Performance assessment of the epiCS® epidermal model after technology transfer to a new production site

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Abstract

Since 2019 the epiCS Reconstructed Human Epidermis is integral part of the OECD Test Guideline 439 for in vitro skin irritation testing of chemicals. Also in 2019, the Henkel AG & Co. KGaA took over the epiCS technology to complement its portfolio of human skin models and to secure the continued supply of epiCS models to third parties. A tiered technology transfer strategy was executed to demonstrate that the epiCS models could be produced at the new production site with the same high quality as at the former production site at SkinInVitro.

After protocol transfer and on-site trainings, 3 batches of epiCS models, each batch grown with keratinocytes from a different donor, were employed in in vitro *skin irritation tests at both production sites with non-classified and skin-irritating chemicals selected from the respective OECD Performance Standards. All chemicals were correctly classified, and all test runs were qualified accordingly. Finally, 30 epiCS model batches were produced at the new production site, and their quality assessed based on tissue architecture, tissue viability (MTT assay), and barrier function (ET50). Irrespective of the keratinocyte donor, tissue viability and ET50 values met the predefined ranges as listed in the OECD TG 439. Taken together, the epiCS technology has been successfully transferred to Henkel. Tissue viability, barrier function and performance in the in vitro skin irritation test match the mandatory prerequisites defined in the OECD TG 439.*

Keywords: Skin irritation test; reproducibility; quality assessment; barrier function; donor variability

Introduction

Analyzing the skin-irritating potential of chemicals is a key component in the toxicological risk assessment process not only for cosmetic products, but for all substances that intentionally or by accident come into contact with skin. Since 2007, this analysis can be performed with an *in vitro* skin irritation test (SIT), which is based on reconstructed human epidermis (RHE), and which is regulatory accepted as a full replacement of the Draize Skin test (OECD, 2021).

The *in vitro* skin irritation tests developed with the EpiSkin and the EpiDerm EPI-200, both considered as validated reference methods (VRM), were the first ones to become integrated in an OECD test guideline (TG 439) in 2007 (Spielmann et al., 2007; OECD, 2021). The VRM test results were then taken to define performance standards for the development and validation of me-too methods, meaning test methods which address the same endpoint, and which are similar in terms of mechanism, function and prediction model (OECD, 2015). In the latest version of the TG 439, 7 different commercially available Reconstructed Human Epidermis models (RHE's) are listed (OECD, 2021). All models have proven their reproducibility and their predictive capacity in the SIT in multicentric validation studies and hence can be used for the *in vitro* skin irritation test of chemicals and formulations according to the users' experiences and preference.

In 2019 the epiCS RHE was included into the TG 439 after a successful validation study, based on the EURL ECVAM/OECD Performance Standards for *in vitro* skin irritation testing using reconstructed human epidermis (OECD, 2015) and subsequent ESAC peer review (ESAC, 2016). The epiCS SIT was finally approved for the TG 439 by the OECD WNT (OECD, 2019). The detailed standard operation procedure, including acceptance criteria, controls, and spreadsheet templates for data analysis, was published in the DB-ALM Protocol n° 212: epiCS® Skin Irritation Test (epiCS® SIT; 2019).

Also in 2019, the Henkel AG & Co. KGaA in Düsseldorf, Germany, found agreement with the owner of the epiCS technology (SkinInVitro GmbH, Troisdorf, Germany) to take over the asset (epiCS technology) and to secure the continued supply of epiCS to third parties. With this plan Henkel aimed at complementing its already existing portfolio of innovative human full-thickness skin models, marketed under the brand name Phenion, with an OECD-approved RHE.

Following the mutual agreement between Henkel and SkinInVitro, a tiered technology transfer plan was jointly developed, comprising 3 phases of increasing complexity. This technology transfer plan was of overarching importance because it had

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to be demonstrated that the epiCS models could be produced at a new production site with the same high-quality standards as in the SkinInVitro production facility, taking correct tissue growth and differentiation as well as its performance in the SIT into account. For future ambitions to transfer production of reconstructed tissues to different sites or teams the following plan can serve as a role model.

In phase 1, all production protocols were transferred to Henkel to allow for an in depth-preparation of the responsible Henkel personnel. Detailed discussions between both teams were followed by an on-site-training including the supervised production of two epiCS tissue batches by Henkel personnel.

Phase 2 comprised the production of 3 batches of epiCS models using three different cell donors simultaneously at both sites with identical keratinocytes and consumables, followed by *in vitro* skin irritation proficiency exercises with irritating (UN GHS Cat.2) and non-irritating chemicals (UN GHS No Cat) selected from the OECD TG 439 and the OECD Performance Standards (OECD, 2015; 2021). The three skin irritation tests at both production sites were expected to correctly classify all chemicals according to the OECD Performance Standards (OECD, 2015), irrespective of production site and team or the employed cell donor.

During phase 3, 30 independent epiCS batches with keratinocytes from 3 different donors were produced at the new production site. Tissue quality and reproducibility were assessed based on tissue viability, epidermal architecture, and barrier function, which must match the quality criteria defined in the respective DB-ALM Protocol n° 212 (2019).

The recent paper summarizes the results of the technology transfer plan which can also serve as guidance for future production transfers to different teams or sites. These results confirm the successful technology transfer between the two production sites, which is the prerequisite for the seamless supply with qualified epiCS epidermal tissues.

Materials & Methods

epiCS® Skin Model

The epiCS® model is an in vitro reconstructed human epidermis comprising primary human keratinocytes grown on an inert polycarbonate filter (pore size 0.4 µm; ∅ 0.6 cm^2) at the air-liquid interface (ALI) in a chemically defined medium. The keratinocytes were provided from Lifeline Cell Technology, (Frederick, MD, USA). Tissue viability, barrier function and histological architecture were evaluated for all epiCS® production batches.

Chemicals

Thiazolyl Blue Tetrazolium Bromide (MTT), Triton X-100 and paraffin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (D-PBS^-) without calcium and magnesium was purchased from Lonza (Basel, Switzerland), formaldehyde from Roth (Karlsruhe, Germany), and hematoxylin and eosin from Richard Allan Scientific (Kalamazoo, MI, USA).

Histology

epiCS® models were fixed in 4% formaldehyde overnight, drained and embedded in paraffin. Cross sections (5 µm) were subsequently stained with hematoxylin-eosin for histological analysis of the tissue architecture.

Viability test

The assay used for quantifying tissue viability was the MTT assay. Dehydrogenase activity of viable cells in the epiCS® models reduces the yellow tetrazolium salt solution (MTT) into an insoluble blue formazan precipitate, which is then extracted from the tissues using isopropanol.

Tissues were transferred into a 24-well plate containing 0.3 mL of 1 mg/mL MTT solution and incubated for 3 h at 37°C, 5% CO_2 and 95% humidity. After 3 h incubation, the tissues were removed from the MTT solution and immersed into 2000 µl of isopropanol at room temperature for 2 h. Optical density (OD) of the formazan extracts was measured in a spectrophotometer at $\lambda = 570$ nm. The OD of the isopropanol was subtracted from each sample. The pre-defined acceptability range for the epiCS was $0.8 < OD > 2.8$ (OECD TG 439, 2021).

Barrier function

The barrier function property of the epiCS was assessed by determining the exposure time required to reduce the relative cell viability by 50% (ET50) upon application of an aqueous 1% Triton X-100 solution. Relative tissue viability was determined at 4 different time points: 0, 2, 3.5, and 5 hours. D-PBS-treated epiCS served as a negative control, which was the basis for the ET50 calculation. For each condition 3 tissues were used. After treatment, the tissues were transferred into a 24-well plate to become analyzed with the MTT assay (see above). Viability of Triton X-100- treated tissues at different time points was compared to the concurrent negative control tissues. The pre-defined acceptability range for the ET50 is 2 hours < ET50 > 7 hours (OECD TG 439, 2021).

Proficiency exercise: in vitro skin irritation test

The *in vitro* skin irritation test was conducted with 9 proficiency chemicals as recommended in OECD TG 439, complemented with 2 additional chemicals from the "OECD Performance Standards for the assessment of proposed similar or modified in vitro reconstructed human epidermis (RhE) test methods for skin irritation testing" (OECD, 2015). A total of 5 non-irritating and 6 irritating neat substances, including liquids and solids, were tested (table 1). An aqueous 5% SDS solution was used as positive control, D-PBS as negative control.

Results

In order to assess the successful technology transfer, an *in vitro* skin irritation proficiency exercise was conducted in parallel at the Henkel site in Düsseldorf, Germany, and at SkinInvitro, Troisdorf, Germany. For this purpose, 3 batches of epiCS models were independently produced at each premise with keratinocytes from 3 different donors. The chemicals were selected from the list of the proficiency chemicals recommended by the OECD and com-

Chemical	CAS-No.	State	Category	Score	Source*
naphtalene acetic acid	86-87-3	solid	No Cat	0	PC
isopropanol	67-63-0	liquid	No Cat	0.3	PC
methyl stearate	112-61-8	solid	No Cat	1.0	PC
heptyl butyrate	5870-93-9	liquid	No Cat	1.7	PC
hexyl salicylate	6259-76-3	liquid	No Cat	2.0	PC
1-decanol	112-30-1	liquid	Cat. 2	2.3	PSt
cyclamen aldehyde	103-95-7	liquid	Cat. 2	2.3	PC
2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCL	86604-75-3	liquid	Cat. 2	2.7	PSt
potassium hydroxide (5% aq)	1310-58-3	liquid	Cat. 2	3.0	PC
1-methyl-3-phenyl-1-piperazine	5271-27-2	solid	Cat. 2	3.3	PC
heptanal	111-71-7	liquid	Cat. 2	3.4	PC

Table 1: Chemicals used in the proficiency exercise. For each chemical the CAS number, state (liquid or solid), GHS category (No Cat: not classified / Cat. 2: skin irritating) and in vivo score is indicated. *The chemicals were taken from 2 sources: PC – Proficiency chemicals according to OECD TG 439/ PSt – OECD Performance Standards (OECD, 2015)

The proficiency exercise strictly followed the protocol as outlined in the DB-ALM Protocol n° 212 (2019). The prediction model as well as the acceptance criteria for qualified test runs are described in the respective OECD TG 439. The prediction model is defined as follows:

rel. tissue viability ≤ 50%	skin-irritating (GHS Cat. 2)
rel. tissue viability < 50%	non-irritating (No Cat. / non-classified)

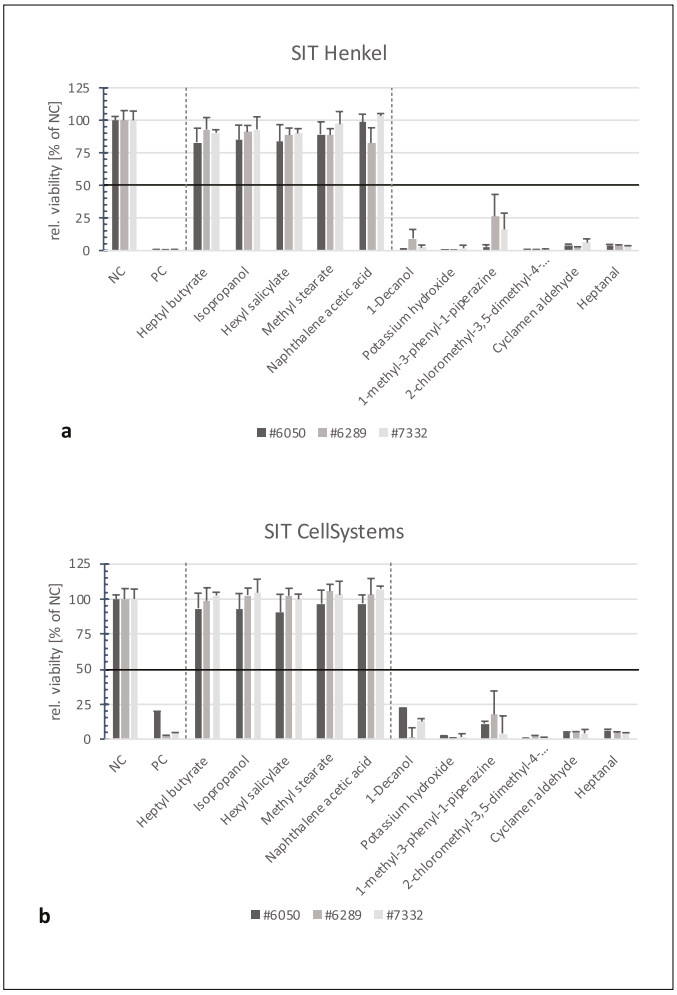

Figure 1: Results of the *in vitro* skin irritation proficiency exercise with selected non-irritating and irritating test chemicals, expressed as relative tissue viability (% tissue viability compared to the negative control). All chemicals were tested in 3 independent valid runs with 3 epiCS each. For each run, a different keratinocyte donor was used. The columns indicate the mean rel. tissue viability ± standard deviation for triplicates. The black line highlights the 50% threshold of the prediction model. The dotted vertical lines separate controls, non-irritants, and irritants. a) Results generated at the Henkel premises; b) Results generated at the SkinInVitro facility. NC – negative control; PC – positive control

plemented with chemicals from the list of reference chemicals for the validation of me-too methods (OECD 2015; 2021). They comprised 5 non-irritating and 6 skin-irritating chemicals.

All epiCS batches used in the proficiency exercise matched the quality criteria as defined in the OECD TG 439 in respect of tissue architecture, optical density of the formazan extracts ($0.8 \leq OD \geq 2.8$) and ET50 values ($2.0\ h \leq ET50 \geq 7.0\ h$). Thus, they were qualified for being used in an *in vitro* skin irritation proficiency exercise.

At the Henkel production site, all proficiency chemicals were predicted correctly according to their *in vivo* skin irritation potential (figure 1a). With the epiCS models treated with known non-irritants, tissue viabilities did not decrease below 75% of the negative control, thus, they were all predicted correctly. All skin-irritating chemicals were classified correctly, too. The standard deviations for the mean data were lower than 18% and thus fulfilled the prerequisites for qualified test runs (OECD, 2021).

The results observed at Henkel matched those generated in parallel at SkinInVitro, the original developer of the epiCS model (figure 1b). All chemicals were unambiguously classified correctly, independently of the keratinocyte donor.

With the proficiency exercise it was also proven that all 3 keratinocyte lots were qualified for epiCS model production. Thus, these keratinocytes were used for phase 3 of technology transfer, intended to demonstrate robustness and reliability of tissue production for several batches over a longer period of time. Three different quality control parameters were analyzed for 30 independently produced epiCS batches: i) tissue architecture, based on H&E-stained tissue slides; ii) tissue viability, determined with the MTT viability assay; and iii) the robustness of the skin barrier, indicated with the ET50 value.

Tissue architecture:

In figure 2 histological representative sections through 3 different epiCS models from 3 independently produced batches are depicted. For each of the tissue lots presented here keratinocytes from a different donor were employed. All epiCS models comprised 6–10 layers of viable keratinocytes which revealed the

phenotype characteristic for the respective state of differentiation. In the stratum basale, the basal keratinocytes, or keratinocyte stem cells, exhibited a pronounced cell polarity and were arranged in a palisade-like pattern. The tissue was covered by a multi-layered stratum corneum. With this tissue architecture, the epiCS models matched the predefined quality requirements based on historical histological data.

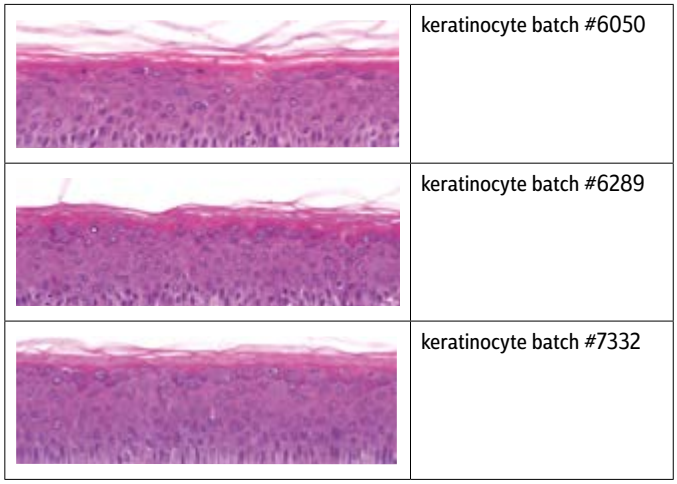

Figure 2: Histological sections through 3 different epiCS models. The tissues were formalin-fixed, paraffin-embedded, cut and stained with H&E. The respective keratinocyte batch is indicated in the right column.

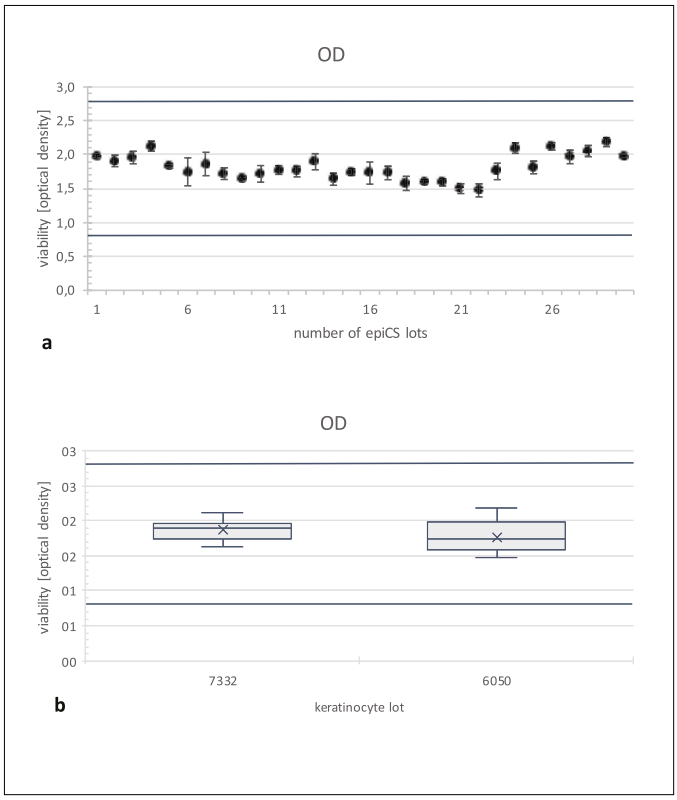

Figure 3: Tissue viability data, assessed with the MTT viability assay. The optical density (OD) of the isopropanolic formazan extraction solution was determined spectrophotometrically at a wavelength of λ = 570 nm. a) individual OD data for epiCS models of each production lot in the fully differentiated state; b) box plot analysis of the OD data for the most often used keratinocyte batches. The box plots highlight the median (line separating the upper and lower box), the mean (X), the upper and lower quartile and the 25% of values above and below the box, respectively (whiskers). The dark lines indicate the lower and upper limits of optical density (0.8 ≤ OD ≥ 2.8).

The tissue viabilities of all assessed batches are shown in figure 3. Figure 3a lists the optical density values for each individual production lot, covering a range of values between 1.4 and 2.2 OD units. As keratinocytes from 2 different donors were taken for most epiCS batches during the transfer phase, the viability data were analyzed separately for each of them in a box plot diagram (figure 3b).

The OD values of epiCS models produced with keratinocyte batches #6050 and #7332 did not differ significantly ($p > 0.1$), mean and median values were in the same range of values. Two epiCS batches were also produced with the keratinocytes #6289, with revealed similar OD values. Independently of the used keratinocytes, all OD values were within the pre-defined range of values for epiCS qualification ($0.8 \leq OD \geq 2.8$). All data are summarized in table 1.

In addition to the overall tissue viability the robustness of the skin barrier, developed under air-liquid interface culture conditions was analyzed. It is expressed as the time at which tissue viability is decreased to 50% after topical exposure with the surfactant TRITON X-100, compared to untreated tissues (ET50).

The ET50 values for all epiCS batches were confined to an interval between 2 and 5 hours (figure 4a). To analyze possible reasons for the variations observed for the individual batches, the

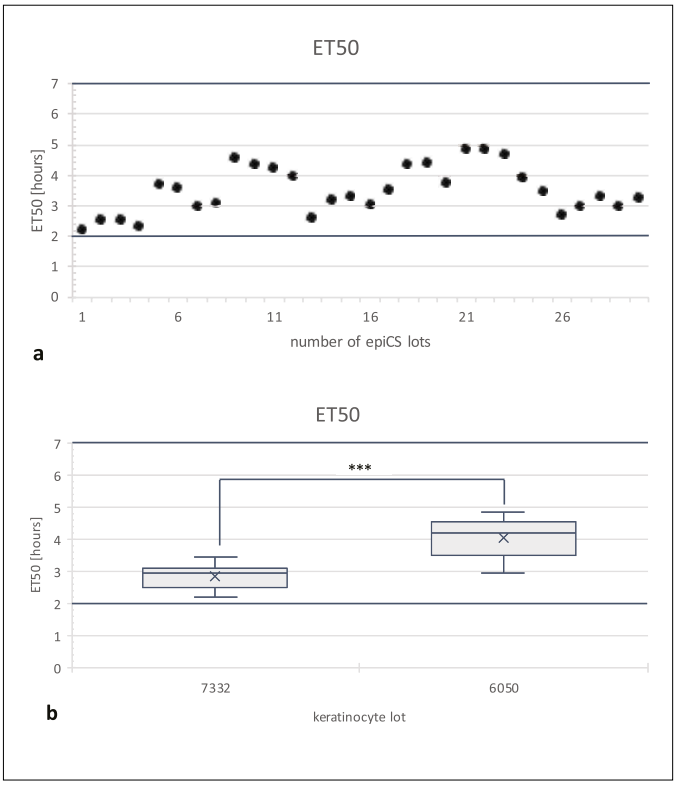

Figure 4: ET50 values, based on tissue viability data after topical treatment of the epiCS models with a 1% TRITON X-100 solution. The ET50 values were calculated with 2nd order polynomial equations derived from regression analyses of each ET50 experiment. a) Individual ET50 data for epiCS models of each production lot in the fully differentiated state; the dark horizontal lines indicate the lower and upper ET50 limits according to the pre-defined quality criteria. b) Box plot analysis of the ET50 data for each used keratinocyte batch. The box plots highlight the median (line separating the upper and lower box), the mean (X), the upper and lower quartile and the 25% of values above and below the box, respectively (whiskers). The asterisks indicate statistically significant differences ($p < 0.05$). The dark lines indicate the lower and upper limits of ET50 (2.0 hours ≤ ET50 ≥ 7.0 hours).

lot #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OD	1,977	1,910	1,960	2,128	1,824	1,749	1,865	1,722	1,645	1,719	1,774	1,752	1,897	1,640	1,746
SD	0,030	0,085	0,093	0,074	0,022	0,206	0,173	0,086	0,030	0,122	0,063	0,078	0,119	0,089	0,048
ET50	2,23	2,54	2,54	2,34	3,67	3,59	2,99	3,07	4,57	4,34	4,24	3,95	2,61	3,21	3,31
cell #	7332	7332	7332	7332	6289	6289	7332	7332	6050	6050	6050	6050	7332	7332	7332

lot #	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
OD	1,731	1,732	1,579	1,602	1,593	1,500	1,475	1,755	2,099	1,814	2,125	1,969	2,055	2,185	1,983
SD	0,163	0,100	0,105	0,037	0,054	0,072	0,097	0,122	0,077	0,094	0,054	0,100	0,083	0,065	0,007
ET50	3,03	3,53	4,35	4,38	3,73	4,84	4,85	4,66	3,9	3,47	2,68	2,95	3,31	2,97	3,22
cell #	7332	6050	6050	6050	6050	6050	6050	6050	6050	7332	7332	7332	6050	6050	6050

Table 2: Compilation of tissue viability data (mean OD +/- SD), ET50 values (hours) and the respective keratinocyte donor (cell #) for all epiCS batches produced during technology transfer.

ET50 values were assessed depending on the keratinocyte lot employed in the respective epiCS models. As indicated in figure 4b, the cell lots #6050 and #7332 differed significantly from each other. Whereas epiCS models based on keratinocytes #6050 were characterized by high ET50 values with a median of 4.1 hours, the median value of epiCS models constructed from #7332 cells was about 2.8 hours. The epiCS models constructed with the third keratinocyte donor, #6289, resulted in ET50 values of approx. 3.6 hours, an intermediate value between those of #7332 and #6050. However, all batches matched the pre-defined quality criteria for the epiCS (2–7 hours), independently of the used keratinocytes. The ET50 values for the single batches are listed in table 2.

Discussion

Transferring an ambitious technology from one production site to another poses serious challenges in respect of guaranteeing the original quality of the product based on this technology. Thus, it is key to conduct a thoroughly monitored technology transfer and consequently to assess the product quality at the new facility according to pre-defined standards. The transfer of the epiCS technology from SkinInVitro to the Henkel AG & Co. KGaA was performed in a tiered approach, following the logic outlined below. Phase 1 comprised the protocol transfer and laboratory personnel theoretical and practical training at the existing production location. Phase 2 included the demonstration of proficiency of the new personnel which was monitored and controlled by the production and quality control team of the existing site. With the 3rd phase, the reproducibility of epiCS production for 30 independently generated batches was assessed. The results achieved in phases 2 and 3 are the subject of the current manuscript. With this approach, we closely pursued the evaluation process which has been described by De Vecchi and co-authors (2018). In their paper the authors present results of a study conducted to demonstrate proficiency and tissue quality following the implementation of the SkinEthic™ RHE tissue model technology in Brazil. For this reason, tissue viability of a series of newly produced batches and the barrier function of the respective tissues were assessed and compared with the value ranges of the SkinEthic™ RHE models produced at the headquarter in France. For final approval, a proficiency exercise for *in vitro* skin irritation testing according to the OECD TG 439 (OECD, 2021) and with a selection of chemicals from the respective TG (Table 1: Proficiency Substances; OECD, 2021) was conducted. The quality assessment published by De Vecchi et al. (2018) served as a template for our own analyses during the epiCS technology transfer.

As part of the epiCS transfer, 30 batches of tissue models were produced with keratinocytes from 3 different donors. All batches matched the pre-defined quality criteria in respect of histological architecture, tissue viability and barrier function, expressed as the ET50 value. The respective value ranges are listed in the OECD TG 439 for *in vitro* skin irritation testing. They belong to the "Functional Conditions" within the "RHE Test Method Components". Only epidermal models matching the conditions listed in the respective guideline are qualified to be used in the *in vitro* assay for regulatory purposes.

The tissue viabilities varied to a certain extent, which must be attributed to the normal variability of biological systems. However, all values were within specification, and no statistically significant differences were observed regarding the keratinocyte lots used. Although the value ranges of ET50 differed significantly between those 2 donors, which were used for most of the epiCS batches, all values fell within the lower and upper thresholds which are mandatory to qualify the tissues for shipment to the customers, and which are an integral requirement in the OECD TG 439. This is a clear indication for the robustness of the epiCS production process which enables epiCS batches within specification over a long period of time and even with keratinocytes from different origins.

An *in vitro* skin irritation proficiency exercise according to the TG 439 was chosen as an integral proof for successful technology transfer. Whereas we tested all 5 non-classified substances (UN GHS No Category) from the list of proficiency chemicals, we deviated from this list of the classified ones (UN GHS Category 2; or skin irritants). Instead of 1-bromohexane (in vivo score 2.7), 1-decanol (*in vivo* score 2.3) and 2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl (*in vivo* score 2.7) were chosen from the "Minimum List of Reference Chemicals for Determination of Reproducibility and Predictive Capacity of similar or modified RhE Skin Irritation Test Methods" (OECD, 2015) and included in our selection. This decision was made based on the documented results of an *in vivo* skin irritation patch test study on volunteers (Jírová et al., 2010). Only 16 of 30 volunteers developed an irritation reaction after topical application of 1-bromohexane, a clear indication that the dermal response to this chemical depends on

the individual skin properties and the genetic background, respectively, of the exposed humans. However, due to the majority principle (16 x Cat 2 versus 14 x No Cat) the chemical was classified Cat 2 in this study. The ambiguous *in vivo* situation was mirrored in some *in vitro* studies conducted to evaluate or validate epidermal models for the *in vitro* skin irritation test according to OECD TG 439. In the catch-up validation study with the OS-REp epidermal model the classification of 1-bromohexane depended on the keratinocytes, and hence on the keratinocyte donor, used to construct the tissues (Mewes et al., 2016; Groeber et al., 2016). In some cases, it was predicted as a non-irritant, a false-negative result in terms of the GHS classification which was originally derived from Draize skin tests (ECETOC, 1995). A similar situation was reported for the KeraSkin™-VM validation study, where 1-bromohexane was also falsely classified as a non-irritant (Jung et al., 2014). The authors speculated about ethnicity-depending differences in dermal physiology as a possible reason for this deviation from the GHS classification. With the Labcyte EPI-MODEL, 1-bromohexane was classified false-negative, too, based on the MTT viability test only (Katoh et al., 2009). Taking that into consideration the inclusion of 1-bromohexane in the list of proficiency and validation chemicals must be considered critically.

To our knowledge, no respective concerns were raised for the substitute chemicals, 1-decanol and 2-Chloromethyl-3,5-dimethyl-4-methoxypyridine HCl, as the classification of both substances as irritants was unambiguous in the *in vitro* tests published so far. With the epiCS models, both chemicals were inconspicuous and hence classified correctly as irritants with keratinocytes from different donors. Selectivity of the epiCS *in vitro* skin irritation test was as recommended, with very high relative tissue viability values for the non-classified and very low values for the true positive chemicals. No relative viability values in the vicinity of the 50% prediction model threshold (borderline values) were observed. The same proficiency exercise was conducted simultaneously at the "old" production site, with the same keratinocyte donors and identical chemicals from the same lot. The results from both premises were nearly identical, both in terms of the pure OD data and the classification.

Taken together, all results generated during the epiCS technology transfer demonstrate technical proficiency and the unaltered high quality of the produced tissue equivalents at the new location. Tissue viability, barrier function and performance in an *in vitro* skin irritation test are in accordance with the standard operation procedures defined by the developer company and with the mandatory performance standards as listed in the OECD TG 439.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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