



Assessment of Human Skin Irritation: Validation of *In Vitro* Models

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Executive Summary

The worldwide drive to refine, reduce and/or replace animal-based testing of cosmetics and personal care products has accelerated and is quickly transitioning from "laudable goal" into validated studies targeted to the interests of the regulatory community in the United States and European Union.

This white paper summarizes the scientific findings from two recently published studies performed in support of the EU's efforts to validate reconstructed skin kits (*in vitro* skin models) as alternative methods for the assessment of several parameters, including human skin irritation [1] [2]. The 3 year project, '*Testing and Improvement of Reconstructed Skin Kits in Order to Elaborate European Standards*', is a comprehensive multi-laboratory, multi-country project headed by L'Oreal (France), and including Beiersdorf (Germany), Wella Cosmital (Switzerland) and Leiden University (The Netherlands) sponsored by the European Union (EC DG XII). Importantly, there is precedence for data generated in EU (ECVAM) studies forming the core data set for submission to US regulators thru the ICCVAM process [3].

The project's purpose was to characterize several commercially available *in vitro* skin models with regard to tissue architecture and lipid composition, and to assess those models' utility in skin irritation, percutaneous absorption and metabolism studies. With regard to skin irritation, the major goals were to identify the *in vitro* test method endpoint(s) that best correlated *in vitro* test results to skin irritation symptoms observed in human subjects (best *in vivo* to *in vitro* correlation) and to identify the *in vitro* skin model(s) that demonstrated both high relevance and high reproducibility.

MatTek Corporation's EpiDerm™ human cell-derived, organotypic *in vitro* skin model was evaluated in both studies and performed exceedingly well in both. Key points from these studies as they relate to EpiDerm and the assessment of skin irritation include:

- **Best Endpoint Relevance** - The MTT ET-50 Tissue Viability Assay¹ was found to be the most predictive endpoint for human skin irritation (the best *in vivo* to *in vitro* correlation).
- **Best Skin Model Relevance** - EpiDerm achieved the best *in vivo* to *in vitro* correlation of all of the *in vitro* skin models tested (R = 0.94). Ranking was based on MTT ET-50 Tissue Viability Assay.
- **Best Skin Model Reproducibility** - EpiDerm had the best intra-lot and inter-lot reproducibility (lowest coefficients of variation) of all the *in vitro* skin models tested, based on MTT ET-50 Tissue Viability Assay results.

¹ MTT ET-50 Assay: a quantitative method for evaluating a tissue's viability in response to external factors. ET-50: time of exposure for a test article to reduce tissue viability to 50%.

To date, EpiDerm has been formally validated by European regulators [4], and proposed for formal acceptance by American regulators [3], as an alternative to animal testing for predicting the dermal corrosivity of new chemical and cosmetic formulations. EpiDerm is also performing superbly in ongoing phototoxicity validation testing.

EpiOcular™, another MatTek organotypic *in vitro* tissue model designed to assess ocular irritancy, is in the process of being formally validated as an alternative to the Draize Rabbit Eye Test. This effort is being sponsored by Colgate-Palmolive [5] via the ICCVAM [6] process in the United States. Colgate organized this formal multi-laboratory initiative with participation by 3M Corporation and Kimberly-Clark [7]. Avon Products recently presented EpiOcular-based research that established a reference database of *in vitro* ocular irritation scores for a cross-section of currently marketed cosmetic and personal care products [8]. Avon researchers also found that data generated using the EpiOcular model allowed them to distinguish differences in mild, milder and mildest ("sub-draize") formulations.

In Europe, ECVAM has identified EpiOcular as a promising alternative approach for ocular irritation testing [5] with preliminary studies by ZEBET (Germany's Center for Documentation and Evaluation of Alternative Methods to Animal Experiments).

What do these study results mean to cosmetics and personal care product manufacturers, and to their ingredient suppliers? If they sell their products in the European Union, their Product Development/Product Safety Departments should be actively considering alternative toxicological test methods. They should also seek assurance that those alternative test methods are based on *in vitro* models that are either accepted by regulators, or are performing well in ongoing validation testing that meets regulatory criteria, such as MatTek's EpiDerm *in vitro* skin model and EpiOcular *in vitro* model for ocular irritation.

Introduction

With the publication of *The Principles of Humane Experimental Technique*, William Russell and Rex Burch [9] introduced a progressive concept that would lay down the tenets for new international laws dealing with the use of animals in both research and regulatory process. The book describes the three R's - *reduction* as a means of lowering the number of animals used to obtain information of a given amount and precision, *refinement* as any development leading to a decrease in the incidence or severity of procedures applied to those animals which have to be used and *replacement* as any scientific method employing non-sentient material which may replace methods which use conscious living vertebrates. In 1991, the European Commission and Member States implemented a legislative policy specifically to encourage research into the development and validation of alternative techniques which could provide the same level of information as that obtained in experiments using animals, or would involve fewer animals or would entail less painful procedures *Directive 86/609/EEC* [10]. The legislation led to the creation of the European Centre for Validation of Alternative Methods (ECVAM).

Additional emphasis was placed on the third R: replacement, in 1993, when the Council of Ministers to the European Communities passed a "Cosmetics Directive" which banned "the marketing in Europe of cosmetic products or ingredients tested on animals after January 1, 1998, unless alternative methods are insufficiently validated"[11]. Along the animal ethics continuum, animals used for the purpose of medical research can be rationalized more easily in minds of the populace than those used for cosmetic and personal care product testing. The policy boosted the amount of public and private research and resources devoted to the development of viable alternatives to the animal tests routinely used for the purpose of approving new products [12].

The Directive was, however, partly a political response to public opinion and it turned out that the deadline set by the ministers was overly optimistic from a technological perspective. The early-1990s had marked the early development phase for the science of *in vitro* toxicology. Those first generation *in vitro* skin models considered for approval produced inconsistent results, and thus made them unacceptable in terms of their ability to assure the public safety. As a result, these first generation models tested never entered the validation process. Over the following decade, technological advances prompted the development of better and more complex models [13]. For example, EpiDerm, a 3-dimensional, organotypic skin model that closely parallels human skin, was created during this later generation of *in vitro* models.

The EpiDerm model, produced by MatTek Corporation, is derived from cultured normal human epidermal keratinocytes (NHEK) grown in a chemically defined cell culture medium that promotes the cells to develop into highly differentiated epidermis. EpiDerm has organized basal, spinous, granular, and cornified layers [14] [15]. The cultured cells are mitotically and metabolically active, and histochemical analysis reveals the presence of keratohyalin granules, tonofilament bundles, desmosomes, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns characteristic of *in vivo* epidermis.

MatTek has been manufacturing the highly reproducible skin model for over 10 years. EpiDerm overcomes shortcomings of previous models in terms of providing tissue that enables more realistic basic research and *in vitro* toxicological studies of epidermal phenomena as varied as apoptosis [16], anti-microbial peptides [17] and genotoxicity due to carcinogenesis [18]. EpiDerm also reduces the number of uncertainties associated with the extrapolation of toxicology results from non-human species.

Validation Studies

Since its inception, ECVAM has evolved into the agency that coordinates the validation of alternative test methods at the European Union level. ECVAM has come to occupy a pivotal position in overseeing the development of alternative test methods and acquiring regulatory acceptance and application for them. The generally accepted definition of validation is "the process by which the credibility of a candidate test is established for a specific purpose with reliability and reproducibility verified" [19].

Based on experience gained during several large-scale validation studies, and in consultation with various international experts, ECVAM has defined the practical and logistical aspects of assuring an alternative test method's credibility [20]. They describe five main stages in the evolution of new test methods: test development; pre-validation; validation (involving a formal inter-laboratory study with the testing of coded chemicals); independent assessment; and progression toward regulatory acceptance [21].

Two studies performed as part of a European Commission-sponsored pre-validation project demonstrated EpiDerm's effectiveness as a non-animal means to assess several dermal toxicology parameters, and its superior performance relative to other models evaluated. The studies [1] [2], published last year, were part of the EC DG XII funded project entitled 'Testing and Improvement of Reconstructed Skin Kits in order to Elaborate European Standards' (Project SMT4-CT 97-2174). This comprehensive multi-year, multi-country project, headed by L'Oreal (France) and involving Beiersdorf (Germany), Wella Cosmital (Switzerland) and Leiden University (The Netherlands), characterized the skin models (tissue architecture and lipid composition) and assessed the models' utility in skin irritation, percutaneous absorption and metabolism studies.

During the development of a new cosmetic formulation, the irritation (or reversible inflammatory response) potential is investigated in order to identify chemicals that might induce adverse skin reactions. The two studies summarized in this white paper addressed the reproducibility of cutaneous irritation testing in reconstructed skin kits [1] and the predictive ability of reconstructed skin kits in assessing the skin irritation levels of cosmetics [2].

Reproducibility Study

In order to be accepted as a replacement for an animal model, a validated non-animal test must be easily implemented in different laboratories, and must be able to produce consistent credible results with few false positives or negatives. C. Faller and M. Bracher, researchers at Cosmital (the research arm of Wella, AG Germany) conducted the initial study comparing the reproducibility of different *in vitro* toxicity endpoints obtained from four skin models (EpiDerm™, EpiSkin™, Skinethic™, and Cosmital) following exposure to 1% sodium lauryl sulfate (SLS), a known irritant.

The time course protocol was applied to six different batches of each skin model using triplicate tissue culture per test conditions. SLS (1%) was applied at 0.5, 1, 2, 3, 6, and 16 hours. *In vitro* toxicity endpoints included levels of MTT; the extracellular release of pro-inflammatory mediator IL-1 α ; and cytosolic enzymes GOT (glutamate oxaloacetate transaminase) and LDH (lactate dehydrogenase).

MTT reduction is a quantitative colorimetric assay for mammalian tissue viability. It measures cell respiration based on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells. The reaction forms a blue formazan product--the amount of formazan produced is proportional to the number of living cells present in the tissue. IL-1 α is an immunogenic cytokine produced in response to irritation. Leakage of the cytosolic enzymes GOT and LDH into the cell medium are an indication of damage to the cell membrane. Of the four, MTT proved to be the most reproducible of the endpoints.

According to Faller and Bracher, EpiDerm was the most resistant to the SLS (1%) treatment, and at the same time, the most reproducible model, with MTT defined as the most reproducible endpoint [1]. For the MTT assay, MatTek's EpiDerm had the highest ET-50 (most resistant to damage by SLS) and the best intra-lot and inter-lot reproducibility [lowest coefficients of variation (CV)] while Skinethic had the lowest ET-50 and the highest intra-lot and inter-lot variability.

MTT Assay ET-50, Intra and Inter-lot Variability, SLS (1%)

Skin Model	ET-50 hr	Avg. Intra-lot CV (%)	Avg. Inter-lot CV (%)
EpiDerm	3.02	10.1	20.4
EpiSkin	1.38	22.0	35.6
Cosmital	1.23	19.5	79.5
Skinethic	0.48	27.8	85.9

IL-1 α had a relatively high intra-lot and inter-lot variability in all the models. Similar results were obtained for LDH and GOT. Although basal levels of IL-1 α , LDH, and GOT were released into the assay medium, amounts distinctly different from the negative controls were only detectable at exposure times showing a significantly decreased tissue viability (at least 50%). Based on these results, the investigators proposed IL-1 α and LDH as additional parameters to be used only in combination with the measurement of cytotoxicity based on the MTT reduction assay (MTT ET-50).

Relevance Study

With the performance of the different *in vitro* endpoints defined, questions about concordance between the *in vitro* models and human skin *in vivo* could be addressed in a second study. The goals of the second study were not only to examine concordance between human *in vivo* and *in vitro* skin irritation classifications, but also to evaluate the correlations between the different parameters and, if possible, develop a prediction model based on the combination of the *in vitro* parameters [2].

Two research groups from two different industrial laboratories (Cosmital and L'Oreal Life Sciences) took part in the study.

Twenty two cosmetic products (6 surfactants, 5 shampoos, 2 mascaras, 2 emulsions, 2 gels, 2 creams, 3 cosmetic base formulations) covering the full range of *in vivo* scores and representing different cosmetic product classes were tested *in vivo* using the modified Frosch-Kligman Soap Chamber Patch Test [22] with repetitive occlusive application on human test subjects. The definitive round of *in vitro* testing was performed using two epidermis equivalents commercially available as kits (EpiDerm™ and EPISKIN™) and a third in-house model (Cosmital).

Frosch-Kligman Soap Chamber Patch Test
<i>In Vivo Parameters / Human Test Subjects</i>
Erythema
Dryness
Fissures
Skin Redness
Transepidermal Water Loss (TEWL)

In vivo skin reactions (erythema, dryness and fissures) were visually evaluated, and skin redness and transepidermal water loss (TEWL) were measured [2]. The parameters measured *in vitro* were the percent cell viability in the MTT reduction assay, with ET-50 determination, and the extracellular release of the pro-inflammatory mediator IL-1 α and of the cytosolic enzyme LDH, into the culture medium collected after topical application of the products for different exposure times (time-course assay). Measurement of GOT release was performed, but the results were not statistically analyzed.

The study demonstrated very good concordance between the *in vitro* and *in vivo* classifications as irritant and non-irritant. The most discriminating *in vitro* parameter was MTT viability after 16 hours of exposure and ET-50. *In vivo*, it was the sum of visual scores and chromametric values at day 5. Direct comparison of the two variables demonstrated that the best correlation between *in vivo* (sum of scores on Day 5) and *in vitro* (MTT ET-50) results were obtained using the EpiDerm tissue. The MTT ET-50 and the Day 5 total score (Day 5 sum) were plotted and the correlation coefficient, *r*, was determined using linear regression. EpiDerm had an *r* of 0.94.

MTT ET-50 assay and sum of <i>in vivo</i> scores on Day 5	
Skin Model	Correlation coefficient, r
EpiDerm	0.94
EpiSkin	0.84
Cosmital	0.90
Skinethic	N/A*

* Not included in relevance study

The experimental data were subjected to more extensive statistical analysis. The tables below provide the Spearman rank correlation coefficients relative to the *in vitro* results and the sum of the *in vivo* scores on Day 5. EpiDerm and MTT ET-50 achieved the highest Spearman rank correlation coefficient (0.92).

<i>EpiDerm Skin model</i>	
Endpoint	Spearman rank correlation coefficient, rho
MTT ET-50	-0.92
16 hr viability	-0.77
IL-1a	0.75
LDH	0.33

<i>Cosmital Skin model</i>	
Endpoint	Spearman rank correlation coefficient, rho
MTT ET-50	-0.85
16 hr viability	-0.77
IL-1a	0.77
LDH	0.36

<i>EpiSkin Skin model</i>	
Endpoint	Spearman rank correlation coefficient, rho
MTT ET-50	-0.85
16 hr viability	-0.77
IL-1a	0.76
LDH	Cannot be measured*

<i>Skinethic Skin model</i>	
Endpoint	Spearman rank correlation coefficient, rho
MTT ET-50	Not Tested
16 hr viability	Not Tested
IL-1a	Not Tested
LDH	Not Tested

The study demonstrated the usefulness of reconstructed human epidermis equivalents for the *in vitro* assessment of the irritation potential for a series of cosmetic products. The authors proposed a general strategy for screening purposes. They suggest testing all new products with an exposure time of 16 hours and classifying those with MTT viability greater than 50% as non-irritant. Faller *et al* pointed out that the usefulness of the *in vitro* approaches resides in their ability to screen formulations before human *in vivo* dermatological evaluation, and in the early selection of the least irritating formulations during the development process.

Continued Optimization Efforts

It was a basic understanding of the cellular mechanisms of skin irritation that enabled the development of relevant *in vitro* test systems for identification of skin irritation. Therefore, it stands to reason that efforts to further optimize these tests would include continued investigation into the biochemical events that occur within the cell during the skin irritation response. Proteomic and microarray technologies have augmented the ongoing search for more and better toxicity endpoints. Several examples of this advanced skin irritation research involving MatTek's EpiDerm skin model follow.

In an ongoing project funded by COLIPA, S. Fletcher *et al* [23] (Unilever) conducted a proteomic and microarray survey in an effort to identify skin irritation proteins (and genes encoding proteins) which may be involved in the skin irritation response using the EpiDerm model exposed to SLS. The cultures were treated in triplicate with non-cytotoxic dose of SLS (0.1mg/ml, determined by histology and MTT) for 15min, 1h, 2h, 4h and 24h. Two dimensional-gel electrophoresis (Multiphor II and DALT system, APB) and mass spectrometry were performed to investigate the protein expression profile and identify proteins of interest. Western blotting and ELISA were used to confirm changes on selected proteins of interest. In addition, microarray analysis was performed on EpiDerm samples using DermArray (Research Genetics) covering 4,000 human genes of relevance to skin biology.

From the proteomic experiments, 67 proteins of potential interest were selected and identified from a range of 2D-gels (1st dimension: pH 4-7, 4-5, 5-6, 6-9 and 6-11). Of the proteins selected, 35 proteins were up-regulated, 19 down-regulated and the expression of 4 remained unchanged (controls). The data indicated that post-translational modification occurred at an early time point (15min) for calmodulin-like skin protein and involucrin following exposure of EpiDerm to SLS. The results demonstrate the differential regulation of a number of proteins in response to SLS that could provide potential new *in vitro* markers of skin irritation.

Since paracrine signaling between dermal fibroblasts and epidermal keratinocytes is also thought to play an important role in wound healing and cancer progression, EpiDermFT (EpiDerm Full Thickness) is an effective basic research tool. For example, P.J. Hayden *et al* [24] of MatTek Corporation recently presented data at the Society of Investigative Dermatology Annual Meeting, demonstrating the induction of cyclo-oxygenase 2 (COX-2) expression in the EpiDerm Skin Model after the tissue had been exposed to ultraviolet radiation (UVR). *In vivo*, COX-2 activity is up-regulated in skin cancers and COX inhibitors reduce UVR-induced tumor formation. The investigator detected up-regulation of COX-2 in the *in vitro* skin model using ELISA and other sensitive detection methods. Additional experiments were conducted to study regulation of COX expression by cytokines in the absence of UVR. IL-1 α , IL-1 β or TNF α induced expression of COX-2 and actions influencing the COX-2 regulation were specific, indicating that the alternative COX-1 pathway was not affected.

The study demonstrated EpiDerm's ability to behave similarly to *in vivo* human skin with respect to regulation of COX-2 by solar UVR and cytokines. Finally, the results indicate that *in vitro* human skin equivalents are useful models for study of COX regulation by UVR, and related aspects of human skin carcinogenesis, and wound healing and collagen deposition / disruption studies that require a fully developed basement membrane.

Conclusions

The European Union, as can be seen from the project and supporting studies cited in this white paper, is moving inexorably toward the elimination of animal-based toxicological testing in the production of new cosmetics and personal care products, and their ingredients. Although the process to validate alternative test methods can seem never-ending at times, the effort is actually accelerating due to the concerted efforts of the governing bodies, their scientific partners, and the rapid technological advances in *in vitro* skin models made by companies like MatTek Corp.

Cosmetics and personal care product manufacturers that sell their products in the European Union, as well as the manufacturers of the ingredients used to produce these products, should redouble their efforts to meet these rapidly approaching regulations.

Product Development/Product Safety Departments at these cosmetics and personal care product companies, and their ingredient suppliers, should be actively considering alternative toxicological test methods. They should seek assurance that those alternative test methods are based on products that are either validated, or are performing very well in ongoing validation testing, such as MatTek's EpiDerm *in vitro* skin model and EpiOcular *in vitro* corneal model. For obvious scientific, commercial and ethical reasons, alternative test methods with a proven record of advancement in regulatory settings should be given preference in the test method / model selection process.

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MTT ET-50 Tissue Viability Assay - Still The Gold Standard

MTT expression has come under close scrutiny because it has emerged as the most reproducible indicator relative to *in vitro* skin irritation models. Questions about whether its predictive abilities are limited to a specific cell layers within a three-dimensional culture were raised when E. Boelsma [25] revealed that MTT was reduced primarily in the basal layer. Because measurement relative to necrosis of the upper superficial cell layers is very important to an irritation assay, investigators have taken a closer look at the MTT expression pattern. Most recently, S.D. Gettings and B.C. Jones presented evidence at the Society of Toxicology Meeting (2003) that demonstrated MTT expression was significant within the viable cell layers of the tissue constructs [26].

Gettings and Jones evaluated the MTT assay's ability to measure the cell viability in tissue equivalent models using two commercially available human corneal models (MatTek EpiOcular OCL-200 and SkinEthic HCE) and multiple tissue viability markers, (i.e., formazan, propidium iodide, rhodamine 123, histological assessment).

MatTek's EpiOcular™ corneal model consists of normal, human cell-derived epidermal keratinocytes, which have been cultured to form a stratified, squamous epithelium similar to that found in the cornea. The epidermal cells, which are cultured on specially prepared cell culture inserts using serum free medium, differentiate to form a multi-layered structure, which closely parallels the corneal epithelium. EpiOcular, like the EpiDerm *in vitro* skin model, is mitotically and metabolically active and releases many of the pro-inflammatory agents (cytokines) known to be important in ocular irritation and inflammation.

The researchers determined that the amount of formazan formed by healthy, non-treated tissue constructs increased linearly between 0.5 and 3 hours of exposure to MTT. The reaction forms a blue formazan product and the amount of formazan produced is proportional to the number of living cells present in the tissue. Histological analysis of cryo-preserved tissue models incubated with MTT revealed that all nucleated cell layers in both tissue models reduced MTT. Hence the assay was active in all viable cell layers.

An opposite staining pattern was visualized in tissue models treated with propidium iodide, a dye that penetrates nonviable cells, with only cells in the upper most (granular) layer retaining the dye. Further, rhodamine 123, which localizes to the mitochondria of viable cells, produced a similar distribution pattern to MTT in both tissue models. Therefore, the substrate for the assay appears to be present again in the appropriate cell layers.

When the tissue constructs were treated with Triton X-100 to induce cellular damage, the researchers measured expected decreases in MTT reduction after 10, 30 and 60 min exposures. Visual assessment of histological sections after Triton X-100 supported the quantifiable values with an approximate amount of cells retaining the formazan crystals. The experiments demonstrated that the traditional MTT method exhibited conversion linearly with time and by multiple cell layers in the tissue constructs. In addition, results from the MTT method were comparable to other cytotoxicity dyes.

The study supports the use of MTT as a tissue viability marker, even as a stand-alone method.

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