厚生労働科学研究費補助金（医薬品・医療機器等レギュラトリーサイエンス総合研究事業）

分担研究報告書

皮膚刺激性及び眼刺激性試験代替法のバリデーションに関する研究

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研究要旨
皮膚刺激性試験代替法 LabCyte EPI-MODEL24 を用いた方法及び眼刺激性試験代替法 STE 法のバリデーションを実施し、どちらも計画通りのバリデーションを実施できた。これらのバリデーション報告書を OECD 事務局に提出した。

キーワード：皮膚刺激性試験、眼刺激性試験、バリデーション、
動物実験代替法

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A．研究目的

2008-2009 年に掛け、日本製の培養表皮モデル LabCyte EPI-MODEL24 を用いた方法の有用性を検証するため[1]、日本動物実験代替法学会バリデーション委員会が皮膚刺激性試験代替法のバリデーション研究を実施した。このバリデーション研究を通して、目的であった LabCyte EPI-MODEL24 を用いた皮膚刺激性試験で得られる皮膚刺激性の判定が，複数の施設間でどの程度一致するか（施設間再現性），ECVAM（European Center for the Validation of Alternative Methods）で認証されている EPISKIN で得られた判定結果とどの程度一致するか（同等性），動物実験結果とどの程度一致するか（代替可能性），という 3 つの課題への解答を，多施設での実験を通して満たすことができた。

この試験法の第三者評価が OECD 第三者評価委員会において実施され、その結果として、1-bromohexane の偽陰性等の結果により、本モデルを用いた皮膚刺激性評価は不十分と判断された。そこで、製造元である株式会社　ジャパン・ティッシュ・エンジニアリング（J-TEC）において試験法の見直しが行われ、プロトコルの改良がなされた。この改良はプロトコルにとって重要な問題であると JaCVAM（Japanese Center for the Validation of Alternative Methods）運営委員会が判断したことから、厚生労働科学研究の支援を受け、この改良点を盛り込んだ改訂プロトコルを用いたバリデーション研究を実施することになった。

一方、STE（Short Time Exposure）試験は、ウサギ角膜由来の SIRC 細胞に被験物質を一定濃度、5 分間曝露した際の細胞生存率をエンドポイントとした簡便な眼刺激性試験代替法である。2008-2009 年に、日本動物実験代替法学会において、STE 試験の技術易習得性、施設間再現性と代替可能性を評価するため、5 施設によるバリデーション研究が実施され、高い技術易習得性が確認された。さらに、コード化された 25 被験物質によるバリデーションが実施され、

良い施設間再現性が確認された。本研究では、先回のバリデーション研究と合わせて被験物質の GHS 区分のバランスが最適になるように、主に Category2 の物質から選択したより多くの被験物質を用いて、先回のバリデーションに参加した 3 施設の協力を得て、代替可能性の再評価を目的とした。

B. 研究方法

B-1) 培養表皮モデル（LabCyte EPI-MODEL24）のバリデーション

OECD Guidelines for the Testing of Chemicals Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method内の[2] performance standardに記載されている20物質をコード化して参加3施設に配布し、改訂プロトコルを用いたバリデーションを実施した。

B-1-1) バリデーション実行委員会

委員長：小島　肇（国立衛研）、
委　員：加藤雅一（J-TEC）、大森　崇（同志社大学）

B-1-2) 参加施設

小林製薬株式会社、ファンケル、DSTC（薬物安全性試験センター）

B-1-3) トレーニング

2010年7月27日に国立衛研にて、改良プロトコルを用い、J-TECより技術指導がなされた。

B-1-4) 予備試験

各施設において、1-bromohexaneが陽性になるよう改良プロトコルのマスターのための予備試験が数回実施された。

B-1-5) 実施期間

バリデーションは2010年9月～11月の間に実施された。

B-1-6)改良プロトコルの概要

本研究は、先回のバリデーションで用いた同一の試験プロトコルに基づいて実施した。コード化された20被験物質をモデルに15分間処理した後、42時間後培養を行い、MTTアッセイで細胞生存率を求めた。50％細胞毒性を基準に陰陽性の判定を実施した。

改訂の主な点は、被験物質の洗い流し方である。洗う回数などに変更はないが、モデルの底面に水流を当てない、洗浄毎に洗浄液を切ることを徹底しない、水分除去に利用していたコットンパッドを利用しないなどが改良された。

B-2) STE法のバリデーション

B-2-1) バリデーション実行委員会

小島　肇（国立衛研）、林和彦、坂口斉（花王）、森本隆史（住友化学株式会社）、ICCVAM（Interagency Coordinating Committee on the Validation of Alternative Methods）代表、ECVAM代表

・データ解析チーム

大森　崇、音泉　卓（同志社大学）、寒水孝司（京都大学）

・被験物質管理チーム

小島　肇（国立衛研）、林和彦（花王）、森本隆史（住友化学株式会社）

B-2-2) 参加施設

株式会社カネボウ化粧品、ポーラ化成工業株式会社、ライオン株式会社

B-2-3) 実施期間

バリデーションは2010年8月～10月の間に実施された。

B-2-4) プロトコル概要

本研究は、先のSTE試験法バリデーションで用いた同一の試験プロトコルに基づいて実施した。コード化された40被験物質を用い、それぞれの試料の溶解性に基づき、生理食塩水、5%DMSO含有生理食塩水、及びミネラルオイルのいずれかを溶媒として選択し、5%、0.05%溶液を作成し、SIRC細胞に5分間暴露した後、MTTアッセイにより細胞生存率を求めた。計3回の実験を行い、平均値を求めた。

まず、5%試料液で評価し、STE試験における非刺激物、刺激物の刺激区分を行い、その刺激区分とGHS区分（非刺激物、刺激物）との一致性を評価した。次にSTE試験の予測モデルを用いた眼刺激ランクとGHSカテゴリー分類（非刺激物、刺激物：Category2及び刺激物：Category1）との一致性を評価した。

B-2-5) 追加実験

一部物質で施設間の結果が食い違った。その原因を明らかにするため、各施設で追加実験が実施された。

（倫理面への配慮）

倫理的な問題が生じる実験を実施しておらず、特に配慮すべき問題はない。

C. 研究結果

C-1) 培養表皮モデル（LabCyte EPI-MODEL24）のバリデーション　詳細な報告書を資料１に添付した。

- 追加バリデーションの結果、すべての施設で、1-bromohexane は陽性となった。他の物質の判定結果に変更はなかった。
- 3 回の SD が 18%を超えた場合もすべての施設で見られた。なお、これら物質はいずれも再試験で基準を満たした。
- その他の陰性、陽性対照物質は、すべての場合に基準を満たしていた。
- ３参加施設の内、１施設（Lab a）がコ

ード番号 4 を陽性と判定したため、specificity が、わずかに performance standard の基準(acceptance criteria 及び success criteria) を達成できなかった。 その他の結果は、すべて基準を満たしていた。

C-2) STE 法のバリデーション

詳細な報告書を資料 2 に添付した。

- 施設 2 及び 3 に試験不成立が見られた。不成立原因の多くは、陽性対照の基準範囲の逸脱であった。
- また、3 回の試験の SD が 15%を超えた場合も施設 2 及び施設 3 で見られた。なお、これら物質はいずれも再試験で基準を満たした。
- ブランク、溶媒対照、陽性対照値の施設間再現性は高かったが、やや施設 2 のバラツキが大きかった。
- コード化した被験物質については、3 施設がともに実施した 10 物質ではすべて同じ判定結果が得られ、先のバリデーション同様、施設間再現性が確認できた。
- また、先のバリデーションでも用いられた 2 物質も同様の判定結果となり、バリデーション間の再現性が確認された。
- 40 物質の中で、GHS 区分との比較では、コード番号 01 及び 25 の 2 物質の判定結果が異なった（GHS カテゴリー分類との比較では、コード番号 01、02 及び 25 の 3 物質が異なった） 。これらの物質の結果が異なった原因を明らかにするために行った追加実験で、コード番号 01 の原因は、溶媒の選択及び被験物質の溶解状態の相違であることがわかった。その他の物質については、カットオフ値（70%）前後の細胞毒性による陰陽性結果の違いと判断した。
- 先のバリデーション結果と結果が確定した 60～61 物質により一致率を算出したところ、 GHS 区分の一致率は 78%以上（61 物質）と高かった
  （ GHS カテゴリー分類との比較では 65%（60 物質）であった）

D. 考察

今回実施したバリデーションは、どちらも計画通り実験を実施でき、想定内の結果を得ることができた。

これらの結果を受け、皮膚刺激性試験代替法 LabCyte EPI-MODEL24 を用いた方法については、2011 年 1 月末にバリデーション報告書（資料 1）をまとめ、OECD 事務局に送付した。

一方、眼刺激性試験代替法 STE 法については、バリデーション報告書（資料 2）をまとめ、SPSF（資料 3）とともに、OECD に 2 月中旬に送付した。4 月の WNT にて、OECD TG の work plan に受け入れられた後が次のステップの始まりである。

すでに、今夏より、ICCVAM での peer review を予定しており、BRD（背景評価文書）の作成を花王とともに進めている。

E. 結論

皮膚刺激性試験代替法 LabCyte EPI-MODEL24 を用いた方法及び眼刺激性試験代替法 STE 法のバリデーションを実施し、どちらも計画通り実験を実施でき、想定内の結果を得ることができた。これらの結果を受け、両バリデーション報告書を OECD 事務局に提出した。

どちらのバリデーションも追加実験であったこともあり、開始から半年でバリデーション報告書まで作成することができた。

F. 参考文献

1） Masakazu Katoh, Fumiyasu Hamajima, Takahiro Ogasawara, and Ken-ichiro Hata (2009) Assessment of the Human Epidermal Model LabCyte EPI-MODEL for In Vitro Skin Irritation Testing According to the ECVAM-Validated Protocol , Journal of Toxicological Science, 34(3) 327-334.

2） OECD (2010) OECD Guidelines for the Testing of Chemicals No. 439: In vitro Skin Irritation assay. Paris, France: Organisation for Economic Cooperation and Development. [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html/].

3） Takahashi Y, Koike M, Honda H, Ito Y. Sakaguchi H, Suzuki H and Nishiyama N. (2008) Development of the short time exposure (STE) test: an in vitro eye irritation test using SIRC cells. Toxicology in Vitro 22:760-770.

4) Takahashi Y, Hayashi T, Watanabe S, Hayashi K, Koike M, Aisawa N, Ebata S, Sakaguchi H, Nakamura T, Kuwahara H and Nishiyama N. (2009) Inter-laboratory study of short time exposure (STE) test for predicting eye irritation potential of chemicals and correspondence to globally harmonized system (GHS) classification. The Journal of toxicological sciences 34:611-626.

5) Takahashi Y, Hayashi T, Koike M,

Sakaguchi H, Kuwahara H and Nishiyama N. (2010) An interlaboratory study of the short time exposure (STE) test using SIRC cells for predicting eye irritation potential. Cutaneous and ocular toxicology 29:77-90.
6) Sakaguchi H, Ota N, Omori T, Kuwahara H, Sozu T, Takagi Y, Takahashi Y, Tanigawa K, Nakanishi M, Nakamura T, Morimoto T, Wakuri S, Okamoto Y, Sakaguchi M, Hayashi T, Hanji T, Watanabe S (2011) Validation study of the Short Time Exposure (STE) test to assess the eye irritation potential of chemicals.,Toxicol In Vitro. 2011 Feb 1

G. 研究発表

1. 論文発表

1) Kojima, H.: Commentary to the Discussion on Topics 3, " In Vitro Test Approaches with Bettre Predictivity" at the 5th International Workshop on GenotoxicityTesting, Genes and Environment, 32(2), 40-42 (2010)
2) 小島肇夫：総合評価の方法、有用性化粧品の処方とその活用、鈴木正人監修、シーエムシー出版、東京、pp. 147-151 (2010)
3) Kojima, H, Takeyosh, i M, Sozu, T, Awogi, T, Arima, K, Idehara, K, Ikarashi, Y, Kanazawa, Y, Maki, E, Omori, T, Yuasa, A, Yoshimura, I.:Inter-laboratory validation of the modified murine local lymph node assay based on 5-bromo-2'-deoxyuridine incorporation. J Appl Toxicol. 31(1)63-74 (2010)
4) Yamamoto, N, Hirano, K, Kojima, H, Sumitomo, M, Yamashita, H, Ayaki, M, Taniguchi, K, Tanikawa, A, Horiguchi, M.: Cultured human corneal epithelial stem/progenitor cells derived from the corneal limbus. In Vitro Cell Dev Biol Anim. 46(9):774-80 (2010)
5) Kojima, H.: 3Rs Activities in Japan, AVLR8 Alternative Testing strategies, Progress report 2010, 266 (2010)
6) 小島肇夫、パイロジェン試験、大阪医薬品協会 会報第745号31-63 (2011)
7) 小島肇夫：動物実験代替法の現状と展望、創薬研究のストラテジー、pp. 41-48、株式会社金芳堂、東京 (2011)
8) 小島肇夫：動物実験の3Rにおける国内外の動向、ドージンニュース No. 138、1-9 (2011)
9) 柘植英哉、森充生、大庭澄明、大内正、寺田三郎、五島隆志、田邊豊重、山影康次、田中憲穂、渡辺美香、畔上二郎、大向英夫、小島肇、平成21年度「日本薬局方の試験法に関する研究」研究報告、輸液用ゴム栓試験法の見直し（第3報）－細胞毒性試験法の検討－、医薬品医療機器レギュラトリーサイエンス 42 (3) 258-271 (2011)

2. 学会発表

1) 山本直樹、谷川篤宏、内藤紘策、綾木雅彦、小島 肇、平野耕治、堀口正之：マウス水晶体上皮細胞の不死化細胞の作出、第114回 日本眼科学会総会 (2010.4)
2) 小島 肇：ヒトiPS細胞を用いた新規 *in vitro* 毒性評価系の構築、日本製薬工業協会セミナー (2010.5)
3) 小島 肇：パネルディスカッション 新しい感作性及び局所刺激性（皮膚・眼）試験法のOECDテストガイドライン, 日本トキシコロジー学会学術年会, 沖縄 (2010.6)
4) 小島 肇：医薬部外品、化粧品の Regulatory Science の展望, 第11回光老化研究会, 東京慈恵会医科大学 (2010.7)
5) Kojima.H., Global impact of 3'Rs on regulatory process: sharing experiences and future trends. XII International Congress of Toxicology, Barcelona, Spain (2010.7)
6) Kojima.H., Inoue, T. and Ohno.Y., JaCVAM's role of new alternatives to animal testing and international harmonization. XII International Congress of Toxicology, Barcelona, Spain (2010.7)
7) 小島 肇：昨今の国際バリデーション研究の進捗, 皮膚基礎研究クラスターフォーラム 第5回教育セミナー (2010.8)
8) 小島 肇：皮膚感作性試験のインビトロ代替法の現状, 日本免疫毒性学会学術大会, 独立行政法人国立環境研究所 大山記念ホール (2010.9)
9) Kojima, H., Arai, S. and Hojyo M., Importance of each human model and the optimal protocol for regulatory use of skin irritation assay. The 23rd Annual and International Meeting of the Japanese Association for Animal Cell Technology, Hokkaido University, Sapporo, Japan (2010.9)

10) 小島　肇：パイロジェン試験，大阪医薬品協会技術研究委員会，大阪 (2010)

11) 小島　肇、北條麻紀：3次元培養表皮モデルを用いるコメットアッセイの条件検討　第3報，日本環境変異原学会第39回大会、つくば(2010. 11)

12) 宇野芳文、小島　肇：インビボコメットアッセイ　JaCVAM国際バリデーション試験の進捗状況報告（第2報），日本環境変異原学会第39回大会、つくば（2010. 11)

13) 小島　肇、中村　牧、山口能宏、泉瑠名、鈴木民恵、萩原沙織、篠田伸介、加藤雅一 培養皮膚モデルLabCyte EPI-MODEL24を用いた皮膚刺激性試験代替法のバリデーション研究、日本動物実験代替法学会第23回大会、東京(2010. 12)

14) 小島　肇、桑原裕史、林卓巳、坂口眞由美、豊田明美、後藤　悠、中村恒彰 、渡辺真一、阿彦恭子 、大森　崇、音泉　卓、寒水孝)、森本隆史、林　和彦、坂口　斉：眼刺激性試験代替法（STE試験）バリデーション研究　第3報、日本動物実験代替法学会第23回大会、東京（2010. 12)

15) 小島　肇、北條麻紀：3次元培養表皮モデルを用いるコメットアッセイの条件検討日本動物実験代替法学会第23回大会、東京（2010. 12)

16) 小島　肇：S5　化学物質の有害性評価に関する代替試験法開発ー発癌性、発生毒性、免疫毒性ー今後の展望、日本動物実験代替法学会第23回大会、東京（2010. 12)

17) 小島　肇：培養皮膚モデルを用いた皮膚刺激性評価の現状、第10回ヒューマンサイエンス研究資源バンクセミナー、大阪（2011. 1)

18) 小島　肇：動物実験代替法における国際動向、日本動物実験代替法学会・JaCVAM合同ワークショップ　動物実験の3Rにおける国際動向、東京(2011. 2)

19) 小島　肇：皮膚細胞研究の応用とその可能性、日本化粧品技術者会大阪支部第15回勉強会　ワークショップ、大阪（2011. 2)

20) Kojima, H.: The Japanese Center for the Validation of Alternative Methods (JaCVAM): Recent ICATM contributions and Future Plans, Information Session: The International Cooperation on Alternative Test Methods (ICATM): Translating Science to Provide Improved Public Health Safety Assessment Tools, 50th Annual SOT meeting, Washington D.C.(2011)

21) Kojima, H. and Hojyo, M.: Optimal conditions for performance of the comet assay using a three-dimensional human epidermal model, 50th Annual SOT meeting, Washington D.C.(2011)

22) W Casey, P Ceger, F Deal, D Allen, G Clark, P Pazos, E Grignard, J de Lange, S Bremer, M Nakamura, H Kojima, A Ono, W Stokes.: Final Results of an International Validation Study of an *In Vitro* ER TA Test Method in BG-1 cells, 50th Annual SOT meeting, Washington D.C.(2011)

23) F Deal, W Casey, P Ceger, D Allen, C Yang, M Nakamura, H Kojima, A Ono, HJ Yoon, SY Ha[7], W Stokes:　International Validation Study of an *In Vitro* Cell Proliferation Test Method for Screening Potential Estrogenic Agonists and Antagonists in MCF-7 cells, 50th Annual SOT meeting, Washington D.C.(2011)

24) J Kulpa-Eddy, R McFarland, R Isbrucker, M Halder, H Kojima, B Jones, NW Johnson, D Allen, E Lipscomb, S Morefield, W Casey, W Stokes: International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing, 50th Annual SOT meeting, Washington D.C.(2011)

25) 小島　肇：日本における動物実験代替法の現状、シンポジウムS2H27　アジアにおける動物実験代替法の展開、第84回日本薬理学会年会、パシフィコ横浜（2011. 3)

26) 小島　肇：動物実験代替法の行政的受け入れと国際協調、シンポジウムS30 レギュラトリーサイエンスは社会にどう役立っているかー薬学系人材の役割と活躍の場を知るー、日本薬学会第131回年会、静岡（2011. 3)

G. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

H. 添付文書

資料１）JaCVAM Report: Me-too Validation Study of *in vitro* Skin Irritation Test using LabCyte EPI-MODEL24 (LabCyte EPI-MODEL24 SIT)

資料２）Short Time Exposure (STE) Test, 2nd Phase Validation Study Report (Version 1.1)

資料３）SPSF

JaCVAM Report:

Me-too Validation Study of *in vitro* Skin Irritation Test using LabCyte EPI-MODEL24

(LabCyte EPI-MODEL24 SIT)

January 24, 2011

LabCyte Validation Management Team

Members of LabCyte Validation Management Team

Mr. Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods
Mr. Masakazu Katoh, Japan Tissue Engineering Co. Ltd.
Mr. Toshiro Yokouchi, Japan Tissue Engineering Co. Ltd.

Biostatistian group

Mr. Takashi Omori, Doshisya Univ.

Participant laboratories

KOBAYASHI Pharmaceutical Co., Ltd. (Mr. Yoshihiro Yamaguchi and Ms. Maki Nakamura)

Fancl Corp. (Ms. Tamie Suzuki and Ms. Runa Izumi)

Drug Safety Testing Center Co., Ltd. (Mr. Shinsuke Shinoda and Ms. Saori Hagiwara)

Abbreviations

CAS: Chemical Abstracts Service
ECVAM: European Centre for the Validation of Alternative Methods
ESAC: ECVAM Scientific Advisory Committee
GHS: Globally Harmonised System
GLP: Good Laboratory Practice
ISO: International Organization for Standardization
JaCVAM: Japanese Centre for the Validation of Alternative Methods
JSAAE: Japanese Society for Alternative to Animal Experiments
J-TEC: Japan Tissue Engineering Co. Ltd.
NIHS: National Institute of Health Sciences
OECD: Organisation for Economic Co-operation and Development
QC: Quality control
PBS: Phosphate buffered saline
PS: Performance Standard
RhE: Reconstructed human epidermis
SD: Standard deviation
SLS: Sodium lauryl sulphate
SPSF: Standard Project Submission Form
TG: Test Guideline
UN: United Nations
VMT: Validation management team
VRM: Validated reference method
WNT: National Coordinators of the Test Guideline Project

Contents

Appendices:

1. Validation study of in vitro skin irritation test using LabCyte EPI-MODEL24 (FINAL report)
2. Summary report of the peer review panel on labCyte EPI-MODEL24 in vitro test method for the assessment of skin irritation potential of chemicals
3. Additional validation of the RhE tests: LabCyte EPI-MODEL24 acute skin irritation prediction
4. Skin Irritation test protocol using the reconstructed human model "LabCyte EPI-MODEL24" (ver.8.2)
5. Original data in the catch-up validation study
6. Test records and data sheet in KOBAYASHI Pharmaceutical Co., Ltd. (example: in Japanese)
7. Result of QC for each lot of LabCyte EPI-MODL24
8. Detailed review documents on the "LabCyte EPI-MODEL24"
9. Masakazu Katoh, Fumiyasu Hamajima, Takahiro Ogasawara, and Ken-ichiro Hata (2009) Assessment of the Human Epidermal Model LabCyte EPI-MODEL for *In Vitro* Skin Irritation Testing According to the ECVAM-Validated Protocol , Journal of Toxicological Science, 34(3) 327-334.
10. OECD TG 439, *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method

1. Goal statement

- The ultimate goal of the test strategy is to replace the regulatory Draize skin irritation test to meet the OECD (Organisation for Economic Co-operation and Development) TG (Test Guideline) 404 (OECD, 2002).
- The primary goal of this catch-up validation study is to evaluate the ability of *in vitro* tests to reliably discriminate skin irritant (I) from non-irritant (NI) chemicals, as defined by the OECD and UN (United Nations) proposal for GHS (Globally Harmonised System) for the classification and labelling of skin irritation (category 1/category 2; no category; Anon., 2003).

2. Objective

The OECD Working group of the National Coordinators of the Test Guideline Project (WNT) accepted the TG No.439: *in vitro* skin irritation test guideline in March 2010. This TG addresses the human health endpoint of skin irritation. Three validated test methods currently adhere to this TG. Prevalidation, optimization and validation studies have been completed for an *in vitro* test method that uses a Reconstructed human epidermis (RhE) model. This method is commercially available as EpiSkin™ and has been designated as the Validated Reference Method (VRM). Two other commercially available *in vitro* skin irritation RhE test methods, namely the EpiDerm™ SIT (EPI-200) and SkinEthic™ RHE test methods, have shown similar results to the VRM according to Performance Standard (PS) - based validation.

On the other hand, another *in vitro* test system that employs a RhE model (LabCyte EPI-MODEL24) has progressed through protocol optimization as a skin irritation test. A multi-laboratory assessment of this system was performed according to several ECVAM (European Centre for the Validation of Alternative Methods) performance standards (ESAC: ECVAM Scientific Advisory Committee statement, 2007, 2008, 2009). The present objective added the Japanese RhE and other similar models to adhere to the OECD TG 439. A me-too validation study was conducted to assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of this test system. The study included a challenging set of 20 test chemicals that would meet the performance standard set forth in the TG No.439. The validation study was undertaken in accordance with the principles and criteria documented in the OECD *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment* (No. 34, OECD, 2005) and according to the Modular Approach to validation (Hartung *et al.* 2004).

3. Background

Researchers in Japan aimed to include the Japanese RhE (LabCyte EPI-MODEL24: LabCyte EPI-MODEL24 SIT) *in vitro* skin irritation test in the TG as an addition to other similar models in the SPSF (Standard Project Submission Form) that were submitted to OECD WNT by EU delegate in April 2008. The validation study described herein was performed between April 2008 and January 2009 by the Validation Management Team (VMT), with financial support from the Japanese Society for Alternative to Animal Experiments (JSAAE).

The study conducted by VMT referenced the original ECVAM performance standard (ECVAM 2007), in which a range of appropriate models was described as one of the acceptance criteria. After completion of the first phase of the study in August 2008, the VMT discussed the criteria for the Labcyte EPI MODEL24 SIT. The VMT decided that the criteria were not set because there was not enough data to define this kind of range at that time. Furthermore, the pre-specification was considered to have too narrow a range and the draft OECD TG came under review at the time of the discussions. As a result, the reliability of the model was considered to be high. Therefore, the VMT decided that criteria for the range may not be needed for this model, while the check for variation should be done.

Based on validation results of the Labcyte EPI-MODEL24 SIT, a member of the Japanese WNT submitted an SPSF of it to OECD in January 2009 and the OECD WNT accepted this assay in its working plan in May 2009. The VMT submitted the first validation report to the OECD secretary in July 2009. On the other hand, we confirmed an additional validation study in reference to the new ECVAM performance standards (ESAC statement, 2009) to be revised for the TECD TG between

April and May of 2009 and submitted the second validation report and Background Review Documents to the OECD secretary in August 2009.

Using these documents, OECD performed a peer review and we received the peer review report from the OECD secretary in March 2010. The OECD peer review on the LabCyte EPI-MODEL24 SIT *in vitro* test method for the assessment of skin irritation potential of chemicals was performed with the validation report and background review documents. In the summary report, the peer review panel indicated that the issue of misclassifying 1-bromohexane should be resolved.

To resolve this issue, the protocol was revised by Japan Tissue Engineering (J-TEC). To confirm general versatility with the revised protocol, we planned an additional validation study according to the OECD performance standard.

4. Test methods

4-1. Reconstructed human cultured epidermal model

LabCyte EPI-MODEL24 is a new, commercially available RhE model produced by J-TEC. It consists of normal human epidermal keratinocytes whose biological origin is neonate foreskin. In order to expand human keratinocytes while maintaining their phenotype, the cells are cultured with 3T3-J2 cells as a feeder layer (Rheinwald and Green, 1975; Green, 1978). Reconstruction of a human cultured epidermis is achieved by cultivating and proliferating keratinocytes on an inert filter substrate (surface area 0.3 cm^2) at the air-liquid interface for 13 days with an optimized medium containing 5% fetal bovine serum. The process generates a multilayer structure consisting of a fully differentiated epithelium with features of the normal human epidermis, including a stratum corneum. LabCyte EPI-MODEL24 is embedded in an agarose gel containing nutrient solution and shipped in 24-well plates at around 18 °C (Kato, 2009: Appendix 4).

4-2. MODEL supplier

According to OECD Good Laboratory Practice (GLP) Consensus Document No.5 "*Compliance of Laboratory Suppliers with GLP Principles*", responsibility for the quality and fitness for use of equipment and materials rests entirely with the management of the test facility (OECD, 1999).

The acceptability of equipment and materials in laboratories complying with GLP must therefore be guaranteed to any regulatory authority to which studies are submitted. In some countries where GLP has been implemented, suppliers belong to national regulatory or voluntary accreditation schemes (for laboratory animals) that can provide users with additional documentation proving that they are using a test system of defined quality.

Audits performed during the study focused on procedures established to guarantee a defined quality of the tissue models.

5. Validation management structure

The management structure of the study is shown in Figure 1.

5-1. Validation management team

The VMT played a central role in overseeing the conduct of the validation study, including implementation of the following aspects of the study:

1) Goal statement
2) Project plan including objective
3) Study protocol / amendments
4) Outcome of QC (Quality Control) audits
5) Test chemicals
6) Data management procedures
7) Timeline / study progression
8) Data collection and analysis
9) Study interpretation and conclusions
10) Reports and publications

The VMT made the final decision on which laboratories would participate in the validation study.

Responsible VMT members:

Chair (Hajime Kojima, JaCVAM: Japanese Centre for the Validation of Alternative Methods)
The sponsor representative, LabCyte EPI-MODEL 24 suppliers and lead lab (Masakazu Katoh: J-TEC)

5-2. Chemical selection, acquisition, coding, and distribution

1) Definition of selection criteria
2) Chemical selection
3) Liaise with suppliers
4) Final check of chemicals provided
5) Acquisition
6) Coding
7) Distribution

Responsible VMT member:
Hajime Kojima, JaCVAM

5-3 . Independent biostatisticians

1) Approve spreadsheets

Responsible VMT member:
Takashi Omori: Doshisya Univ.

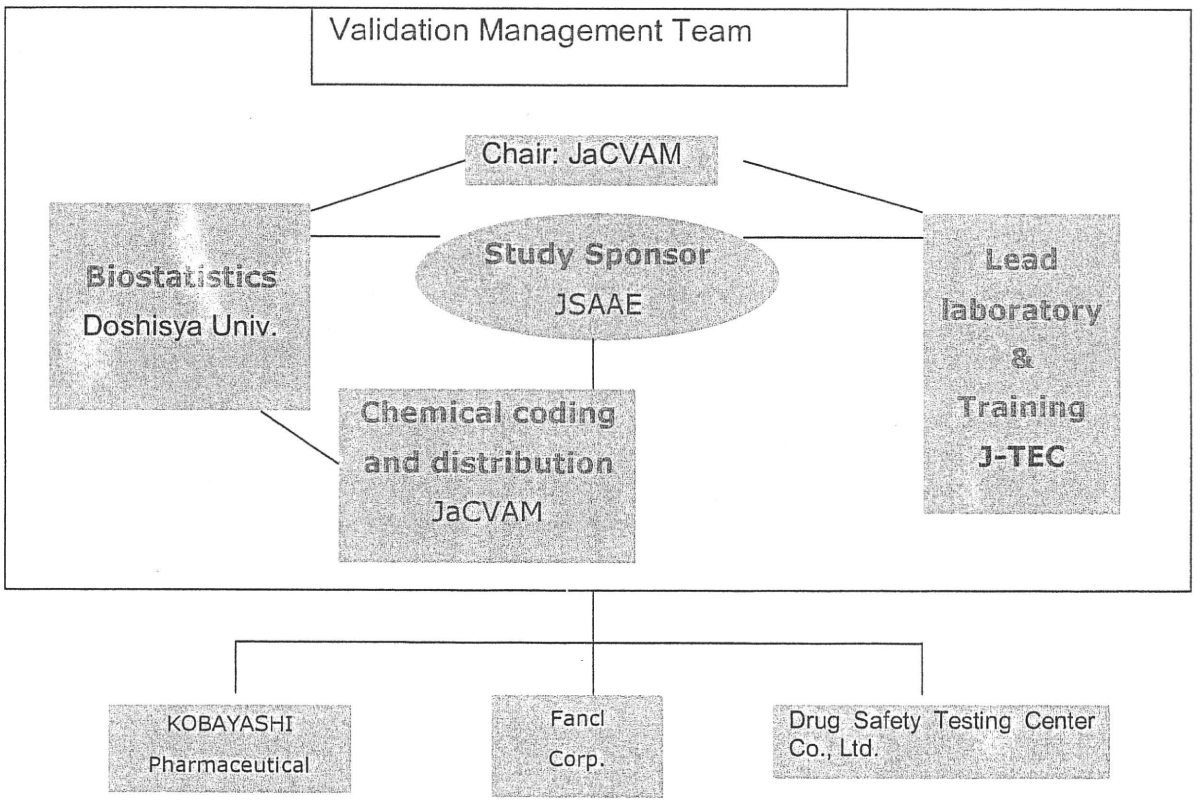

Fig. 1. Management structure of me-too validation study on the LabCyte EPI-MODEL24 SIT

5-4. Participating laboratories

The laboratories participating in the study are shown in **Fig. 1**.

The following three laboratories participated in the validation study for the evaluation of the LabCyte EPI-MODEL 24 assays:

- Laboratory a — KOBAYASHI Pharmaceutical Co., Ltd. (Yoshihiro Yamaguchi and Maki Nakamura)
- Laboratory b — Fancl Corp. (Tamie Suzuki and Runa Izumi)
- Laboratory c — Drug Safety Testing Center Co., Ltd. (Shinsuke Shinoda and Saori Hagiwara)

A lead laboratory was also identified as J-TEC (Mr. Masakazu Kato and Mr Toshihiro Yokouchi). This laboratory did not participate in the validation study.

Each laboratory was responsible for complying with GLP principles and specifying QC aspects of the study.

5-5. Sponsorship

The study was managed and financed by JaCAM and J-TEC.

1) JaCVAM financially supported the following activities:
- management of the study (VMT meetings)
- provision of independent statistical support (VMT meetings)
- purchase, coding, and distribution of chemicals to the laboratories
- independent QC audit of the data
- publication of the study results

2) J-TEC supported the following aspects of the study:
- the lead laboratories for the test method
- training of the participating laboratories
- independent QC audit of the LabCyte EPI-MODEL24 SIT
- financial assistance of the participated laboratories

6. Study design and test period

Before initiation of the validation study, J-TEC delegates conducted a training course on using LabCyte EPI-MODEL24 SIT with a revised protocol (ver.8.1) at the National Institute of Health Sciences (NIHS) on July 27, 2010. All technicians from each laboratory participated in this training course. Furthermore, all laboratories participated in preliminary testing. After this preliminary test, the protocol was revised to ver.8.2 to include detailed descriptions of the washing protocol. Three laboratories attended an additional validation study after one laboratory was not able to obtain a positive test result with 1-bromohexane.

The preliminary test was conducted by three laboratories between August and September of 2010. The duration of validation study was between September and November of 2010.

7. Test chemicals

7-1. Chemical selection

To meet the OECD performance standard, the VMT selected 20 chemicals for testing (Table 1). The final approval of the chemicals proposed by JaCVAM was the responsibility of the VMT. To avoid any potential for bias in the final selection, laboratory representatives on the VMT did not participate in these discussions, nor were they made aware of the chemicals finally approved for testing in the validation study.

Table 1. Minimum List of Reference Chemicals for Determination of Accuracy and Reliability Values for Similar or Modified RhE Skin Irritation Test Methods and Codes

No.	Name	CAS number	UN GHS *in vivo* Cat.	Storage	Chemical code		
					Lab a	Lab b	Lab c
1	1-bromo-4-chlorobutane	6940-78-9	No Cat.	RT	B-261	D-281	G-301
2	diethyl phthalate	84-66-2	No Cat.	RT	B-262	D-282	G-302
3	naphthalene acetic acid	86-87-3	No Cat.	RT	B-263	D-283	G-303
4	allyl phenoxy-acetate	7493-74-5	No Cat.	RT	B-264	D-284	G-304
5	isopropanol	67-63-0	No Cat.	RT	B-265	D-285	G-305
6	4-methylthio-benzaldehyde	3446-89-7	No Cat.	RT	B-266	D-286	G-306
7	methyl stearate	112-61-8	No Cat.	RT	B-267	D-287	G-307
8	heptyl butyrate	5870-93-9	No Cat. (*Optional Cat. 3*)	RT	B-268	D-288	G-308
9	hexyl salicylate	6259-76-3	No Cat. (*Optional Cat. 3*)	RT	B-269	D-289	G-309
10	cinnamaldehyde	104-55-2	No Cat. (*Optional Cat. 3*)	2-8C	B-270	D-290	G-310
11	1-decanol	112-30-1	Cat.2	RT	B-271	D-291	G-311
12	cyclamen aldehyde	103-95-7	Cat.2	RT	B-272	D-292	G-312
13	1-bromohexane	11-25-1	Cat.2	RT	B-273	D-293	G-313
14	2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl	86604-75-3	Cat.2	RT	B-274	D-294	G-314
15	di-n-propyl disulphide	629-19-6	Cat.2	RT	B-275	D-295	G-315
16	potassium hydroxide 5%	1310-58-3	Cat.2	RT	B-276	D-296	G-316
17	benzynethiol,5-(1,1-dimethylethyl) -2-methyl	7340-90-1	Cat.2	RT	B-277	D-297	G-317
18	1-methyl-3-phenyl-1-piperazine	5271-27-2	Cat.2	RT	B-278	D-298	G-318
19	heptanal	111-71-7	Cat.2	RT	B-279	D-299	G-319
20	1,1,1-trichloroethane	71-55-6	Cat.2	RT	B-280	D-300	G-320

1) CAS No.: Chemical abstracts service registry number.

7-2. Chemical coding and distribution

Independent coding and distribution of chemicals were contracted out by JaCVAM to an independent laboratory. The company's name is certified according to ISO (International Organization for Standardization) 9001 and GLP, and has proven experience of reliable services. The codes were provided by JaCVAM.

8. Protocol

8-1. Protocol of the skin irritation test with LabCyte EPI-MODEL24 SIT

According to the suggestion of the OECD peer review panel, J-TEC resolved the false-negative issues of 1-bromohexane. The SOP (Standard Operating Procedure: ver.8.1) included a modified washing protocol in a revision dated June 30, 2010. Modifications to the washing protocol are shown in Table 2 (Detailed process described in Appendix 7). Using the revised SOP, the validation study was performed to show clear data and addressed comments of the OECD peer review panel.

Table 2. Modification points of washing protocol between SOP ver.7.1 and SOP ver.8.2.

Modification points	SOP ver.7.1	SOP ver.8.2
1. Handling the PBS stream from washing bottle	It was not defined.	The revision specified to avoid hitting the tissue surface directly with the PBS stream.
2. Removal of PBS by tapping	It was not defined.	It was briefly defined.
3. Correct use of the cotton pad	It was not defined.	It was defined to avoid touching the tissue surface directly with the cotton pad.

LabCyte EPI-MODEL 24 tissues were shipped from the supplier on Mondays and delivered to recipients on Tuesdays. Upon receipt, the tissues were aseptically removed from the transport agarose medium, transferred into 24-well plates (BD Biosciences, CA, USA) with the assay medium (0.5 mL), and incubated overnight (37°C, 5% CO_2 humidified atmosphere). On the following day, the tissues were topically exposed to the test chemicals. Liquids (25 µL) were applied with a micropipette, and solids (25 mg) were applied from microtubes and moistened with 25-µL sterile water. If necessary, the mixture was gently spread over the surface of the epidermis with a microspatula. Viscous liquids were applied using a cell-saver-type tip with a micropipette. Each test chemical was applied to three tissues. In addition, three tissues serving as negative controls were treated with 25-µL distilled water, and three tissues serving as positive controls were exposed to 5% SLS (sodium lauryl sulphate). After a 15-minute exposure, each tissue was carefully washed with PBS (phosphate buffered saline, Invitrogen, CA, USA) 10 times using a washing bottle to remove any remaining test chemical from the surface. The blotted tissues were then transferred to new 24-well plates containing 1 mL of fresh assay medium.

The treated and control tissues were incubated for 42 hours (37 °C, 5% CO_2 humidified atmosphere). When the 42-hour incubation period was complete, blotted tissues were transferred to new 24-well plates containing 0.5 mL of freshly prepared MTT medium (1 mg/mL; Dojindo Co., Kumamoto, Japan) for the MTT assay. Tissues were incubated for 3 hours (37 °C, 5% CO_2 humidified atmosphere) and then transferred to microtubes containing 0.3 mL isopropanol, which completely immersed the tissue. Formazan extraction was performed at room temperature, and the tissues were allowed to stand overnight. Subsequently, 200-µL extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and 650 nm as a reference absorbance, with isopropanol as a blank.

The tissue viability was calculated as a percentage relative to the viability of the negative controls. The median of three values from identically treated tissues was used to classify a chemical according to the prediction model.

8-2. Prediction model of skin irritation

In this study, the prediction model (acceptability criteria and positive criteria) of skin irritation potential with LabCyte EPI-MODEL24 SIT was set to refer to the conditions for the OECD TG 439 and its Performance Standards.

8-2-1) Acceptance criteria on the RhE test method components

According to paragraph 27; acceptability criteria in the OECD TG 439, tissues treated with the negative controls and positive controls, *i.e.* 5% aqueous SLS, should reflect their ability to respond to an irritant chemical under the conditions of the test method. Associated and appropriate measures of variability between tissue replicates should be defined.

1) OD_{NC} of the negative control is greater than 0.7.
2) The viability of the positive control (5% aqueous SLS) is less than 40%.
3) If standard deviations (SDs) are used they should be within the one-sided 95% tolerance interval calculated from historical data; for the VRM SD < 18%.

8-2-2) Positive criteria

The OD values obtained with each test sample can be used to calculate the percentage of viability normalized to the No Category, which is set to 100%. The cut-off value for percentage of cell viability distinguishing irritant from non-classified test chemicals and the statistical procedure(s) used to evaluate the results and identify irritant chemicals, should be clearly defined, documented, and proven to be appropriate. The cut-off values for the prediction of irritation are given below:
The test chemical is considered to be an irritant to skin in accordance with GHS category 2 if the tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50%.
Depending on the country and regional regulatory requirements, the test chemical may be considered as a No Category if the tissue viability after exposure and post-treatment incubation is more than (>) 50%.

8-2-3) Study acceptance criteria

It is possible that one or several tests with one or more test chemicals do not meet test acceptance criteria for the test and control chemicals or are not acceptable for other reasons. To complement missing data, a maximum number of two additional tests for each test chemical is admissible ("retesting"). Because retesting requires concurrent testing with a positive control and negative control, a maximum number of two additional runs may be conducted for each test chemical.

It is conceivable that even after retesting, the minimum number of three valid runs required for each tested chemical is not obtained for every Reference Chemical in every participating laboratory, leading to an incomplete data matrix. In such cases the following three criteria should all be met in order to consider the datasets acceptable:

1. All 20 Reference Chemicals should have at least one complete run sequence.
2. In each of at least three participating laboratories, a minimum of 85% of the run sequences need to be complete (for 20 chemicals, three invalid run sequences are allowed in a single laboratory).
3. A minimum of 90% of all possible run sequences from at least three laboratories need to be complete (for 20 chemicals tested in three laboratories, a total of six invalid run sequences are allowed).

8-2-4) Rules

The calculation of the reliability and accuracy values of the proposed test method should be done considering all four criteria below, ensuring that values for reliability and relevance are calculated in a predefined and consistent manner:

1. Only data of runs from complete run sequences qualify for calculation of within- and between-laboratory variability and predictive capacity (accuracy) of the test method.
2. The final classification for each Reference Chemical in each participating laboratory should be obtained by using the mean value of viability over the different runs of a complete run sequence.
3. Only data obtained for chemicals that have complete run sequences in all participating laboratories qualify for calculation of between-laboratory variability of the test method.
4. Calculation of the accuracy values should be done on the basis of individual laboratory predictions obtained for the 20 Reference Chemicals by the different participating laboratories.

In this context, a **run sequence** consists of three independent runs from one laboratory for one test chemical. A **complete run sequence** is a run sequence from one laboratory for one test chemical where all three runs are valid. This means that any single invalid run invalidates an entire run sequence of three runs.

Within-laboratory reproducibility

An assessment of within-laboratory reproducibility should show that the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of 20 Reference Chemicals within one single laboratory is equal to or higher than (≥) 90%.

Between-laboratory reproducibility

An assessment of between-laboratory reproducibility is not essential if the proposed test method is to be used in a single laboratory only. For methods to be transferred between laboratories, the concordance of classifications (UN GHS Category 2 and No Category) obtained in different, independent test runs of 20 Reference Chemicals between preferentially a minimum of 3 laboratories should be equal or higher than (≥) 80%.

Predictive capacity (accuracy)

The accuracy (sensitivity, specificity and overall accuracy) of the proposed similar or modified test method should be comparable or better to that of the VRM, taking into consideration information relating the species of interest (Table 3). The sensitivity should be equal to or higher than (≥) 80%. However, an additional restriction applies to the sensitivity of the proposed *in vitro* test method; only two *in vivo* Category 2 chemicals, *1-decanol* and *di-n-propyl disulphide,* may be misclassified as a No Category by more than one participating laboratory. The specificity should be equal to or higher than (≥) 70%. No restrictions with regard to specificity of the proposed *in vitro* test method were applied; any participating laboratory may misclassify any *in vivo* No Category chemical as long as the final specificity of the test method is within the acceptable range. The overall accuracy should be equal to or higher than (≥) 75%. Although the sensitivity of the VRM calculated for the 20 Reference Chemicals listed in Table 1 is equal to 90%, the defined minimum sensitivity value required for any similar or modified test method to be considered valid is set at 80% because both *1-decanol* (a borderline chemical) and *di-n-propyl disulphide* (a false negative of the VRM) are known to be non-irritant chemicals in humans, although they have been identified as irritants in the rabbit test. Since RhE models are based on cells of human origin, they may predict these chemicals as non-irritant (UN GHS No Category).

Table3. Required predictive values for sensitivity, specificity and overall accuracy for any similar or modified test method to be considered valid.

Sensitivity	Specificity	Overall Accuracy
≥ 80%	≥ 70%	≥ 75%

8-3. Data collection, handling, and analysis

The independent biostatistician for the study collected and organized the data using specific data collection software (Datasheet5.0:20090430.xls). They worked in close collaboration with JaCVAM (Hajime Kojima). After decoding the data, JaCVAM performed statistical analyses. The data management procedures and statistical tools applied were approved by the VMT.

8-4. Quality assurance, GLP

Laboratories

All participating laboratories conducted research following OECD GLP-like principles.

QC aspects

JaCVAM (Hajime Kojima) assured the quality of all the data and records. After the validation study, all study documents were submitted to the chairperson of VMT and only data sheets were forwarded by e-mail to the biostatistician. All data sheets from one participating laboratory, KOBAYASHI Pharmaceutical Co., Ltd. are provided as an example in Appendix 6. The chairperson reviewed the contents of the study documents and clarified illegible or unclear content by contacting each group by e-mail or telephone.

9. Results

9-1 Comments in the datasheets

A few comments from each laboratory are listed in Table 4. Application of potassium hydroxide (5%aq) (B276, B296, and B288) caused the model's layers to be desquamated. Upon application of B301, B304, B306, and B310, the cups were discoloured and crystallized. The VMT judged that these occurrences had no effect on the results of the study.

Table 4. Comments on the datasheets (Viability)

Lab	Exp.No.	Lot	Date	Comments
a	Main-1	LCE24-100906-A	2010/9/9	The model's layers treated by B-276 were desquamated
b	Main-1	LCE24-100906-A	2010/9/8	The model's layers treated by B-296 were desquamated
b	Main-2	LCE24-100913-A	2010/9/15	The model's layers treated by B-288 & B-296 were desquamated
c	Main-1	LCE24-100830-A	2010/9/6	Cups treated by B-301, 304, 306 and 310 were discoloured.
c	Main-2	LCE24-100913-A	2010/9/20	Cups treated by B-301, 304, 306 and 310 were discoloured.
c	Main-3	LCE24-100920-A	2010/9/27	Cups treated by B-301, 304, 306 and 310 were discoloured.

9-2 Negative control

Table 5 shows the absorbance values for the negative control. All data for the negative control met the acceptance criteria.

Table 5. Viability of negative control

Laboratory	Average OD/ triplicate tissues	Average, SD at all OD
Lab a	0.88	0.91±0.05
	0.87	
	0.92	
	0.98	
Lab b	1.03	1.03±0.06
	1.02	
	1.13	
	0.98	
	0.98	
Lab c	1.09	1.07±0.09
	0.98	
	1.01	
	1.06	
	1.20	