.3 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays Based on a Weight-of-Evidence Approach[a] by Chemical using Data from the ECVAM and other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™	EpiDerm™ (EPI-200) (prediction model 2)	Corrositex®
Number of Chemicals	122	60	24	163
Overall Sensitivity[b]	94% (51/54)	82% (23/28)	92% (11/12)	85% (76/89)
Overall Specificity[c]	71% (48/68)	84% (27/32)	83% (10/12)	70% (52/74)
Overall Accuracy[d]	81% (99/122)	83% (50/60)	92% (22/24)	79% (128/163)
False Positive Rate	29% (20/68)	16% (5/32)	17% (2/12)	30% (22/74)
False Negative Rate	6% (3/54)	18% (5/28)	8% (1/12)	15% (13/89)
Test Chemical Inter-laboratory Coefficient of Variation	34.7[e] 3.8-322[f] 120[g]	11.3[e] 3.9-148.8[f] 20[g]	12.3[e] 0.9-51.2[f] 144[g]	30.3[e] 7.7-252.5[f] 180[g]

[a] A chemical is first classified as positive or negative for corrosivity within each laboratory based on the majority of test results obtained (when replicate testing was conducted). Next, the chemical is classified as positive or negative for corrosivity based on the majority of test results obtained in multiple laboratories (when multiple laboratory studies were conducted). In instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls within or across laboratories), the chemical was eliminated from inclusion in the performance calculations.

[b] Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test.

[c] Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test.

[d] Accuracy (concordance) is defined as the proportion of correct outcomes of a method.

[e] Median values

[f] Range of values

[g] The total number of independent values, which is calculated as the number of chemicals tested multiplied by the number of participating laboratories.

Table 2.4 General comparison of the Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) assays from independent test results in the ECVAM validation studies (Fentem et al., 1998; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™ (prediction model B)	EpiDerm™ (EPI-200) (prediction
Number of Chemicals Tested in ECVAM Validation Study	60 (Fentem et al., 1998)	60/24[a] (Fentem et al., 1998)	24 (Liebsch et al., 2000)
Sensitivity[b]	88% (140/159)	83% (201/243) / 88% (87/99)	88% (63/72)
Specificity[b]	72% (142/196)	80% (237/297) / 79% (92/117)	86% (62/72)
Accuracy[b]	79% (282/355)[c]	81% (438/540) / 83% (179/216)	87% (125/144)
False Positive Rate[b]	28% (54/196)	20% (60/297) / 21% (25/117)	14% (10/72)
False Negative Rate[b]	12% (19/159)	17% (42/243) / 12% (12/99)	13% (9/72)
Number of Trials[f]	355	540 / 216	144
Test Chemical Inter-laboratory Coefficient of Variation	34.7[d] 10-322[e] 360[f]	30.2[d] 7.7-252.5[e] 540[f]	12.3[d] 0.9-51.2[e] 144[f]

[a] The first numbers for accuracy, sensitivity, specificity, false positive rate, and false negative rate correspond to the 60 chemicals tested in the ECVAM Skin Corrosivity Test using EPISKIN™ (Barratt et al., 1998; Fentem et al., 1998); the latter values correspond to a direct comparison of EpiDerm™ (EPI-200) and EPISKIN™ for the same 24 materials tested in both systems (Liebsch et al., 2000).

[b] Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method. False positive rate is defined as the proportion of all negative chemicals or chemical mixtures that are falsely identified as positive. False negative rate is defined as the proportion of all positive chemicals or chemical mixtures that are falsely identified as negative.

[c] The percentages are based on the number of correct trials among the total number of trials (i.e., independent tests) provided in parenthesis.

[d] Median values

[e] Range of values

[f] The total number of trials conducted in the validation study minus the non-qualified (NQ) results. This number is equal to the number of chemicals multiplied by the number of participating laboratories multiplied by the number of replicate tests.

Table 2.5 Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000)

No.[a]	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN™ [b]	EpiDerm™ (EPI-200)
1	Hexanoic acid	ORGAC	R34/II&III	R35	R35	N/A
29	65/35 Octanoic/decanoic acid	ORGAC	R34/II&III	R34	R35	N/A
36	2-Methylbutyric acid	ORGAC	R34/II&III	R35	R34	N/A
40	Octanoic acid (caprylic acid)	ORGAC	R34/II&III	R35	R34/C	C
47	60/40 Octanoic/decanoic acids	ORGAC	R34/II&III	R34	R34/C	C
50	55/45 Octanoic/decanoic acids	ORGAC	R34/II&III	R35	R34	N/A
7	3,3'-Dithiodipropionic acid	ORGAC	NC	NC	NC	N/A
12	Dodecanoic acid (lauric acid)	ORGAC	NC	NC	NC	NC
26	Isotearic acid	ORGAC	NC	NC	NC	NC
34	70/30 Oleine/octanoic acid	ORGAC	NC	NC	NC	N/A
58	10-Undecenoic acid	ORGAC	NC	NC	R34	N/A
2	1,2-Diaminopropane	ORGBA	R35/I	R35	R34/C	C
15	Dimethyldipropylenetriamine	ORGBA	R35/I	R35	R34/C	C
38	Tallow amine	ORGBA	R35/II	2R34/2NC/2NQ	NC	N/A
55	1-(2-Aminoethyl)piperazine	ORGBA	R34/II	R35	NC	N/A
13	3-Methoxypropylamine	ORGBA	R34/II&III	R35	R34	N/A
17	Dimethylisopropylamine	ORGBA	R34/II&III	R35	R34/C	C
45	n-Heptylamine	ORGBA	R34/II&III	R35	NC	C
10	2,4-Xylidine (2,4-Dimethylaniline)	ORGBA	NC	R34	R34	N/A
35	Hydrogenated tallow amine	ORGBA	NC	NC	NC	NC
59	4-Amino-1,2,4-triazole	ORGBA	NC	NC	NC	NC
8	Isopropanol	NORG	NC	NC	NC	N/A
11	2-Phenylethanol	NORG	NC	NC	NC	N/A
16	Methyl trimethylacetate (referred to as Methyl 2,2-dimethylpropanoate in EpiDerm™ (EPI-200)	NORG	NC	NC	NC	C
19	Tetrachloroethylene	NORG	NC	NC	NC	NC
22	n-Butyl propionate	NORG	NC	NC	NC	N/A
27	Methyl palmitate	NORG	NC	NC	NC	N/A
44	Benzyl acetone	NORG	NC	NC	NC	NC
51	Methyl laurate	NORG	NC	NC	NC	N/A
56	1,9-Decadiene	NORG	NC	NC	NC	NC
3	Carvacrol	PHEN	R34/II&III	R34	R34	N/A
23	2-tert-Butylphenol	PHEN	R34/II&III	R35	R34/C	C
9	*o*-Methoxyphenol (Guaiacol)	PHEN	NC	NC	R34	N/A
30	4,4-Methylene-bis-(2,6-di-tert-butylphenol)	PHEN	NC	NC	NC	N/A
49	Eugenol	PHEN	NC	NC	NC	NC

Table 2.5 (continued)

No.[a]	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN™ [b]	EpiDerm™ (EPI-200)
4	Boron trifluoride dihydrate	INORGAC	R35/I	R35	R35/C	C
28	Phosphorus tribromide	INORGAC	R35/I	R35	R35/C	C
32	Phosphorus pentachloride	INORGAC	R35/I	R35	R34	N/A
25	Sulfuric acid (10% wt.)	INORGAC	R34/II&III	R34	R34	N/A
57	Phosphoric acid	INORGAC	R34/II	R35	R34	N/A
43	Hydrochloric acid (14.4% wt)	INORGAC	R34/II&III	R35	R34	N/A
53	Sulfamic acid	INORGAC	NC	R34	R34/C	C
18	Potassium hydroxide (10% aq.)	INORGBA	R34/II&III	R35	R34/C	C
42	2-Mercaptoethanol,Na salt (45%aq.)	INORGBA	R34/II&III	R35	NC	N/A
21	Potassium hydroxide (5% aq.)	INORGBA	NC	R35	R34	N/A
24	Sodium carbonate (50% aq.)	INORGBA	NC	R34	NC	NC
20	Ferric [iron (III)] chloride	INORGSAL	R34/II	R35	R34	N/A
52	Sodium bicarbonate	INORGSAL	NC	R34	NC	N/A
54	Sodium bisulfite	INORGSAL	NC	3R34/3NC	NC	N/A
5	Methacrolein	ELECTRO	R34/II&III	NC	R34/C	NC
14	Allyl bromide	ELECTRO	R34/II&III	R35	R34	N/A
48	Glycol bromoacetate (85%)	ELECTRO	R34/II&III	NC	R34/C	C
6	Phenethyl bromide	ELECTRO	NC	NC	NC	N/A
31	2-Bromobutane	ELECTRO	NC	3R34/3R35	NC	N/A
33	4-(Methylthio)-benzaldehyde	ELECTRO	NC	NC	NC	N/A
39	2-Ethoxyethyl methacrylate	ELECTRO	NC	NC	NC	N/A
46	Cinnamaldehyde	ELECTRO	NC	NC	NC	N/A
37	Sodium undecylenate (33% aq.)	SOAP	NC	R35	R34	N/A
41	20/80 Coconut/palm soap	SOAP	NC	NC	NC	N/A
60	Sodium lauryl sulfate (20% aq.)	SOAP	NC	R35	NC	NC

Overall corrosivity classifications were determined by the majority of the reported results obtained from each assay. If results do not show a majority, a definitive classification could not be determined.

Definitions are as follows: C = Corrosive; NC = Noncorrosive; R34 is equivalent to packing groups II and/or III; R35 is equivalent of packing group I, except for tallow amine (R35/II); NQ = Non-qualified; N/A = Not applicable because not tested; ORGAC = Organic acid; ORGBA = Organic base; NORG = Neutral organics; PHEN = phenol; INORGAC = Inorganic acid; INORGBA = Inorganic base; INORGSAL = Inorganic salt; ELECTRO = Electrophile; SOAP = Soap surfactant

[a] Number assigned each chemical by the ECVAM Management Team.

[b] For EPISKIN™, prediction model B was the more complex prediction model and was the only model considered in detail by the ECVAM Management Team (Fentem et al., 1998).

SUMMARY CONCLUSIONS AND RECOMMENDATIONS

ECVAM concluded that EPISKIN™ was an *in vitro* replacement assay for *in vivo* corrosivity testing. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that EPISKIN™ was both reliable and reproducible. NICEATM concurs with that conclusion. For some chemical or product classes (e.g., industrial chemicals, cleaners and detergents), the small number of chemicals and/or the unbalanced distribution of corrosive and noncorrosive chemicals does not allow accurate conclusions to be made on the performance of EPISKIN™ for those chemical classes.

The two major questions to be addressed for *in vitro* corrosivity assays are:

1. Has the assay been evaluated sufficiently and is its performance satisfactory to support the proposed use for assessing the corrosivity potential of chemicals and chemical mixtures?

2. Does the assay adequately consider and incorporate, where scientifically feasible, the 3Rs of animal use (refinement, reduction, and replacement alternatives)? Does the assay offer advantages with respect to animal welfare considerations?

EPISKIN™ skin model was adequate for assigning packing groups according to the EU skin corrosion hazard classes (R34/R35) and the UN packing group classifications (I and II/III). However, since the performance of EPISKIN™ was not assessed for distinguishing between UN packing group II and packing group III, all R34 classifications would be conservatively classified as packing group II.

In response to the second question, EPISKIN™ sufficiently considers and incorporates the 3Rs. Specifically, the use of EPISKIN™ offers advantages with respect to animal welfare considerations, including animal use refinement, reduction, and replacement. Similarly, the use of this assay as part of an integrated approach reduces and refines the use of animals by providing a basis for decisions on further testing. When this method is used as part of the integrated testing strategy for corrosivity/irritation, there is a reduction in the number of animals required because positive results usually eliminate the need for animal testing, and when further testing in animals is determined to be necessary, only one animal could be required to identify a corrosive chemical (one animal is used if the *in vitro* test is negative).

ECVAM Protocol for EPISKIN™: an *In Vitro* Assay for Assessing Dermal Corrosivity

Original Draft: March 1997
Confirmed: January 2002

NOTE: This protocol presents the standard operating procedure used in the ECVAM Skin Corrosivity Validation Study (1996/1997). ECVAM confirmed the accuracy of the SOP in October 2000, and this protocol was supplied by Dr. Andrew Worth of ECVAM via email on May 22, 2001.

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EPISKIN™ Test

The corrosivity potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the EPISKIN™ reconstituted human epidermis.

Objectives and Application

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency, hazard identification
PURPOSE OF TESTING	:	classification and labelling

Proposed replacement for the *in vivo* Draize rabbit skin corrosivity test to be used for hazard identification and classification of corrosive potential to fulfil international regulatory requirements pertaining to the handling, packing and transport of chemicals.

Basis of the Method

Most international regulatory classification schemes define chemically induced dermal corrosion as full thickness destruction (necrosis) of the skin tissue, while some extend the definition of corrosion to include any irreversible alterations caused to the skin. The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. The determination of skin corrosion potential is therefore included in international regulatory requirements for the testing of chemicals, for example, in OECD testing guideline 404 (Anon., 1992); Annex V of Directive 67/548/EEC (Anon., 1992) and in the U.S. Code of Federal Regulations (Anon., 1991). Corrosivity is usually determined *in vivo* using the Draize rabbit skin test (Draize *et al*., 1944).

The present test is based on the experience that corrosive chemicals show cytotoxic effects following short-term exposure of the stratum corneum of the epidermis. The test is designed to predict and classify the skin corrosivity potential of a chemical by assessment of its effect on a reconstituted human epidermis.

EPISKIN Standard Model™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin corrosivity testing involves topical application of test materials to the surface of the skin, and the subsequent assessment of their effects on cell viability. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT. (Fentem *et al*., 1998)

Experimental Description

Endpoint and Endpoint Detection	**:**	cell viability as determined by reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT
Test System	**:**	EPISKIN™ reconstructed human epidermis system *

Test materials are applied to the stratum corneum of the epidermal model (one epidermis unit per test material) for three different exposure periods: 3 minutes, 1 hour, and 4 hours. Exposure to the test chemical was terminated by rinsing with PBS (phosphate buffered saline). EPISKIN cultures exposed to the control compounds for 240 min serve as the controls for all three exposure periods. For each test material, three independent tests with three different batches of EPISKIN are to be undertaken.

The viability of the epidermis is assessed by measuring the mitochondrial activity. The tissues are incubated for 3 hours with MTT solution (0.3 mg/l; 2.2 ml per well). MTT, a yellow-coloured tetrazolium salt, is reduced by succinate dehydrogenase into a blue formazan precipitate in the mitochondria of living cells. The precipitated formazan is extracted overnight by using acidified isopropanol (0.85 ml), and is then quantified spectrophotometrically at a wavelength between 545nm and 595nm.

All experimental procedures have to be conducted at room temperature (18-28°C); if the temperature is below 20°C, the 3-hour MTT incubation should be carried out in a warmer environment of 20-28°C. NaCl (50 µl) and glacial acetic acid (50 µl) are used as negative and positive controls, respectively.

Some highly reactive chemicals can produce fumes, which may affect adjacent units in the same plate. It is recommended that if there is any suspicion that a material could cause fumes, it should be tested alone in a single plate. It is particularly important that the negative control units are not exposed to fumes from other units, hence it is recommended to routinely incubate positive and negative controls in a separate plate.

NOTE: The commercial availability of EPISKIN (SADUC-Biomatériaux Imedex, Chaponost, France) was restricted following the completion of the validation study to enable new production facilities to be completed. It is likely to be available again during 2000. In a subsequent small catch up study, the EPIDERM nodel has been tested and accepted for the assessment of the corrosive potential of chemical substances (INVITTOX No. 119).

Test Compounds

A total of 60 test compounds, consisting of 11 organic acids, 10 organic bases, 9 neutral organics, 5 phenols, 7 inorganic acids, 4 inorganic bases, 3 inorganic salts, 8 electrophiles, 3 soaps/surfactants have been tested in the ECVAM validation study.

Details of the test compounds and test results are available in **dbVas** of ECVAM SIS.

Prediction Model

The test results are interpreted on the basis of the exposure time needed to cause cell viability to decrease below 35%. The determination of the UN packing groups and EU classifications is summarized in the table reported in the section 4.1. **"Interpretation of test results"** of the present standard operating procedure.

Status

This method has been evaluated in the **ECVAM Skin Corrosivity Validation Study** conducted under the auspices of ECVAM during 1996 and 1997 (Fentem *et al*., 1998). The ECVAM Scientific Advisory Committee (ESAC) agreed that the results obtained with the EPISKIN™ test in the ECVAM international validation study on *in vitro* tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The test proved applicable to testing of all the above reported chemical classes of different physical forms. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good.

The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied; it was also able to distinguish between known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. Based on the outcome of the study, the ESAC unanimously endorsed the statement that the EPISKIN test was scientifically validated for use as a replacement for the animal test and that this test was ready to be considered for regulatory acceptance (10th meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 1998). (Anon., 1998b).

- The 27th meeting of the Committee for Adaptation to Technical Progress of "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" agreed that the human skin model assays, which meet certain criteria, would form part of "Annex V method B.40. Skin Corrosion", February 2000 (Commission Directive 2000/33/EC). Furthermore, these models are now under consideration for inclusion in the OECD Guidelines.
  Further details on the ECVAM Validation Study are available in **dbVas** of the ECVAM SIS.

Last update: May 2000

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Procedure Details, March 1997*

EPISKIN™ TEST

NOTE: This protocol presents the standard operating procedure used in ECVAM Skin Corrosivity Validation Study (1996/1997).

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* The accuracy of the SOP has been confirmed in October 2000.

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1. Introduction

Product Description

The EPISKIN-SM™ (Standard Model) kit contains 12 reconstructed epidermis units. Each reconstructed epidermis unit consists of a human collagen (Types III and I) matrix, representing the dermis, covered with a film of Type IV human collagen, upon which stratified differentiated epidermis derived from human keratinocytes has been laid. Test materials can be applied directly to the stratum corneum.

Precautions

The epidermal cells are taken from healthy volunteer donors negative to anti-HIV 1 and 2, and to hepatitis C, antibodies, and to hepatitis B antigens. Nevertheless, normal handling procedures for biological materials should be followed:

(a) it is recommended that gloves are worn during handling; and
(b) after use, the epidermis, the material in contact with it, and the culture medium, should be decontaminated (for example, by using a 10% solution of bleach or a 1% solution of pyosynthene), prior to disposal.

Quality Control

EPISKIN-SM kits are manufactured according to defined quality assurance procedures (certified ISO 9001). All biological components of the epidermis and the kit culture medium have been tested for the presence of viruses, bacteria and mycoplasma. The quality of the final product is assessed by undertaking an MTT cell viability test and a cytotoxicity test with sodium dodecylsulphate (SDS). For reasons connected with the nature of the product, it is shipped before all of the necessary checks have been completed. A release form certifying the conformity (or otherwise) of the batch is sent to the user, by fax, on the day of delivery of the kit.

2. Materials

2.1. KIT CONTENTS

DESCRIPTION	USE
1 EPISKIN-SM plate containing 12 reconstructed epidermis units (area: 0.38cm^2)	each reconstructed epidermis is attached to the base of a tissue culture vessel with an O-ring set and maintained on nutritive agar for transport
1 12-well assay plate	for assays
1 flask of sterile assay medium	basic medium for use in assays
1 EPISKIN-SM biopsy punch	for easy sampling of epidermis

1 lot of "MTT reagents":

1 flask MTT reagent	to reconstitute
1 flask PBS 10x wash solution	to dilute
1 flask 4N NaOH	to adjust pH of wash solution
1 flask extraction solution of isopropanol acid (ready to use)	

1 flask negative control (NaCl, 9g/l) 1 flask positive control (glacial acetic acid)	specific controls for the corrosivity test

2.2. MATERIALS NOT PROVIDED WITH THE KIT

- 500ml wash bottle
- 5ml glass tubes with corks
- 200µl micropipette
- Multidispenser micropipette (2.2ml)
- 50µl or 100µl positive displacement micropipette (for applying thick or viscous samples)
- Vacuum source and Pasteur pipettes
- Small forceps
- Timers
- Microplate reader with filter of 545-595nm and 96-well microplates; or spectrophotometer and 1ml microcells
- Vortex mixer
- Non-sterile ventilated cabinet

3. Experimental Procedures and Timing

Details of the kit and assay procedures should be registered on the reporting form (Annex 1).

3.1. RECEIPT OF TEST KIT

Check the date of dispatch written on the package. Before opening the EPISKIN-SM kit:
(a) inspect the colour of the agar medium used for transport and check that its pH is acceptable: orange colour = good; yellow or violet colour = not acceptable;
and
(b) inspect the colour of the temperature indicator to verify that the kit has not been exposed to a temperature above 40°C: the indicator changes from white to grey at 40°C.
In the event of any anomaly, immediately contact the Sales Administration Department at SADUC (Tel: +33 78 56 72 72; Fax: +33 78 56 00 48).

Place the assay medium supplied with the kits at 2-8°C. Leave the EPISKIN-SM kits in their packaging at room temperature until the assays are to be undertaken.

3.2. APPLICATION AND RINSING

Safety precautions: MTT and corrosive materials are dangerous. Work in a non-sterile, ventilated, cabinet, wear protective gloves, and a mask and safety glasses, as necessary. Pre-warm the assay medium to 37°C. An approximate timing for conducting the test procedure is given below as a guide.

9.30: proceed with the application of test material for the 4-hour samples

(a) Fill the appropriate number of wells of an assay plate with pre-warmed culture medium (2.2ml per well). Mark the plate lids with the application time (4 hours) and the code numbers of the chemicals to be tested (1 well per chemical), or negative control (3 wells) or positive control (3 wells).
(b) Open the EPISKIN-SM kits and place an epidermis unit into each prepared well. Mark each epidermis unit with the appropriate code number.

9.45: application of the products during **4 hours:**

(c) Add 50µl of test material to each well by using the positive displacement pipette.
(d) In the case of solids, the material should be crushed to a powder, if necessary, and 20mg applied evenly to the epidermal surface (with difficult materials, use sufficient to cover the epidermal surface); add 100µl NaCl (9g/l saline) to ensure good contact with the epidermis.
(e) Add 50µl NaCl (9g/l saline) to each of the three negative control wells.
(f) Add 50µl glacial acetic acid to each of the three positive control wells.
(g) Replace the lid on the plate and incubate for 4 hours (± 5 minutes) in a ventilated cabinet at room temperature (18-28°C).

Note: The negative and positive controls incubated for 4 hours will act as controls for all of the incubation times.

10.00: proceed with the application of test material for the 1-hour samples

(a) Fill the appropriate number of wells of an assay plate with pre-warmed culture medium (2.2ml per well). Mark the plate lids with the application time (1 hour) and the code numbers of the chemicals to be tested (1 well per chemical).
(b) Open the EPISKIN-SM kits and place an epidermis unit into each prepared well. Mark each epidermis unit with the appropriate code number.

10.15: application of the products during **1 hour**

(c) Add 50µl of test material to each well by using the positive displacement pipette.
(d) In the case of solids, apply 20mg and add 100µl of NaCl (9g/l), as described previously for the 4-hour samples.
(e) Replace the lid on the plate and incubate for 1 hour (± 5 minutes) in a ventilated cabinet at room temperature (18-28°C).

10.30: proceed with the application of test material for the 3-minute samples

(a) Prepare the MTT solution (0.3mg/ml; enough for 2.2ml per well for the entire assay) and the PBS 1x wash solution, as indicated in the "MTT reagents" leaflet accompanying the test kit.
(b) Fill the appropriate number of wells of an assay plate with pre-warmed culture medium (2.2ml per well). Mark the plate lids with the application time (3 minutes) and the code numbers of the chemicals to be tested (1 well per chemical).
(c) Open the EPISKIN-SM kits and place an epidermis unit into each prepared well. Mark each epidermis unit with the appropriate code number.

10.45: application of the products during **3 minutes**

(d) Add 50µl of test material to each well by using the positive displacement pipette. Proceed well by well at 20-second intervals, with the aid of multiple timers (test a maximum of 5 or 6 materials at a time). Ensure that the exposure period is exactly 3 minutes for each well
(e) In the case of solids, apply 20mg and add 100µl of NaCl (9g/l), as described previously for the 4-hour samples.
(f) Remove the EPISKIN-SM unit and rinse thoroughly with PBS 1x solution, to remove all of the test material from the epidermal surface.
(g) Replace the EPISKIN-SM unit in the culture medium.
(h) When all of the units have been rinsed:
- remove the culture medium
- place the units on absorbent paper, or remove the rest of the PBS from the epidermal surface with a Pasteur pipette linked to a vacuum source (be careful not to touch the epidermis)
- add 2.2ml of the MTT solution (0.3mg/ml) to each well
- replace the lid on the plate. If the ambient temperature is 20-28°C, leave to incubate

for 3 hours (± 5 minutes) in a ventilated cabinet at room temperature, **protected from light**. If the ambient temperature is below 20°C, then leave to incubate for 3 hours (± 5 minutes) at temperature of 20-28°C, **protected from light**. An incubator (with or without CO_2), or a warm location within the laboratory, may be used. It is important that all the samples from each exposure time are treated identically.

11.15: rinse the 1-hour samples and replace the culture medium with 2.2ml of MTT solution (0.3mg/ml), as described above.

11.45: place 0.85ml of acidified isopropanol into labelled glass tubes (one tube corresponding to one well of the tissue culture plate). Label each tube with the name of the test material and the incubation time.

13.45: rinse the 4-hour samples and replace the culture medium with 2.2ml of MTT solution (0.3mg/ml), as described above.

3.3. FORMAZAN EXTRACTION

At the end of each incubation with MTT (*14.15*, *14.45* and *17.00*), the formazan extraction should be undertaken:

(a) place the units on absorbent paper
(b) remove the MTT solution from each well
(c) take a biopsy of the epidermis by using the biopsy punch, by placing the epidermis unit on the plate lid
(d) separate the epidermis from the collagen matrix with the aid of forceps, and place both parts (epidermis and collagen matrix) into the acidified isopropanol
(e) cork each tube and mix thoroughly by using a vortex mixer
(f) ensure that the acidified isopropanol is in good contact with all of the material
(g) store at room temperature overnight, protected from light.

3.4. ABSORBANCE/OPTICAL DENSITY MEASUREMENTS

Following the formazan extraction (left overnight):

(a) mix each tube by using a vortex mixer
(b) let the solution settle for 1-2 minutes, so that any cell fragments do not interfere with the absorbance readings
(c) place a 200µl sample from each tube into the wells of a 96-well plate (labelled appropriately)
(d) read the optical densities (OD) of the samples at a wavelength between 545nm and 595nm using acidified isopropanol solution as the blank.
(e) record the results on the template given in Annex 2.

Note: if a spectrophotometer is used rather than a plate reader, place a 500µl sample from each tube and 500µl isopropanol (not acidified) in a 1ml microcell and read the OD at 545-595nm using the acidified isopropanol solution as the blank.

4. Calculations of viability percentages and acceptability criteria

Record all calculations on the Data Report Form (Annex 3).

Viability (%) = 100 x (OD test material/mean OD negative control at 4 hours)

(a) calculate the mean OD of the 3 negative control values: this corresponds to 100% viability. Based on historical data the minimum acceptable mean OD for negative controls is 0.115 (mean ± 2SD). The maximum acceptable mean OD for the negative control is 0.4 (to allow for incubations at 28°C).

(b) calculate the mean OD of the 3 positive control values: the % viability of the positive control is calculated relative to the mean negative control. Based on historical data (mean ±2SD), the acceptable mean percentage viability range for positive controls is 0-20%.

(c) calculate the % viability following exposure to the test material at each incubation time as the OD expressed as a percentage of the mean negative control value.

(d) assay acceptability criteria*:* for an assay to be acceptable, the mean positive and negative control values should fall within the ranges given above.
In those cases where the mean values fall outside the range, the assay should be repeated, except in cases where the same chemical has been tested on at least two other occasions (with acceptable control values) and the results of all of the tests give the same corrosivity classification.

4.1 INTERPRETATION OF TEST RESULTS

The test results are interpreted on the basis of the exposure time needed to cause cell viability to decrease below 35%. The determination of the packing group is summarized in the following table:

Classification	**Packing group**	**Criteria for *In Vitro* interpretation**
UN	Corrosive class I	If viability < 35% after 3 min exposure
	Corrosive class II	If viability ≥ 35% after 3 min exposure and < 35% after 1 hour exposure
	Corrosive class III	If viability ≥ 35% after 1 hour exposure and < 35% after 4 hours exposure
	Non corrosive	If viability ≥ 35% after 4 hours exposure
EU	Corrosive class R35	If viability < 35% after 3 min exposure
	Corrosive class R34	If viability ≥ 35% after 3 min exposure and < 35% after 4 hours exposure
	Non-corrosive	If viability ≥ 35% after 4 hours exposure

In cases where the viability values from individual skin units are highly variable, causing different corrosivity classifications, the chemical should normally be re-tested. If one or more sets of data are considered to be incorrect (or inconsistent with data from other runs), the results should be replaced by those generated in a repeat run.

In cases where the viability values fall below 35%, but longer exposure times give values of >35% (or values higher than the earlier time point), the results should be considered to be doubtful. The run should normally be repeated.

Annex 1

ECVAM SKIN CORROSIVITY VALIDATION STUDY

EPISKIN ™

Assay report form

Experimental center: .....

Kit Reception

Lot number

Date of Receipt

Observations.

Assay

Date of Assay.

Code of tested product

Observations

Annex 2

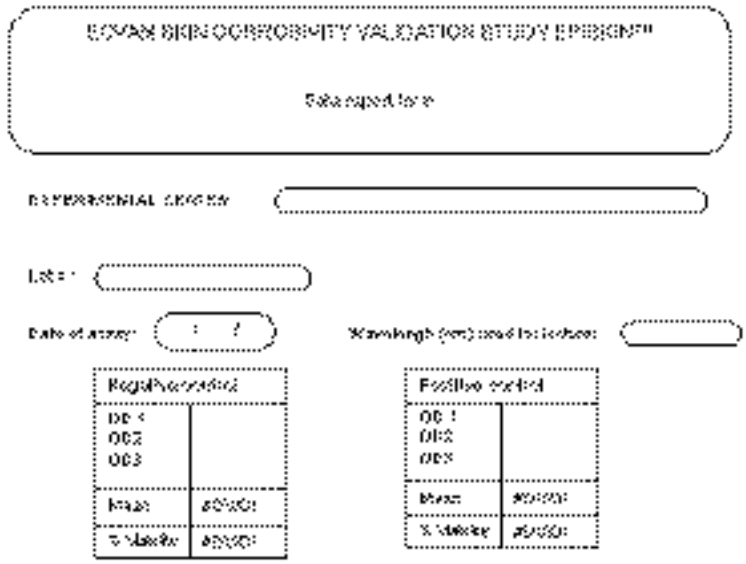
FEUILLE DE DEPOTS

Date (jour/mois/an) |_|_| |_|_| |_|_|_|_|

N. de plaque _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Annex 3



Product Code	Physical Appearance	3 min exposure		1 hour exposure		4 hours exposure	
		OD	% viability	OD	% viability	OD	% viability
			#DIV/0!		#DIV/0!		#DIV/0!
			#DIV/0!		#DIV/0!		#DIV/0!
			#DIV/0!		#DIV/0!		#DIV/0!
			#DIV/0!		#DIV/0!		#DIV/0!
			#DIV/0!		#DIV/0!		#DIV/0!
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			#DIV/0!		#DIV/0!		#DIV/0!
			#DIV/0!		#DIV/0!		#DIV/0!

Annex 4

ECVAM SKIN CORROSIVITY VALIDATION STUDY EPISKIN™

Data compilation form

EXPERIMENTAL CENTER:

Product Code	Physical Appearance	Lot N. Episkin	% viability 3 min	% viability 1hr	% viability 4 hrs

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Section 3.0

EPIDERM™ (EPI-200)

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Summary Report of the EpiDerm™ (EPI-200) *In Vitro* Assay for Assessing Dermal Corrosivity

Prepared for

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Evaluation of Alternative Toxicological Methods (NICEATM)
National Institute of Environmental Health Sciences
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Prepared by

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Supporting the NICEATM

Revised: June 5, 2002

PURPOSE

This report focuses on the performance of EpiDerm™ (EPI-200) to determine the usefulness and limitations of the assay for the identification of potential human corrosive chemicals. This report discusses also how EpiDerm™ (EPI-200) compares to EPISKIN™, a mechanistically related *in vitro* human skin model system, and to other validated *in vitro* corrosivity tests (Rat Skin Transcutaneous Electrical Resistance [TER] and Corrositex®). The data and assessments reviewed for this report included the European Centre for the Validation of Alternative Methods (ECVAM) formal pre-validation/validation study on EpiDerm™ (EPI-200) (Liebsch et al., 2000) and additional information formally submitted by MatTek, the commercial source of the assay, to ICCVAM for consideration (see MatTek Submission to ICCVAM; Appendix F, September 13, 2000).

EVALUATION OF REGULATORY AND SCIENTIFIC RATIONALE

EpiDerm™ (EPI-200) is one of several *in vitro* corrosivity assays formally evaluated by ECVAM as alternatives to the *in vivo* rabbit corrosivity test (Fentem et al., 1998; Liebsch et al., 2000). The assay is a three-dimensional human skin model that uses cell viability as a measure of toxicity (i.e., corrosivity). Because EpiDerm™ (EPI-200) is a human skin model, it may be more relevant to assessing human skin corrosivity potential than a test based on skin from another species. Also, the mode of application (topical) of the test material mimics the route of human exposure.

EpiDerm™ (EPI-200) has been approved by the ECVAM Scientific Advisory Committee for use in corrosivity testing in Europe (Balls and Hellsten, 2000). This method has also been adopted for regulatory use within the European Union (EU) by the European Commission (EU, 2000).

EVALUATION OF THE TEST METHOD

A standard kit contains media, reagents, and 24 tissues. The tissues provided in the test kit consist of normal, human epidermal keratinocytes cultured in a chemically defined medium to produce a stratified, highly differentiated, organotypic tissue model of the human epidermis. An EpiDerm™ (EPI-200) kit is equipped with sufficient amounts of medium, washing solutions, and sterile, disposable tissue culture plasticware to test four test materials and concurrent negative and positive controls. For use in corrosivity testing, the test material (liquids: 50 µL; solids: 25 mg) is topically applied to a tissue for 3 and 60 minutes. Per test compound, replicate plates are used for each test period. Cell viability is assessed by measuring mitochondrial activity using the MTT (a tetrazolium salt) assay. A test chemical is classified as corrosive if it induces a 50% or greater decrease in relative cell viability at 3 minutes or an 85% or greater decrease in relative cell viability at 60 minutes. The scientific rationale for these decision criteria are based on a correlative analysis of the ability of a number of corrosive (C) and noncorrosive (NC) chemicals to induce histopathological necrosis and an associated reduction in cell viability (Perkins et al., 1996). EpiDerm™ (EPI-200) will complement EPISKIN™, an ECVAM-validated *in vitro* corrosivity method, by providing an alternative and commercially available method.

Information on differences and similarities between EpiDerm™ (EPI-200) and EPISKIN™ are detailed in **Table 3.1**.

EVALUATION OF TEST METHOD DATA QUALITY

The performance of EpiDerm™ (EPI-200) was evaluated in three phases (Liebsch et al., 2000). Phase I was conducted by ZEBET (Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments, Berlin, Germany), and involved protocol and prediction model refinement using 50 chemicals. Phase II involved the transfer of the protocol to a second laboratory (Huntington Life Sciences) and the reproducibility of the assay was assessed by the repeat testing of 11 chemicals. In addition, in Phase II, ZEBET tested those chemicals classified as false negative in Phase I, aiming to refine the protocol and prediction model by increasing test sensitivity. Phase III was a formal evaluation of the reliability and performance of the assay using three laboratories (ZEBET, Huntington Life Sciences, and BASF AG), in which a blind trial conducted with 24 test chemicals was performed using the refined final protocol. In designing the Phase III study, ECVAM based its validation process on experimental results demonstrating that the EpiDerm™ (EPI-200) and EPISKIN™ assays were mechanistically identical (Roguet et al., 1999). For Phase III, ECVAM selected a subset of 24 chemicals from the 60 chemicals tested in the EPISKIN™ ECVAM validation study (Fentem et al., 1998). The selection of the 60 chemicals in the original validation study was based on unequivocal animal data (Barratt et al., 1998). Care was taken to ensure a balanced representation of the chemical classes in this subset, as well as to minimize the number of chemicals previously in Phase I (there was an overlap of 5 chemicals). The 24 chemicals selected included 12 corrosive tested and 12 noncorrosive chemicals -- four organic acids (2 C; 2 NC), six organic bases (4 C, 2 NC), four neutral organic bases (4 NC), two phenols (1 C, 1 NC), three inorganic acids (2 C; 1 NC), two inorganic bases (1 C; 1 NC), two electrophiles (2 C), and one surfactant (1 NC).

Table 3.1 General Protocol Comparison between EPISKIN™ and EpiDerm™ (EPI-200)

	EPISKIN™	EpiDermä (EPI-200)
Assay	Reconstructed human epidermis and a functional stratum corneum (not an animal model). Tissue approximates the barrier of normal human skin.	
Known limits of use	No known restrictions except for chemicals that reduce MTT. Although a relatively small numbers of chemicals have been evaluated in some chemical classes (i.e., cleaners and detergents), classified by ECVAM as otherwise without limits.	
Tissue construct acceptability	QC measures are based on historical laboratory control data.	
Materials, equipment, and supplies needed	Similar	
Replicates	Single tissue (culture)/experiment (ECVAM, 2000b) or 3 replicates/ experiments (OECD, 2001c)	Duplicate tissues/experiment, experiment replication if needed
Dosing procedures	Liquids: 50 µL applied neat Solids: 20 mg + saline	Liquids: 50 µL applied neat Solids: 25 mg + 25 µL H2O
Exposure duration	3 minutes, 1 hour, 4 hours	3 minutes, 1 hour
Endpoint	Relative cell viability compared to concurrent negative control, based on MTT assay (measure of mitochondrial function); assay based on optical density.	
Negative and positive controls	No vehicle control (undiluted test material used) Negative control: saline Positive control: glacial acetic acid	No vehicle control (undiluted test material used) Negative control: water Positive control: 8.0 N KOH
Acceptable range of control responses	Negative control: 4-hour optical density at 545-595 nm = 0.113-0.309 for MTT incubations at 20-28°C. Positive control: viability at 4 hours must be 0-20%.	Negative control: 3-min and 1-hour optical density at 570 or 540 nm = ≥0.8. Positive control: viability at 3 min must be ≤30%.
Data analysis	Determination of relative viability at each exposure duration. No statistical analysis.	
Positive response	Relative cell viability <35% at any exposure duration (=packing group).	Relative cell viability <50% after 3 minutes and/or <15% after 60 minutes.
Criteria for accepting or rejecting a test	Acceptable control values Test repeated if inconsistent toxicity response pattern across exposure durations (i.e., less toxicity at a longer exposure duration) or if corrosivity classification is variable	Acceptable control values Test repeated if difference in viability between duplicate tissues >30% and the corrosivity classification is variable, or (recommended) if the resulting viability is near to a classification cut-off.

The tests were conducted in the "spirit" of GLP. Each chemical was tested twice using independent lots of tissue by each of three different laboratories. A formal audit of the ECVAM data by a Quality Assurance Unit was not conducted; however, it was stated that all data submitted by the participating laboratories were verified against the original data sheets by ECVAM staff.

EVALUATION OF TEST METHOD PERFORMANCE

For this summary report, an analysis was conducted, similar to the performance analysis conducted for the ICCVAM Peer Review of Corrositex®; the current analysis evaluated the performance characteristics of the EpiDerm™ (EPI-200) assay against the corresponding *in vivo* rabbit corrosivity data and the corresponding *in vitro* corrosivity data generated by EPISKIN™. The database used in the evaluation of the performance characteristics of EpiDerm™ (EPI-200) consisted of data from the ECVAM pre-validation/validation study only (Liebsch et al., 2000); other data were not located.

For ease of comparison, chemicals evaluated in the EpiDerm™ (EPI-200) assay were classified into the same chemical and product class designations used in the Corrositex® evaluation. A weight-of-evidence approach was used for classifying discordant results within or between laboratories; in instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls), the chemical was eliminated from inclusion in the performance calculations.

Based on the database of 24 chemicals and chemical mixtures used in the validation study and using a weight-of-evidence approach to classify the corrosivity results (**Tables 3.2** and **3.4**), EpiDerm™ (EPI-200) had an accuracy of 92% (22/24 chemicals or chemical mixtures), a sensitivity of 92% (11/12 chemicals or chemical mixtures), a specificity of 83% (10/12 chemicals or chemical mixtures), a false positive rate of 17% (2/12 chemicals or chemical mixtures), and a false negative rate of 8% (1/12 chemicals or chemical mixtures). From these data, which met pre-study acceptance criteria of no more than 20% false negatives and 20% false positives, the ECVAM concluded that EpiDerm™ (EPI-200) was valid for use as a replacement for the *in vivo* rabbit skin test for distinguishing between corrosive and noncorrosive chemicals for all of the chemical classes studied (Liebsch et al., 2000). As for EPISKIN™, due to the relatively small numbers of chemicals evaluated in some chemical classes, definitive conclusions as to the adequacy of EPISKIN™ or EpiDermä (EPI-200) for some classes of chemicals were difficult to make with a high degree of confidence. However, taking into account the relative simplicity of the mechanism of action of corrosives, ECVAM concluded that the EpiDerm™ (EPI-200) method would be generally applicable across all chemical classes (Fentem et al., 1998; Liebsch et al., 2000). A comparison of the ability of EpiDerm™ (EPI-200) and EPISKIN™ to correctly identify corrosive and noncorrosive chemicals among the 24 chemicals tested in Phase III is provided in **Table 3.2**. Both assays are nearly identical in their performance (see also **Table 3.4**).

Table 3.2 Summary of Results for EpiDerm™ (EPI-200) and EPISKIN™ Compared to *In Vivo* Rabbit Results

Material	EPISKIN™	EpiDerm™ (EPI-200)
Corrosive	11/12	11/12
Noncorrosive	11/12	10/12

EVALUATION OF TEST METHOD RELIABILITY (REPEATABILITY/ REPRODUCIBILITY)

The inter- and intra-laboratory reliability of EpiDerm™ (EPI-200) was evaluated in the ECVAM pre-validation/validation study (Liebsch et al., 2000). In Phase III, each chemical was tested twice using different tissue lots in each of three laboratories (i.e., 144 tests were conducted). Of 72 replicate tests, 5 (6.9%) did not replicate. Regarding inter-laboratory reproducibility, three of the 24 chemicals (12.5%) were not predicted by all three laboratories (i.e., the performance characteristics of the three laboratories were nearly identical). Intra- and inter-laboratory reliability was evaluated formally using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors), scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Based on the results obtained, ECVAM concluded that EpiDerm™ (EPI-200) provided excellent reliability (Liebsch et al., 2000). After reviewing the intra- and inter-laboratory evaluations conducted by ECVAM, it was concluded by NICEATM that the analyses were appropriate and that the conclusions were accurate.

OTHER SCIENTIFIC REVIEWS

In May 2001, a search of the open literature was conducted to locate additional EpiDerm™ (EPI-200) studies. Four databases (PubMed, Web of Science, Toxline, and Current Contents Connect) were searched using the key terms "EpiDerm", and "Epi" within one word of "derm". Additional references were obtained from the MatTek technical references section at www.mattek.com. The search found no additional relevant studies conducted with EpiDerm™ (EPI-200).

OTHER CONSIDERATIONS

Like EPISKIN™, the EpiDerm™ (EPI-200) kit contains all of the necessary materials to conduct the test and does not require additional preparation. No animals are used in this test. The cost for conducting EpiDerm™ (EPI-200) is reported by MatTek (e-mail communication from Mitch Klausner, MatTek Corporation) to be approximately $800 per kit or $200 per test chemical (**Table 3.3**). This cost is less than the *in vivo* rabbit skin test and similar to that for the other validated *in vitro* corrosivity assays (Fentem et al., 1998). The time needed to conduct the EpiDerm™ (EPI-200) is similar to EPISKIN™.

RELATED ISSUES

Refinement, Reduction, and Replacement

Since the method is designed as a replacement for animals, EpiDerm™ (EPI-200) would clearly reduce the requirement for animal testing for corrosivity. Therefore, it has the potential to eliminate the use of animals for the determination of corrosivity. If used in an integrated testing approach, EpiDerm™(EPI-200) provides for reduction and refinement of animal use.

Comparison to Other *In Vitro* Assays

General comparative information on EpiDerm™(EPI-200) compared to Rat Skin TER, EPISKIN™, and Corrositex® is provided in **Tables 3.3** through **3.6**. In contrast to Corrositex® and EPISKIN™, EpiDerm™(EPI-200), like Rat Skin TER, cannot be used to identify packing group classifications.

Table 3.3 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays

	Rat Skin TER	EPISKIN™ (prediction model B)	EpiDerm™ (EPI-200) (prediction model 2)	Corrositex®
Test Method Description	Acceptable	Acceptable	Acceptable	Acceptable
Adequacy/Completeness of Protocol	Acceptable	Acceptable	Acceptable	Acceptable
Usefulness for Assessing Corrosivity/Non-corrosivity	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Usefulness for Determining Packing Groups	Not Acceptable (Fentem et al., 1998)	Can group as UN packing group II/III or I (Fentem et al., 1998)[a]	Not Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Repeatability and Reproducibility	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (Fentem et al., 1998; ICCVAM, 1999)
Animal Use Refinement, Reduction, and Replacement Considerations	Refines and reduces animal use when used as a stand-alone test or in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.	Refines and reduces animal use when used in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.
Cost	~$500-850/test	~$450/test kit[b]	~$200/test chemical	~$300/test chemical
Study duration	2 work-days	1 work-day	1 work-day	≤ 4 hr/chemical

[a] Since the performance of EPISKINä was not assessed for distinguishing between UN packing groups II and III, all R34 classifications would be conservatively classified as UN packing group II.

[b] One to three chemicals may be tested per test kit; however, it is recommended by the supplier that each test chemical be assayed using 3 different skin batches/kits which equates to a total cost of ~$430/ test chemical.

Table 3.4 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays Based on a Weight-of-Evidence Approach[a] by Chemical using Data from the ECVAM and other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™	EpiDerm™ (EPI-200) (prediction model 2)	Corrositex®
Number of Chemicals	122	60	24	163
Overall Sensitivity[b]	94% (51/54)	82% (23/28)	92% (11/12)	85% (76/89)
Overall Specificity[b]	71% (48/68)	84% (27/32)	83% (10/12)	70% (52/74)
Overall Accuracy[b]	81% (99/122)	83% (50/60)	92% (22/24)	79% (128/163)
False Positive Rate	29% (20/68)	16% (5/32)	17% (2/12)	30% (22/74)
False Negative Rate	6% (3/54)	18% (5/28)	8% (1/12)	15% (13/89)
Test Chemical Inter-laboratory Coefficient of Variation	34.7[c] 3.8-322[d] 120[e]	11.3[c] 3.9-148.8[d] 20[e]	12.3[c] 0.9-51.2[d] 144[e]	30.3[c] 7.7-252.5[d] 180[e]

[a] A chemical is first classified as positive or negative for corrosivity within each laboratory based on the majority of test results obtained (when replicate testing was conducted). Next, the chemical is classified as positive or negative for corrosivity based on the majority of test results obtained in multiple laboratories (when multiple laboratory studies were conducted). In instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls within or across laboratories), the chemical was eliminated from inclusion in the performance calculations.

[b] Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method.

[c] Median values

[d] Range of values

[e] The total number of independent values, which is calculated as the number of chemicals tested multiplied by the number of participating laboratories.

Table 3.5 General Comparison of the Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays from Independent Test Results in the ECVAM Validation Studies (Fentem et al., 1998; Liebsch et al., 2000)

	TER	EPISKIN™ (prediction model B)	EpiDerm™ (EPI-200) (prediction
Number of Chemicals Tested in ECVAM Validation Study	60 (Fentem et al., 1998)	60/24[a] (Fentem et al., 1998)	24 (Liebsch et al., 2000)
Sensitivity[b]	88% (140/159)	83% (201/243) / 88% (87/99)	88% (63/72)
Specificity[b]	72% (142/196)	80% (237/297) / 79% (92/117)	86% (62/72)
Accuracy[b]	79% (282/355)[c]	81% (438/540) / 83% (179/216)	87% (125/144)
False Positive Rate[b]	28% (54/196)	20% (60/297) / 21% (25/117)	14% (10/72)
False Negative Rate[b]	12% (19/159)	17% (42/243) / 12% (12/99)	13% (9/72)
Number of Trials[d]	155	540 / 216	144
Test Chemical Inter-laboratory Coefficient of Variation	34.7[d] 10-322[e] 155[f]	30.2[d] 7.7-252.5[e] 540[f]	12.3[d] 0.9-51.2[e] 144[f]

[a] The first numbers for accuracy, sensitivity, specificity, false positive rate, and false negative rate correspond to the 60 chemicals tested in the ECVAM Skin Corrosivity Test using EPISKIN™ (Barratt et al., 1998; Fentem et al., 1998); the latter values correspond to a direct comparison of EpiDerm™ (EPI-200) and EPISKIN™ for the same 24 materials tested in both systems (Liebsch et al., 2000).

[b] Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method. False positive rate is defined as the proportion of all negative chemicals or chemical mixtures that are falsely identified as positive. False negative rate is defined as the proportion of all positive chemicals or chemical mixtures that are falsely identified as negative.

[c] The percentages are based on the number of correct trials among the total number of trials (i.e., independent tests) provided in parenthesis.

[d] Median values

[e] Range of values

[f] The total number of trials conducted in the validation study minus the non-qualified (NQ) results. This number is usually equal to the number of chemicals multiplied by the number of participating laboratories multiplied by the number of replicate tests.

Table 3.6 Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000)

No.[a]	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN™ [b]	EpiDerm™ (EPI-200)
1	Hexanoic acid	ORGAC	R34/II&III	R35	R35	N/A
29	65/35 Octanoic/decanoic acid	ORGAC	R34/II&III	R34	R35	N/A
36	2-Methylbutyric acid	ORGAC	R34/II&III	R35	R34	N/A
40	Octanoic acid (caprylic acid)	ORGAC	R34/II&III	R35	R34/C	C
47	60/40 Octanoic/decanoic acids	ORGAC	R34/II&III	R34	R34/C	C
50	55/45 Octanoic/decanoic acids	ORGAC	R34/II&III	R35	R34	N/A
7	3,3'-Dithiodipropionic acid	ORGAC	NC	NC	NC	N/A
12	Dodecanoic acid (lauric acid)	ORGAC	NC	NC	NC	NC
26	Isotearic acid	ORGAC	NC	NC	NC	NC
34	70/30 Oleine/octanoic acid	ORGAC	NC	NC	NC	N/A
58	10-Undecenoic acid	ORGAC	NC	NC	R34	N/A
2	1,2-Diaminopropane	ORGBA	R35/I	R35	R34/C	C
15	Dimethyldipropylenetriamine	ORGBA	R35/I	R35	R34/C	C
38	Tallow amine	ORGBA	R35/II	2R34/2NC/2NQ	NC	N/A
55	1-(2-Aminoethyl)piperazine	ORGBA	R34/II	R35	NC	N/A
13	3-Methoxypropylamine	ORGBA	R34/II&III	R35	R34	N/A
17	Dimethylisopropylamine	ORGBA	R34/II&III	R35	R34/C	C
45	n-Heptylamine	ORGBA	R34/II&III	R35	NC	C
10	2,4-Xylidine (2,4-Dimethylaniline)	ORGBA	NC	R34	R34	N/A
35	Hydrogenated tallow amine	ORGBA	NC	NC	NC	NC
59	4-Amino-1,2,4-triazole	ORGBA	NC	NC	NC	NC
8	Isopropanol	NORG	NC	NC	NC	N/A
11	2-Phenylethanol	NORG	NC	NC	NC	N/A
16	Methyl trimethylacetate (referred to as Methyl 2,2-dimethylpropanoate in EpiDerm™ (EPI-200))	NORG	NC	NC	NC	C
19	Tetrachloroethylene	NORG	NC	NC	NC	NC
22	n-Butyl propionate	NORG	NC	NC	NC	N/A
27	Methyl palmitate	NORG	NC	NC	NC	N/A
44	Benzyl acetone	NORG	NC	NC	NC	NC
51	Methyl laurate	NORG	NC	NC	NC	N/A
56	1,9-Decadiene	NORG	NC	NC	NC	NC
3	Carvacrol	PHEN	R34/II&III	R34	R34	N/A
23	2-tert-Butylphenol	PHEN	R34/II&III	R35	R34/C	C
9	*o*-Methoxyphenol (Guaiacol)	PHEN	NC	NC	R34	N/A
30	4,4-Methylene-bis-(2,6-di-tert-butylphenol)	PHEN	NC	NC	NC	N/A

Table 3.6 (continued)

No.[a]	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN™ [b]	EpiDerm™ (EPI-200)
49	Eugenol	PHEN	NC	NC	NC	NC
4	Boron trifluoride dihydrate	INORGAC	R35/I	R35	R35/C	C
28	Phosphorus tribromide	INORGAC	R35/I	R35	R35/C	C
32	Phosphorus pentachloride	INORGAC	R35/I	R35	R34	N/A
25	Sulfuric acid (10% wt.)	INORGAC	R34/II&III	R34	R34	N/A
57	Phosphoric acid	INORGAC	R34/II	R35	R34	N/A
43	Hydrochloric acid (14.4% wt)	INORGAC	R34/II&III	R35	R34	N/A
53	Sulfamic acid	INORGAC	NC	R34	R34/C	C
18	Potassium hydroxide (10% aq.)	INORGBA	R34/II&III	R35	R34/C	C
42	2-Mercaptoethanol, Na salt (45% aq.)	INORGBA	R34/II&III	R35	NC	N/A
21	Potassium hydroxide (5% aq.)	INORGBA	NC	R35	R34	N/A
24	Sodium carbonate (50% aq.)	INORGBA	NC	R34	NC	NC
20	Ferric [iron (III)] chloride	INORGSAL	R34/II	R35	R34	N/A
52	Sodium bicarbonate	INORGSAL	NC	R34	NC	N/A
54	Sodium bisulfite	INORGSAL	NC	3R34/3NC	NC	N/A
5	Methacrolein	ELECTRO	R34/II&III	NC	R34/C	NC
14	Allyl bromide	ELECTRO	R34/II&III	R35	R34	N/A
48	Glycol bromoacetate (85%)	ELECTRO	R34/II&III	NC	R34/C	C
6	Phenethyl bromide	ELECTRO	NC	NC	NC	N/A
31	2-Bromobutane	ELECTRO	NC	3R34/3R35	NC	N/A
33	4-(Methylthio)-benzaldehyde	ELECTRO	NC	NC	NC	N/A
39	2-Ethoxyethyl methacrylate	ELECTRO	NC	NC	NC	N/A
46	Cinnamaldehyde	ELECTRO	NC	NC	NC	N/A
37	Sodium undecylenate (33% aq.)	SOAP	NC	R35	R34	N/A
41	20/80 Coconut/palm soap	SOAP	NC	NC	NC	N/A
60	Sodium lauryl sulfate (20% aq.)	SOAP	NC	R35	NC	NC

Definitions are as follows: C = Corrosive; NC = Noncorrosive; R34 is equivalent to packing groups II and/or III; R35 is equivalent of packing group I, except for tallow amine (R35/II); NQ = Non-qualified; N/A = Not applicable because not tested; ORGAC = Organic acid; ORGBA = Organic base; NORG = Neutral organics; PHEN = phenol; INORGAC = Inorganic acid; INORGBA = Inorganic base; INORGSAL = Inorganic salt; ELECTRO = Electrophile; SOAP = Soap surfactant
Overall corrosivity classifications were determined by the majority of the reported results obtained from each assay. If results do not show a majority, a definitive classification could not be determined.

[a] Number assigned each chemical by the ECVAM Management Team.

[b] For EPISKIN™, prediction model B was the more complex prediction model and was the only model considered in detail by the ECVAM Management Team (Fentem et al., 1998).

SUMMARY CONCLUSIONS AND RECOMMENDATIONS

ECVAM concluded that EpiDerm™(EPI-200) was an *in vitro* replacement assay for *in vivo* corrosivity testing. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that EpiDerm™ (EPI-200) was both reliable and reproducible; NICEATM concurs with that conclusion.

The two major questions to be addressed for *in vitro* corrosivity assays are:

1. Has the assay been evaluated sufficiently and is its performance satisfactory to support the proposed use for assessing the corrosivity potential of chemicals and chemical mixtures?
2. Does the assay adequately consider and incorporate, where scientifically feasible, the 3Rs of animal use (refinement, reduction, and replacement alternatives)? Does the assay offer advantages with respect to animal welfare considerations?

In response to the first question, the performance characteristics of the EpiDerm™ (EPI-200) method indicates, in specific testing circumstances, that this test may be considered useful as part of an integrated testing strategy for assessing the dermal corrosion potential of chemicals.

In response to the second question, EpiDerm™ (EPI-200) sufficiently considers and incorporates the 3Rs. Specifically, the use of EpiDerm™ (EPI-200) offers advantages with respect to animal welfare considerations, including animal use refinement, reduction, and replacement. Similarly, the use of the EpiDerm™ (EPI-200) assay as part of an integrated approach reduces and refines the use of animals by providing a basis for decisions on further testing. When these methods are used as part of an integrated testing strategy for corrosivity, there is a reduction in the number of animals required because positive results typically eliminate the need for animal testing, and when further testing in animals is determined to be necessary, only one animal is required to confirm a corrosive chemical. Follow-up testing using *in vivo* methods, when deemed necessary, could also employ test agent dilution schemes to minimize possible pain in any individual animal.

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ECVAM Protocol for EPIDERM™ (EPI-200): an *In Vitro* Assay for Assessing Dermal Corrosivity

Original Draft: October 24, 1997
Confirmed: January 2002

NOTE: This protocol presents the standard operating procedure used in the ECVAM Prevalidation of EpiDerm™ (EPI-200), Skin Corrosivity test (1997-1998). ECVAM confirmed the accuracy of the SOP in October 2000, and this protocol was supplied by Dr. Andrew Worth of ECVAM via email on May 22, 2001.

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EPIDERM™ (EPI-200) Skin Corrosivity Test

The corrosivity potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the Epiderm™ (EPI-200) human epidermal model.

Objectives

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency, hazard identification
PURPOSE OF TESTING	:	classification and labelling

Proposed replacement for the *in vivo* Draize rabbit skin corrosivity test (OECD testing guideline 404, Anon., 1992b; and Annex V of Directive 67/548/EEC, Anon., 1992a) to be used for hazard identification and classification of corrosive potential to fulfil international regulatory requirements pertaining to the handling, packing and transport of chemicals.

Basis of the Method

The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. The two major mechanisms of skin corrosion are the destruction (erosion or solubilisation) of the skin penetration barrier (stratum corneum) including the viable skin cells underneath, and the rapid penetration of highly cytotoxic chemicals through the skin barrier without involving its destruction.

The present test is based on the experience that corrosive chemicals show cytotoxic effects following short-term exposure of the stratum corneum of the epidermis. The test is designed to predict and classify the skin corrosivity potential of a chemical by assessment of its effect on a reconstituted three-dimensional human epidermis model. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT.

Experimental Description

Endpoint and Endpoint Detection	:	Cell viability as determined by reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT
Test Parameter	:	50% viability
Test System	:	EpiDerm™ (EPI-200) human epidermal model system

On day of receipt EpiDerm™ (EPI-200) tissues are placed in the refrigerator. Next day, at least one hour before starting the assay, the tissues are transferred to 6-well plates with assay medium, which is immediately replaced before the test is started. The test is performed on a total of 4 tissues per test material, together with a negative control and a positive control.

Two tissues are used for a three-minute exposure to the test chemical and two for a one-hour exposure. 50 µl of the undiluted test material (liquids, semi-solids) or ~ 25 mg solid +25 µl H_2O are added into the MILLICELL® insert on top of the EPI-200 tissues. The remaining tissues are concurrently treated with 50µl distilled water (negative control) and with 50µl 8N-KOH (positive control). After the exposure period, the tissues are washed with phosphate buffered saline (PBS) to remove residual test material. Rinsed tissues are kept in 24-well plates (holding plates) in 300 µl serum free assay medium until 12 tissues (=one application time) have been dosed and rinsed. The assay medium is then replaced with 300 µl MTT-medium and tissues are incubated for three hrs (37°C, 5% CO_2). After incubation, tissues are washed with PBS and formazan is extracted with 2 ml isopropanol (either for 2 hrs or overnight). The optical density of extracted formazan is determined spectrophotometrically at 570 nm (or 540 nm) and cell viability is calculated for each tissue as a % of the mean of the negative control tissues. The skin corrosivity potential of the test materials is classified according to the remaining cell viability following exposure to the test material for either of the two exposure times.

Test Compounds

A total of 24 test compounds were chosen from the 60 chemicals tested in the ECVAM Skin Corrosivity Validation Study (1996/1997). These compounds included 4 organic acids, 6 organic bases, 4 neutral organics, 2 phenols, 3 inorganic acids, 2 inorganic bases, 2 electrophiles and 1 soap/surfactant.

Prediction Model

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after exposure compared to the negative control tissues concurrently treated with H_20. A chemical is classified “corrosive”, if the relative tissue viability after 3 min exposure to a test material is decreased below 50% (PM1). In addition, those materials classified "non corrosive" after 3 min (viability ≥ 50%) are classified "corrosive" if the relative tissue viability after 1 hr treatment with a test material is decreased below 15 % (PM2). For details see the section 4. **"Evaluation, Prediction Models (PM1 and PM2)"** reported in the present standard operating procedure.

Status

Following presentation of the outcome to the Management Team of the ECVAM Skin Corrosivity Validation Study on 22 April 1998, it was recommended to carry out a small catch up study of the EpiDerm™ (EPI-200) test rather than a formal validation study. This "Prevalidation Study of the EpiDerm™ (EPI-200) Skin Corrosivity Test" (March 1997-April 1998) has successfully been concluded (Liebsch *et al.*, 2000). Based on the outcome of the study (Botham & Fentem, 1999), ESAC unanimously endorsed the statement that the EpiDerm™ (EPI-200) human skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the EU and draft OECD test guidelines on skin corrosion (14th meeting at ECVAM of the ECVAM Scientific Advisory Committee,

European Commission, March 2000; Anon., 2000b).

> The 27th meeting of the Committee for Adaptation to Technical Progress of "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" agreed that the human skin model assays, which meet certain criteria, would form part of "Annex V method B.40. Skin Corrosion", February 2000 (Commission Directive 2000/33/EC). Furthermore, these models are now under consideration for inclusion in the OECD Guidelines.

Further details may be obtained from the contact person.

Remarks

After in 1993/94 two *in vitro* assays (Corrositex and Skin^2 ZK 1350) had achieved limited regulatory acceptance (exemptions for the use with specified chemical classes) by the US DOT, an international prevalidation study on three *in vitro* tests for skin corrosivity was performed in 1996. As a follow-up to this study, a formal validation study, initiated and sponsored by ECVAM, has been conducted (1996/97). Tests being evaluated were the rat skin Transcutaneous Electrical Resistance (TER) assay; CORROSITEX™ test; Skin^2 ZK 1350 and EPISKIN™ test (protocol numbers: 115, 116, 117, 118 respectively). As an outcome of this Validation Study, two tests (TER assay and EPISKIN™) have scientifically been validated as a replacement to the animal test. Following the successfully conclusion of this study, the production of the two *in vitro* 3-D models of reconstructed human skin/epidermis (Skin^2 and EPISKIN) was interrupted by the manufactors. Skin^2 is no longer produced, while EPISKIN will be available again shortly.

The present EpiDerm™ (EPI-200) assay is, therefore, used as a substitute for the two models. The need for a substitute test is supported by experience of a similar performance of different models in skin corrosivity testing (ECETOC, 1995) and by the OECD tier strategy for the classification of skin irritancy/corrosivity, developed by the US EPA and the German BgVV which includes the use of validated *in vitro* tests (OECD, 1996) for positive classification.

Details on the validation study are available in **dbVas** of the ECVAM SIS.

Last update: October 2000

Procedure Details, 24 October 1997

EPIDERM™ (EPI-200) SKIN CORROSIVITY TEST

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Note: The protocol presents the standard operation procedure used in the Prevalidation of EpiDerm™ (EPI-200)Skin Corrosivity test (1997-1998).

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1. Introduction and remarks

The SOP is based on a method developed at Procter & Gamble in 1996. The SOP was drafted at ZEBET in Phase I of the prevalidation study and a database comprising 96 tests with 50 chemicals was produced using the first Draft SOP. The SOP was then refined according to discussions with P&G and with the partner laboratories participating in phases II and III (Huntingdon Life Sciences, UK and BASF AG, D) which lead to the attached final SOP.

2. Materials

2.1 MATERIALS, NOT PROVIDED WITH THE KITS

Sterile, blunt-edged forceps	For transferring tissues from agarose
500 ml wash bottle	For rinsing tissue after test material exposure
200 ml beaker	For collecting PBS washes
Sterile disposable pipettes, pipette tips and pipetters	For diluting, adding, and removing media and test materials. For topically applying test materials to tissues
37°C incubator 5% CO_2	For incubating tissues prior to and during assays
Vacuum source/trap (optional)	For aspirating solutions
Laminar flow hood (optional)	For transferring tissues under sterile conditions
37°C water bath	For warming Media and MTT solution
Mortar and Pestle	For grinding granulars
Adjustable Pipet 1 ml	For pipetting assay medium under inserts (0.9 ml)

Pipet 300 µl	For pipetting MTT medium into 24-well plates
Pipet 2 ml	For pipetting MTT extraction solution into 24-well plate
Pipet 200 µl	For pipetting extracted formazan from 24-well plate into 96 well plate to be used in a plate photometer
Pipet 50 µl	For application of liquid test materials
Positive displacement pipet 50 µL	For application of semi-solid test materials
Sharp spoon (NaCl weight: 25±1 mg) Aesculap, Purchase No.: FK623	For application of solids
(bulb headed) sound	To aid levelling the spoon (spoonful)
Laboratory balance	For pipette verification and checking spoonful weight
96-well plate photometer 570 or 540 nm	For reading OD
Shaker for microtiter/MILLICELL® plates	For extraction of formazan
Stop-watches	To be used during application of test materials
Potassium Hydroxyde, 8 N (Sigma # 17-8)	To be used as positive control with each kit
Dulbeccos PBS (ICN # 196 0054) or (ICN # 196 1054) or (ICN # 176 0020) or (ICN # 176 0022)	Use for rinsing tissues Use as ready solution or dilute from 10x concentrate or prepare from PBS powder
HCl	For pH adjustment of PBS
NaOH	For pH adjustment of PBS
H_2O, pure (distilled or aqua pur)	To be used as negative control with each kit
Two additional 24-well plates	Use for preparing the "holding plates"

2.2. EPI-200 KIT COMPONENTS

Examine all kit components for integrity. If there is a concern call MatTek Corporation immediately (Mitch Klausner, ☎ +1-508-881-6771, Fax +1-508-879-1532).

1	Sealed 24-well plate	Contains 24 inserts with tissues on agarose
2	24-well plates	Use for MTT viability assay
4	6-well plates	Use for storing inserts, or for topically applying test agents
1 bottle	Maintenance Medium	Do not use in present assay
1 bottle	Serum-Free Assay Medium	DMEM-based medium
1 bottle	PBS Rinse Solution (100 ml)	Use for rinsing the inserts in MTT assay

1 vial	1% Triton X-100 Solution (10 ml)	Skin irritant reference chemical **Do not use in present assay**
1	MTT Assay Protocol	MatTek Corporation: steps are included in the present protocol

2.3. MTT-100 ASSAY KIT COMPONENTS

1 vial, 2 ml	MTT concentrate	
1 vial, 8 ml	MTT diluent (supplemented DMEM)	For diluting MTT concentrate prior to use in the MTT assay
1 bottle, 60 ml	Extractant Solution (Isopropanol)	For extraction of formazan crystals

3. Methods

3.1. EXPIRATION AND KIT STORAGE

Epi-200 kits are shipped from Boston on Monday. If possible, make sure that they are arriving in the laboratory on Tuesday. Upon receipt of the EpiDermä (EPI-200) tissues, place the sealed 24 well plates and the assay medium into the refrigerator (4°C). Place the MTT concentrate containing vial in the freezer (-20°C) and the MTT diluent in the refrigerator (4°C).

Part #	**description**	**conditions**	**shelf life**
Epi-200	EpiDermä (EPI-200) cultures	refrigerator (4°C)	until Friday, of the week of delivery
Epi-100	assay medium	refrigerator (4°C)	7 days
MTT-099	MTT diluent	refrigerator (4°C)	7 days
MTT-100	MTT concentrate	freezer (-20°C)	2 months

Record lot numbers of all components shown on the lot/production label on sealed tray in the Methods Documentation Sheet (see ANNEX)

3.2. QUALITY CONTROLS

3.2.1. Assay Acceptance Criterion 1: Negative Controls

The absolute OD_{570} or OD_{540} of the negative control tissues in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after the shipping and storing procedure and under specific conditions of the assay. Tissue viability is meeting the acceptance criterion if the mean OD of the two tissues is $OD \geq 0.8$.

3.2.2. Assay Acceptance Criterion 2: Positive Control

Potassium Hydroxyde as 8.0 normal ready made solution (Sigma # 17-8) is used as positive reference and has to be tested with each kit according to section 3.4. A 3 minutes application of 8.0 n KOH will reveal a mean relative tissue viability of ~20%.
An assay is meeting the acceptance criterion if mean relative tissue viability of the 3 min Positive Control is ≤ 30%.

3.2.3. Maximum inter tissue viability difference

In the present test protocol each chemical is tested on 2 tissues per application time (3 min and 1 hr). Thus, in contrast to the first test version (which used only 3 min application on 4 tissues) statistically outlying tissues cannot be identified any more. According to the historical data base existing at ZEBET the mean difference between untreated tissue duplicates is 9% ± 7% (S.D.).
A difference > 30% between two tissues treated identically should be regarded as a rejection criterion, and re-testing of the chemical is recommended if the resulting viability is near to a classification cut-off.
Note: If necessary, calculate % difference between the mean of the 2 tissues (= 100%) and one of the two tissues. If this difference is > 15% then rejection should be considered.

3.3. PREPARATIONS

3.3.1. MTT solution (prepare freshly on day of testing)

Thaw the MTT concentrate (MTT-100) and dilute with the MTT diluent (MTT-099). Store the remaining MTT solution in the dark at 4°C for later use on the same day (do not store until next day).
Note: Some test chemicals may reduce MTT, which will result in a blue colour without any involvement of cellular mitochondrial dehydrogenase. Although in the present assay the test chemicals are rinsed off and the DMEM medium beneath the tissues is changed before contact with MTT medium, some amount of a test chemical may be released by the tissues into the MTT medium and directly reduce the MTT, which would be interpreted as "tissue viability".

To check MTT reducing capability a solution of MTT in DMEM (1.0 mg/ml) can be prepared and ~100 µL (liquid test material) or 30 mg (solid test material) added to 1 ml MTT medium. If the mixture turns blue/purple after about 1 hr at room temperature, the test material is presumed to have reduced the MTT. This check can only be used to explain unexpected results, but it can not be used for quantitative correction of results.

3.3.2. Dulbecco's PBS

Using ICN FLOW 10× DPBS (Cat. no. see section 2.1 **"Materials, not provided with the kits"**) dilute 1 in 10 with distilled water and adjust to pH 7.0 with either NaOH or HCl. Record the pH adjustment in the MDS. If PBS powder is used: prepare PBS according to supplier instructions.
Note: 1 Litre is sufficient for all rinsing performed with one kit. If PBS is prepared from 10x concentrates or powder and not sterilised after preparation do not use PBS for more than one week.

3.3.3. Test materials

Safety Instruction

1. For handling of non-coded test chemicals follow instructions given in the Material Safety Data Sheet.
2. If coded chemicals are supplied from BIBRA, no information regarding the safe handling will be provided. Therefore, all test materials must be treated as if they

were corrosives and work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear gloves, protect eyes and face).

Except solids all test materials are applied neat (undiluted):

Liquids	**:**	Dispense 50 µl directly atop the Epi-200 tissue. If necessary spread to match size of tissue. Record the use of spreading in the MDS.
Semi-solids	**:**	Dispense 50 µl using a positive displacement pipet directly atop the Epi-200 tissue. If necessary spread to match size of tissue. Record the use of spreading in the MDS.
Solids	**:**	Crush and grind test material in a mortar with pestle wherever this improves the consistency. Fill 25 mg application spoon (see section 2.1. **"Materials not provided with the kits"**) with fine ground test material. Level the "spoonful" by gently scratching the excess material away with an appropriate aid, avoiding compression ("packing") of the test material [#]. Add 25 µl H_20 for wetting of the test material (increase volume of H_20 in case of materials where this is not enough for wetting). If necessary spread to match size of tissue. Record in the MDS if grinding was not used and if spreading or increasing H_20 volume was necessary.

[#] **Note**: "Packing" can be avoided by using a rod shaped sound instead of a flat spatula. If a bulb headed sound is used the bulb can be used to empty the spoon completely.

3.4 EXPERIMENTAL PROCEDURE

Note: Since the present test is a short term test which makes use of the epidermis model over a period of only 5 hours, sterility is not as important as is in other applications of EpiDerm™ (EPI-200). Nevertheless, it is important to keep assay media sterile and to keep risk of contamination at a low level.

Day prior to testing

1. Upon receipt of the EpiDermä (EPI-200) kit(s), place the sealed 24 well plates containing the tissues and the assay medium into the refrigerator (4° C). Place the vial containing the MTT concentrate in the freezer (-20°C).
2. Preparation of PBS according to section 3.3.2 **"Dulbecco's PBS"**.

Day of testing

Introductory note: One kit is used for testing 4 test chemicals, negative control and positive control, each of them applied both for 3 min and 1 hr to two tissue replicates. Thus, the experimental design can be either that the 3 min applications are completed first and subsequently the 1 hr experiment is performed, or, alternatively, that the 3 min applications are performed during the exposure period of the 1 hr experiment. The following steps are describing the latter option.

1). Before treatment pre-warm the assay medium in a 37°C waterbath.
2). Pipet 0.9 ml of the assay medium into each well of four sterile 6-well plates.
3). At least 1 hour before dosing, remove the EpiDermä (EPI-200) tissues from the refrigerator. Under sterile conditions using sterile forceps, transfer the inserts into four 6-well plates containing the pre-warmed assay medium.

Note: Care should be taken to remove all adherent agarose sticking to the outside of the inserts. Any air bubbles trapped underneath the insert should be released. Label the 6 well plates (lid and bottom) indicating the test material.

4). Place the four 6-well plates containing the tissues into a humidified (37°C, 5% CO_2) incubator for at least 1 hour prior to dosing (pre-incubation).
5). Prepare MTT solution according to section 3.3.1 **"MTT solution"**.
6). Before pre-incubation is complete, prepare two 24-well plates to be used as "holding plates", one for the 3 min experiment, the other for the 1 hr experiment. In addition, prepare two 24-well plates for the MTT assay: Use the plate design shown below. Pipette 300 µL of either pre-warmed assay medium or MTT medium in each well. Place the 4 plates in the incubator.

24-well plate design (used as "holding plates" and for MTT assay)

NC	C1	C2	C3	C4	PC
NC	C1	C2	C3	C4	PC

3 min

NC	C1	C2	C3	C4	PC
NC	C1	C2	C3	C4	PC

1 hr

NC = Negative Control
C1-C4 = Test Chemical 1,2,3,4
PC = Positive Control

7). After pre-incubation is completed (at least 1 hr) replace medium by 0.9 ml fresh assay medium in all four 6-well plates. Place two 6-well plates (3 min experiment) back into the incubator, the other two 6-well plates are used for the 1 hour experiment. Use the following plate design:

6-well plate design (chemical treatment and incubation)

Negative control	test material 1	test material 2
Negative control	test material 1	test material 2
Test material 3	test material 4	positive control

plate A (3 min)

negative control	Test material 1	test material 2
negative control	Test material 1	test material 2
test material 3	Test material 4	positive control

plate C (1 hour)

Test material 3	test material 4	positive control

plate B (3 min)

test material 3	Test material 4	positive control

plate D (1 hour)

Note: To avoid experimental errors it is recommended to use NC and PC at **identical** positions in all experiments. In contrast, test chemicals should be positioned differently in the two independent experiments.

8). **1 hour experiment**: Add 50 µL H_2O (negative control) into the first insert atop the EpiDermä (EPI-200) tissue. Set the timer to 1 hr and start it, repeat the procedure with the second tissue. Proceed with test material 1 - 4 and the positive control in the same manner until all 12 tissues are dosed and rinsed. Place both 6-well plates into the incubator (37°C, 5 % CO_2). Record start time in the MDS.
9) **3 minutes experiment**: Add 50 µL H_2O (negative control) into the first insert atop the EpiDermä (EPI-200) tissue. Set the timer to 3 min and start it. Repeat the procedure with the second tissue. Important: keep a constant time interval between dosing (e.g. 40 sec.). After 3 min of application, with forceps, remove the first insert immediately from the 6-well plate. Using a wash bottle gently rinse the tissue with PBS (20 times) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom with blotting paper. Place insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all 12 tissues are dosed and rinsed.
10) **3 minutes:** once all tissues have been dosed and rinsed and are in the holding plate, remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plate in the incubator, record start time of MTT incubation in the MDS and incubate for the plate for 3 hours (37°C, 5% CO_2).
11) **1 hour:** after the 1 hour period of test material exposure (in the incubator) is completed with forceps remove the first insert from the 6-well plate. Using a wash bottle gently rinse the tissue with PBS (20 times) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom with blotting paper. Place insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all 12 tissues are rinsed.
12) **1 hour:** once all tissues have been rinsed and are in the holding plate, remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plate in the incubator, record start time of MTT incubation in the MDS and incubate for the plate for 3 hours (37°C, 5% CO_2).
13) **3 minutes:** After the 3 hour MTT incubation period is complete, aspirate MTT medium from all 12 wells (e.g. gently using a suction pump), refill wells with PBS and aspirate PBS. Repeat the rinsing twice and make sure tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.
14) **3 minutes:** Immerse the inserts by gently pipetting 2 ml extractant solution (isopropanol) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.
15) **3 minutes:** Seal the 24 well plate (e.g. with a zip bag) to inhibit isopropanol evaporation. Record start time of extraction in the MDS. Extract either over night without shaking at room temperature or, alternatively, 2 hours with shaking (~120 rpm) at room temperature.
16) **1 hour:** After the 3 hour MTT incubation period is complete, aspirate MTT medium

from all 12 wells (e.g. gently using a suction pump), refill wells with PBS and aspirate PBS. Repeat the rinsing twice and make sure tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.
17) **1 hour:** Immerse the inserts by gently pipetting 2 ml extractant solution (isopropanol) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.
18) **1 hour:** Seal the 24 well plate (e.g. with a zip bag) to inhibit isopropanol evaporation. Record start time of extraction in the MDS. Extract either over night without shaking at room temperature or, alternatively, 2 hours with shaking (~120 rpm) at room temperature.

Second day of testing (only if formazan has been extracted over night!)
19) After the extraction period is complete for both, the **3 min** and the **1 hr** experiment, pierce the inserts with an injection needle (~ gauge 20, ~0.9 mm diameter) and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Place the 24-well plates on a shaker for 15 minutes until solution is homogeneous in colour.
20) Per each tissue transfer 3 × 200µL aliquots * of the blue formazan solution into a 96-well flat bottom microtiter plate, both from the **3 min** exposure and from the **1 hr** exposure. For the 96 well plate, use exactly the plate design given next page, as this configuration is used in the data spreadsheet. Read OD in a plate spectrophotometer at 570 nm, without reference filter. Alternatively, ODs can be read at 540 nm.

* **Note**: In contrast to normal photometers, in plate readers pipetting errors influence the OD. Therefore, 3 formazan aliquots shall be taken from each tissue extract. In the data sheet these 3 aliquots will be automatically reduced to one value by calculating the mean of the three aliquots. Thus, for calculations from each single tissue only one single mean OD-value is used.

Note: Readings are performed without reference filter, since the "classical" reference filter often used in the MTT test (630 nm) is still within the absorption curve of formazan. Since filters may have a ± tolerance in some cases the reference filter reduces the dynamics of the signal (OD) up to 40%.

Fixed 96 well-plate design (for OD reading in plate photometer, 3 aliquots per tissue)

NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	3 min
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	1 hour
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
tissue 1	tissue 2	tissue 1	tissue 2	tissue 1	tissue 2	tissue 1	tissue 2	tissue 1	tissue 2	Tissue 1	Tissue 2	

3.5. DOCUMENTATION

3.5.1. Method Documentation Sheet, MDS (see ANNEX)

The MDS allows to check the correct set up, calibration and function of the equipment as well as correct weights, applications etc. The MDS is designed as a paper document "in the spirit of

GLP". For each kit, make a hardcopy of the MDS, fill in and sign the requested information, starting the day prior to testing and ending after the test has been conducted.

Note (1): If several tests are performed per week, pipette verification (weighing H_20 on a balance) is only necessary once at the beginning of each week. Nevertheless, if adjustable pipettes are used the correct adjustment shall be checked and recorded in the MDS before each test.

Note (2): If solids cannot be sufficiently ground to a fine powder, it is recommended to check the weight of the levelled application spoon and record this weight in the MDS.

3.5.2. Data Spreadsheet

The MS EXCEL spreadsheet "C-SPREAD.XLS" is provided by ZEBET. Data files of optical densities (ODs) generated by the microplate reader are copied from the reader software to the Windows Clipboard and then pasted into the first map of the EXCEL spreadsheet in the 96-well format given above (Note: Only 72 wells of the 96 wells are used!).
The spreadsheet consists of three maps, named Import, MDS_Information and Spread**.** The first map (Import) is used for pasting the OD values (cursor position: A20!). Use the second map (MDS information) for the entry of the requested information (tissue lot-no., test material codes, date...), they will be copied from there to the other maps. The third map (Spread) does the calculations and provides a column graph of the results.

File names to be used in prevalidation phase III:
Since each single XLS file contains the data of 4 test chemicals, each of them coded by BIBRA with a four digit number there is no way to use "intelligent" file names which would allow to recognise the test chemicals from the file names. Therefore, file names should first give the testing laboratory name (3 digits), then a dash (1 digit) and then the test number (2 digits):
BAS-01.XLS, BAS-02.XLS, ...BAS-12.XLS
HLS-01.XLS, HLS-02.XLS, ...HLS-12.XLS
ZEB-01.XLS, ZEB-02.XLS, ...ZEB-12.XLS

4. Evaluation, Prediction Models (PM 1 and PM 2)

Note: The mathematical rule for the prediction or classification of *in vivo* skin corrosivity potential from the *in vitro* data is called Prediction Model (PM). For the present test two prediction models are defined, one definitive model (PM 1), based on published data (Perkins et al., 1996) which have been confirmed by extensive testing at ZEBET during Phase I of the present prevalidation study.

Nevertheless, the data base obtained in Phase I indicated that sensitivity was a bit too low (71%) to be used as a full animal replacement test, whereas the specificity of the test was very high (89%). Since a shift of the cut-off for classification would not have sufficiently increased the sensitivity, the test design was changed by including a second, longer application time of 1 hr for the test chemicals. This changed test design was experimentally tested at ZEBET when the prevalidation study had already proceeded to Phase II. Therefore, ZEBET was able to test only those chemicals again, which were classified negative with the 3 min EpiDerm™ (EPI-200) protocol. The data indicated that the sensitivity was increased (some false negatives were predicted now correct as corrosives) but the influence of this change on the total predictive capacity of the assay could not be sufficiently investigated. Therefore, a second, tentative prediction model (PM 2) was defined, which has to be verified / falsified by the data obtained in Phase III of the present prevalidation study.

4.1. PREDICTION MODEL 1

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after 3 min treatment compared to the negative control tissues concurrently treated with H_20. A chemical is classified "corrosive", if the relative tissue viability after 3 min treatment with a test material is decreased below 50 %:

mean tissue viability (% negative control)	**Prediction C / NC**
< 50	Corrosive
≥ 50	Non-corrosive

4.2. PREDICTION MODEL 2

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after 3 min treatment compared to the negative control tissues concurrently treated with H_20. A chemical is classified "corrosive" in any case, if the relative tissue viability after 3 min treatment with a test material is decreased below 50 %. In addition, those materials classified "non corrosive" after 3 min (viability ≥ 50%) are classified "corrosive" if the relative tissue viability after 1 hr treatment with a test material is decreased below 15 %.

mean tissue viability (% negative ctrl.)	**Prediction C / NC**
3 min: < 50	Corrosive
3 min:. ≥ 50 and 1 hour: < 15	Corrosive
3 min:. ≥ 50 and 1 hour: ≥ 15	Non-corrosive

ANNEX: METHODS DOCUMENTATION SHEET (MDS)

ASSAY No:.....DATE:.....
XLS file name:.....

Kit receipt

EpiDerm™ (EPI-200) kit received (day/date):	Day used:
EpiDerm™ (EPI-200) Lot no.:	Production date:
Epi-100 Assay medium Lot no.:	Expiration date:
MTT concentrate Lot no.:	Date:
MTT diluent Lot no.:	Date:
MTT extractant Lot no.:	Date:
Booked in by (ID):	

PBS preparation

DPBS Lot no.:	Expiration date:	
Vol 10x DPBS:	Vol water:	Initial pH:
NaOH used to adjust pH:		Final pH:
HCl used to adjust pH:		Final pH:
Prepared by (ID):		

Incubator verification

Incubator #	CO_2 (%)	Temperature (°C)	Check water in reservoir (✓)

ID / date:

Pipette verification (triplicate weightings)
Note: Perform pipette verification only once per week and refer to it in all assays of this week.
But: If adjustable pipettes are used, check correct adjustment daily and mark with (✓).

Verification	0.9 ml	300 µL	200 µL	25 µL	50 µL
	H_20 weight (mg)				
1. 2. 3.					

ID / date:

Dosing procedure
Please mark (✓) the type of application. Also, mark (✓) wetting with H_20. If significantly more than 25 µL of H_20 had to be used for wetting solids record ~ volume. REMARKS: record, if spreading was necessary or if crushing and grinding was not used (because it did not improve consistence of test material).

TEST MATERIAL CODE	LIQUID 50 µl (✓)	SEMI-SOLID	SOLID spoon (✓) + x µl H_2O		Material Characterisation §	REMARKS
Neg. Control						
Pos. Control						

§ use your own wording, like: "highly viscous"

Record experimental design of the 6-well plates

plate A (3 min)

Negative control		
Negative Control		

plate C (1 hour)

negative control		
negative control		

plate B (3 min)

		positive control
		positive control

plate D (1 hour)

		Positive Control
		Positive Control

(record code numbers of test materials) ID / date:

Time protocols:

Procedure	**Start**	**Stop**
1 hr pre-incubation of tissues		
1 hr chemical application (incubator)		
3 hrs MTT incubation (1 hr experiment)		
3 hrs MTT incubation (3 min experiment)		
Formazan extraction		

ID / Date:

Check plate photometer filter (_)

reading filter: 570 nm	
reading filter: 540 nm	

ID / Date:

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Section 4.0

Rat Skin Transcutaneous Electrical Resistance (TER) Assay

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Summary Report of the Rat Skin Transcutaneous Electrical Resistance (TER) *In Vitro* Assay for Assessing Dermal Corrosivity

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Revised: June 6, 2002

PURPOSE

This report focuses on the performance of the Rat Skin Transcutaneous Electrical Resistance (TER) assay to determine the usefulness and limitations of the assay for the identification of potential human corrosive chemicals. This report also discusses how Rat Skin TER compares to the *in vivo* rabbit skin corrosivity test and to other *in vitro* corrosivity tests (EPISKIN™, EpiDerm™ [EPI-200], and Corrositex®). The data and assessments reviewed included an inter-laboratory trial (Botham et al., 1992), a prevalidation study (Botham et al., 1995), and a validation study (Barratt et al., 1998; Fentem et al., 1998). Additionally, an independent analysis of the Rat Skin TER performance data, taking into account the totality of the database, was conducted.

EVALUATION OF REGULATORY AND SCIENTIFIC RATIONALE

The Rat Skin TER assay has been in use for over five years (Botham et al., 1995). This assay is one of several *in vitro* corrosivity assays evaluated as alternatives to the *in vivo* rabbit corrosivity test by the European Centre for the Validation of Alternative Methods (ECVAM) in a formal validation study (Fentem et al., 1998).

The assay has been approved by the ECVAM Scientific Advisory Committee for use in corrosivity testing in Europe (Balls and Corcelle, 1998) and has also been evaluated and accepted for its intended use by the European Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) (SCCNFP, 1999). This method has been adopted for regulatory use within the European Union (EU) by the European Commission (EU, 2000).

EVALUATION OF THE TEST METHOD

In the Rat Skin TER assay, test materials (liquids: 150 µL; solids 100 mg plus 150 µL of water) are applied for 2 and 24 hours to the epidermal surfaces of skin discs obtained from the skin of humanely killed young rats. Nine to 15 discs can be prepared from one rat pelt. Pelts must give a resistance value greater than 10 kΩ to be acceptable for use in the test. To test each chemical, three skin discs are used per time period, in addition to a concurrent positive and negative control. Corrosive materials are identified by the ability of the chemical to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction of the inherent transcutaneous electrical resistance below a predetermined threshold level of 5 kΩ. The validation protocol developed by ECVAM included a dye-binding assay, which is used to reduce the number of false positives encountered in the prevalidation study for surfactants and solvents. The scientific and mechanistic basis of the test and the rationale for using a 5 kΩ criterion for identifying potential human corrosivity were not discussed by Botham et al. (1995) or Fentem et al. (1998).

EVALUATION OF TEST METHOD DATA QUALITY

The Rat Skin TER assay was evaluated in three studies: an inter-laboratory trial (Botham et al., 1992), a prevalidation study (Botham et al., 1995), and an ECVAM validation study (Fentem et al., 1998). The inter-laboratory trial was based on an evaluation of 20 chemicals (6 corrosives/14 noncorrosives), while the prevalidation and ECVAM validation studies evaluated 50 chemicals (25C/25NC) and 60 chemicals (27C/33NC), respectively. The main

criterion for including chemicals in the study was that their corrosivity classification was based on unequivocal animal data (Barratt et al., 1998). The ECVAM validation chemical test set included organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1 NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC), and soaps/surfactants (3NC). Despite the small numbers of chemicals in some categories, ECVAM concluded that the set of test chemicals used in the validation study represented the best possible group for evaluating the performance characteristics of the *in vitro* assays, given the limited availability of unequivocal animal data (Barratt et al., 1998).

In the validation study, each chemical was tested twice in each of three different laboratories. The tests were stated to have been conducted in the "spirit" of GLP (Fentem et al., 1998). A formal audit of the ECVAM data by a Quality Assurance Unit was not conducted; however, it was stated that all data submitted by the participating laboratories were verified against the original data sheets by ECVAM staff on at least three separate occasions.

EVALUATION OF TEST METHOD PERFORMANCE

For this summary report, an analysis was conducted, similar to the performance analysis conducted for the ICCVAM Peer Review of Corrositex®; the current analysis evaluated the performance characteristics of the Rat Skin TER assay against the corresponding *in vivo* rabbit corrosivity data. The database used in the Rat Skin TER evaluation consisted of data from three published sources (Botham et al., 1992; Botham et al., 1995; Fentem et al., 1998). For ease of comparison, chemicals evaluated in the Rat Skin TER assay were classified into the same chemical and product class designations used in the Corrositex® evaluation. A weight-of-evidence approach was used for classifying discordant results within or between laboratories; in instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls), the chemical was eliminated from inclusion in the performance calculations.

The results of the overall performance analysis for the Rat Skin TER assay are presented in **Table 4.1**. Based on a database of 122 chemical and chemical mixtures, this assay had an accuracy of 81% (99/122 chemicals or chemical mixtures), a sensitivity of 94% (51/54 chemicals or chemical mixtures), a specificity of 71% (48/68 chemicals or chemical mixtures), a false positive rate of 29% (20/68), and a false negative rate of 6% (3/54). These performance characteristics were not different when the Botham et al. (1992 and 1995) studies were evaluated independently of the ECVAM validation study (Fentem et al., 1998) (**Tables 4.2** and **4.3**, respectively). The performance characteristics for the Rat Skin TER assay remained consistent when evaluated against various chemicals classes, including organic and inorganic acids and bases, organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixtures. Based on the validation study results, which met pre-study acceptance criteria of no more than 20% false negatives and 20% false positives, the ECVAM concluded that the Rat Skin TER assay was valid for use as a replacement for the *in vivo* rabbit skin test for distinguishing between corrosive and noncorrosive chemicals for all of the chemical types studied (Fentem et al., 1998; Balls and Corcelle, 1998). ECVAM concluded also that the Rat Skin TER assay

was not capable of classifying chemicals or chemical mixtures by packing group (i.e., it could not distinguish between known R35/I and R34/II & III chemicals). However, it was stated that taking into account the relative simplicity of the mechanism of action of corrosives, this method would be generally applicable across all chemical classes (Fentem et al. 1998).

EVALUATION OF TEST METHOD RELIABILITY (REPEATABILITY/ REPRODUCIBILITY)

The Rat Skin TER assay has been evaluated for repeatability and/or reproducibility in three different studies. In the Botham et al. (1992) inter-laboratory trial, no statistically significant level of inter-laboratory variability was found for corrosives (6 compounds), noncorrosives (14 compounds), or for all test materials (20 compounds); variability among the three independent laboratories was assessed using ANOVA. An intra-laboratory analysis was not possible. In the prevalidation study (Botham et al., 1995), the agreement for the classifications obtained by both participating laboratories was 92% (23 of 25 C and 23 of 25 NC chemicals).

In the ECVAM validation study (Fentem et al., 1998), the 60 chemicals were each tested twice by each of three laboratories. Intra- and inter- laboratory reliability was evaluated using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors), scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Based on their analyses, ECVAM concluded that inter- and intra-laboratory variability was approximately equivalent, with no evidence of systematic differences between experiments within a laboratory. Of the 60 chemicals tested, 37 gave the same corrosivity classification in both experiments in all three laboratories. For ten of the remaining 23 chemicals, only one experiment resulted in a classification differing from the other 5 predictions. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that the Rat Skin TER assay was reliable and reproducible. Due to the lack of quantitative data for individual chemicals in the published studies, no independent evaluation of repeatability or reproducibility for the Rat Skin TER assay could be conducted. However, after reviewing the intra- and inter-laboratory evaluations conducted by ECVAM, it was concluded by NICEATM that the analyses were appropriate and that the conclusions were accurate.

Table 4.1 Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Overall)[1]

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	122	81	(99/122)	94	(51/54)	71	(48/68)
Organic and Inorganic Acids and Bases[2]	64	91	(58/64)	98	(44/45)	74	(14/19)
Organic and Inorganic Bases and Base Mixtures[3]	27	93	(25/27)	100	(20/20)	71	(5/7)
Organic and Inorganic Acids and Acid Mixtures	31	94	(29/31)	100	(20/20)	82	(9/11)
Amines	21	95	(20/21)	100	(15/15)	83	(5/6)
Inorganic Bases and Base Mixtures	6	83	(5/6)	100	(5/5)	0	(0/1)
Acid Derivatives	6	67	(4/6)	80	(4/5)	0	(0/1)
Surfactants	21	62	(13/21)	100	(4/4)	53	(9/17)
Industrial Chemicals	26	73	(19/26)	50	(1/2)	75	(18/24)
Cleaners and Detergents	7	86	(6/7)	100	(2/2)	80	(4/5)

[1]This analysis contains data from Fentem et al. (1998), Botham et al. (1995), and Botham et al. (1992).

[2]This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives.

[3]This chemical class includes amines, inorganic bases, and base mixtures.

Table 4.2 Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Fentem et al., 1998)

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	58	81	(47/58)	93	(25/27)	71	(22/31)
Organic and Inorganic Acids and Bases[1]	39	85	(33/39)	96	(24/25)	64	(9/14)
Organic and Inorganic Bases and Base Mixtures[2]	13	85	(11/13)	100	(9/9)	50	(2/4)
Organic and Inorganic Acids and Acid Mixtures	20	90	(18/20)	100	(11/11)	78	(7/9)
Amines	9	89	(8/9)	100	(6/6)	67	(2/3)
Inorganic Bases and Base Mixtures	4	75	(3/4)	100	(3/3)	0	(0/1)
Acid Derivatives	6	67	(4/6)	80	(4/5)	0	(0/1)
Surfactants	5	60	(3/5)	NA	(0/0)	60	(3/5)
Industrial Chemicals	10	80	(8/10)	100	(1/1)	78	(7/9)
Cleaners and Detergents	1	100	(1/1)	NA	(0/0)	100	(1/1)

NA = Not applicable

[1]This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives.

[2]This chemical class includes amines, inorganic bases, and base mixtures.

Table 4.3 Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Botham et al., 1992; 1995)

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	65	82	(53/65)	96	(27/28)	70	(26/37)
Organic and Inorganic Acids and Bases[1]	26	100	(26/26)	100	(21/21)	100	(5/5)
Organic and Inorganic Bases and Base Mixtures[2]	14	100	(14/14)	100	(11/11)	100	(3/3)
Organic and Inorganic Acids and Acid Mixtures	12	100	(12/12)	100	(10/10)	100	(2/2)
Amines	12	100	(12/12)	100	(9/9)	100	(3/3)
Inorganic Bases and Base Mixtures	2	100	(2/2)	100	(2/2)	NA	(0/0)
Acid Derivatives	0	NA	(0/0)	NA	(0/0)	NA	(0/0)
Surfactants	16	63	(10/16)	100	(4/4)	50	(6/12)
Industrial Chemicals	16	69	(11/16)	0	(0/1)	73	(11/15)
Cleaners and Detergents	6	83	(5/6)	100	(2/2)	75	(3/4)

NA = Not applicable

[1]This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives.

[2]This chemical class includes amines, inorganic bases, and base mixtures.

OTHER SCIENTIFIC REVIEWS

In March 1999, a search of the open literature was conducted to locate additional Rat Skin TER studies. Six databases (Medline, Toxline, Embase, Biosis, Caba, and LifeSci) were searched using the key terms "Transcutaneous" within one word of "electrical" within one word of "resistance"; and "TER" and "rat" or "rats". The search found no additional relevant studies conducted with this assay. In May 2001, another search was performed to locate additional TER studies. Four databases (PubMed, Web of Science, Toxline, and Current Contents Connect) were searched using the same search strategy and no additional relevant studies were found.

OTHER CONSIDERATIONS

The cost for conducting the Rat Skin TER assay is reported by Syngenta Corporation (e-mail communication from Phil Botham, Syngenta CTL) to be approximately $500-800 per test. When compared to other *in vitro* methods (EPISKIN™, EpiDerm™ (EPI-200), and Corrositex®), the cost and the time necessary to conduct the Rat Skin TER assay are greater (**Table 4.4**). Additionally, TER requires the use of animals, whereas EPISKIN™ and Corrositexâ do not.

RELATED ISSUES

Refinement, Reduction, and Replacement

The Rat Skin TER assay does not eliminate the use of animals. However, if used in an integrated approach, TER provides for the reduction and refinement of animal use.

Comparison to Other *In Vitro* Assays

General comparative information on the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositexâ assays is provided in **Tables 4.4** through **4.7**.

Table 4.4 General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays

	Rat Skin TER Assay	EPISKIN™ (prediction model B)	EpiDerm™ (EPI-200) (prediction model 2)	Corrositex®
Test Method Description	Acceptable	Acceptable	Acceptable	Acceptable
Adequacy/Completeness of Protocol	Acceptable	Acceptable	Acceptable	Acceptable
Usefulness for Assessing Corrosivity/Non-corrosivity	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Usefulness for Determining Packing Groups	Not Acceptable (Fentem et al., 1998)	Can group as UN packing group II/III or I (Fentem et al., 1998)[a]	Not Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Repeatability and Reproducibility	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (Fentem et al., 1998; ICCVAM, 1999)
Animal Use Refinement, Reduction, and Replacement Considerations	Refines and reduces animal use when used as a stand-alone test or in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.	Refines and reduces animal use when used in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.
Cost	~$500-850/test	~$450/test kit[b]	~$200/test chemical	~$300/test chemical
Study duration	2 work-days	1 work-day	1 work-day	≤ 4 hr/chemical

[a] Since the performance of EPISKINä was not assessed for distinguishing between UN packing groups II and III, all R34 classifications would be conservatively classified as UN packing group II.

[b] One to three chemicals may be tested per test kit; however, it is recommended by the supplier that each test chemical be assayed using 3 different skin batches/kits which equates to a total cost of ~$430/ test chemical.

Table 4.5 General Comparison of the Rat Skin TER Assay, EPISKIN™, EpiDerm™ (EPI-200), and Corrositex® Assays Based on a Weight-of-Evidence Approach[a] by Chemical using Data from the ECVAM and Other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™	EpiDerm™ (EPI-200) (prediction model 2)	Corrositex®
Number of Chemicals	122	60	24	163
Overall Sensitivity[b]	94% (51/54)	82% (23/28)	92% (11/12)	85% (76/89)
Overall Specificity[b]	71% (48/68)	84% (27/32)	83% (10/12)	70% (52/74)
Overall Accuracy[b]	81% (99/122)	83% (50/60)	92% (22/24)	79% (128/163)
False Positive Rate	29% (20/68)	16% (5/32)	17% (2/12)	30% (22/74)
False Negative Rate	6% (3/54)	18% (5/28)	8% (1/12)	15% (13/89)
Test Chemical Inter-laboratory Coefficient of Variation	34.7[c] 3.8-322[d] 120[e]	11.3[c] 3.9-148.8[d] 20[e]	12.3[c] 0.9-51.2[d] 144[e]	30.3[c] 7.7-252.5[d] 180[e]

[a] A chemical is first classified as positive or negative for corrosivity within each laboratory based on the majority of test results obtained (when replicate testing was conducted). Next, the chemical is classified as positive or negative for corrosivity based on the majority of test results obtained in multiple laboratories (when multiple laboratory studies were conducted). In instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls within or across laboratories), the chemical was eliminated from inclusion in the performance calculations.

[b] Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method.

[c] Median value

[d] Range of values

[e] The total number of independent values, which is calculated as the number of chemicals tested multiplied by the number of participating laboratories.

Table 4.6 General Comparison of the Rat Skin TER, EPISKIN™ and EpiDerm™ (EPI-200), Assays from Independent Test Results in the ECVAM Validation Studies (Fentem et al., 1998; Liebsch et al., 2000)

	Rat Skin TER Assay	EPISKIN™ (prediction model B)	EpiDerm™ (EPI-200) (prediction
Number of Chemicals Tested in ECVAM Validation Study	60 (Fentem et al., 1998)	60/24[a] (Fentem et al., 1998)	24 (Liebsch et al., 2000)
Sensitivity[b]	88% (140/159)	83% (201/243) / 88% (87/99)	88% (63/72)
Specificity[b]	72% (142/196)	80% (237/297) / 79% (92/117)	86% (62/72)
Accuracy[b]	79% (282/355)[c]	81% (438/540) / 83% (179/216)	87% (125/144)
False Positive Rate[b]	28% (54/196)	20% (60/297) / 21% (25/117)	14% (10/72)
False Negative Rate[b]	12% (19/159)	17% (42/243) / 12% (12/99)	13% (9/72)
Number of Trials[d]	355	540 / 216	144
Test Chemical Inter-laboratory Coefficient of Variation	34.7[d] 10-322[e] 360[f]	30.2[d] 7.7-252.5[e] 540[f]	12.3[d] 0.9-51.[e] 144[f]

[a] The first numbers for accuracy, sensitivity, specificity, false positive rate, and false negative rate correspond to the 60 chemicals tested in the ECVAM Skin Corrosivity Test using EPISKIN™ (Barratt et al., 1998; Fentem et al., 1998); the latter values correspond to a direct comparison of EpiDerm™ (EPI-200) and EPISKIN™ for the same 24 materials tested in both systems (Liebsch et al., 2000).

[b] Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method. False positive rate is defined as the proportion of all negative chemicals or chemical mixtures that are falsely identified as positive. False negative rate is defined as the proportion of all positive chemicals or chemical mixtures that are falsely identified as negative.

[c] The percentages are based on the number of correct trials among the total number of trials (i.e., independent tests) provided in parenthesis.

[d] Median value

[e] Range of values

[f] The total number of trials conducted in the validation study minus the non-qualified (NQ) results. This number is equal to the number of chemicals multiplied by the number of participating laboratories multiplied by the number of replicate tests.

Table 4.7 Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ (EPI-200) Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000)

No.[a]	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN™ [b]	EpiDerm™ (EPI-200)
1	Hexanoic acid	ORGAC	R34/II&III	R35	R35	N/A
29	65/35 Octanoic/decanoic acid	ORGAC	R34/II&III	R34	R35	N/A
36	2-Methylbutyric acid	ORGAC	R34/II&III	R35	R34	N/A
40	Octanoic acid (caprylic acid)	ORGAC	R34/II&III	R35	R34/C	C
47	60/40 Octanoic/decanoic acids	ORGAC	R34/II&III	R34	R34/C	C
50	55/45 Octanoic/decanoic acids	ORGAC	R34/II&III	R35	R34	N/A
7	3,3'-Dithiodipropionic acid	ORGAC	NC	NC	NC	N/A
12	Dodecanoic acid (lauric acid)	ORGAC	NC	NC	NC	NC
26	Isotearic acid	ORGAC	NC	NC	NC	NC
34	70/30 Oleine/octanoic acid	ORGAC	NC	NC	NC	N/A
58	10-Undecenoic acid	ORGAC	NC	NC	R34	N/A
2	1,2-Diaminopropane	ORGBA	R35/I	R35	R34/C	C
15	Dimethyldipropylenetriamine	ORGBA	R35/I	R35	R34/C	C
38	Tallow amine	ORGBA	R35/II	2R34/2NC/2NQ	NC	N/A
55	1-(2-Aminoethyl)piperazine	ORGBA	R34/II	R35	NC	N/A
13	3-Methoxypropylamine	ORGBA	R34/II&III	R35	R34	N/A
17	Dimethylisopropylamine	ORGBA	R34/II&III	R35	R34/C	C
45	n-Heptylamine	ORGBA	R34/II&III	R35	NC	C
10	2,4-Xylidine (2,4-Dimethylaniline)	ORGBA	NC	R34	R34	N/A
35	Hydrogenated tallow amine	ORGBA	NC	NC	NC	NC
59	4-Amino-1,2,4-triazole	ORGBA	NC	NC	NC	NC
8	Isopropanol	NORG	NC	NC	NC	N/A
11	2-Phenylethanol	NORG	NC	NC	NC	N/A
16	Methyl trimethylacetate (referred to as Methyl 2,2-dimethylpropanoate in EpiDerm™)	NORG	NC	NC	NC	C
19	Tetrachloroethylene	NORG	NC	NC	NC	NC
22	n-Butyl propionate	NORG	NC	NC	NC	N/A
27	Methyl palmitate	NORG	NC	NC	NC	N/A
44	Benzyl acetone	NORG	NC	NC	NC	NC
51	Methyl laurate	NORG	NC	NC	NC	N/A
56	1,9-Decadiene	NORG	NC	NC	NC	NC
3	Carvacrol	PHEN	R34/II&III	R34	R34	N/A
23	2-tert-Butylphenol	PHEN	R34/II&III	R35	R34/C	C
9	*o*-Methoxyphenol (Guaiacol)	PHEN	NC	NC	R34	N/A
30	4,4-Methylene-bis-(2,6-di-tert-butylphenol)	PHEN	NC	NC	NC	N/A
49	Eugenol	PHEN	NC	NC	NC	NC
4	Boron trifluoride dihydrate	INORGAC	R35/I	R35	R35/C	C

Table 4.7 (continued)

No.[a]	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN™ [b]	EpiDerm™ (EPI-200)
28	Phosphorus tribromide	INORGAC	R35/I	R35	R35/C	C
32	Phosphorus pentachloride	INORGAC	R35/I	R35	R34	N/A
25	Sulfuric acid (10% wt.)	INORGAC	R34/II&III	R34	R34	N/A
57	Phosphoric acid	INORGAC	R34/II	R35	R34	N/A
43	Hydrochloric acid (14.4% wt)	INORGAC	R34/II&III	R35	R34	N/A
53	Sulfamic acid	INORGAC	NC	R34	R34/C	C
18	Potassium hydroxide (10% aq.)	INORGBA	R34/II&III	R35	R34/C	C
42	2-Mercaptoethanol, Na salt (45% aq.)	INORGBA	R34/II&III	R35	NC	N/A
21	Potassium hydroxide (5% aq.)	INORGBA	NC	R35	R34	N/A
24	Sodium carbonate (50% aq.)	INORGBA	NC	R34	NC	NC
20	Ferric [iron (III)] chloride	INORGSAL	R34/II	R35	R34	N/A
52	Sodium bicarbonate	INORGSAL	NC	R34	NC	N/A
54	Sodium bisulfite	INORGSAL	NC	3R34/3NC	NC	N/A
5	Methacrolein	ELECTRO	R34/II&III	NC	R34/C	NC
14	Allyl bromide	ELECTRO	R34/II&III	R35	R34	N/A
48	Glycol bromoacetate (85%)	ELECTRO	R34/II&III	NC	R34/C	C
6	Phenethyl bromide	ELECTRO	NC	NC	NC	N/A
31	2-Bromobutane	ELECTRO	NC	3R34/3R35	NC	N/A
33	4-(Methylthio)-benzaldehyde	ELECTRO	NC	NC	NC	N/A
39	2-Ethoxyethyl methacrylate	ELECTRO	NC	NC	NC	N/A
46	Cinnamaldehyde	ELECTRO	NC	NC	NC	N/A
37	Sodium undecylenate (33% aq.)	SOAP	NC	R35	R34	N/A
41	20/80 Coconut/palm soap	SOAP	NC	NC	NC	N/A
60	Sodium lauryl sulfate (20% aq.)	SOAP	NC	R35	NC	NC

Overall corrosivity classifications were determined by the majority of the reported results obtained from each assay. If results do not show a majority, a definitive classification could not be determined.

Definitions are as follows: C = Corrosive; NC = Noncorrosive; R34 is equivalent to packing groups II and/or III; R35 is equivalent of packing group I, except for tallow amine (R35/II); NQ = Non-qualified; N/A = Not applicable because not tested; ORGAC = Organic acid; ORGBA = Organic base; NORG = Neutral organics; PHEN = phenol; INORGAC = Inorganic acid; INORGBA = Inorganic base; INORGSAL = Inorganic salt; ELECTRO = Electrophile; SOAP = Soap surfactant

[a] Number assigned each chemical by the ECVAM Management Team.

[b] For EPISKIN™, prediction model B was the more complex prediction model and was the only model considered in detail by the ECVAM Management Team (Fentem et al., 1998).

SUMMARY CONCLUSIONS AND RECOMMENDATIONS

ECVAM concluded that the Rat Skin TER assay was an *in vitro* replacement assay for *in vivo* corrosivity testing (Fentem et al., 1998). NICEATM concurs with the ECVAM conclusion that the Rat Skin TER assay is both reliable and reproducible. For some chemical or product classes (e.g., cleaners and detergents), the small number of chemicals and/or the unbalanced distribution of corrosive and noncorrosive chemicals does not allow accurate conclusions to be made on the performance of this assay for these chemical classes.

The two major questions to be addressed for *in vitro* corrosivity assays are:

1. Has the assay been evaluated sufficiently and is its performance satisfactory to support the proposed use for assessing the corrosivity potential of chemicals and chemical mixtures?

2. Does the assay adequately consider and incorporate, where scientifically feasible, the 3Rs of animal use (refinement, reduction, and replacement alternatives)? Does the assay offer advantages with respect to animal welfare considerations?

In response to the first question, the performance characteristics of the Rat Skin TER assay indicates, in specific testing circumstances, that this test may be considered useful as part of an integrated testing strategy for assessing the dermal corrosion potential of chemicals.

In response to the second question, the Rat Skin TER assay sufficiently considers and incorporates the 3Rs. The assay offers animal welfare advantages, including animal use refinement and reduction; this method reduces the number of animals used as skin from one humanely killed rat may be used to test up to five chemicals. Similarly, the use of the Rat Skin TER assay as part of an integrated approach reduces and refines the use of animals by providing a basis for decisions on further testing. Follow-up testing using *in vivo* methods, when deemed necessary, could employ fewer animals and test agent dilution schemes to minimize possible pain in any individual animal.

ECVAM Protocol for Rat Skin Transcutaneous Electrical Resistance: an *In Vitro* Assay for Assessing Dermal Corrosivity

Original Draft: July 1996
Confirmed: January 2002

NOTE: This protocol presents the standard operating procedure used in the ECVAM Skin Corrosivity Validation Study (1996/1997). ECVAM confirmed the accuracy of the SOP in October 2000, and this protocol was supplied by Dr. Andrew Worth of ECVAM via email on May 22, 2001.

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Rat Skin Transcutaneous Electrical Resistance (TER) Test

The corrosivity potential of a chemical may be predicted from its effects on the transcutaneous electrical resistance of rat skin and from its effects on the penetration of sulforhodamine B dye through the skin.

Objectives and Applications

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency
PURPOSE OF TESTING	:	hazard identification, classification and labelling

Proposed replacement for the *in vivo* method, the Draize rabbit skin corrosivity test, to be used for hazard identification and classification of corrosive potential to fulfil international regulatory requirements pertaining to the handling, packing and transport of chemicals.
When used in screening mode, the TER test is employed to predict corrosivity potential rather than the degree of corrosive effect (i.e. potency) (Fentem *et al.,* 1998).

Basis of the Method

Most international regulatory classification schemes define chemically induced dermal corrosion as full thickness destruction (necrosis) of the skin tissue, while some extend the definition of corrosion to include any irreversible alterations caused to the skin. The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals.

The determination of skin corrosion potential is therefore included in international regulatory requirements for the testing of chemicals, for example, in OECD testing guideline 404 (Anon., 1992); Annex V of Directive 67/548/EEC (Anon., 2000) and in the U.S. Code of Federal Regulations (Anon., 1991).

Corrosivity is usually determined *in vivo* using the Draize rabbit skin test (Draize *et al.,* 1944). The present test is based on the experience that transcutaneous electrical resistance (TER) measurements are believed to be of value in predicting severe cutaneous effects *in vivo*. The TER assay developed and evaluated by Oliver and coworkers (Barlow *et al.,* 1991; Oliver *et al.,* 1986; 1988; Oliver, 1990) has been used successfully as a routine in-house test for several years (Fentem *et al.,* 1998).

As an outcome of the ECVAM prevalidation study for protocol optimization, a second endpoint, dye binding (sulforhodamine B) has been added to reduce the number of false positive predictions encountered previously with surfactants and neutral organics.

Experimental Description

Endpoint and Endpoint Detection	:	- changes in transcutaneous electrical resistance (kΩ); - dye binding (sulforhodamine B) determined by optical density measurements;
Test System	:	isolated rat skin.

Liquid or solid test material is applied to the inner epidermal surface of discs of freshly isolated rat dorsal skin. After the exposure periods of 2 and 24 hours, the skin is washed and transcutaneous electrical resistance is measured. If the electrical resistance values are <5kΩ and the substance is a surfactant or neutral organic, then the sulforhodamine B dye is applied to the epidermal surface of each skin disc. The discs are washed and then subjected to a dye extraction procedure. The amount of dye extracted is determined from optical density measurements. The changes in the endpoints are then compared to HCl and H_2O, the positive and negative controls.

Test Compounds

A total of 60 test compounds, consisting of 11 organic acids, 10 organic bases, 9 neutral organics, 5 phenols, 7 inorganic acids, 4 inorganic bases, 3 inorganic salts, 8 electrophiles and 3 soaps/surfactants were tested in the ECVAM validation study. Details on the test compounds and test results are available from dbVas of the ECVAM SIS.

Prediction Model

Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a predetermined threshold level.

If the transcutaneous electrical resistance readings are ≤5kΩ at either of the contact periods, and the substance is a surfactant or neutral organic, then the dye penetration results are considered.

For detailed information see section 11, “Interpretation of results” of the present standard operating procedure.

Discussion

The TER assay is robust, requires inexpensive and readily available equipment, and can be performed by most laboratory personnel provided that care is taken during the critical steps of disc preparation and washing. The assay is inexpensive to perform in comparison with the three-dimensional tissue culture models and the CORROSITEX assay, and the technology is not protected by patent. These factors support the overall applicability of the TER assay in routine testing. The validation study has demonstrated the accuracy of the TER assay in identifying C and NC chemicals (Fentem *et al.*, 1998).

Status

The TER assay has been evaluated in intralaboratory and interlaboratory studies (Botham *et al.,* 1992; Oliver *et al.,* 1986, 1988), and it performed creditably in the prevalidation study conducted during 1993 and 1994 (Botham *et al.,* 1995). This method has been evaluated in the ECVAM Skin Corrosivity Validation Study conducted in 1996 and 1997 (Fentem *et al.,* 1998). Based on the outcome of the study, the ECVAM Scientific Advisory Committee (ESAC) concluded that the results obtained with the rat skin TER test in the "ECVAM Skin Corrosivity Validation Study" were reproducible. The test proved applicable to testing all the above reported chemical classes of different physical forms. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good.

ESAC unanimously endorsed the statement that the rat skin TER test was scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and non-corrosive chemicals, and that this test was ready to be considered for regulatory acceptance (10th meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 1998; Anon., 1998). The 27th meeting of the Committee for Adaptation to Technical Progress of "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" agreed that the TER Test would form part of "Annex V method B.40. Skin Corrosion", February 2000 (Directive 2000/33/EC). Furthermore, this test is now under consideration for inclusion in the OECD Guidelines. Further details on the ECVAM Validation Study are available from dbVas of the ECVAM SIS.

Last update: May 2000

Procedure Details, July 1996*

RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST

NOTE: This protocol presents the standard operating procedure evaluated in the ECVAM Skin Corrosivity Validation Study (1996/1997).

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* The accuracy of the SOP has been confirmed in October 2000.

1. INTRODUCTION AND OBJECTIVES

The purpose of this technique is to assess the degree of the skin corrosive potential of a test chemical *in vitro*. The results obtained from the transcutaneous electrical resistance (TER) measurements are believed to be of value in predicting severe cutaneous effects (degree of skin corrosive potential) *in vivo*. As a prelude to formal validation, the TER assay was evaluated in a prevalidation study (Botham *et al.*, 1995). Preliminary evaluation of the results indicated that the TER test required optimisation, to enable differentiation between different classes of corrosive materials, and to reduce the number of over-predictions (false positives). The results of this optimisation (Hadfield & Lewis, 1996; unpublished data), indicated that the modified electrical resistance test was able to differentiate between classes of corrosive materials (R35/R34) and, by the addition of a second endpoint, dye binding, was able to reduce the number of false positive predictions. The following protocol was therefore devised for use in the ECVAM international validation study on *in vitro* tests for skin corrosivity (Barratt *et al.*, 1998; Fentem *et al.*, 1998).

2. SAFETY PRECAUTIONS

Standard local safety precautions should be adopted. All materials should be handled in accordance with their potential hazards.

3. ANIMALS AND HUSBANDRY

20-23 day old Wistar rats are purchased for use in the test. Animals are acclimatised for a minimum of one night, depending on the day of delivery. On the day after arrival they are shaved and washed: animals are held securely and the dorsal flank hair is carefully removed with small animal clippers. The animals are then washed by careful wiping, whilst submerging the area in a one-litre volume of antibiotic solution (see following section 4). Animals are washed again on the third or fourth day following the first wash, and they are then used within 3 days (animals must

not be older than 31 days for pelt preparation).

4. PREPARATION OF ANTIBIOTIC SOLUTION

An antibiotic solution is prepared by adding streptomycin, penicillin, chloramphenicol and amphotericin B to 1 litre of luke-warm deionised water. The resulting antibiotic solution should contain the following concentrations: 8mgml^{-1} streptomycin; 800µgml^{-1} penicillin; 10µgml^{-1} chloramphenicol; and 10µgml^{-1} amphotericin B. Streptomycin, penicillin, chloramphenicol and amphotericin B are available from standard laboratory suppliers. It is also acceptable to use mixtures of antibiotics containing glutamine which are commercially available. Appropriate inhalation safety procedures should be followed when handling antibiotics.

5. PREPARATION OF SKIN AND MOUNTING ON *IN VITRO* APPARATUS

Animals are humanely killed by inhalation of a rising concentration of CO_2 followed by cervical dislocation. The dorsal skin of each animal is then removed and stripped of excess fat by carefully peeling it away from the skin by using the thumb and forefinger covered with paper towel. The pelt is placed over the end of a 10 mm diameter polytetrafluoroethylene (PTFE) tube ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place, and excess tissue is trimmed away with a scalpel blade. Tube and 'O ' ring dimensions are shown in Figure 3. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly (or soft paraffin wax), applied with a scalpel blade. The tube is supported by a spring ("Terry") clip inside a plastic receptor chamber containing 10ml of magnesium sulphate solution (154mM; see Figure 1). The PTFE tube is uniquely numbered with a label prior to test substance application.

Skin discs of approximately 0.79cm^2 can be obtained from any number of animals. However, the viability of each pelt must be assessed prior to use in the test by using the following method: two discs are taken from each pelt and prepared as described above. Electrical resistance measurements are then taken for each disc (see section 7). Both discs must produce resistance values of ≥10kΩ. The two discs are then discarded and the remainder of the pelt is used in the test. If both discs fall below the 10kΩ threshold, the pelt is discarded. If one disc falls below this threshold, another is tested; if this also falls below the threshold, the pelt is discarded. If the disc produces a TER measurement of ≥10kΩ, the pelt can be used in the test.

PTFE tubes and rubber 'O' rings are available from IMS, Dane Mill, Broadhurst Lane, Congleton, Cheshire CW12 1LA, UK.

6. TEST CHEMICAL APPLICATION AND REMOVAL

A measured volume of liquid test material (0.15ml) is applied to the inner epidermal surface (see Figure 1). When using solid test materials, a sufficient amount of solid material is applied to the surface of the disc ensuring that the whole surface of the epidermis is covered. Deionised or distilled water (0.15ml) is then added on top of the solid material and the tubes are shaken. Three skin discs are used for each time point per chemical. Test chemicals are applied for contact periods of 2 and 24 hours. After the required contact time, the test chemical is removed by washing with a jet of tap water, at room temperature, for approximately 10 seconds or until no further test material can be removed.

Control substances for the TER test and the dye binding assay:

Positive	-	36% HCI
Negative	-	DH_2O

All to be tested at the 24-hour contact period only.

Test substances should have maximum contact with the skin. For some solids this may be achieved by warming up to 30°C to melt the test substance, or by grinding to produce a granular material or powder.

Where measured test substance TER values are higher than the negative (water) control values (for example, waxy solids which may become liquids at approximately room temperature), the skin surface can be washed with water at up to 37°C. The skin should be visually inspected to determine if the skin is coated with test substance. The TER value should then be re-measured. If the value is less than or equal to the upper limit of the negative (water) control range, and if the skin disc appears to be free of residue, it can be accepted. If the TER value does not reduce to the upper limit of the negative control range after washing with the warm water, the disc should be rejected.

7. TRANSCUTANEOUS ELECTRICAL RESISTANCE MEASUREMENTS

The transcutaneous electrical resistance is measured using an AIM electronic databridge 401 or 6401 (available from H. Tinsley and Co., 275 King Henry's Drive, New Addington, Croydon, Surrey CR0 0AE, UK).

Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a small volume of 70% ethanol sufficient to cover the epidermis. After approximately 3 seconds, the ethanol is removed by inverting the tube. The PTFE tube is then replaced in the receptor chamber and the tissue is hydrated by the addition of 3ml of magnesium sulphate solution (154mM) to the inside of the PTFE tube; any air bubbles are dislodged by slight tapping.

The stainless steel electrodes of the databridge are then placed on either side of the skin disc to take the resistance measurement in kΩ/skin disc (see Figure 2). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 3. The inner (thick) electrode clip is rested on the top of the PTFE tube during resistance measurement, to ensure that a consistent length of electrode is submerged in the $MgSO_4$ solution. The outer (thin) electrode is positioned inside the receptor chamber, so that it rests on the bottom of the chamber. The distance between the bottom of the Terry clip and the bottom of the PTFE tube is set at 7.0 cm, to reduce the variability of resistance measurements between individual skin discs, which is influenced by the distance between the electrodes. The electrical resistance is then recorded from the databridge display.

If the reading falls above 20kΩ this may be due to the test material coating the epidermal surface of the skin disc. Removal of this coating can be performed by holding a gloved thumb over the end of the tube and shaking it for approximately 10 seconds; the $MgSO_4$ solution is then poured away. If any test material is present it may be seen as a residue in the $MgSO_4$ solution. The transcutaneous electrical resistance of the skin is then measured as described previously.

8. SULFORHODAMINE B DYE APPLICATION AND REMOVAL

If the electrical resistance values are ≤5kΩ at the 2- and/or 24-hour contact periods, an assessment of dye penetration is carried out on the 24-hour contact period tissues. If the skin disc was punctured during the jet washing procedure to remove the test chemical, then that particular tube is excluded from further testing.

150µl of a 10% (w/v) dilution of sulforhodamine B dye in DH_2O is applied to the epidermal surface of each skin disc for 2 hours. To remove any excess/unbound dye, the skin discs are then jet-washed with tap water at room temperature for approximately 10 seconds (or until the water runs clear). Each skin disc is carefully removed from the PTFE tube and placed in a 20ml scintillation vial containing 8ml of deionised water. The vials are agitated gently for 5 minutes to remove any further excess/unbound dye. This rinsing procedure is then repeated. Each skin disc is removed and placed into another 20ml scintillation vial containing 5ml of 30% (w/v) sodium dodecyl sulphate (SDS) in DH_2O and is incubated overnight at 60°C.

After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged in a 15ml centrifuge tube at 1000rpm for 8 minutes at 21°C (relative centrifugal force ≈ 175g). A 1ml sample of the supernatant is then placed into another 15ml centrifuge tube and diluted 1 in 5 (v/v) (i.e. 1ml + 4ml) with 30% (w/v) SDS in DH_2O. The optical density of the solution is determined at 565.5nm and the results are recorded.

Sulforhodamine B (90% dye content) and SDS are available from Sigma Chemical Company, Poole, UK.

9. FURTHER INFORMATION

Experience with the TER assay has shown that there are two critical stages. Experienced users pay particular attention to: a) skin disc preparation, ensuring removal of all fatty tissues and a complete seal of the skin on the PTFE tube; b) washing of the disc to remove as much of the test substance as possible. Residues of test substance remaining on the skin may affect the resistance values (for example, waxy substances, which solidify on the skin's surface). The positive controls TER values can drift with time (within days) if the samples are not fresh aliquoted from the stock acid maintained according to the storage recommendations on the label.

10. CALCULATION OF DYE CONTENT/DISC

The dye content, in µg/disc, is calculated from the optical density values as follows:

Sulforhodamine B dye molar extinction coefficient = 8.7×10^4,
Molecular Weight = 580,
No correction for the purity of the dye is made,
Optical density = 0.973,

$$\frac{0.973 \leftrightarrow 10^{-4}}{8.7} = 0.112 \leftrightarrow 10^{-4} = 11.2 \leftrightarrow 10^{-6} = 11.2 \mu M = 11.2 \mu\text{mol/l}$$

$$11.2 \leftrightarrow 580 \leftrightarrow 10^{-6} = 6496 \leftrightarrow 10^{-6}\ \textbf{\textit{g/l}} = 6.496 \leftrightarrow 10^{-3}\ \textbf{\textit{g/l}}$$

Dye was extracted into 5ml of solvent:

$$\frac{6.496 \times 10^{-3}}{200} = 0.325 \times 10^{-4}\ g/l = 32.5 \times 10^{-6}\ g/l$$

Solution was diluted 1 in 5 (v/v):

$$32.5 \times 10^{-6} \times 5 = 162.5 \times 10^{-6} = 162.5 \mu g/disc$$

The sulforhodamine B dye content is determined for each skin disc. A mean dye content is then calculated for the three skin discs at 24 hours. If a skin disc is punctured during the washing procedure used to remove the dye, then the individual dye content is recorded but it is excluded from the calculation of the mean.

All results are recorded on the data sheet shown in Appendix 1.

11. INTERPRETATION OF RESULTS

a) Results are accepted on condition of adherence to the ranges given below. If the positive and negative control results for the experiment do not fall within the accepted ranges, the data on the test substance cannot be interpreted and the experiment must be repeated.

Dye binding assay		TER assay	
36% HCl positive control range (µg/disc)	Distilled water negative control range (µg/disc)	36% HCl positive control range (kΩ)	Distilled water negative control range (kΩ)
40 - 100	15 – 35	0.5 - 1.0	10 - 25

b) If the transcutaneous electrical resistance readings obtained for all test substance contact periods are >5kΩ, then the substance is classified as non-corrosive.

c) If the transcutaneous electrical resistance readings are ≤5kΩ after a 2-hour contact period, and the substance is not a surfactant or neutral organic, then the substance is classified as corrosive (R35).

d) If the transcutaneous electrical resistance readings are ≤5kΩ after a 24-hour contact period (but >5kΩ after 2 hours contact), and the substance is not a surfactant or neutral organic, then the substance is classified as corrosive (R34).

e) If the transcutaneous electrical resistance readings are ≤5kΩ at either of the contact periods, and the substance is a surfactant or neutral organic, then the dye penetration results are considered.

f) If the mean disc dye content is ≥mean disc dye content of the 36% HCI positive control obtained concurrently in the experiment at the 24-hour contact period, then the substance is a true positive and is therefore classified as corrosive (R34).

g) If the mean disc dye content is < mean disc dye content of the 36% HCI positive control obtained concurrently in the experiment at the 24-hour contact period, then the substance is a false positive and is therefore classified as non-corrosive.

A flow diagram for interpretation of the results is attached.

Figure 1

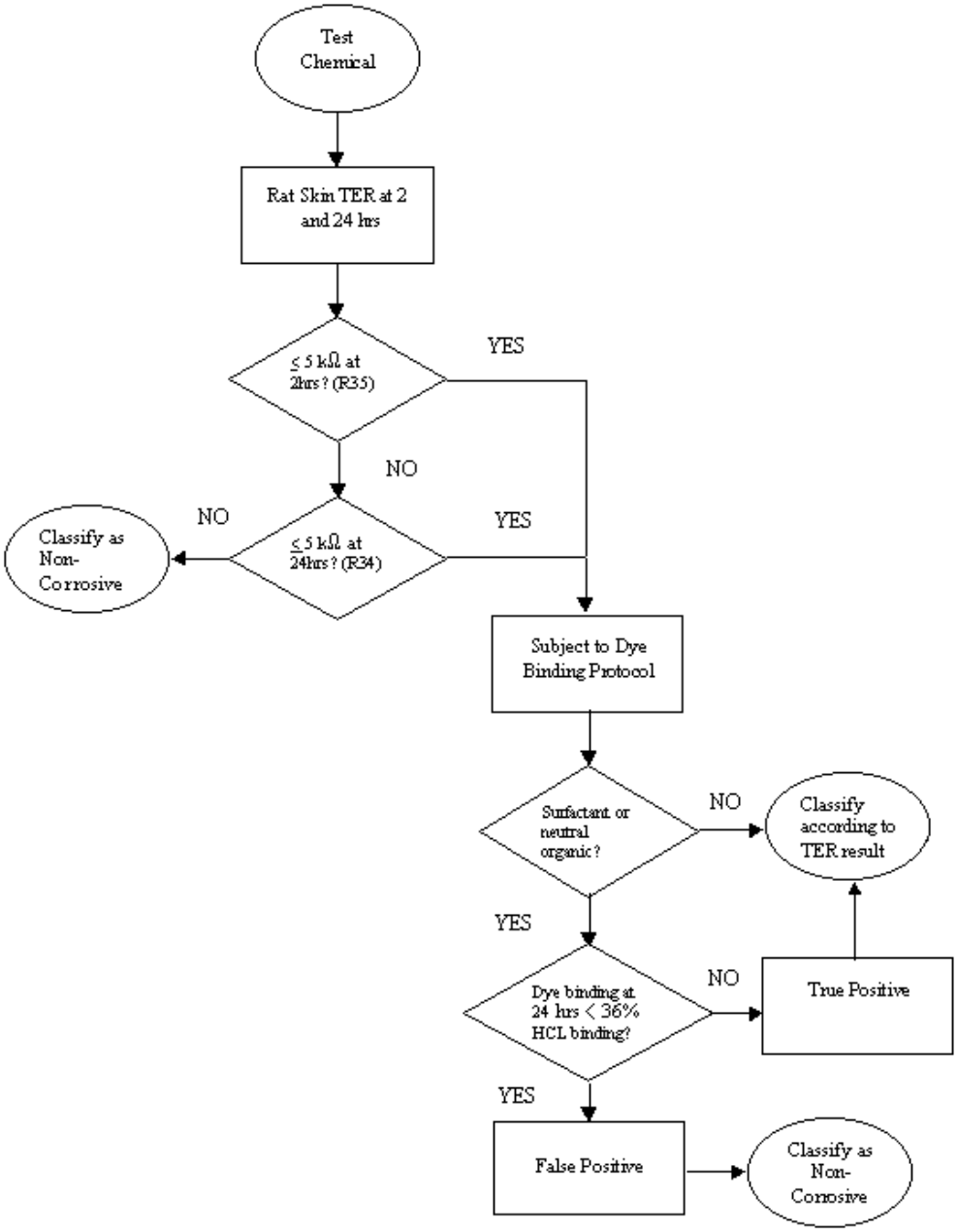

Test Chemical
Rat Skin TER at 2 and 24 hrs
≤ 5 kΩ at 2hrs? (R35)
YES
NO
≤ 5 kΩ at 24hrs? (R34)
NO
YES
Classify as Non-Corrosive
Subject to Dye Binding Protocol
Surfactant or neutral organic?
NO
Classify according to TER result
YES
Dye binding at 24 hrs < 36% HCL binding?
NO
True Positive
YES
False Positive
Classify as Non-Corrosive

Figure 2

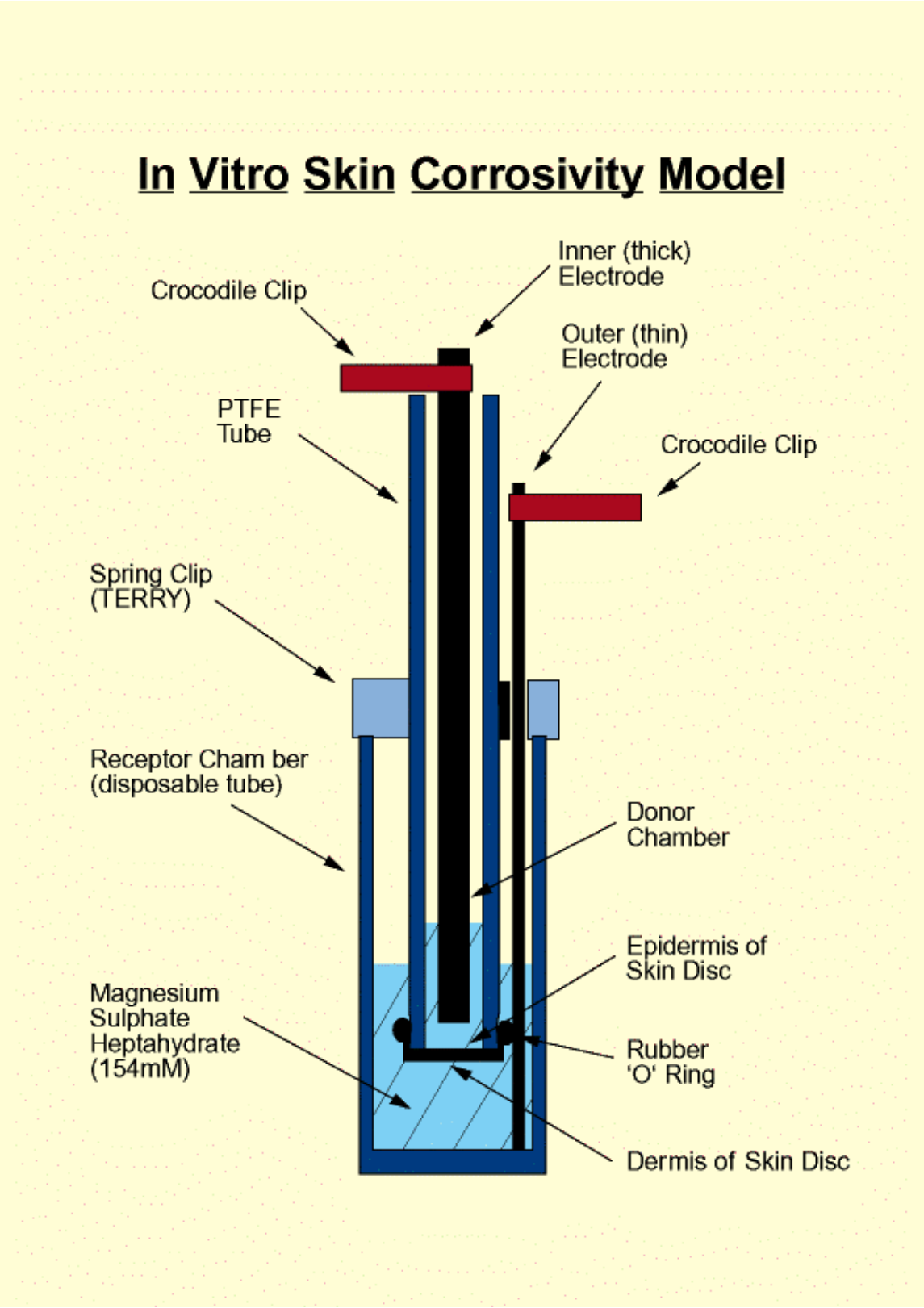
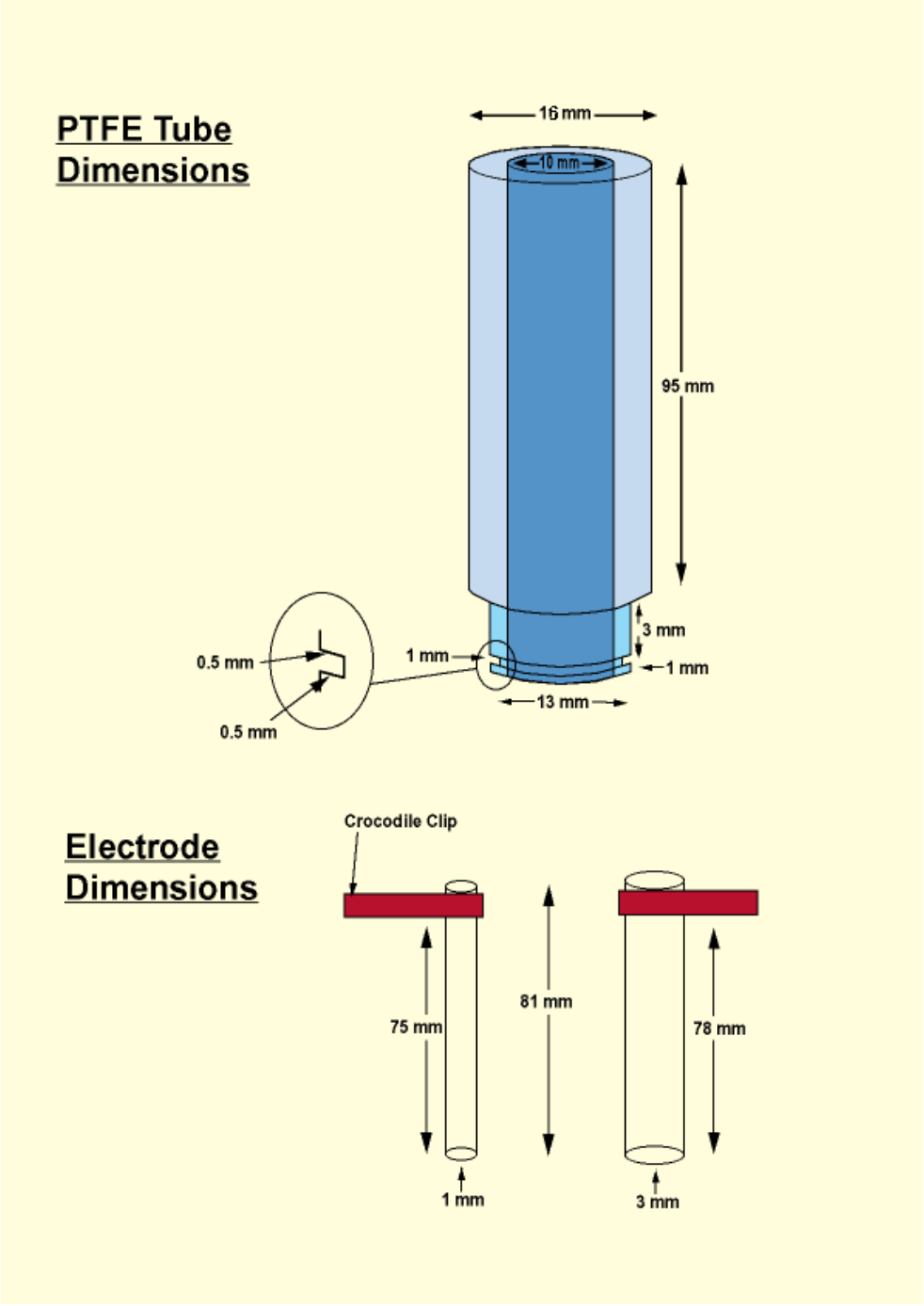

In Vitro Skin Corrosivity Model
Inner (thick) Electrode
Crocodile Clip
Outer (thin) Electrode
PTFE Tube
Crocodile Clip
Spring Clip (TERRY)
Receptor Cham ber (disposable tube)
Donor Chamber
Epidermis of Skin Disc
Magnesium Sulphate Heptahydrate (154mM)
Rubber 'O' Ring
Dermis of Skin Disc

Figure 3


PTFE Tube Dimensions
16 mm
10 mm
95 mm
3 mm
1 mm
13 mm
0.5 mm
1 mm
0.5 mm
Electrode Dimensions
Crocodile Clip
75 mm
81 mm
78 mm
1 mm
3 mm

APPENDIX 1: ECVAM Validation Study

Transcutaneous Electrical Resistance Test/Dye Binding - Results Summary Sheet

Laboratory Study Number: _____ Laboratory Name: _____ Experiment Number _____

Test Chemical and Appearance	Contact Time (hours)	Tissue Number	Electrical Resistance (kohms)	Mean TER ± S.D. (kohms)
	2			
	24			

Dye Contact Time (hours)	Optical Density at 956.5 nm (dissolved in 5)	Dye Concentration (µg/dcm)	Mean Dye Concentration ± S.D. (µg/dcm)	10% PCL Positive Control Mean Dye Concentration ± S.D. (µg/dcm)	Classification	
2					R5	PG I
					R24	PG I/II
					NC	NC

Signature: _____ Date: _____

Checked by: _____ Date: _____

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APPENDIX A

Federal Regulations and Guidelines for Corrosivity

A-1 Summary Table of Guidelines and Regulations for Dermal Corrosivity.....A-3

A-2 OPPTS 870.2500 Acute Dermal Irritation / U.S. EPA Health Effects Test Guidelines A-5

A-3 Chapter 8 (Precautionary Labeling) of U.S. EPA, Office of Pesticide Programs, Label Review Manual.....A-13

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GUIDELINES AND REGULATIONS FOR DERMAL CORROSIVITY

AGENCY OR ORGANIZATION	GUIDELINES AND REGULATIONS[1]	COMMENTS
Consumer Product Safety Commission (CPSC)	**16CFR1500** **§1500.3**-Definitions **§1500.4**-Human experience with hazardous substances **§1500.41**-Method of testing primary irritant substances	The method involves the application of the test substance on the hair-free intact and abraded skin of at least 6 albino rabbits. http://www.access.gpo.gov/nara/cfr/cfr-retrieve.html#page1
Occupational Safety and Health Administration (OSHA)	**29CFR1910** **§1910.1200** Definition of Corrosive and Hazard Communication (includes Appendix A)	OSHA accepts determination of dermal corrosivity by Corrositex® and other *in vitro* tests. States that OSHA does not require chemical manufacturers or importers to conduct animal tests for the evaluation of the hazard potential of chemical products.
US Department of Transportation (US DOT)	Exemption allowing use of Corrositex® as an alternative test method. **49CFR 173** **§173.136**(a)(1) Class 8 Definitions **§173.137**(a), (b), (c)(1) Class 8 Assignment of Packing Group **§173 Subpart J** – Appendix A to part 173 – Method of Testing Corrosion to Skin **49CFR172** **§172.442** Corrosive Label **§172.558** Corrosive Placard	Original exemption granted 28 April 1993. Current exemption expires 31 October 2002. §173.137 requires determination of the packing group based on data from tests conducted in accordance with 1992 OECD Guideline for Testing of Chemicals, No. 404, Acute Dermal Irritation/Corrosion (OECD Draft Document, March 2000).
US Environmental Protection Agency (EPA), Office of Solid Waste and Emergency Response	**62CFR32452** (13 June 1997) (final rule) affecting 40 CFR Parts 260, 264, 265, and 266. Hazardous Waste Management System; Testing and Monitoring Activities. Incorporates by reference update III of "Test Methods for Evaluating Solid Waste, Physical/Chemical Methods," EPA Publication SW-846, 3rd ed. SW-846 Method 9040 (**40CFR261.22**) determines corrosivity by the pH extremes (≤ 2 or ≥ 11.5).	State-of-the-art analytical technologies for RCRA-related testing include Method 1120, Dermal Corrosion, which describes the use of the Corrositex® test kit. http://www.epa.gov/epaoswer/hazwaste/test/pdfs/1120.pdf http://www.epa.gov/epaoswer/hazwaste/test/sw846.htm

EPA, Office of Pollution Prevention and Toxic Substances (OPPTS)	**OPPTS 870.2500** Acute Dermal Irritation (included as Appendix A-2)	EPA Health Effects Test Guidelines http://www.epa.gov/docs/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Drafts/
EPA, Office of Pesticides	**40CFR152** **§152.170** Criteria for restriction to use by certified applicators **40CFR156** **§156.10** Labeling requirements (skin corrosives are assigned toxicity category I) **40CFR157** §157 Subpart B - Child-Resistant Packaging (§157.22 states requirement for pesticides corrosive to the eyes or skin) **40CFR158** **§158.690** (acute dermal toxicity testing requirement for biochemical pesticides is waived if corrosive to skin or falls within the corrosive pH ranges)	
US Food and Drug Administration (US FDA)	**21CFR 70** **§70 Subpart C** - Safety Evaluation. §70.42(b) Criteria for evaluating the safety of color additives	Corrosivity not mentioned *per se*. Sensitization and primary irritation mentioned. No other formal regulations found with respect to corrosivity.
Organization for Economic Cooperation and Development (OECD)	Harmonized integrated hazard classification system for human health and environmental effects of chemical substances.	Provides a tiered testing strategy for the evaluation of dermal corrosivity. http://www.oecd.org/ehs/Class/HCL6.htm

[1]Unless otherwise specified in the comments column, guidelines may be accessed via the US Government Printing Office (GPO) Code of Federal Regulations database http://www.access.gpo.gov/nara/cfr/cfr-table-search.html.

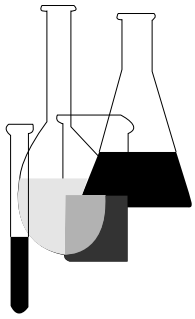
United States
Environmental Protection
Agency

Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–98–196
August 1998

Health Effects Test Guidelines

OPPTS 870.2500
Acute Dermal Irritation



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512–0132. This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (http://www.epa.gov/epahome/research.htm) under the heading ''Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines.''

OPPTS 870.2500 Acute dermal irritation.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline are 40 CFR 798.4470 Primary Dermal Irritation; OPP 81–5 Primary Dermal Irritation (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation; Human and Domestic Animals) EPA report 540/09–82–025, 1982; and OECD 404 Acute Dermal Irritation/ Corrosion .

(b) **Purpose.** Determination of the irritant and/or corrosive effects on skin of mammals is useful in the assessment and evaluation of the toxic characteristics of a substance where exposure by the dermal route is likely. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance. Data on primary dermal irritation are required by 40 CFR part 158 to support the registration of each manufacturing-use product and end-use product. See specifically §§158.50 and 158.340 to determine whether these data must be submitted.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

Dermal corrosion is the production of irreversible tissue damage in the skin following the application of the test substance.

Dermal irritation is the production of reversible inflammatory changes in the skin following the application of a test substance.

Pharmacological effect means any chemically induced physiological changes in the test animal.

Target organ means any organ of a test animal showing evidence of an effect of chemical treatment.

(d) **Principle of the test methods.** (1) The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control (except when severe irritation/corrosion is suspected and the stepwise procedure is used (see paragraph (f)(1)(iii) of this guideline)). The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 14 days.

(2) When testing solids (which may be pulverized if considered necessary), the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

(e) **Initial considerations.** (1) Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less, or 11.5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties.

(2) It is unnecessary to test materials which have been shown to be highly toxic (LD_{50} less than 200 mg/kg) by the dermal route or have been shown not to produce irritation of the skin at the limit test dose level of 2000 mg/kg body weight.

(3) It may not be necessary to test *in vivo* materials for which corrosive properties are predicted on the basis of results from well validated and accepted *in vitro* tests. If an *in vitro* test is performed before the *in vivo* test, a description or reference to the test, including details of the procedure, must be given together with results obtained with the test and reference substances.

(4) It may not be necessary to test materials for which corrosive potential is predicted from structure-activity relationships.

(f) **Test procedures—**(1) **Animal selection—**(i) **Species and strain.** The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

(ii) **Number of animals.** At least three healthy adult animals (either sex) should be used unless justification/reasoning for using fewer animals is provided. It is recommended that a stepwise procedure be used to expose one animal, followed by additional animals to clarify equivocal responses.

(iii) **Stepwise exposure of animals**. A single rabbit may be used if it is suspected that the test material might produce severe irritation/ corrosion. Three test patches are applied concurrently or sequentially to the animal. The first patch is removed after 3 min. If no serious skin reaction is observed, the second patch is removed after 1 hour. If observations indicate that exposure can be continued humanely, the third patch is removed after 4 hours and the responses graded. If a corrosive effect is observed after either 3 min or 1 hour of exposure, the test is immediately terminated by removal of the remaining patches. If a corrosive effect is observed after an exposure of up to 4 hours, then further animal testing is not required. If no corrosive effect is observed in one animal after a 4–hour exposure, the test is completed using two additional animals, each with one patch

only, for an exposure period of 4 hours. If it is expected that the test substance will not produce severe irritancy or corrosion, the test may be started using three animals, each receiving one patch for an exposure period of 4 hours.

(2) **Control animals.** Separate animals are not recommended for an untreated control group. Adjacent areas of untreated skin of each animal may serve as a control for the test.

(3) **Dose level.** A dose of 0.5 mL of liquid or 500 mg of solid or semisolid is applied to the test site.

(4) **Preparation of test area.** Approximately 24 h before the test, fur should be removed from the test area by clipping or shaving from the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

(5) **Application of the test substance.** (i) The recommended exposure duration is normally 4 hours unless corrosion is observed (see paragraph (f)(1)(iii) of this guideline). Longer exposure may be indicated under certain conditions (e.g. expected pattern of human use and exposure). At the end of the exposure period, residual test substance should generally be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

(ii) When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should by taken into account. If a vehicle is used, it should not alter the absorption, distribution, metabolism, retention or the chemical properties of the test substance nor should it enhance, reduce, or alter its toxic characteristics. Although water or saline is the preferred agent to be used for moistening dry test materials, other agents may be used providing the use is justified. Acceptable alternatives include: gum arabic, ethanol and water, carboxymethyl cellulose, polyethylene glycol, glycerol, vegetable oil, and mineral oil.

(iii) The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with nonirritating tape. In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semiocclusive dressing for the duration of the exposure period. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

(6) **Observation period.** The duration of the observation period need not be rigidly fixed. It should be sufficient to fully evaluate the reversibility or irreversibility of the effects observed. It need not exceed 14 days after application.

(7) **Clinical examination and scoring.** (i) After removal of the patch, animals should be examined for signs of erythema and edema and the responses scored within 30–60 min, and at 24, 48, and 72 hours after patch removal.

(ii) Dermal irritation should be scored and recorded according to the grades in the following Table. 1. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any lesions and other toxic effects should be fully described.

Table 1.—Evaluation of Skin Reaction

	Value
Erythema and Eschar Formation:	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible	4
Edema Formation:	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure	4
Maximum possible	4

(g) **Data and reporting**—(1) **Data summary.** Data should be summarized in tabular form, showing for each individual animal the irritation scores for erythema and edema at 30 to 60 min, and 24, 48, and 72 hours after patch removal, any other dermal lesions, a description of the degree and nature of irritation, corrosion and reversibility, and any other toxic effects observed.

(2) **Evaluation of results.** The dermal irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed as reference values which are only meaningful when supported by a full description and evaluation of the observations.

(3) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

(i) Species, strain, sex, age and source of test animal.

(ii) Rationale for selection of species (if species is other than the species preferred or required by OPP's toxicology data requirements for pesticide registration).

(iii) Tabulation of erythema and edema data and any other dermal lesions/responses for each individual animal at each observation time point (e.g. 30–60 minutes and 24, 48, and 72 hours until end of test/reversibility).

(iv) Description of any lesions observed.

(v) Narrative description of the degree and nature of irritation or corrosion observed.

(vi) Description of any systemic effects observed.

(vii) Description of any pre-test conditioning, including diet, quarantine and treatment of disease.

(viii) Description of caging conditions including number (and any change in number) of animals per cage, bedding material, ambient temperature and humidity, photoperiod, and identification of diet of test animal.

(ix) Manufacturer, source, purity, and lot number of test substance.

(x) Physical nature, and, where appropriate, concentration and pH value for the test substance.

(xi) Identification and composition of any vehicles (e.g., diluents, suspending agents, and emulsifiers) or other materials used in administering the test substance.

(xii) A list of references cited in the body of the report, i.e., references to any published literature used in developing the test protocol, performing the testing, making and interpreting observations, and compiling and evaluating the results.

(h) **References.** The following references should be consulted for additional background information on this test guideline.

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(3) Dixon, W.J. ed. Biomedical Computer Programs (BMD). 2nd edition, University of California Press: Los Angeles (1970).

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(5) Draize, J.H. et al. Methods for the Study of Irritation and Toxicity of Substances Applied Topically to the Skin and Mucous Membranes, *Journal of Pharmacology and Experimental Therapeutics.* 83:377–390 (1944).

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(7) Galen, R.S. and S.R. Gambino. Beyond Normality (The Predictive Value and Efficiency of Medical Diagnosis). Wiley, New York (1975).

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(9) Marzulli, F.N. and Maibach, H.I. Dermatotoxicology and Pharmacology, *Advances in Modern Toxicology.* Vol. 4. (New York: Hemisphere Publishing Corp., 1977).

(10) National Academy of Sciences. Principles and Procedures for Evaluating the Toxicity of Household Substances. A report prepared by the Committee for the Revision of NAS Publication 1138, Under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC, (1978).

(12) U.S. EPA, Atlas of Dermal Lesions, Office of Pesticides and Toxic Substances, Report 20T–2004, August 1990.

(13) World Health Organization. Part I. Environmental Health Criteria 6, *Principles and Methods for Evaluating the Toxicity of Chemicals.* Geneva, World Health Organization (1978).

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United States Environmental Protection Agency

Office of Pesticide Programs

Label Review Manual
Chapter 8: Precautionary Labeling

Introduction

Precautionary labeling provides the pesticide user with information regarding the potential toxicity, irritation and sensitization hazard associated with the use of a pesticide. The precautionary labeling also identifies the precautions necessary to avoid exposure, any personal protective equipment (PPE) which should be used when handling a pesticide and first aid in case of accidental exposure.

This chapter is organized into the following five major parts:

- Background Information
- Determination of Products Subject to the Worker Protection Standard
- Precautionary Labeling
- First Aid (Statements of Practical Treatment)
- Optional Labeling and Deviations

Background Information

1. ACUTE TOXICITY DATA: The precautionary labeling which includes the signal word, personal protective equipment and first aid statements is normally determined by six acute toxicity studies and product composition. The acute oral, acute dermal and acute inhalation studies measure the lethality of a product via the designated route of exposure. The primary eye irritation and primary skin irritation studies measure the severity of irritation or corrosivity caused by a product. The dermal sensitization study determines whether a product is capable of causing an allergic reaction. With the exception of the dermal sensitization study each acute toxicity study is assigned a toxicity category (See Table 1 below).

Table 1 - Toxicity Categories

Study	Category I	Category II	Category III	Category IV
Acute Oral	Up to and including 50 mg/kg	> 50 thru 500 mg/kg	> 500 thru 5000 mg/kg	> 5000 mg/kg
Acute Dermal	Up to and including 200 mg/kg	> 200 thru 2000 mg/kg	> 2000 thru 5000 mg/kg	> 5000 mg/kg
Acute Inhalation[1]	Up to and including 0.05 mg/liter	> 0.05 thru 0.5 mg/liter	> 0.5 thru 2 mg/liter	> 2 mg/liter
Eye Irritation	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days	Corneal involvement or irritation clearing in 8-21 days	Corneal involvement or irritation clearing in 7 days or less	Minimal effects clearing in less than 24 hours
Skin Irritation	Corrosive (tissue destruction into the dermis and/or scarring)	Severe irritation at 72 hours (severe erythema or edema)	Moderate irritation at 72 hours (moderate erythema)	Mild or slight irritation (no irritation or slight erythema)

[1)] 4 hr exposure

2. GUIDANCE DOCUMENTS USED TO DETERMINE LABELING: The Code of Federal Regulations specifies both acute toxicity categories (40 CFR 156.10(h)(1)(i)) and precautionary labeling statements associated with each toxicity category (40 CFR 156.10(h)(2)). These acute toxicity categories and precautionary labeling statements are not currently being used by the Agency as they are less detailed and provide less protection for pesticide users than other guidance. The 40 CFR 156.10(h)(2)(i) states that precautionary labeling statements listed therein can be modified or expanded to reflect specific hazards. The precautionary labeling provided in the Federal Register notice issued on 9/26/84 entitled Proposed Rule on Labeling Requirements (Volume 49, Number 188) is being used because it is more detailed and provides better protection. The acute toxicity categories listed in the proposed rule are also being used with one exception. The acute inhalation toxicity categories currently used are from a 2/l/94 Health Effects Division paper entitled "Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies. The Worker Protection Standard issued in 1992 is the major guidance document for labeling of agricultural products. That document is also being used to determine type of respiratory protective equipment for products which are not subject to the WPS.

Determination of Products Subject to the Worker Protection Standard (WPS)

Review this section to determine whether the label under review involves a product, which is subject to the WPS. WPS does not apply to manufacturing use products, or to unregistered pesticides used under an experimental use permit issued under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) section 5 or under an exemption issued under FIFRA section 18. This determination is important when reviewing the following sections of this manual because the personal protective equipment for WPS products is more specific and there are some additional labeling requirements.

- Does the product bear directions for use involving the production of an agricultural plant on a farm, forest, nursery, or greenhouse or does the product bear labeling which could reasonably permit such a use?

  - **NO:** The product is not subject to the WPS. Go to the next section on Precautionary Labeling.

  - **YES:** Does the product meet any of the exceptions listed below?

EXCEPTIONS: Does the product bear directions solely for any of the following uses?

- For mosquito abatement, Mediterranean fruit fly eradication, or similar wide-area public pest control programs sponsored by governmental entities.

- On livestock or other animals, or in or about animal premises.

- On plants grown for other than commercial or research purposes, which may include plants in habitations, home fruit and vegetable gardens, and home greenhouses.

- On plants that are in ornamental gardens, parks, golf courses, and public or private lawns and grounds and that are intended only for aesthetic purposes or climatic modification.

- In a manner not directly related to the production of agricultural plants, including, but not limited to, structural pest control, control of vegetation along rights-of-way and in other non-crop areas, and pasture and rangeland use.

- For control of vertebrate pests.

- As attractants or repellents in traps.
- For research uses of unregistered pesticides.

- **NO**: The product IS subject to the WPS.
- **YES**: The product is NOT subject to the WPS.

Please Remember:

In some cases it is not clear whether or not a product is "in-scope" of the WPS. If the intention is to remove the product from the scope of the WPS, language should be used that limits where this product can be applied, rather than who may apply it. This can be done by adding one of the following exclusionary statements. For example:

> "Not for use on turf being grown for sale or other commercial use as sod, or for commercial seed production, or for research purposes."
> or
> "For use only on home lawns."

For further details, see PR Notice 93-11, Supplement F.

Label Reviewer: Be aware, that PR Notice 93-11, Supplement G provides guidance on splitting the product label to allow the non-agricultural uses to be labeled separately and avoid WPS requirements.

Precautionary Labeling

If toxicity categories are known: Use the toxicity categories to determine the appropriate labeling identified in the following sections of this chapter.

If toxicity categories are not known, as in the case of many me-too submissions, the label review will essentially involve a comparison of the draft label against the cited label. **Cited labels often contain errors themselves. To ensure that these errors are not passed on to the Me-too label, review the following sections of this chapter to verify that the draft and cited labels are correct.**

1.SIGNAL WORD: Review the following to determine the correct signal word and its location:

A. Correct Signal Word: The signal word is determined by the most severe toxicity category assigned to the five acute toxicity studies or by the presence of special inerts (methanol in concentrations of 4% or more). Refer to the acute toxicity data review to determine the most severe toxicity category. Also check the confidential statement of formula to determine if methanol is present. If acute toxicity categories are not known, the signal word on the label under review must be identical to the signal word on the cited product. Signal words are as follows:

> Toxicity Category I - DANGER
> Toxicity Category II - WARNING
> Toxicity Categories III & IV - CAUTION

B. Required Location: The signal word may only appear in three places on the label. It must appear on the front panel of the label immediately below the child hazard warning statement, in the precautionary labeling section immediately below the subheading "Hazards to Humans and Domestic Animals" and in the WPS posting statement if one is on the label.

C. Other Requirements: Make sure that the label text does not contain the terms "caution," "warning" or "danger" except as the signal word for that label. (e.g., "CAUTION: Wash hands before eating, or smoking" on a "WARNING" label). Another example is the statement required by California's Proposition 65 which normally requires the term "warning." Registrants should use the term "notice" or "attention" instead so that it does not conflict with the EPA required signal word. Make sure that the signal word does not appear on the same line with the child hazard warning and that the signal word runs parallel with other label text. It is preferred that the signal word appears in all capital letters.

D. WPS Products: Products subject to the WPS which are classified as toxicity category I or II must also bear the corresponding Spanish signal word and the Spanish statement provided below. The Spanish signal word and the statement below must appear in close proximity to the English signal word. The Spanish signal word for toxicity category I is "PELIGRO" and the Spanish signal word for toxicity category II is "AVISO." The statement which must appear on toxicity category I and II WPS products is as follows:

"Si usted no entiende la etiqueta, busque a alguien para que se la explique a usted en detalle. (If you do not understand the label, find someone to explain it to you in detail.)"

2. POISON - SKULL & CROSSBONES DETERMINATION: The word "POISON" and the skull and crossbones symbol are required whenever a product is classified as toxicity category I due to the results of either the acute oral, acute dermal, or acute inhalation toxicity studies (40 CFR 156.10(h)(1)(i)) or if the inert (Methanol 4% or more) are present in the product [1984 proposed labeling rule, Part 156.50].

A. Required Location: The word "Poison" and the skull and crossbones symbol must appear in immediate proximity to the signal word which must be "DANGER."

B. Display Requirements: "Poison" must appear in red on a contrasting background. If the proposed label does not indicate these display requirements, include this requirement in your response to the registrant.

Table 2 - Acute Toxicity Category Determination for Sample Products

Type of Study	Product A	Product B	Product C	Product D	Product E
Acute Oral	III	IV	I	III	II
Acute Dermal	IV	III	III	IV	II
Acute Inhalation	III	IV	III	III	II
Primary Eye	III	II	I	I	II
Primary Skin	IV	IV II	IV	II	
Special Inert	No	No	No	No	Yes
Correct Signal Word	CAUTION	WARNING	DANGER*	DANGER	DANGER*

*Product C and Product E must also bear additional labeling (Skull & Crossbones symbol in close proximity to the word "POISON" which must appear in red on a contrasting background). Product C must bear the additional labeling as a result of the toxicity category I classification for the acute oral toxicity study. Product E must bear

the additional labeling because it contains a special inert (Methanol) which is described above in the first paragraph.

3. CHILD HAZARD WARNING STATEMENT: The phrase "Keep Out Of Reach Of Children" is required on almost all product labels. The child hazard warning statement may be completely omitted for manufacturing use products. A modified child hazard warning statement may be used for products where child contact is expected. For products requiring a modified statement, make sure that the statement is appropriate for the use pattern. Examples of appropriate statements are as follows: "Do not allow children to apply product" or "Do not allow children to play with pet collar" (1984 proposed labeling rule, 156.46).

   A. Required Location: The child hazard warning statement must appear on the front panel (40 CFR 156.10(h)(1)(ii)).

   B. Other Requirements: It is also preferred that the child hazard warning appears on a separate line above the signal word. The CFR does not include this specific requirement. However, it is included in the 1984 proposed labeling rule. When the signal word and child hazard warning appear on the same line, a pesticide user may assume that the signal word is intended more so for children rather than as a general precaution for all persons. If the label under review has the signal word and child hazard warning on the same line, instruct the registrant to revise the label. Also make sure that the child hazard warning statement runs parallel with other label text.

      Make sure that the "Precautionary Statements" and the "Directions for Use" do not contain any statements which imply that the product may be used by children. For example, draft labels of products intended to repel insects may contain instructions such as "Do not allow use by small children without close adult supervision." Such labeling is unacceptable as it implies that a child can apply the product as long as an adult watches. Such a statement conflicts with the child hazard warning statement. Pesticide products should not be applied by children because they may be incapable of reading and correctly following the directions for use.

4. PRECAUTIONARY STATEMENTS: Precautionary statements are required for each acute toxicity study classified as toxicity category I, II, or III and for products found to be dermal sensitizers.

   A. Required Header: The precautionary statements must appear under the heading "Precautionary Statements" and the appropriate subheading "Hazard to Humans and Domestic Animals." The phrase ". . . and Domestic Animals" may be left off if it is inappropriate or list separately from Hazard to Humans. The signal word must appear after the subheading.

   B. Required Location: The precautionary statement section may appear on any panel. Please note that the precautionary statements must not be included within the "Directions For Use" section. With the exception of PPE for early reentry, all PPE must be located in the precautionary labeling section. Order of Statements: The precautionary statements should be organized so that the most severe routes of exposure as demonstrated by the toxicity category classification are listed first.

   C. Fumigants: Refer to PR Notice 84-5 (Reference: PR-84-05) and Registration Standards and/or Reregistration Eligibility Documents (REDs) for precautionary statements.

   D. Determining Statements For All Other Products: Select precautionary statements from the tables below based on the toxicity category assigned to each study. In cases where the toxicity categories are not known, the precautionary labeling for at least one route of exposure must be consistent with the signal word. Sentences from the various tables may be combined to form a

concise paragraph containing the precautionary labeling statements. Repetitious sentences should be omitted.
(1) Products Not In Scope of WPS: Use the precautionary statements and PPE contained in this section, Tables 3 through 8 and then go to the section entitled First Aid (Statements of Practical Treatment).
(2) Products In Scope of WPS: Use the precautionary statements in this section, Tables 3 through 8. Disregard the PPE contained in the Tables 3 through 8. Refer to Sections 5 through 8 to determine the PPE for WPS products.

Table 3 - Acute Oral Toxicity Study*

Toxicity Category	Signal Word	Precautionary Statements and Personal Protective Equipment
I	DANGER Skull & Crossbones required	Fatal if swallowed. Wash thoroughly with soap and water after handling and before eating, drinking, or using tobacco.
II	WARNING	May be fatal if swallowed. Wash thoroughly with soap and water after handling and before eating, drinking or using tobacco.
III	CAUTION	Harmful if swallowed. Wash thoroughly with soap and water after handling.
IV	CAUTION	No statements are required. However, if the registrant chooses to use category III labeling that is acceptable.

*Products Containing 4% or more of Methanol: Add the following to the precautionary statements: "Methanol may cause blindness."

Table 4 - Acute Dermal Toxicity Study

Toxicity Category	Signal Word	Precautionary Statements and Personal Protective Equipment
I	DANGER Skull & Crossbones required	Fatal if absorbed through skin. Do not get in eyes, on skin, or on clothing. Wear protective clothing and gloves (specify protective clothing and type of gloves). Wash thoroughly with soap and water after handling and before eating, drinking, or using tobacco. Remove contaminated clothing and wash before reuse.
II	WARNING	May be fatal if absorbed through skin. Do not get in eyes, on skin, or on clothing. Wear protective clothing and gloves (specify protective clothing and type of gloves). Wash thoroughly with soap and water after handling and before eating, drinking or using tobacco. Remove contaminated clothing and wash clothing before reuse.
III	CAUTION	Harmful if absorbed through skin. Avoid contact with skin, eyes or clothing. Wash thoroughly with soap and water after handling.
IV	CAUTION	No statements are required. However, if the registrant chooses to use category III labeling that is acceptable.

Table 5 - Acute Inhalation Toxicity Study

Toxicity Category	Signal Word	Precautionary Statements and Personal Protective Equipment
I	DANGER Skull & Crossbones required	Fatal if inhaled. Do not breathe (dust, vapor, or spray mist).* [Identify specific respiratory protective device approved by the Mine Safety and Health Administration and the National Institute for Occupational Safety and Health.]** Remove contaminated clothing and wash clothing before reuse.
II	WARNING	May be fatal if inhaled. Do not breathe (dust, vapor or spray mist).* Wear a mask or pesticide respirator jointly approved by the Mine Safety and Health Administration and the National Institute for Occupational Safety and Health. Remove contaminated clothing and wash clothing before reuse.
III	CAUTION	Harmful if inhaled. Avoid breathing (dust, vapor or spray mist).* Remove contaminated clothing and wash clothing before reuse.
IV	CAUTION	No statements are required. However, if the registrant chooses to use category III labeling that is acceptable.

* Choose the word which appropriately describes the product during use. **Refer to Section to determine the specific respiratory protective device. This section can be used for both WPS and Non-WPS products.

Table 6 - Primary Eye Irritation Study

Toxicity Category	Signal Word	Precautionary Statements and Personal Protective Equipment
I	DANGER	Corrosive.* Causes irreversible eye damage. Do not get in eyes or on clothing. Wear protective eyewear (goggles, face shield, or safety glasses).** Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash clothing before reuse.
II	WARNING	Causes substantial but temporary eye injury. Do not get in eyes or on clothing. Wear protective eyewear (goggles, face shield, or safety glasses).** Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash clothing before reuse.
III	CAUTION	Causes moderate eye irritation. Avoid contact with eyes or clothing. Wash thoroughly with soap and water after handling.
IV	CAUTION	No statements are required. However, if the registrant chooses to use category III labeling that is acceptable.

*The term "corrosive" is not required if only eye irritation (redness) was observed during the study and was still present at day 21.

**Use the term "safety glasses" in the precautionary labeling for residential use products.

Table 7 - Primary Skin Irritation Study

Toxicity Category	Signal Word	Precautionary Statements and Personal Protective Equipment
I	DANGER	Corrosive. Causes skin burns. Do not get in eyes or on clothing. Wear protective clothing and gloves (specify protective clothing and type of gloves)*. Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash clothing before reuse.
II	WARNING	Causes skin irritation. Do not get on skin or on clothing. Wash thoroughly with soap and water after handling. Remove contaminated clothing and wash clothing before reuse.
III	CAUTION	Avoid contact with skin or clothing. Wash thoroughly with soap and water after handling.
IV	CAUTION	No statements are required. However, if the registrant chooses to use category III labeling that is acceptable.

*The need for rubber (homeowner products) or chemical resistant gloves must be determined on an individual basis. Some products cause blistering if confined under clothing.

Table 8 - Dermal Sensitization Study

Study Results	Precautionary Statement
Product is a sensitizer or is positive for sensitization.	Prolonged or frequently repeated skin contact may cause allergic reactions in some individuals.
Product is not a sensitive or is negative for sensitization.	No labeling is required for this hazard.

5. WPS PERSONAL PROTECTIVE EQUIPMENT (PPE) REQUIREMENTS: Personal protective equipment is required for both pesticide handlers as well as workers who reenter treated areas prior to the expiration of the restricted entry interval (REI).

   A. Determining Toxicity Categories for Each Route of Exposure: If all acute toxicity categories are known, skip to Section B. If any acute toxicity categories are unknown, review this section to determine the preferredorder for selecting alternate data to establish a toxicity category for the missing data:
      (1) If available, use the toxicity categories assigned to the acute dermal toxicity, acute inhalation toxicity, primary eye irritation, and primary skin irritation data on the end-use product.
      (2) If either the acute dermal toxicity or acute inhalation toxicity data are missing, use the toxicity category assigned to the acute oral toxicity data.
      (3) If the acute oral, acute dermal and acute inhalation toxicity data are missing, use the product signal word to determine the equivalent toxicity category.

   B. WPS Products: Use the toxicity categories to determine from Table 9 whether the label under review contains the appropriate PPE.

      Statements for Contaminated Personal Protective Equipment

      Use the following statement if the signal word is "Danger" or "Warning" and the product is a concentrate (diluted before use, or is an ultra-low-volume or low volume concentrate, or contains more than 50% active ingredient):

      > "Discard clothing and other absorbent materials that have been drenched or heavily contaminated with this product's concentrate. Do not reuse them."

Table 9 - Personal Protective Equipment for WPS Products

	Toxicity Category by Route of Exposure of End-Use Product[1]			
Route of Exposure	I (DANGER)	II (WARNING)	III (CAUTION)	IV (CAUTION)
Dermal Toxicity or Skin Irritation Potential[1]	Coveralls worn over long-sleeved shirt and long pants Socks Chemical-resistant footwear Gloves[3]	Coveralls worn over short-sleeved shirt and short pant Socks Chemical-resistant footwear Gloves[3]	Long-sleeved shirt and long pants Socks Shoes Gloves[3]	Long-sleeved shirt and long pant Socks Shoes No minimum[5]

	Toxicity Category by Route of Exposure of End-Use Product[1]			
Inhalation Toxicity	Respiratory protection device[4]	Respiratory protection device[4]	No minimum[5]	No minimum[5]
Eye Irritation Potential	Protective eyewear	Protective eyewear	No minimum[5]	No minimum[5]

[1]Refer to section 6 for PPE for product using Water Soluble Packaging.
[2]If dermal toxicity and skin irritation toxicity categories are different, PPE shall be determined by the more severe toxicity category of the two. If dermal toxicity or skin irritation is category I or II, refer to Section C below to determine if additional PPE is required beyond that specified in Table 9.
[3]Refer to Section 7 to determine the specific type of chemical-resistant glove.
[4]Refer to Section 8 to determine the specific type of respiratory protection.
[5]Although no minimum PPE is required for these toxicity categories and routes of exposure, the Agency may require PPE on a product-specific basis.

C. PPE for Dermal Protection: Additional PPE is required for products which are classified as toxicity category I or II for acute dermal toxicity or skin irritation. If the label under review does not involve a category I or II classification for either of these studies, skip this section and go to Section 6. If the label under review does involve a category I or II classification for either the acute dermal toxicity or skin irritation, review the following table to determine the additional PPE which must appear on the label under review.

Table 10 - Additional Dermal toxicity and/or Skin Irritation PPE

Conditions Requiring Additional PPE and Labeling	Required PPE and Labeling
All products which are not ready to use and do not require a chemical resistant suit must bear the corresponding statement:	"When mixing and loading wear a chemical resistant apron."
All products having applications which might involve overhead exposure must bear the corresponding statement:	"For overhead exposure wear chemical-resistant headgear."
All products involving use of equipment other than the product container to mix, load or apply the product must bear the corresponding statement:	"When cleaning equipment add a chemical-resistant apron."

6. ENGINEERING CONTROL SYSTEMS SECTION: If the product in the water soluble bag is subject to the Worker Protection Standard, then the following language should be placed in the engineering control section using the placement in PR Notice 93-7.

A. This label language is to be used for end-use products classified as category I or II for either acute dermal toxicity OR skin irritation potential. (NOTE: If either of these data is not available, use the end-use product signal word as a surrogate. Signal words DANGER and WARNING indicate toxicity category I and II respectively.)

"When handlers use closed systems, enclosed cabs, or aircraft in a manner that meets the requirements listed in the Worker Protection Standard (WPS) for agricultural pesticides (40 CFR 170.240(d)(4-6), the handler PPE requirements may be reduced or modified as specified in the WPS."

"Water-soluble packets when used correctly qualify as a closed loading system under the WPS. Handlers handling this product while it is enclosed in intact water-soluble packets may elect to wear reduced PPE of long-sleeved shirt, long pants, shoes, socks, a chemical-resistant apron, and chemical-resistant gloves."

"IMPORTANT: When reduced PPE is worn because a closed system is being used, handlers must be provided all PPE specified above for "applicators and other handlers" and have such PPE immediately available for use in an emergency, such as a spill or equipment break-down."

B. This label language is to be used for end-use products classified as category III or IV for acute dermal toxicity AND skin irritation potential. (NOTE: if either of these data is not available, use the end-use product signal word as a surrogate. Signal word CAUTION indicates toxicity category III or IV.)

"When handlers use closed systems, enclosed cabs, or aircraft in a manner that meets the requirements listed in the Worker Protection Standard (WPS) for agricultural pesticides (40 CFR 170.240(d)(4-6), the handler PPE requirements may be reduced or modified as specified in the WPS."

"Water-soluble packets when used correctly qualify as a closed loading system under the WPS. Handlers handling this product while it is enclosed in intact water-soluble packets may elect to wear reduced PPE of long-sleeved shirt, long pants, shoes, and socks."

"IMPORTANT: When reduced PPE is worn because a closed system is being used, handlers must be provided all PPE specified above for "applicators and other handlers" and have such PPE immediately available for use in an emergency, such as a spill or equipment break-down."

7. CHEMICAL RESISTANT GLOVE SELECTION FOR HANDLERS: Chemical resistant gloves are required for all WPS products classified as toxicity category I, II, or III for acute dermal toxicity or primary skin irritation. Review the types of chemical resistant gloves below, and determine if the label lists the appropriate glove type based on the product formulation. Please note that the registrant can specify another chemical resistant glove type other than those specified below if information is available that indicates that another glove type provides greater protection. If the label bears another chemical resistant glove type and the registrant has indicated that it is more protective based on available information, allow that glove type to remain on the label. If the label bears another chemical resistant glove type than those listed below and the registrant has not indicated that it is more protective based on available information, request that the registrant verify that the appropriate chemical resistant glove type is on the label. The label must indicate the specific type of chemical resistant glove (such as nitrile, butyl, neoprene, and/or barrier laminate). See the solvent list in PR Notice 93-7, pp. 13-15. For those solvents not listed contact the Chemistry and Exposure Branches (CB-I or II). Listed below are the standard glove types required by the WPS.

A. Solid Formulations applied as solids or formulations containing only water as the solvent or solvents other than water less than 5%, the glove statement shall specify "waterproof gloves." (Reference: Supplement III, Main Labeling Guidance, Page 11, of PR Notice 93-7.

B. Aqueous-Based Formulations applied as formulated or diluted solely with water for application, the glove statement shall specify "waterproof gloves."

C. Other Liquid Formulations which are formulated or diluted with liquids other than water (constitutes more than 5% of the end-use product), the glove statement shall specify "chemical-resistant (such as nitrile or butyl) gloves."

D. Gaseous Formulations or Formulations applied as Gases will retain any existing glove statement established before 10/20/92 including any glove prohibition statement. If no glove statement or glove prohibition currently exist on the label, then the glove statement shall be "chemical-resistant (nitrile or butyl) gloves."

8. SPECIFIC RESPIRATORY PROTECTION DEVICE (RPD) SELECTION FOR HANDLERS: RPD(s) are required for all products classified as toxicity category I or II for acute inhalation. Review the RPD types below and determine if the label lists the appropriate type based on the product description and toxicity category. Please note that if the registrant has submitted information to support the selection of another type of RPD, which is more protective, allow the registrant to retain that RPD requirement on the label under review. Information that could support an alternate RPD could be the submission of the product vapor pressure indicating that the RPD specified in the list below would not provide adequate protection or may pose an increased risk to the user unnecessarily.

A. Gases Applied Outdoors: Products that are formulated or applied as a gas (space and soil fumigants) and that may be applied outdoors must bear labeling specifying the following RPD requirements and statement:

"For handling activities outdoors, use either a respirator with an organic-vapor-removing cartridge with a prefilter approved for pesticides (MSHA/NIOSH approval number prefix TC-23C) or a canister approved for pesticides (MSHA/NIOSH approval number prefix TC-14G)."

B. Gaseous Products Used in Enclosed Areas: Products that are formulated or applied as a gas (space and soil fumigants) and that may be used in greenhouses or other enclosed areas must bear labeling specifying the following RPD requirements and statement:

"For handling activities in enclosed areas, use either a supplied-air respirator with MSHA/NIOSH approval number prefix TC-19C, or a self-contained breathing apparatus (SCBA) with MSHA/NIOSH approval number TC-13F."

C. Solid Products: Products that are formulated and applied as solids must bear labeling specifying the following RPD requirements and statement:

"For handling activities, use a dust/mist filtering respirator (MSHA/NIOSH approval number prefix TC-21C)."

D. Liquid Products in Toxicity Category I: Products that are formulated and applied as liquids must bear labeling specifying the following RPD requirements and statement:

"For handling activities, use either a respirator with an organic-vapor-removing cartridge with a prefilter approved for pesticides (MSHA/NIOSH approval number prefix TC-23C), or a canister approved for pesticides (MSHA/NIOSH approval number prefix 14G)."

E. Liquid Products in Toxicity Category II: Products that are formulated or applied as liquids must bear labeling specifying the following RPD requirements and statement:

"For handling activities during [insert applicable terms based on directions for use: airblast, mistblower, pressure greater than 40 p.s.i. with fine droplets, smoke, mist, fog, aerosol or direct overhead] exposures, wear either a respirator with an organic vapor-removing cartridge with a prefilter approved for pesticides (MSHA/NIOSH approval number prefix TC-23C), or a canister

approved for pesticides (MSHA/NIOSH approval number prefix 14G). For all other exposures, wear a dust/mist filtering respirator (MSHA/NIOSH approval number prefix TC-21C)."

9. REQUIRED LOCATION FOR PPE LABELING FOR APPLICATORS AND OTHER HANDLERS: PPE statements for applicators and other handlers must appear in the "PRECAUTIONARY STATEMENTS" section of the labeling. Refer to PR Notice 93-7 Supplement 3-A, Part 1 Product Worksheet. All products subject to the WPS must bear the following statements:

   "Follow the manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables, use detergent and hot water. Keep and wash PPE separately from other laundry."

10. LABELING AND PPE FOR EARLY REENTRY WORKERS - WPS Products

   A. All products subject to the WPS must bear the following statements for workers who reenter the treated area prior to the expiration of the reentry interval:

"PPE Required for early entry to treated areas that is permitted under the Worker Protection Standard and that involves contact with anything that has been treated, such as plants, soil, or water, wear: (Insert all PPE required for applicators and other handlers. Omit any respiratory protective devices)."

   B. Additional Early Reentry Worker PPE Requirements: The following modifications must be made to the early reentry worker labeling and PPE required in Section A. above:

   - If the handler body clothing requirement is a long-sleeved shirt and long pants, then the early-entry worker requirement shall be "coveralls."

   - If there is no handler requirement for or against gloves, then the early-entry requirement shall be "waterproof gloves."

   C. Required Location for Early-Reentry Worker Labeling and PPE Labeling: PPE statements for early-reentry workers must appear in the "DIRECTIONS FOR USE" section of the labeling under the heading "AGRICULTURAL USE REQUIREMENTS" immediately after the restricted-entry statement.

11. USER SAFETY RECOMMENDATIONS: If the product falls within the scope of WPS, then a User Safety Recommendations box, as indicated in PR Notice 93-7, Supplement Three, must also appear in a separate box on the label containing appropriate user safety information. The preferred location for this box is at the end of the section headed "Hazards to Humans (and Domestic Animals).

First Aid (Statement of Practical Treatment)

A first aid statement is required for each route of exposure (oral, dermal, inhalation, eye and skin) where the toxicity study has been classified as category I, II, or III. It is acceptable for the registrant to include first aid statements (category III statements) for studies that are classified as category IV.

1. REQUIRED HEADER: Any of the following headings are acceptable: First Aid, Statement of Practical Treatment or Practical Treatment. The heading "Antidote" cannot be used unless a specific antidote is recommended. The label should bear the heading which is most readily recognized by the intended users of the product. This determination will be made by the registrant.

2. CONTENT AND CLARITY: First aid statements must be brief, clear, simple and in straightforward language so that the average person can easily and quickly understand the instructions. First aid statements should be appropriate for all ages or when necessary, should include distinctions between the treatments for different ages, i.e., children vs. adults. The first aid statements should be such that any reasonably competent individual could perform them. First aid statements should not include procedures which must be performed by medical personnel or require specialized equipment (See Note to Physician) section.

   If the product contains an organophosphate (i.e., an organophosphorus ester that inhibits cholinesterase) or an N-methyl carbamate (i.e., an N-methyl carbamic acid ester that inhibits cholinesterase), the appropriate term shall be shown in the First Aid statement.

3. ORDER OF STATEMENTS: First aid statements should be organized so that the most severe routes of exposure as demonstrated by the toxicity category classification are listed first.

4. REQUIRED LOCATION: First aid statements must appear on the front panel of the label for all products classified as toxicity category I for acute oral, acute dermal, or acute inhalation exposure (40 CFR 156.10(h)(1)(i)). First aid statements triggered by any other exposure classification may appear on the front, side or back panel of the product label. However, any time first aid statements appear somewhere other than on the front panel, a referral statement such as "see side/back panel for first aid" must appear on the front panel in close proximity to the signal word. Furthermore, first aid statements on the side or back panel should be grouped near the other precautionary labeling, yet set apart or distinguishable from the other label text.

5. FIRST AID STATEMENTS FOR FUMIGANTS: Refer to PR Notice 84-5 (Reference: PR-84-05) and Registration Standards/REDs.

6. FIRST AID STATEMENTS FOR ALL OTHER PRODUCTS: Review the following sections to determine the appropriate first aid statements for each route of exposure.

A. Acute Oral: Use the following flow chart to determine the appropriate oral first aid statements. Please note that oral first aid statements are controversial and there are differing opinions within the medical community concerning whether emesis (vomiting) should be recommended. Until the Agency resolves this issue, in situations where the registrant has recommended an oral first aid statement that differs from those on the flowchart, instruct the registrant to modify the statement according to the flowchart or provide a justification for the use of the alternate statement.

   If the registrant's justification indicates that the proposed first aid statement was based on medical staff evaluation of the product, let the registrant retain the proposed first aid statement as long as it meets the requirements set forth in "Content and Clarity of Statements" and does not involve the use of salt water for emesis (PR Notice 80-2 (Reference: PR-80-02)). If the registrant indicates that the statement was selected by simply referring to another product, request that the registrant revise the statement based on the flow chart.

Determining the Acute Oral Exposure First Aid Statement

Is the active ingredient: zinc phosphide? IF NO | | |	IF YES---->	Use the following Statement: IF SWALLOWED: Immediately call a Poison Control Center or doctor, or transport the patient to the nearest hospital. Do not drink water. Do not administer anything by mouth or make the patient vomit unless advised to do so by a doctor."
Is the product tox category I or II for oral toxicity? IF NO | |	IF YES---->	Use the below for "All other Products."
Is product corrosive (pH < 2 or > 11.5) or is product toxicity categoryI or II for eye or dermaL irritation? IF NO | | |	IF YES---->	Use the following statement: IF SWALLOWED: Call a doctor or get medical attention. Do not induce vomiting or give anything by mouth to an unconscious person. Drink promptly a large quantity of milk, eggwhites, gelatin solution, or if these are not available, drink large quantities of water. Avoid alcohol."
Does the product contain > 10% petroleum distillates? IF NO | |	IF YES---->	Use the following statement: IF SWALLOWED: Call a doctor or get medical attention. Do not induce vomiting. Do not give anything by mouth to an unconscious person. Avoid alcohol.
All other Products. NOTE: Products in tox category IV for oral toxicity, do not require a first aid statement. However, if the registrant chooses, he may use either of these statements on his label.	IF YES---->	Use either of the following statements: IF SWALLOWED: Call a physician or Poison Control Center. Drink1 or 2 glasses of water and induce vomiting by touching back of throat with finger. If person is unconscious, do not give anything by mouth and do not induce vomiting. OR IF SWALLOWED: Call a physician or Poison Control Center. Drink 1 or 2 glasses of water and induce vomiting by touching back of throat with finger, or if available by administering syrup of ipecac. If person is unconscious, do not give anything by mouth and do not induce vomiting.

NOTE: The criteria in this flowchart are listed in priority order, with the highest at the top. If you have a product/chemical that meets more than one criteria, use the criteria that appears first on the chart. Do not use more than one criteria!

B. Acute Dermal and Primary Skin Irritation: Since both these studies focus on the dermal route of exposure, the first aid statements for these two studies can be combined when required for both studies. If a statement is required for both studies, use the first aid statement required for the acute dermal toxicity study if both studies are in the same acute toxicity or for the more severe acute toxicity category if the studies are in different acute toxicity categories.

Table 11 - Skin Irritation Statements

Toxicity Category	Required First Aid Statement
I	IF ON SKIN: Wash with plenty of soap and water. Get medical attention.
II	Same as above
III	IF ON SKIN: Wash with plenty of soap and water. Get medical attention if irritation persists.
IV	Statement not required. Registrant may use category III statements, if he chooses

Table 12 - Dermal Toxicity Statements

First Aid Statement Based on Acute Dermal Toxicity Study	
Toxicity Category	Required First Aid Statement
I	IF ON SKIN: Wash with plenty of soap and water. Get medical attention.
II	Same as above
III	Same as above
IV	Statement not required. Registrant may use category III statements, if he chooses

C. Acute Inhalation: Selection of the first aid statement is straightforward and is based on the toxicity category assigned to the particular study.

Table 13 - Acute Inhalation Statements

Toxicity Category	Required First Aid Statement
I	IF INHALED: Remove victim to fresh air. If not breathing, give artificial respiration, preferably mouth-to-mouth. Get medical attention.
II	Same as above
III	Same as above
IV	Statement not required. Registrant may use category III statements, if he chooses

D. Primary Eye Irritation: Selection of first aid statement is straightforward and is based on the toxicity category assigned to the particular study.

Table 14 - Eye Irritation Statements

Toxicity Category	Required First Aid Statement
I	IF IN EYES: Hold eyelids open and flush with a steady, gentle stream of water for 15 minutes. Get medical attention.
II	Same as above
III	IF IN EYES: Flush eyes with plenty of water. Call a physician if irritation persists.
IV	Statement not required. Registrant may use category III statements, if he chooses

7. NOTE TO PHYSICIAN

A. When Required: The "Note to Physician" is not required nor mentioned in the 40 CFR. If the label under review is for a product which is a fumigant, refer to PR Notice 84-5 (Reference: PR-84-05) or relevant Registration Standards for the "Note to Physician." For all other products, we are currently requiring a "Note to Physician" as specified in the proposed rule for the following types of products:

- All products that are classified as toxicity category I.
- Any product which is corrosive or classified as toxicity category I for eye or skin.
- Products that are in acute oral toxicity category III, and contain > 10% petroleum distillate.
- Any product that produces physiological effects requiring specific antidotal or medical treatment such as: Cholinesterase Inhibitors (e.g., carbamates and phosphorothioates, and organophosphates); Metabolic Stimulants (e.g., dichlorphenols); Anticoagulants (e.g., warfarin).

B. Contents of Note: The proposed rule does not provide specific notes to physicians except for corrosive and toxicity category I and II eye and skin irritants. The proposed rule does provide the following guidance concerning the content of notes to physicians. Check the label under review to make certain that it addresses the following information:

- technical information on symptomatology;
- use of supportive treatments to maintain life functions; medicine that will counteract the specific physiological effects of the pesticide;
- company telephone number to specific medical personnel who can provide
- specialized medical advice.

C. Specific Note for Corrosive or Toxicity Category I Eye or Skin Irritants:

Use the following Note to Physician:
"Note to Physician: Probable mucosal damage may contraindicate the use of gastric lavage."

D. Required Location: The "Note to Physician" should be located in close proximity to the "First Aid Statement" but should be clearly distinguished from it. In other words, it should not be mixed in with the "First Aid Statement" but should appear below the last first aid statement.

8. LABELING STATEMENTS FOR SPECIAL SITUATIONS

A. Chemigation statement (PR Notice 93-7, Supplement 3, page 39)
B. Soil incorporation/injection/seed treatment (PR Notice 93-7, Supplement 3, page 39)
C. Engineering control statements (PR Notice 93-7, Supplement 3, page 50)
D. ULV and LV uses (PR Notice 93-7, Supplement 3, page 40)

Optional Labeling and Deviations

EPA will consider precautionary statements that deviate from the requirements of this chapter, provided they meet the conditions as described below:

1. USE DILUTIONS: Additional precautionary wording and first aid statements on the label are allowed on concentrated products that correspond with a use dilution of the product that is consistent with the product's directions for use. EPA policy allows such labeling provided data are submitted to support

such statements. The policy provided guidelines for the submission and review of such data and for the content and placement of associated labeling (Reference: LP96-03).

A. Data Requirements: All data and draft labeling for use dilution precautionary wording and first aid statements should be sent to the appropriate product manager with a request for pesticide amendment.

   Use dilution precautionary wording or first aid statements triggered by systemic toxicity (acute oral, dermal or inhalation toxicity) can be supported by calculations. Most systemic toxicity categories span an order of magnitude (10-fold). Therefore, in most cases, if dilutions are an order of magnitude or more, except for category I, they will bump up at least one toxicity category. For example, if the end product toxicity category is II and it will be diluted 10-fold, the diluted product will be at toxicity category III; if it is diluted 100-fold, it will be at toxicity category IV.

   Use dilution precautionary wording or first aid statements triggered by skin or eye irritation or dermal sensitization must be supported by new or cited studies. If another registered dilute product (such as a ready to use formulation) has acceptable data and is found similar to the concentrated product after it has been diluted, that data may be used to support revised labeling.

B. Labeling Requirements: It is important to remember that it is not our intent to allow dual sets of precautionary statements and/or first aid statements on the label. Rather, we will allow certain "additional" statements that are applicable to the most concentrated use dilution only. These additional statements (triggered by the toxicity category of the use dilution) should be placed directly after the required statements for the concentrate. Following are some examples (in parentheses)of how use dilution labeling should appear on product labeling: (actual use dilution statements will depend on data submitted).

   Precautionary Statements:

   "Causes substantial but temporary eye injury. Do not get in eyes or on clothing. Wear goggles or face shield. Wash thoroughly with soap and water after handling. Remove contaminated clothing before reuse. (After product is diluted in accordance with the directions for use, goggles or face shield are not required").

   First Aid:

   "If on skin: Wash with plenty of soap and water. Get medical attention. (If product, diluted in accordance with the directions for use, gets on skin, medical attention is not required.)

2. TOXICITY CATEGORY IV PRECAUTIONARY LABELING: If the product is all toxicity category IV (non-sensitizer), precautionary labeling statements are normally not required. However, if a registrant desires to place precautionary labeling on such a product, they may do so. To promote labeling consistency it is recommended that the registrant use precautionary statements triggered by toxicity category III. Registrants may propose alternate labeling, which should be reviewed by precautionary labeling reviewers.

3. ME-TOO DEVIATIONS: If a me-too product is citing a product that has optional precautionary statements on the label, such as 1. and/or 2. above, those statements are not required on the me-too if the acute results are available. If you have any questions about the availability of the acute studies, check with the precautionary labeling reviewers.

4. OTHER DEVIATIONS: Registrants may submit WPS and non-WPS precautionary labeling that differ from the requirements in this chapter. Such requests must be supported by data (or substantive justification) should be routed to precautionary labeling reviewers or the Chemistry and Exposure Branches (CB-I or II) (for PPE). For example, the statement "Do not remove contact lenses, if worn. Get immediate medical attention." cannot be approved by the Agency without supporting data or rationale.

APPENDIX B

European Commission Evaluations and Statements of *In Vitro* Skin Corrosivity Methods

B-1 Statements by the ECVAM Scientific Advisory Committee (ESAC)

- **Statement on Rat Skin TER and EPISKIN™ B-3**
- **Statement on EpiDerm™ B-13**

B-2

- **SCCNFP's Opinion on *In Vitro* Skin Corrosivity Methods.....B-15**
- **SCCNFP's Committee Members B-19**

B-3 European Union Commission Directive for Rat Skin TER and a Human Skin Model Assay.....B-21

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STATEMENT ON THE SCIENTIFIC VALIDITY OF THE RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST
(AN *IN VITRO* TEST FOR SKIN CORROSIVITY)

STATEMENT ON THE SCIENTIFIC VALIDITY OF THE EPISKIN™ TEST
(AN *IN VITRO* TEST FOR SKIN CORROSIVITY)

STATEMENT ON THE SCIENTIFIC VALIDITY OF THE RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST (AN *IN VITRO* TEST FOR SKIN CORROSIVITY)

At its 10th meeting, held on 31 March 1998 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

> The results obtained with the rat skin transcutaneous electrical resistance (TER) test in the ECVAM International validation study on *in vitro* tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The rat skin TER test proved applicable to testing a diverse group of chemicals of different physical forms, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/surfactants. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good. The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied. The Committee therefore agrees with the conclusion from this formal validation study that the rat skin TER test is scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and non-corrosive chemicals, and that this test is ready to be considered for regulatory acceptance.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results and evaluation of the validation study by the Management Team, which is to be published in *Toxicology in Vitro*.[3]

This validation study was conducted in accordance with the general principles laid down in the report of the CAAT[2]/ERGATT[2] workshop held in 1990, [4] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995, [5] criteria laid down by ECVAM and the ECB, [2, 6] criteria recommended at an OECD[2] workshop held in 1996, [7] and the US ICCVAM[2] report on validation and regulatory acceptance. [8] The outcome of a prevalidation study on *in vitro* tests for skin corrosivity was published in 1995, as ECVAM workshop report 6. [9] A separate report on the selection of the test chemicals for the validation study is to be published alongside the Management Team's report in *Toxicology In Vitro*. [10]

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3 April 1998

1. The ESAC was established by the European Commission, and is composed of representatives of the EU Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 31 March 1998:

   Dr B Blaauboer (ERGATT)
   Professor J Castell (Spain)
   Dr B Garthoff (EFPIA)
   Dr C Hendriksen (The Netherlands)
   Professor G Papadopoulos (Greece)
   Dr B Rusche (Eurogroup for Animal Welfare)
   Professor H Spielmann (Germany)
   Professor H. Tritthart (Austria)
   Professor E Walum (Sweden)
   Dr P Botham (ECETOC)
   Dr D Clark (UK)
   Professor A Guillouzo (France)
   Dr R Lorenzini (Italy)
   Professor V Rogiers (Belgium)
   Dr O de Silva (COLIPA)
   Dr O Svendsen (Denmark)
   Dr M Viluksela (Finland)
   Dr F Zucco (Eurogroup for Animal Welfare)

   Professor M Balls (ECVAM)
   Dr J Fentem (ECVAM)
   Ms S Louhimies (DGXI)
   Mr A Van Elst (DGXXIV)
   Mr G Corcelle (DGXI)
   Dr G Fracchia (DGXII)
   Dr M Robert (DGII)

2. CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; ECB: European Chemicals Bureau. Ispra, Italy; ERGATT: European Research Group for Alternatives in Toxicity Testing, Utrecht, The Netherlands; ICCVAM: ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; OECD: Organization for Economic Cooperation and Development, Paris, France; UN: United Nations.

3. Fentem JH, Archer GEB, Balls M, Botham PA, Curren RD, Earl LK, Esdaile DJ, HoIzhütter H-G & Liebsch M (1998) The ECVAM International validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. *Toxicology in Vitro*, in press.

4. Balls M, Blaauboer BJ, Brusick D, Frazier J, Lamb D, Pemberton M, Reinhardt C, Roberfroid M, Rosenkranz H, Schmid B, Spielmann H, Stammati AL & Walum E (1990) Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. ATLA 18: 303-337.

5. Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H & Zucco F (1995) Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. ATLA 23: 129-147.

6. Balls M & Karcher W (1995) The validation of alternative test methods. ATLA 23: 884-886.

7. Anon. (1996) Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. 60pp. Paris: OECD.

8. Anon. (1997) Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. 105pp. Research Triangle Park, NC: NIEHS.

9. Botham, PA, Chamberlain M, Barratt MD, Curren RD, Esdalle DJ, Gardiner JR, Gordon VC, Hildebrand B, Lewis RW, Liebsch M, Logemann P, Osborne R, Ponec M, Régnier J-F, Steiling W, Walker AP & Balls M (1995) A prevalidation study on *in vitro* skin corrosivity testing. The report and the recommendations of ECVAM workshop 6. ATLA 23:219-255.

10. Barratt MD, Brantom PG, Fentem JH, Gerner I, Walker AP & Worth AP (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. *Toxicology in Vitro*, in press.

General Information about the ECVAM skin corrosivity validation study:

A. The study was coordinated from ECVAM, and the Management Team (MT) was chaired by Dr Julia Fentem (ECVAM). The other four MT members acted as representatives of the "lead laboratories" and each took responsibility for one of the four tests included in the validation study: Dr Rodger Curren (Microbiological Associates Inc., USA; CORROSITEX®). Dr Lesley Earl (Unilever, UK; rat skin TER assay), Mr David Esdaile (Rhône-Poulenc Agro, France; EPISKIN™), and Dr Manfred Liebsch (ZEBET, Germany: Skin2^{TM} assay). The study was principally funded by ECVAM, under the terms of 14 separate contracts with the participating organisations. Professor Michael Bails (ECVAM) and Dr Philip Botham (ESAC; ZENECA CTL, UK) represented the sponsors in any contacts with the MT. In addition to ECVAM, the participating organisations were: Agence du Medicament (France), BASF Aktiengesellschaft (Germany), BIBRA International (UK), COVANCE (UK), Humboldt University (Germany), Huntingdon Life Sciences (UK), INRS (France), Microbiological Associates Inc. (USA), Microbiological Associates Ltd (UK), Rhône-Poulenc Agro (France), Sanofi Recherche (France), Unilever Research (UK), ZEBET, BgVV (Germany) and ZENECA CTL (UK).

B. This study began in 1996, as a follow-up to a prevalidation study on *in vitro* tests for replacing the *in vivo* Draize rabbit test for skin corrosivity. The main objectives were to: (a) Identify tests capable of discriminating corrosives (C) from non-corrosives (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only); and (b) determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. The tests selected for inclusion In the validation study were: (a) the rat skin TER assay; (b) CORROSITEX®; (c) the Skin2™ ZK1350 corrosivity test; and (d) EPISKIN™. Each test was conducted in three independent laboratories, according to the principles, criteria and procedures for undertaking validation studies outlined previously by ECVAM in conjunction with International experts in this area. Prediction models for the four tests were clearly defined in the test protocols.

C. A test set of 60 chemicals was selected by an independent Chemicals Selection Sub-Committee, including organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC) and soaps/surfactants (3NC). The first set of ten coded chemicals was distributed independently of the MT and participating laboratories in June 1996. Further to the satisfactory completion of the first phase of the study, the remaining 50 coded chemicals were distributed in September 1996. The results obtained were submitted to ECVAM's statistician, Dr Graeme Archer, for independent analysis in consultation with Dr Hermann-Georg Holzhütter (Humboldt University, Berlin, Germany). Data analysis and preparation of the final reports took place between May and October 1997.

D. The rat skin TER assay has been used successfully as a routine in-house test for several years. When used in screening mode, the TER method is employed to predict corrosivity potential rather than the degree of corrosive effect (i.e. potency), and it has been used primarily to guide humane *in vivo* skin testing. The TER assay has been evaluated in several intralaboratory and interlaboratory studies, and it performed creditably in the prevalidation study conducted during 1993 and 1994. The test protocol evaluated in this validation study had been refined on the basis of recommendations from the prevalidation study, to include a dye binding procedure for reducing the number of false positive predictions obtained previously with test materials containing surfactants and solvents. in outline, test materials are applied for up to 24 hr to the epidermal surfaces of skin discs taken from the pelts of humanely killed young rats. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a predetermined threshold level (5kΩ).

Rat Skin TER Assay Prediction Model:

TER (kΩ)	Treatment time (hours)	Mean disc dye content	C/NC	EU risk phrase	UN packing group
>5	2 & 24	Nm[a]	NC	no label	-
< or = 5	2	-	C	R35	I
	24	-	C	R34	II/III
Surfactants/neutral organics:					
< or = 5	24	> or = +ve control	C	R34	II/III
	24	< +ve control	NC	no label	-

aNM = not measured

E. The prediction model for the rat skin TER test was used to classify the corrosivity potentials of the 60 test chemicals on the basis of the *in vitro* data obtained in the three laboratories conducting the test Comparing these *in vitro* classifications with the *in vivo* classifications independently assigned to the chemicals before the blind trial began gave the following key statistical parameters:

Sensitivity:	C	88%
	R34/II & III	18%
	R35/I	88%
Specificity:	72%	
Predictivity:	C	72%
	R34/II & III	40%
	R35/I	22%
Accuracy:	C/NC	79%
	R35/R34/NC	55%

The underprediction and overprediction rates for the TER test relative to the study objectives were :

Objective (a): C v NC	underprediction rate	12%
	Overprediction rate	28%
Objective (b): R35/I v R34/II & III v NC	underprediction rate	
	R35/I-->NC	6%
	R34/II & III --> NC	14%
	overprediction rate	
	NC --> R35/I	12%
	NC --> R34/II & III	16%
	R34/II & III --> R35/I	69%

* unacceptable according to the criteria defined by the MT before undertaking the data analysis

F. In order for the rat skin TER test to be considered for use for legislative and other purposes, measures will be taken to press for the updating of OECD Testing Guideline 404 and Annex V method B.4 of *Directive 67/548/EEC*.

G. A statement on the scientific validity of the EPISKIN™ assay for skin corrosivity testing was also endorsed by the ESAC on 31 March 1998. The two other methods included in the validation study, CORROSITEX® and Skin2, did not meet all of the criteria for them to be considered acceptable as replacement tests. The corrosivity potentials of about 40% of the test chemicals could not be assessed with CORROSITEX®, although it may be valid for testing specific classes of chemicals (such as organic bases and inorganic acids). The Skin2 assay, as conducted in this validation study, had an unacceptably high underprediction rate (57%), although it had a specificity of 100% it is recognised that both of these methods could be useful if they were incorporated into a tiered testing strategy for skin corrosivity.

STATEMENT ON THE SCIENTIFIC VALIDITY OF THE EPISKIN™ TEST (AN *IN VITRO* TEST FOR SKIN CORROSIVITY)

At its 10th meeting, held on 31 March 1998 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1], unanimously endorsed the following statement:

> The results obtained with the EPISKIN™ test (involving the use of a reconstructed human skin model) in the ECVAM international validation study on *in vitro* tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The EPISKIN test proved applicable to testing a diverse group of chemicals of different physical forms, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/surfactants. The concordances between the skin corrosivity classifications derived

> from the *in vitro* data and from the *in vivo* data were very good. The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied; it was also able to distinguish between known R35 (UN2 packing group I) and R34 (UN packing groups II & III) chemicals. The Committee therefore agrees with the conclusion from this formal validation study that the EPISKIN test is scientifically validated for use as a replacement for the animal test, and that it is ready to be considered for regulatory acceptance.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results and evaluation of the validation study by the Management Team, which is to be published in *Toxicology in vitro*.[3]

This validation study was conducted in accordance with the general principles laid down in the report of the CAAT[2]/ERGATT[2] workshop held in 1990, [4] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,[5] criteria laid down by ECVAM and the ECB, [2, 6] criteria recommended at an OECD[2] workshop held in 1996, [7] and the US ICCVAM[2] report on validation and regulatory acceptance.[8] The outcome of a prevalidation study on *in vitro* tests for skin corrosivity was published in 1995, as ECVAM workshop report 6. [9] A separate report on the selection of the test chemicals for the validation study is to be published alongside the Management Team's report in *Toxicology in vitro*. [10]

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3 April 1998

1. The ESAC was established by the European Commission, and is composed of representatives of the EU Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 31 March 1998:

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   Mr G Corcelle (DGXI)
   Dr G Fracchia (DGXII)
   Dr M Robert (DGII)

2. CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; ECB: European Chemicals Bureau. Ispra, Italy; ERGATT: European Research Group for Alternatives in Toxicity Testing,

Utrecht, The Netherlands; ICCVAM: ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; OECD: Organization for Economic Cooperation and Development, Paris, France; UN: United Nations.

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6. Balls M & Karcher W (1995) The validation of alternative test methods. ATLA 23: 884-886.

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8. Anon. (1997) *Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods.* 105pp. Research Triangle Park, NC: NIEHS.

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10. Barratt MD, Brantom PG, Fentem JH, Gerner I, Walker AP & Worth AP (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the test chemicals. Toxicology *in vitro*, in press.

General information about the ECVAM skin corrosivity validation study:

A. The study was coordinated from ECVAM, and the Management Team (MT) was chaired by Dr Julia Fentem (ECVAM). The other four MT members acted as representatives of the "lead laboratories" and each took responsibility for one of the four tests included in the validation study: Dr Rodger Curren (Microbiological Associates Inc., USA; CORROSITEX®). Dr Lesley Earl (Unilever, UK; rat skin TER assay), Mr David Esdaile (Rhône-Poulenc Agro, France; EPISKIN™), and Dr Manfred Liebsch (ZEBET, Germany: Skin2™ assay). The study was principally funded by ECVAM, under the terms of 14 separate contracts with the participating organisations. Professor Michael Bails (ECVAM) and Dr Philip Botham (ESAC; ZENECA CTL, UK) represented the sponsors in any contacts with the MT. In addition to ECVAM, the participating organisations were: Agence du Medicament (France), BASF Aktiengesellschaft (Germany), BIBRA International (UK), COVANCE (UK), Humboldt University (Germany), Huntingdon Life Sciences (UK), INRS (France), Microbiological Associates Inc. (USA), Microbiological Associates Ltd (UK), Rhône-Poulenc Agro

(France), Sanofi Recherche (France), Unilever Research (UK), ZEBET, BgVV (Germany) and ZENECA CTL (UK).

B. This study began in 1996, as a follow-up to a prevalidation study on *in vitro* tests for replacing the *in vivo* Draize rabbit test for skin corrosivity. The main objectives were to: (a) Identify tests capable of discriminating corrosives (C) from non-corrosives (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only); and (b) determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. The tests selected for inclusion In the validation study were: (a) the rat skin TER assay; (b) CORROSITEX®; (c) the Skin2^{TM} ZK1350 corrosivity test; and (d) EPISKIN^{TM}. Each test was conducted in three independent laboratories, according to the principles, criteria and procedures for undertaking validation studies outlined previously by ECVAM in conjunction with International experts in this area. Prediction models for the four tests were clearly defined in the test protocols.

C. A test set of 60 chemicals was selected by an independent Chemicals Selection Sub-Committee, including organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC) and soaps/surfactants (3NC). The first set of ten coded chemicals was distributed independently of the MT and participating laboratories in June 1996. Further to the satisfactory completion of the first phase of the study, the remaining 50 coded chemicals were distributed in September 1996. The results obtained were submitted to ECVAM's statistician, Dr Graeme Archer, for independent analysis in consultation with Dr Hermann-Georg Holzhütter (Humboldt University, Berlin, Germany). Data analysis and preparation of the final reports took place between May and October 1997.

D. EPISKIN™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin corrosivity testing involves topical application of test materials to the surface of the skin for 3, 60 and 240 min, and the subsequent assessment of their effects on cell viability by using the MTT assay. An in-house evaluation and prevalidation of the test was conducted during 1994-96. On the basis of these studies, the test protocol was refined prior to its inclusion in this validation study.

EPISKIN Prediction Model:

Treatment time (min)	Viability (%)	C/NC	EU risk phrase	UN packing group
3	<35	C	R35	I
3 / 60	> or = 35/>35	C	R34	II
60 / 240	> or = 35 / <35	C	R34	III
240	>35	NC	no label	-

E. The prediction model for the EPISKIN test was used to classify the corrosivity potentials of the 60 test chemicals on the basis of the *in vitro* data obtained in the three laboratories conducting the test. Comparing these *in vitro* classifications with the *in vivo* classifications independently assigned to the chemicals before the blind trial began gave the following key statistical parameters:

Sensitivity:	C	83%
	R34/II & III	75%
	R35/I	39%
Specificity:		80%
Predictivity:	C	77%
	R34/II & III	64%
	R35/I	53%
Accuracy:	C/NC	81%
	R35/R34/NC	74%

The underprediction and overprediction rates for the EPISKIN test relative to the study objectives were:

Objective (a): C v NC	underprediction rate	17%
	overprediction rate	20%
Objective (b): R35/I v R34/II&III v NC	underprediction rate	
	R35/I --> NC	17%
	R34/II & III --> NC	18%
	overprediction rate	
	NC --> R35/I	1%
	NC --> R34/II & III	19%
	R34/II & III -->R35/I	8%

F. In order for the EPISKIN test to be considered for use for legislative and other purposes, measures will be taken to press for the updating of OECD Testing Guideline 404 and Annex V method B.4 of *Directive 67/548/EEC*.

G. A statement on the scientific validity of the rat skin transcutaneous electrical resistance (TER) assay for skin corrosivity testing was also endorsed by the ESAC on 31 March 1998. The two other methods included in the validation study, CORROSITEX® and Skin2 did not meet all of the criteria for them to be considered acceptable as replacement tests. The corrosivity potentials of about 40% of the test chemicals could not be assessed with CORROSITEX®, although it may be valid for testing specific classes of chemicals (such as organic bases and inorganic acids). The Skin2 assay, as conducted in this validation study, had an unacceptably high underprediction rate (57%), although it had a specificity of 100%. It is recognised that both of these methods could be useful if they were incorporated into a tiered testing strategy for skin corrosivity.

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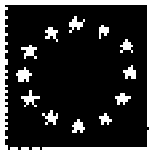

Commission of fine European Union
Joint Research Centre
Institute for Health & Consumer Protection
21020 Iapra (VA)
Italy

ECVAM European Centre for the Validation of Alternative Methods

STATEMENT ON THE APPLICATION OF THE EPIDERM™ HUMAN SKIN MODEL FOR SKIN CORROSVITY TESTING

At its 14th meeting, held on 14-15 March 2000 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

> Following a review of the results of the ECVAM-funded prevalidation study on the EpiDerm™ skin corrosivity test coordinated by ZEBET, it is concluded that the EpiDerm human skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the draft EU and OECD test guidelines on skin corrosion.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular; the report on the results and evaluation of the study prepared for the ESAC,[2] and a report on the study which has been accepted for publication.[3]

The validation study was conducted in accordance with the general principles laid down in the report of the CAAT[4]/ERGATT[4] workshop held in 1990,[5] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,[6] criteria laid down by ECVAM and the ECB,[4,7] criteria recommended at an OECD[4] workshop held in 1996,[8] and the US ICCVAM[4] report on validation and regulatory acceptance.[9]

The status of the draft guidelines referred to is as follows. The Competent Authorities of it EU Member States accepted the draft guideline on skin corrosivity testing[10] into the Annex V guidelines at the 27th Meeting on Adaptation to Technical Process in relation to *Directive 67/548/EEC,*, held in Brussels on 4 February 2000. An equivalent draft guideline[11] has been circulated by the OECD Secretariat for consideration by the OECD Member Countries.

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

Eva Helisten
Head of Unit E.2
Environment Directorate General
European Commission
Brussels

20 March 2000

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Excerpts from the *Opinion on in vitro methods to assess skin corrosivity in the safety evaluation of cosmetic ingredients or mixtures of ingredients adopted by the plenary session of the Scientific Committee for Cosmetic Products, and Non-food Products intended for Consumers (SCCNFP) of 25 November 1998* as obtained from the Internet at
http://europa.eu.int/comm/food/fs/sc/sccp/out47_en.html

Outcome of discussions

Terms of Reference

Two *in vitro* methods developed to assess skin corrosivity of chemicals, the "Rat skin Trancutaneous Electrical Resistance (TER) test" and the "EPISKIN test" have been validated by ESAC (ECVAM Scientific Advisory Committee). The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) has been requested by DG III to advise the Commission on the applicability of the methods to the safety assessment of chemicals used as cosmetic ingredients.

Background

The European Centre for the Validation of Alternative Methods (ECVAM) has conducted in 1996-1997 a validation study of *in vitro* tests developed to assess skin corrosivity of chemicals. This study was a follow-up to a pre-validation study of tests developed for replacing the *in vivo* Draize skin corrosivity test in rabbits. The main objectives of the validation study, as defined by the sponsors and the management team before the study began, were :

(a) to identify tests capable of discriminating corrosives (C) from non corrosive (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only);

(b) to determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals.

Organisation of the study

The study was coordinated from ECVAM. A Management Team (MT) was constituted by four representatives of lead laboratories, each of them being responsible for one of the four tests being evaluated. The tests selected for inclusion in the validation study were the rat transcutaneous electrical resistance (TER) test, Corrositex, the Skin^2 ZK1350 corrosivity test, and Episkin. Each test was conducted in three different laboratories, according to principles, criteria and procedures previously defined by ECVAM. Prediction models for each of the four tests were defined in the test protocols.

Coordination /MT /Laboratories

Sixty chemicals were selected by an independent Chemicals Selection Sub-Committee, and distributed coded to the participating laboratories. These included organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC), and soaps/surfactants (3NC). The selection is fully described in a publication (Ref. 1); the main criterion for including chemicals in the test set was that the corrosivity classifications were based on unequivocal animal data.

The results obtained were analysed by statistician experts. The classifications of the corrosivity potential of the test chemicals, as derived from the *in vitro* data obtained in the three laboratories conducting the test, were compared to the *in vivo* classifications independently assigned to the chemicals before the blind trial, to yield sensitivity, specificity, predictivity and accuracy of the test.

Main results

The full details of the validation study have been published (Ref. 2). Two tests, with a good reproductibility within and between test laboratories, proved applicable to the testing of a diverse group of chemicals: the TER test and Episkin.

In the TER test, test materials are applied for 2 to 24 hours to the epidermal surface of skin discs taken from the pelts of humanely killed young rats, and corrosive chemicals are identified by their ability to produce a loss of normal stratum corneum integrity, which is measured as a reduction of the inherent transcutaneous electrical resistance (below a predetermined threshold level).

Episkin is a tri-dimensional human skin model with a reconstructed epidermis and a functional stratum corneum. When utilised in corrosivity testing, application of test chemicals to the surface of the skin for 3, 60 and 240 min, is followed by an assessment of cell viability.

Sensitivity, specificity, predictivity and accuracy in distinguishing corrosive from non corrosive chemicals were very high for both tests: 88, 72, 72, 79 and 83, 80, 77, 81% respectively for the TER test and Episkin. In addition, Episkin was also able to distinguish between known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals.

Opinion of the SCCNFP

ECVAM Scientific Advisory Committee (ESAC), which had been fully informed of the progression of the validation procedure, reviewed the final results and unanimously endorsed a statement that the rat skin TER test is scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and noncorrosive chemicals, and that Episkin is scientifically validated as a replacement for the animal test, and that these tests are ready for regulatory acceptance.

Sixty chemicals were used for the validation of these two methodologies; twenty of them are used as cosmetic ingredients, according to the "European inventory and common nomenclature of ingredients employed in cosmetic products" (Ref. 3).

SCCNFP reviewed publications from the validation study and ESAC statements, and propose that these two methods could be applied to the safety assessment of chemicals used as cosmetic ingredients. A cosmetic ingredient or mixture of ingredients can be corrosive per se. When corrosivity cannot be excluded, testing for irritancy on animals or humans should be preceeded by a corrosivity test using one of these two validated *in vitro* methodologies.

References

1- Barratt M.D. & al. Toxicology in Vitro (1998) 12, 471-482
2- Barratt M.D. & al. Toxicology in Vitro (1998) 12, 483-524
3- Commission Decision 96/335 EC of 8 May 1996 establishing an inventory and a common nomenclature of ingredients employed in cosmetic products J.O. L 132 of 1 June 1996

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Excerpts from the *alphabetical list of the scientists appointed by the Commission as members of the Scientific Committee for Cosmetic Products, and Non-food Products intended for Consumers (SCCNFP) set up by Decision 97/579/EC of 23 July 1997* as obtained from the Internet at http://europa.eu.int/comm/food/fs/sc/sccp/members_en.html.

- Klaus E. Andersen, Professor of Dermatology, Odense University Hospital, Odense (Danmark)
- Robert Anton, Professeur de Pharmacognosie, Université Louis Pasteur Strasbourg, Faculté de Pharmacie, Illkirch (France)
- Claire Marcia Chambers, Consultant Toxicologist, Chambers Toxicological Consulting, Roundwood (Ireland)
- Alessandro di Domenico, Head of Ecotoxicology Unit, Istituto Superiore di Sanità, Roma (Italia)
- Vassilios M. Kapoulas, Professor emeritus of Biochemistry, University of Ioannina, Halandri (Ellas)
- Fritz H. Kemper, Professor emeritus of Pharmacology and Toxicology, University of Münster, Münster (Deutschland) - Vice-Chair of the Committee
- Christian Laurent, Lecturer, Scientific Director, Université de Liège, Institut de Pathologie, Liège (Belgique)
- Berend A.R. Lina, Study director, Division Quality Consultant, TNO Nutrition and Food Research, Toxicology Division, Zeist (Nederland)
- Nicola Loprieno, Professor emeritus of Genetics, University of Pisa, Pisa (Italia) - Vice-Chair of the Committee
- Jean-Paul Marty, Professor of Dermopharmacology and Cosmetology, Faculty of Pharmacy, University Paris South (Paris XI), Châtenay-Malabry (France)
- José Luis Parra, Research professor, Consejo Superior de Investigaciones Cientificas, Centro de Investigación y Desarrollo, Barcelona (España)
- Thomas Platzek, Senior scientist, Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin (Deutschland)
- Suresh Chandra Rastogi, Senior Research Scientist, National Environmental Research Institute, Roskilde (Danmark)

- Vera M. Rogiers, Professor, Vrije Universiteit Brussel, Faculty of Medicine and Pharmacy, Brussel (België)

- Tore Sanner, Professor, The Norwegian Radium Hospital, Institute for Cancer Research, Oslo (Norge)

- Hans Schaefer, Invited Lecturer for Skin Physiology and Skin Pharmacology, Department of Dermatology, Charité-Hospital, Humboldt-University, Berlin (Deutschland); Former Scientific Director, L'Oréal, Clichy (France)

- Josep Vives-Rego, Professor of Microbiology, Universitat de Barcelona, Facultat de Biologia, Barcelona (España)

- Ian R. White, Director, Consultant Dermatologist, St. Thomas' Hospital, St. John's Institute of Dermatology, London (United Kingdom) - Chair of the Committee

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available by clicking on the #90 at the following link:
http://europa.eu.int/eur-lex/en/archive/2000/l_13620000608en.html

Official Journal
of the European Communities

L 136
Volume 43
8 June 2000

[click on the blue title or page number below to access the *Official Journal* page]

English edition

Legislation

Contents

I *Acts whose publication is obligatory*

* **Commission Directive 2000/32/EC of 19 May 2000** adapting to technical progress for the 26th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (1) 1

* **Commission Directive 2000/33/EC of 25 April 2000** adapting to technical progress for the 27th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (1) **90**

*** Commission Decision of 19 May 2000** correcting Directive 98/98/EC adapting to technical progress for the 25th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (notified under document number C(2000) 1333) **(1)** 108

(1) Text with EEA relevance

EN

Acts whose titles are printed in light type are those relating to day-to-day management of agricultural matters, and are generally valid for a limited period. The titles of all other Acts are printed in bold type and preceded by an asterisk.

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APPENDIX C

OECD Related Test Guidelines and Documents

(*reference only*)

The following documents were used as reference material during the review of EpiDerm™ (Epi-200), EPISKIN™, and the Rat Skin TER Assay. OECD has or will adopt final versions of these in 2002. Current or final adopted versions of the Guidelines are available through the OECD website at http://www1.oecd.org/ehs/test/health.htm.

- **OECD Guideline for Testing of Chemicals Draft Revised Guideline 404: Acute Dermal Irritation/Corrosion (May 2001)**

- **Supplement to TG 404: A Sequential Testing Strategy for Dermal Irritation and Corrosion (May 2001)**

- **OECD Guideline for Testing of Chemicals Draft Revised Guideline 405: Acute Eye Irritation and Corrosion (May 2001)**

- **Supplement to TG 405: A Sequential Testing Strategy for Eye Irritation and Corrosion (May 2001)**

- **OECD Guideline for Testing of Chemicals Draft Proposal for a New Guideline 430: *In Vitro* Skin Corrosion: Transcutaneous Electrical Resistance (TER) Test (March 2002)**

- **OECD Guideline for Testing of Chemicals Draft Proposal for a New Guideline 431: *In Vitro* Skin Corrosion: Human Skin Model Test (March 2002)**

APPENDIX D

Federal Register Notice

Vol. 66, No. 189, pp. 49685-49686, Sept. 28, 2001

EPISKIN™, EpiDerm™, and Rat Skin Transcutaneous Electrical Resistance Methods: *In vitro* Test Methods Proposed for Assessing the Dermal Corrosivity Potential of Chemicals: Notice of Availability of a Background Review Document and Proposed ICCVAM Test Method Recommendations and Request for Public Comment

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Health, 6701 Rockledge Drive, Room 3190, MSC 7848, Bethesda, MD 20892, (301) 435–1507.

Name of Committee: Center for Scientific Review Special Emphasis Panel.

Date: October 11, 2001.

Time: 9:00 am to 5:00 pm.

Agenda: To review and evaluate grant applications.

Place: Holiday Inn, 8120 Wisconsin Avenue, Bethesda, MD 20814.

Contact Person: Richard Marcus, PhD, Scientific Review Administrator, Center for Scientific Review, National Institutes of Health, 6701 Rockledge Drive, Room 5168, MSC 7844, Bethesda, MD 20892, (301) 435–1245, richard.marcus.@nih.gov.

Name of Committee: Center for Scientific Review Special Emphasis Panel.

Date: October 11, 2001.

Time: 1:00 pm to 2:30 pm.

Agenda: To review and evaluate grant applications.

Place: NIH, Rockledge 2, Bethesda, MD 20892, (Telephone Conference Call).

Contact Person: Victor A. Fung, PhD, Scientific Review Administrator, Center for Scientific Review, National Institutes of Health, 6701 Rockledge Drive, Room 4120, MSC 7804, Bethesda, MD 20814–9692, (301) 435–3504, fungv@csr.nih.gov.

(Catalogue of Federal Domestic Assistance Program Nos. 93.306, Comparative Medicine, 93.306; 93.333, Clinical Research, 93.333, 93.337, 93.393–93.396, 93.837–93.844, 93.846–93.878, 93.892, 93.893, National Institutes of Health, HHS)

Dated: September 20, 2001.

LaVerne Y. Stringfield,

Director, Office of Federal Advisory Committee Policy.

[FR Doc. 01–24366 Filed 9–27–01; 8:45 am]

BILLING CODE 4140–01–M

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

National Institute of Environmental Health Sciences (NIEHS) National Toxicology Program (NTP)

EPISKIN™, EpiDerm™, and Rat Skin Transcutaneous Electrical Resistance Methods: In Vitro Test Methods Proposed for Assessing the Dermal Corrosivity Potential of Chemicals; Notice of Availability of a Background Review Document and Proposed ICCVAM Test Method Recommendations and Request for Public Comment.

Summary

The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) announces availability of a background review document (BRD) entitled ''EPISKIN™, EpiDerm™, and Rat Skin Transcutaneous Electrical Resistance (TER) Methods: In Vitro Test Methods for Assessing the Dermal Corrosivity Potential of Chemicals,'' and proposed test method recommendations from the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) on the use of these methods. The NICEATM invites public comment on the BRD and ICCVAM recommendations.

Availability of Background Review Document and Proposed ICCVAM Recommendations

An electronic version of this BRD and proposed ICCVAM test method recommendations may be obtained from the NICEATM/ICCVAM web site at *http://iccvam.niehs.nih.gov*. For a paper copy (a limited number are available), please contact the NICEATM at (919) 541–3398 or via email at *niceatm@niehs.nih.gov*.

Request for Public Comment

NICEATM invites written public comments on the BRD on in vitro corrosivity methods and the proposed ICCVAM recommendations for these methods. The deadline for submission of comments is November 13, 2001. Comments submitted via email are preferred; the acceptable file formats are MS Word (Office 98 or older), plain text, or PDF. Comments should be sent to Dr. William Stokes, Director, NICEATM, NIEHS, MD EC–17, P.O. Box 12233, Research Triangle Park, NC, 27709; telephone 919–541–3398; fax 919–541–0947; email *niceatm@niehs.nih.gov*. Persons submitting written comments should include their contact information (name, affiliation, address, telephone/fax numbers, and email) and sponsoring organization, if any.

Public comments received in response to this **Federal Register** notice will be posted on the NICEATM/ICCVAM web site *http://iccvam.niehs.nih.gov* and provided to the ICCVAM. ICCVAM will consider all comments prior to finalizing its test recommendations on EpiDerm™, EPISKIN™, and Rat Skin TER. In accordance with Public Law 106–545, ICCVAM test recommendations will be forwarded to appropriate Federal agencies and will be made available to the public on the NICEATM/ICCVAM website.

Background

ICCVAM and the ICCVAM Corrosivity Working Group (CWG) recently evaluated three in vitro test methods for assessing the dermal corrosivity potential of chemicals and chemical mixtures—EpiDerm™, EPISKIN™, and Rat Skin TER. EpiDerm™ and EPISKIN™ utilize a three dimensional human skin model comprised of a reconstructed epidermis and a functional stratum corneum. The test chemical is applied to this reconstructed epidenmis for a specified time and subsequent cell viability is measured. Rat Skin TER assesses the skin corrosivity of a chemical by applying the test material to the epidermal surface of a rat skin disc for two and 24 hours; subsequently, the transcutaneous electrical resistance (TER) of the skin disc is measured. NICEATM prepared a background review document summarizing the available data and prior reviews for the three test methods, which was then considered by the CWG and ICCVAM. The CWG concluded, based on the information provided and outcomes of the previous reviews, that further evaluation by an independent scientific peer review panel did not appear necessary, and recommended that these methods undergo ICCVAM evaluation using an expedited review process (ICCVAM, 2001). ICCVAM agreed with the CWG recommendation for expedited review. This process involves the development of a draft ICCVAM position (proposed ICCVAM test recommendations) and publishing the position in the **Federal Register** for public comment. Public comments are considered by ICCVAM, and if no major problems are found, ICCVAM then finalizes its test recommendations and forwards to federal agencies for their determination of regulatory acceptability. If major problems are noted, then ICCVAM will determine an appropriate process for further evaluation, such as an independent peer review panel evaluation.

ECVAM Evaluation

The European Center for the Validation of Alternative Methods (ECVAM) conducted validation studies on these three in vitro methods (Barratt et al., 1998; Fentem et al., 1998; Liebsch et al., 2000). The ECVAM Management Team concluded that EpiDerm™, Rat Skin TER, and EPISKIN™ were scientifically valid for use as replacements for the animal test currently used to distinguish between corrosive and non-corrosive chemicals and for all chemical classes (Fentem et al., 1998; Liebsch et al., 2000).

Other Reviews

The validation status of these three methods was then evaluated by the ECVAM Scientific Advisory Committee (ESAC). The ESAC also concluded that the Rat Skin TER, EpiDerm™, and the EPISKIN™ tests were scientifically

valid for use as replacements for the animal test and were ready to be considered for regulatory acceptance (Balls and Corcelle, 1998; Balls and Hellsten, 2000). The European Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) evaluated the EPISKIN™ and Rat Skin TER and concluded that they were applicable for the safety evaluation of cosmetic ingredients or mixtures of ingredients (Anon., 1999). The European Commission subsequently adopted EpiDerm™, EPISKIN™, and Rat Skin TER (Anon., 2000).

Proposed ICCVAM Recommendations

ICCVAM proposes that these assays can be used to assess the dermal corrosion potential of chemicals in a weight-of-evidence approach in an integrated testing scheme [e.g., OECD Globally Harmonised Classification System (OECD, 1998); OECD Revised Proposals for Updated Test Guidelines 404 and 405: Dermal and Eye Corrosion/ Irritation Studies (OECD, 2001a)]. These integrated testing schemes for dermal irritation/corrosion allow for the use of validated and accepted in vitro methods. In this approach, positive in vitro corrosivity responses do not generally require further testing and can be used for classification and labeling. Negative in vitro corrosivity responses shall be followed by in vivo dermal corrosion/irritation testing. (Note: The first animal used in the irritation/ corrosivity assessment would be expected to identify any chemical corrosives that were false negatives in the in vitro test). Furthermore, as is appropriate for any in vitro assay, there is the opportunity for confirmatory testing if false positive results are indicated on a weight of evidence evaluation of supplemental information, such as pH, structure activity relationships (SAR), and other chemical and testing information.

Additional Information About ICCVAM and NICEATM

ICCVAM, with 15 participating Federal agencies, was established in 1997 to coordinate interagency issues on toxicological test method development, validation, regulatory acceptance, and national and international harmonization. The ICCVAM Authorization Act of 2000 (Public Law 106–545) formally authorized and designated ICCVAM as a permanent committee administered by the NIEHS with specific duties that include the technical evaluation of new and alternative testing methods. ICCVAM is charged with developing test recommendations based on those technical evaluations, and forwarding these to Federal agencies for their consideration. The NICEATM was established in 1998 to coordinate and facilitate ICCVAM activities, to provide peer review for validation activities and to promote communication with stakeholders. The NICEATM is located at the NIEHS, Research Triangle Park, NC. Additional information concerning ICCVAM and NICEATM can be found on the ICCVAM/NICEATM web site at *http://iccvam.niehs.nih.gov*.

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OECD. OECD Revised Proposals for Updated Test Guidelines 404 and 405: Dermal and Eye Corrosion/Irritation Studies. [OECD ENV/JM/TG (2001)2]. OECD Environment Directorate, Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology. Test Guidelines Programme. Circulated in preparation for the 13th Meeting of the Working Group of the National Coordinators of the Test Guidelines Programme, OECD, Paris, France. (2001a)

Dated: September 21, 2001.

Samuel H. Wilson,

Deputy Director, National Institute of Environmental Health Sciences.

[FR Doc. 01–24371 Filed 9–27–01; 8:45 am]

BILLING CODE 4140–01–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

National Institute of Environmental Health Sciences (NIEHS); National Toxicology Program (NTP)

Report of the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity; Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity: Notice of Availability and Request for Public Comment.

Summary

Notice is hereby given of the availability of the reports entitled, ''Report of the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity'' NIH Publication 01–4499 and ''Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity'' NIH Publication 01–4500. The Report provides conclusions and recommendations from expert scientists based on their review of current in vitro methods for assessing acute toxicity at an October 17–20, 2000 workshop. The workshop was organized by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The Guidance Document

APPENDIX E

Public Comments in Response to the *Federal Register* Request for Comments

Comments available at:

http://iccvam.niehs.nih.gov/methods/epiddocs/corrcomm.htm

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