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Pre-validation of an in vitro skin irritation test for medical devices using the reconstructed human tissue model EpiDerm™

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ABSTRACT

Assessment of dermal irritation is an essential component of the safety evaluation of medical devices. Reconstructed human epidermis (RhE) models have replaced rabbit skin irritation testing for neat chemicals and their mixtures (OECD Test Guideline 439). However, this guideline cannot be directly applied to the area of medical devices (MD) since their non-toxicity assessment is largely based on the testing of MD extracts that may have very low irritation potential. Therefore, the RhE-methods previously validated with neat chemicals needed to be modified to reflect the needs for detection of low levels of potential irritants.

A protocol employing RhE EpiDerm was optimized in 2013 using known irritants and spiked polymers (Casas et al., 2013, TIV). In 2014 and 2015 MatTek In Vitro Life Science Laboratories (IVLSL) and RIVM assessed the transferability of the assay. After the successful transfer and standardization of the protocol, 17 laboratories were trained in the use of the protocol in the preparation for the validation. Laboratories produced data with 98% agreement of predictions for the selected references and controls.

We conclude that a modified RhE skin irritation test has the potential to address the skin irritation potential of the medical devices. Standardization and focus on the technical issues is essential for accurate prediction.

1. Introduction

In the 1980s, modified Draize rabbit skin irritation test (Draize et al., 1994) was included in the Tripartite Agreement test matrix for predicting the skin irritation potential of medical devices (OECD, 1988, 2002), in the 1990s, this test was also included in the ISO 10993 standards (ISO 10993-10:, 2010). Skin irritancy testing became one of three biocompatibility tests recommended for all medical devices along with the cytotoxicity and sensitization (ISO 10993-5:, 2009; ISO 10993-10:, 2010).

ISO Technical Committee (TC) 194 which concerns the biological

and clinical evaluation of medical devices, encourages the use of alternative tests if they are appropriately validated, reasonably and practically available, reliable and reproducible (ISO 10993-1:, 2009). If these criteria are met, the in vitro test should be considered for use in preference to in vivo tests' (ISO 10993-2:2006). In 2009, OECD adopted a new test guideline (TG) number 439, allowing for assessment of the skin irritation potential using the reconstructed human epidermis (RhE) models. However, RhE tests proposed by this guideline were developed and designed for the hazard identification, classification and labelling of neat chemicals and mixtures and are predictive only for the induction of significant skin irritation effects of in vivo grade 2.3 and higher

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(OECD, 2004, 2015). These tests are therefore not suitable for testing of extracts from medical devices that are highly diluted solutions of chemicals that may be potentially irritating or cytotoxic.

Other skin irritation protocols, previously developed by the surfactant and cosmetic industry to predict mild and moderate irritation (Faller et al., 2002), exist and they require long exposure time and dose thus providing significantly higher sensitivity towards irritating chemicals. In 2013, Casas et al. published a proof-of concept study that evaluated a modification of the EpiDerm™ skin irritation protocol and confirmed ability of the RhE in vitro assay to identify skin irritants at concentrations similar to those of substances extracted from medical device polymers. This pilot study was the basis for further work described in this paper that also built upon the results from two validation studies of the EpiDerm protocol for skin irritation testing of chemicals (Kandárová et al., 2009; Kandarova and Liebsch, 2017) as well as on the protocols developed and validated for cosmetic testing (Faller et al., 2002, MatTek ET-50 protocols).

It summarizes all optimization steps and considerations that led to the pre-validation and transfer of the EpiDerm skin irritation test for Medical Devices (EpiDerm SIT-MD) to 17 laboratories in preparation for the round robin validation study (de Jong et al., 2017, this TIV issue).

1.1. Organization of the study

The management team (MT) of this project was composed of scientists from Medtronic, MatTek IVLSL, RIVM and Cyprotex (see Fig. 1). The MT was responsible for the planning, protocol development, test materials selection and training of participating laboratories. Furthermore it took care of management, oversight of timelines, and reporting on the study progress at stakeholder's meetings (ISO TC 194 plenary sessions) and for the final data interpretation and publications.

The management team's goals were separated into the following three steps (phases of the pre-validation):

- Phase I: To evaluate the performance of the protocol developed by Casas et al. (2013), then optimize and formalize the technical aspects of the test and produce a formal Standard Operating Procedure (SOP).

- Phase II: To challenge the predictive ability of the optimized test in an inter-laboratory study with polymers spiked with known irritants.

- Phase III: To assure the transfer of the protocol to the laboratories interested in participating in the round robin validation study.

The chronology of the project along with important milestones is summarised in Table 1. Test materials (polymers) that were specially synthetized for purposes of this study, were kindly provided by National Institute of Health Sciences (NIHS) (Tokyo, Japan), Arthrex, Inc. (Naples, Florida, USA) and Medtronic plc (Minneapolis, Minnesota, USA).

In addition to the in vitro testing and trainings conducted at 3 different locations (MatTek IVLSL, MatTek Corp., and Kurabo Industries, Ltd.), controlled human patch testing with several benchmark materials was conducted at National Institute of Public Health (Prague, Czech Republic) in order to assess the irritation potential in human volunteers under conditions similar to the Draize rabbit test and the newly developed EpiDerm SIT-MD (see Kandárová et al., 2017 this TIV issue).

2. Materials and methods

2.1. Test system

The reconstructed human epidermal (RhE) tissue model EpiDerm™ (MatTek, Ashland, USA and MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia – ISO 9001:2008 certified) consists of normal, human-derived epidermal keratinocytes (taken from healthy volunteers negative to HIV and hepatitis) that have been cultured to form a multi-layered, highly differentiated model of the human epidermis. The EpiDerm™ model consists of an organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo (Canon et al. 1994).

The EpiDerm™ tissues (surface area 0.63 cm^2) are cultured on specially prepared cell culture inserts and shipped to customers as kits, containing 12, 18 or 24 tissues on shipping agarose together with necessary amount of culture media and 6-well and 24-well plates. In addition, the MTT kit (containing MTT concentrate, diluent, and extracting solution) is provided by MatTek. For the purpose of the current
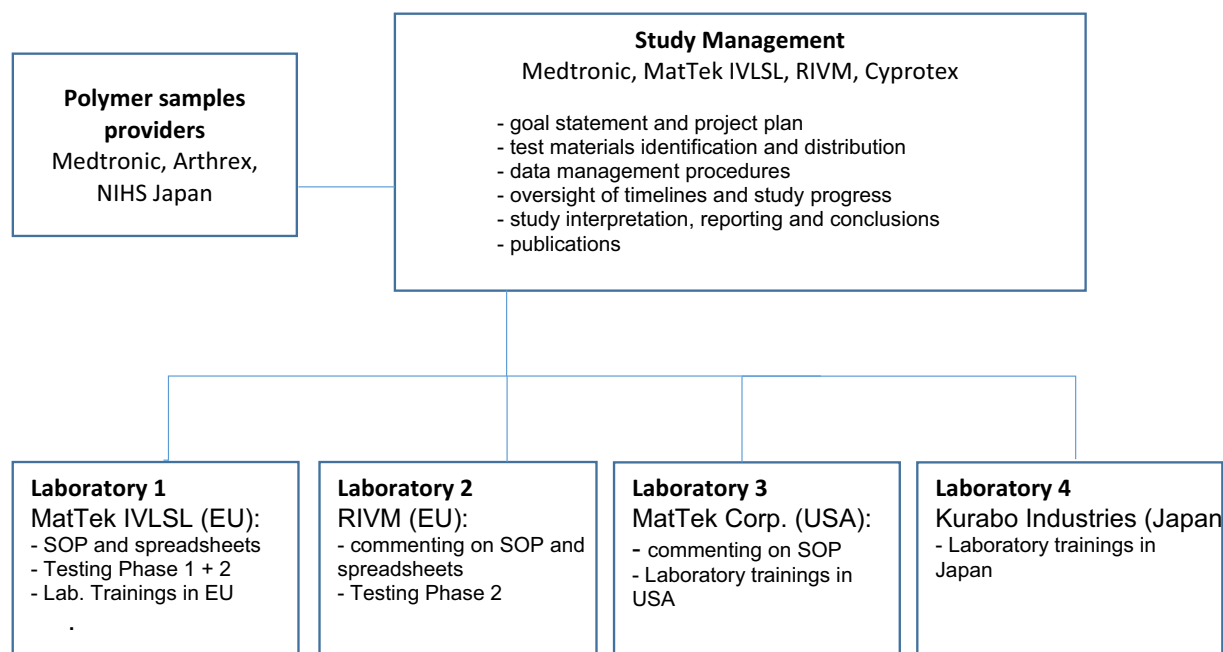

Fig. 1. Management structure of the project.

Table 1
Chronology and milestones achieved in the pre-validation study.

2013	Decision on the way forward in pre-validation based on data published data (Casas et al., 2013)
	- Series of experiments conducted in summer 2013 at MatTek IVLSL leading to the optimization and formalisation of the test protocol and shorter exposure times. Generation of data on benchmarks and positive controls (Testing Phase I)
	- First SOP developed
	- Five laboratories trained in conductance of the test (RIVM, Eurofins Italy, NIOM, Nelson Labs and NAMSA)
2014	Further improvement of the SOP, presentation of the interim results at the ISO TC 194
	- Limiting the exposure time to 18 h to simplify the test
	- Presentation of the interim results to the Working Group 8 Meeting ISO TC 194 – Mishima, Japan April 22, 2014
	- Extended protocol transferability testing between RIVM and MatTek IVLSL (Testing Phase II)
	- Four new laboratories trained in conductance of the test (Boston Scientific, SP Technical Research Institute, Arthrex, Toxicon)
2015	SOT and EUROTOX presentations of the interim data on pre-validation and trainings (Kandarova et al., 2015), detailed report on the study progress at the ISO TC 194
	- Conductance of the Human patch studies at NIH Prague (see Kandárová et al., 2017, this TIV issue)
	- Presentation of the results obtained in Phase I and Phase II at the ISO TC meeting in Sweeden, Lunds, June 08, 2015
	- Three additional laboratories trained in conductance of the test (Yonsei University, Cyprotex, APS)
2016	SOT and EUROTOX presentations of the pre-validation results conducted with 17 materials at RIVM and MatTek and overview of the completed trainings (Kandárová et al., 2016)
	- Final version of the SOP 03/2016 (MatTek SOP, 2016)
	- Teleconference agreement on IL-1α endpoint non-inclusion in the main study (but all laboratories to keep frozen media; lead laboratories to conduct testing, and, if results are promising, all laboratories will determine IL-1α)
	- Four new laboratories trained in conductance of the test (ENVIGO Germany, Kurabo Industries Ltd., UKM Malaysia, NIHS Japan)
	- Start of the round robin study (see de Jong et al., 2017, this TIV issue)

study, specialized kits were developed and provided under the part # EPI-200-SIT-MD.

2.1.1. Quality controls of the test system

The EpiDerm™ System is manufactured according to defined quality assurance procedures compliant with Good Manufacturing Practice. All biological components of the epidermis and the culture medium are tested by the manufacturers for viral, bacterial, fungal and mycoplasma contamination. Barrier properties of each manufactured tissue lot are controlled by MatTek. Per request, MatTek provides detailed information about lot-specific ET50 following exposure to Triton X-100 (1%) (chemical recommended as penetration marker by the OECD TG 439), information regarding tissue viability (MTT test), together with a historical database of results.

Handling procedures for biological materials should be followed. It is recommended that gloves are worn during handling of the skin and kit components. After use, the epidermis, the material and all media in contact with it should be decontaminated prior to disposal (e.g. using 10% bleach or special containers).

2.2. Test materials

The following four test materials were used: Lactic acid (LA) 4% (w/v) solution in saline, Heptanoic acid (HA) 2% (w/v) in sesame oil (so), Polymer Y-4 (extracted in saline and so) and Sodium Dodecyl Sulphate (SDS) 1% (v/v in saline and so) were used as benchmark materials for evaluation of the Protocol developed by Casas et al., 2013 and for its optimization and formalisation of the SOP (Testing Phase I). Polymer Y-4 was extracted into the vehicles in the ratio of 6 cm²/ml of the material/solvent. Extractions were conducted at 37 °C for 72 h (for more details on the benchmarks used see Table 2a).

In the Phase II, conducted between MatTek IVLSL and RIVM with the already optimized test protocol, a number of irritating and non-irritating polymer materials (Table 2b) were prepared by Arthrex and Medtronic and sent to MatTek IVLSL and RIVM for evaluation. Technical preparation of these materials is described in detail by Coleman et al., 2017 (TIV, the same issue). Polymers used in this study were prepared and extracted with saline and sesame oil (pharma grade) as recommended by ISO 10993–12:2012 on sample preparation of reference materials (ISO 10993-12, 2012). Extractions were conducted at 37 °C for 72 h.

2.3. The EpiDerm SIT-MD protocol

The in vitro skin irritation test, with modified dose (100 μL) and exposure conditions (18 h, no post-incubation), was developed in this study using the reconstructed tissue models EpiDerm (EPI-200). This protocol (known as EpiDerm SIT-MD) has its scientific basis in previously published papers on skin irritation testing, namely Faller et al., 2002, Kandárová et al., 2009 and Casas et al., 2013.

Upon the receipt of the parcel containing the EpiDerm kits, tissues were aseptically removed from the transport agarose and conditioned for 1-h incubation in 0.9 mL assay medium per well of a 6-well plate at 37 °C, 5% CO_2 and saturated humidity (i.e. standard tissue culture conditions (STCC)), in order to release transport stress-related compounds and any debris accumulated during shipment. In lieu of actual shipment, tissues were stored overnight at 4 °C at the manufacturers' (MatTek) facility prior to use. The tissues were then transferred to 0.9 mL fresh assay medium (EPI-100-NMM) and further conditioned overnight.

On the next day, the culture medium was exchanged, and the apical surface of the tissues was dosed with 100 μL of the irritant solutions or extracts shown in Tables 2a and 2b. Positive controls (PC, 1% v/v SDS) in saline and in sesame oil (SO)), negative control (NC, Dulbecco's phosphate buffered saline (DPBS)) and vehicle controls (VC, saline and SO) were tested concurrently in the EpiDerm SIT- MD assays. Dosing was performed in 60 s interval. Incubation times were initially set to 4, 18 and 24 h in order to assess the irritation potency of the selected benchmarks and correctly determine the exposure time necessary for the development of the expected irritation effect. The final version of the SOP uses consequently single 18 ± 1 h exposure at STCC.

Following exposure, tissues were rinsed with Ca^{2+} free and Mg^{2+} free DPBS, blotted to remove excess PBS, transferred to 24-well plates containing 0.3 mL freshly prepared MTT medium (1 mg MTT/mL; MatTek MTT-100 kit) per well and incubated for 3 h at STCC. Media from the test was placed into the freezer at −20 °C for IL-1α analysis.

Following incubation with MTT, tissues were blotted and transferred to new 24-well plates. Two millilitres of isopropanol (analytical grade, part of MTT-100 kit) were added to each well to completely immerse the inserts. The plates were sealed with parafilm and formazan extraction was performed either at room temperature for 2 h on a plate shaker or overnight without shaking at 4 °C to prevent evaporation of isopropanol.

Table 2a
Characterization of the test materials used as benchmarks for protocol optimisations and transfer (Testing Phase I).

	CAS number	Chemical/sample	Supplier	Form supplied	Purity	Remark
1	50–21-5	Lactic acid, 4% solution (w/v)	Fluka	Liquid	90%	4% solution
						solution in ultra-pure water
2	111–14-8	Heptanoic acid, 2% (w/v)	Sigma-Aldrich	Liquid	> 95%	2% solution was prepared into sesame oil
3	n.a.	Polymer Y-4	NIHS, Japan	Sheet	n.a.	saline extract
	n.a.	Polymer Y-4	NIHS, Japan	Sheet	n.a.	sesame oil extract
4	151–21-3	SDS, 1%	Sigma-Aldrich	solution	n.a.	1% saline solution
						prepared from 20% stock solution of SDS in water
	151–21-3	SDS, 1%	Sigma-Aldrich	solution	n.a.	1% sesame oil solution was prepared from 20% solution of SDS in water
5	7647–14-5	Saline (0.9% NaCl)		solution	n.a.	–
6	n.a.	Sesame oil		Liquid	n.a.	–

Table 2b
Materials used for the assessment of the predictive capacity of the EpiDerm skin irritation (Phase II).

	Chemical	Affinity to solvent	Expected in vivo outcome
1	Y-4 Polymer (PVC + 5.8% Genapol X-080)	uncertain	Positive
2	25% HA in one-part silicone	non-polar	Positive
3	15% SDS in two-part silicon	polar	Positive
4	17% SDS in two-part silicon	polar	Positive
5	60% LA in one-part silicone[*]	polar	Positive
6	15% SDS in two-part silicone	polar	Positive
7	15% SDS in PVC	polar	Positive
8	7% Genapol X-100 in PVC	polar	Positive
9	PVC control	uncertain	Negative
10	Y-1 control PVC	uncertain	Negative
11	One-part silicone L/N 68021	uncertain	Negative
12	Two-part silicone	uncertain	Negative
13	Polyurethane control	uncertain	Negative

[*] The LA in this sample was in powdered form (Galactic Powder 60. Galactic Inc., Milwaukee, Wisconsin, USA). For more information about these materials see Coleman et al. in this TIV issue.

Thereafter, two 200 μL aliquots of isopropanol extract per tissue were transferred to a 96-well plate and optical density was measured at 570 nm ± 30 nm, with isopropanol as a blank.

Tissue viability was calculated in comparison to the DPBS-treated NCs. Cell viability below 50% in either the SO or saline extracts at any of the time-point was considered to be a sign of irritation. For further details see EpiDerm SIT-MD SOP (MatTek SOP, 2016).

2.4. Interleukin 1 alpha (IL-1α) assessment

Assessment of the interleukin **IL-1α** in the supernatant (i.e. interleukins released by the EpiDerm tissue models into the cell culture media) was conducted using Human IL-1α kit Quantikine ELISA kit from R&D Systems following the manufacturer's SOP.

2.5. Training of testing laboratories

Laboratories that expressed an interest to participate in the round robin (RR) study planned for 2016 (see de Jong et al., 2017, this TIV issue) were first requested to undergo voluntary training in the Epi-Derm SIT-MD protocol at either in the MatTek facilities (EU/USA) or with assistance of Kurabo Industries Ltd. in Japan. The training consisted of 2 training phases:

During the Training Phase I, the EpiDerm SIT-MD test was demonstrated by an experienced scientist who was familiar with technical details of the method. After demonstration of all techniques, participants performed the assay according to the protocol and under the supervision of the trainer. Every participant was trained for all

techniques. The SOP and the assay documentation were discussed, comments and suggestions for improvement of the method and documentation were collected for later inclusion in the SOP. Questions from the laboratories raised during the trainings were discussed and addressed in the later versions of the SOP.

During Training Phase II, which was typically conducted one or two weeks after Phase I, laboratories were asked to demonstrate proficiency by generating their own data on the provided benchmarks. Most of the laboratories conducted the proficiency testing with 4, 18 and 24-h exposure times as requested by MatTek. However, some of the laboratories that joined the training shortly before the start of the RR study, or those that felt sufficiently trained due to the previous experience with the assay, decided to conduct only one of the exposure times (usually 18 h).

A list of the laboratories that underwent voluntary training of the EpiDerm SIT-MD in connection with the RR project is presented in Table 3. Not all of them, however, participated in the RR study.

3. Results

3.1. Phase I: Formalisation of the protocol, SOP development

During Phase I, benchmark materials from Casas et al. (2013) were re-tested in several test designs in order to evaluate/establish:

1. the exposure time that would shorten the assay without compromising sensitivity and in the same time to assure for stability and tissue integrity of the negative controls, vehicle controls and positive controls,
2. positive control that could work both in polar and non-polar solvents,
3. impact of the extraction conditions (24- vs. 72-h extraction), and
4. impact of the different polar vehicles use (DPBS vs. saline).

In the paper published by Casas et al. (2013), 24 and 48 h were suggested as suitable exposure times, however, from MatTek's in house experience it was known that tissues may not tolerate well long exposure times (i.e. 48 h) with relatively high dose of vehicle (here 100 μL) without partial loss of viability in the negative and vehicle controls. Therefore, in the current project, we decided to evaluate whether shorter exposure times could be used with well-established time-to-toxicity protocols that had been developed for testing of cosmetics and surfactants (Faller et al., 2002; MatTek ET-50 SOP).

As shown in Fig. 2, optical densities of the negative controls (NC) at 4, 18 and 24 h are comparable to vehicle controls (VC, SO. and VC, Saline.), however, at 24 h, an overall decrease in the optical densities have been noticed compared to the shorter exposure times. This pattern was also seen in some other experiments (data not shown).

In search for a Positive control, we focused on Sodium dodecyl sulphate (SDS), since as a surfactant it has an affiliation to both polar

Table 3

Seventeen laboratories underwent training in the EpiDerm SIT-MD protocol in connection with the RR project. Cyprotex was not trained in Phase I having sufficient experience with the Casas et al. (2013) studies. UKM did not complete the Phase II training.

| #	Abbrev.	Full name of the institute	Training site	Training year	Phase I	Phase II
1	RIVM	RIVM, National Institute for Public Health and the Environment, Bilthoven, The Netherlands	EU	2013	x	x
2	Eurofins	Eurofins Biolab Srl, Vimodrone, Milan, Italy	EU	2013	x	x
3	NIOM	NIOM, Norway	EU	2013	x	x
4	Nelson	Nelson Laboratories, Inc., Salt Lake City, UT, USA	USA	2013	x	x
5	NAMSA	NAMSA, Northwood, OH, USA	USA	2013	x	x
6	Boston Scientific	Boston Scientific, Marlborough, MA, USA	USA	2014	x	x
7	SP TRI	SP Technical Research Institute of Sweden, Chemistry, Materials and Surfaces, Borås, Sweden	EU	2014	x	x
8	Arthrex	Arthrex Inc., Naples, FL, USA	EU	2014	X	x
9	TOXIKON	Toxikon, Inc., Bedford, MA, USA	USA	2014	X	x
10	LEXAMED	LexaMed, Ltd., Toledo, OH, USA	USA	2015	x	x
11	YONSEI	Yonsei University College of Dentistry, Department & Research Institute for Dental Biomaterials & Bioengineering, Seoul, South Korea	USA	2015	x	x
12	Cyprotex	Cyprotex US LCC, Kalamazoo, MI, USA	USA	2015	–	x
13	APS	American Preclinical Services LLC, Minneapolis, MN, USA	USA	2015	X	x
14	ENVIGO	Envigo CRS GmbH, Rossdorf, Germany	EU	2016	X	x
15	Kurabo	Kurabo Industries, Ltd. Osaka, Japan	EU	2016	X	x
16	NIHS	NIHS, Division of Medical Devices National Institutes of Health Services, Tokyo, Japan	Japan	2016	X	x
17	UKM	School of Bioscience & Biotechnology Faculty of Science & Technology Universiti Kebangsaan Malaysia	EU	2016	X	–
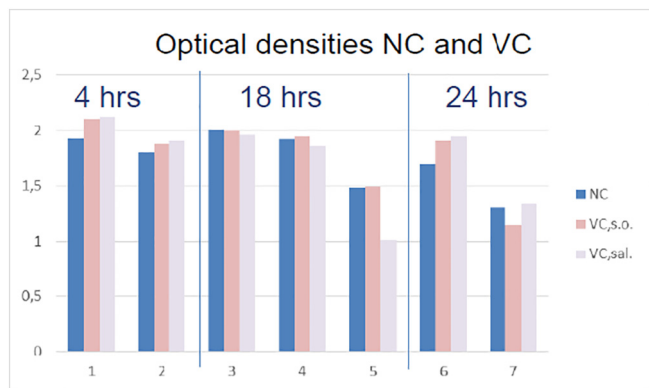
Fig. 2. Optical densities of the non-treated controls and vehicle controls depending on exposure time.

and non-poplar solvents/extracting solutions. SDS is also used as a positive control in human patch tests as well as in OECD TG 439. Several dose-response experiments with different concentrations of SDS were performed in both water and SO with the aim of finding a concentration that would result in a positive response within the proposed time-range of 4, 18 and 24 h. The calculated IC-50 (50% inhibition concentration) for SDS in water at 4, 18 and 24 h were in the range of 0.25–0.3% and in sesame oil in the range of 0.1–0.9%. Viability as well as variability of the SDS in sesame oil was higher compared to the water solutions (see Fig. 3).

Based on the results obtained, and considering the possible variabilities of the assay (e.g. biological variability of the tissues and their responses and operator variability), a positive control concentration of 1% SDS was chosen for both saline and SO extraction solutions. A 1% solution of SDS is known to introduce a mild irritation in some sensitive individuals when exposed topically for longer periods and/or repeatedly.

The optimal exposure time in the EpiDerm SIT MD was established as a function of the controls stability and protocol sensitivity towards irritating benchmark materials. Negative controls (NC) and vehicle controls (VC) were required to have a minimal decrease in the optical density compared to the non-treated controls during the entire exposure time. Positive controls (PC), on the other hand, had to be predicted as irritating in both solvents without extreme damage of the tissues that would be reflected by viabilities close to 0% and detachment of the skin from the supporting membranes of the cell culture inserts.

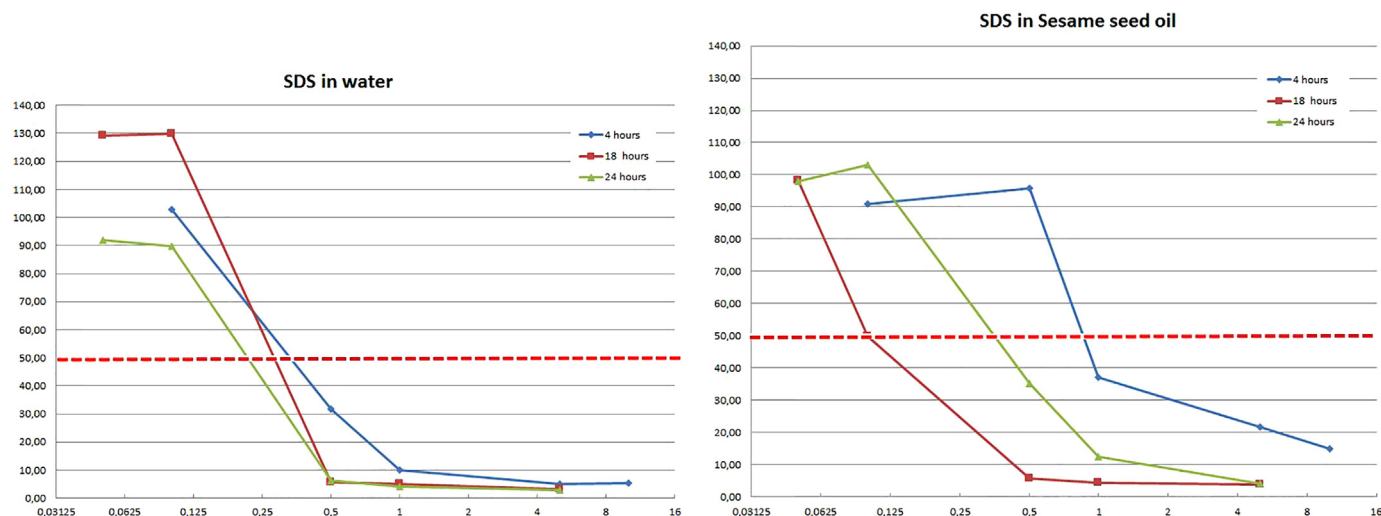In order to find the optimal exposure time, tissues were exposed to

Fig. 3. Determination of the IC-50 value for SDS in water and sesame oil at 4, 18 and 24 h. The calculated IC-50 for SDS in water at 4, 18 and 24 h were in the range of 0.25–0.3% and in sesame oil in the range of 0.1–0.9%. Viability as well as variability of the SDS in sesame oil was higher compared to the water solutions.
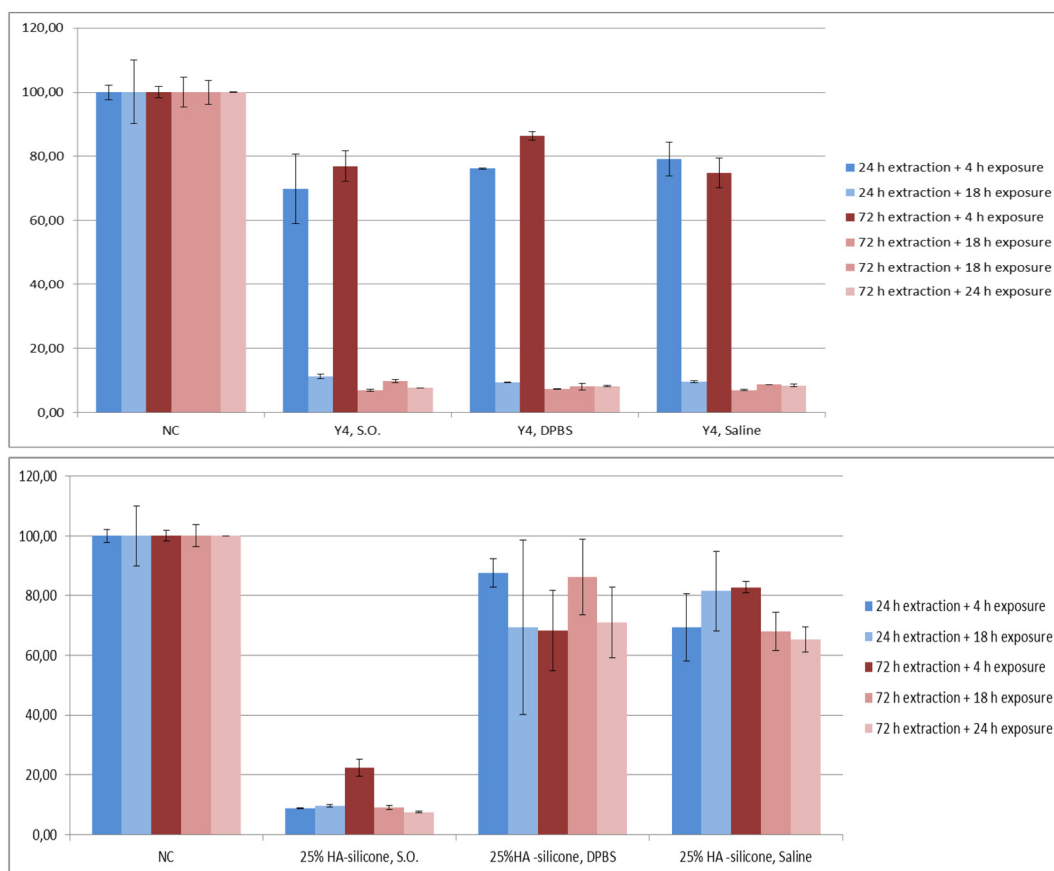
Fig. 4. Two polymers, Y-4 and silicone spiked with 25% HA, were tested in various exposure times (4, 18 and 24 h) and in three different extracting solutions - sesame oil (so), DPBS and saline. Two extraction times (24 and 72 h) were used. Regardless the extraction time (24 vs. 72 h, and the extraction solvents used (DPBS, Saline, Sesame Oil), Polymer Y-4 produced significant cytotoxicity after 18 and 24 h of exposure and can therefore be classified as irritating. Silicone spiked with 25% HA and extracted with sesame oil produced positive responses under all conditions tested and negative response when extracted with saline. These results are due to the different affinity of heptanoic acid to the polar and non-polar extraction solvents.

Lactic acid (LA) 4%, Heptanoic acid (HA) 1% and Y-4 polymer extracts. HA and LA were identified by Casas et al. (2013) as useful benchmarks, but the final concentration for the further protocol optimisation were selected in the same experiments as described for SDS, i.e. IC-50 determination (data not shown). These experiments were conducted for 4, 18 and 24 h exposure times. In addition, we also evaluated how 24- vs. 72-h extraction times influenced the tissue viability results of the Y-4 polymer extracts.

After 4 h exposure time, LA 4% and HA 2% provided positive, but sometimes also borderline predictions, however with 18 and 24 h exposures these materials were clearly classified as irritating. Similar results were obtained with Polymer Y-4 (see Fig. 4), with exception that at 4 h, the material was constantly underpredicted as non-irritating. There were no significant differences between saline and DPBS extracts. Testing of several polymers spiked with the known irritants (SDS and HA, Table 4) with 4, 18 and 24 h exposure confirmed, that the best exposure scenario will be 18 ± 1 h to assure sufficient sensitivity of the test to predict intra-cutaneous irritation.

3.2. Phase II: Inter-laboratory comparison

The aim of the second phase was to challenge the predictive ability of the optimized test in an inter-laboratory study with polymers spiked with known irritants (LA, HA, SDS and Genapol) and polymers used as non-irritating controls (see Table 2b). The materials were sent to MatTek IVLSL and RIVM by Medtronic and the testing was conducted in an approximately similar time-frame. MatTek assessed also IL-1-α profile of all materials tested. Results obtained in this testing phase are

summarised in Table 4, Figs. 5 and 6.

The predictions were in most cases in good agreement between the two laboratories, as can be judged by the mean tissue viability values summarised in Table 4. Only one material, silicone containing Lactic acid, resulted in different predictions between the two testing laboratories. It was also the only one material, where the IL-1α analysis improved the classification. See Fig. 6E.

In most of the experiments, the IL-1α release correlated with the tissue viability decrease. However, the Y-4 polymer's first IL-1α run resulted in a negative result, while the second run produced a positive prediction despite the fact that the tissue viability in both runs were comparably low. These results may be due to the faster cytotoxicity effect in the first evaluation that prevented synthesis and release of the IL-1α by the keratinocytes.

Based on the results obtained in this limited study, we conclude that the vehicle treated controls and materials that are truly non-irritating provide IL-1α release below 40 pg/mL in the EpiDerm protocol. Materials showing increase in the IL-1α above 50 pg/mL (measured in this specific test and with the Quantikine Human IL-1 α kit) should be treated with caution since they may produce inflammation or skin sensitisation in the sensitive patients. Different IL-1 α kits, however, provide different basal levels of IL-1 α, and therefore validation of this procedure is recommended before routine use.

3.3. Phase III: Training of 17 laboratories for the upcoming round robin study

The goal of this final step was to transfer the EpiDerm SIT-MD

Table 4
Result obtained in the inter-laboratory assessment of the 13 polymer materials at MatTek and RIVM.

| No.	Material name	Expected in vivo prediction	Extracting solution	RIVM			MatTek			Prediction based in viability
				Viability mean (%)	SD/Diff	N	Viability mean (%)	Diff	N	
1	Y-4 Polymer	Irritant	Saline	4,3	0,6	3	7,9	1,7	2	Irritant
			Sesame oil	15,2	12,7	3	8,4	2,8	2	
2	25% HA in one-part silicone	Irritant	Saline	29,7	8,5	3	55,1	26,0	2	Irritant (based mainly on sesame oil results)
			Sesame oil	4,4	0,4	3	8,4	1,5	2	
3	15% SDS in two-part silicone	Irritant	Saline	2,5	0,2	3	5,6	n.a.	1	Irritant (based on saline results)
			Sesame oil	67,4	54,7	3	75,7	n.a.	1	
4	17% SDS in two-part silicone	Irritant	Saline	2,5	0,3	3	4,4	n.a.	1	Irritant (based on saline results)
			Sesame oil	71,4	53,9	3	98,9	n.a.	1	
5	60% LA in one-part silicone	Irritant	Saline	96,4	10,0	3	44,7*	79,1	2	Non-irritant/Irritant
			Sesame oil	98,7	4,3	3	82,3	39,0	2	
6	15% SDS in silicone (new type)	Irritant	Saline	3,1	0	2	4,2	1,9	2	Irritant (based on saline results)
			Sesame oil	91,2	10,2	2	76,1	64,0	2	
7	15% SDS in silicone with PVC	Irritant	Saline	2,5	0,2	2	5,1	2,7	2	Irritant (based on saline results)
			Sesame oil	96,45	20,5	2	95,5	31,2	2	
8	7% Genapol in PVC	Irritant	Saline	3,9	0,2	2	7,2	2,8	2	Irritant
			Sesame oil	3,6	0,4	2	16,5	25,8	2	
9	PVC control	Non-irritant	Saline	94,5	6,6	2	77,4	44,6	2	Non-irritant
			Sesame oil	96,3	0,4	2	90,9	22,1	2	
10	Y-1 control PVC	Non-irritant	Saline	101,95	2,7	2	95,7	39,8	2	Non-irritant
			Sesame oil	101,95	4,9	2	86,2	28,2	2	
11	One-part silicone NC L/N 68021	Non-irritant	Saline	97,75	1,3	2	n.a.	n.a.	0	Non-irritant
			Sesame oil	98,85	1,9	2	n.a.	n.a.	0	
12	Two-part silicone NC	Non-irritant	Saline	95,5	1,2	2	n.a.	n.a.	0	Non-irritant
			Sesame oil	96	3,4	2	n.a.	n.a.	0	
13	Polyurethane control	Non-irritant	Saline	93,6	13,2	3	81,6	10,9	2	Non-irritant
			Sesame oil	92,8	10,4	3	92,7	28,8	2	
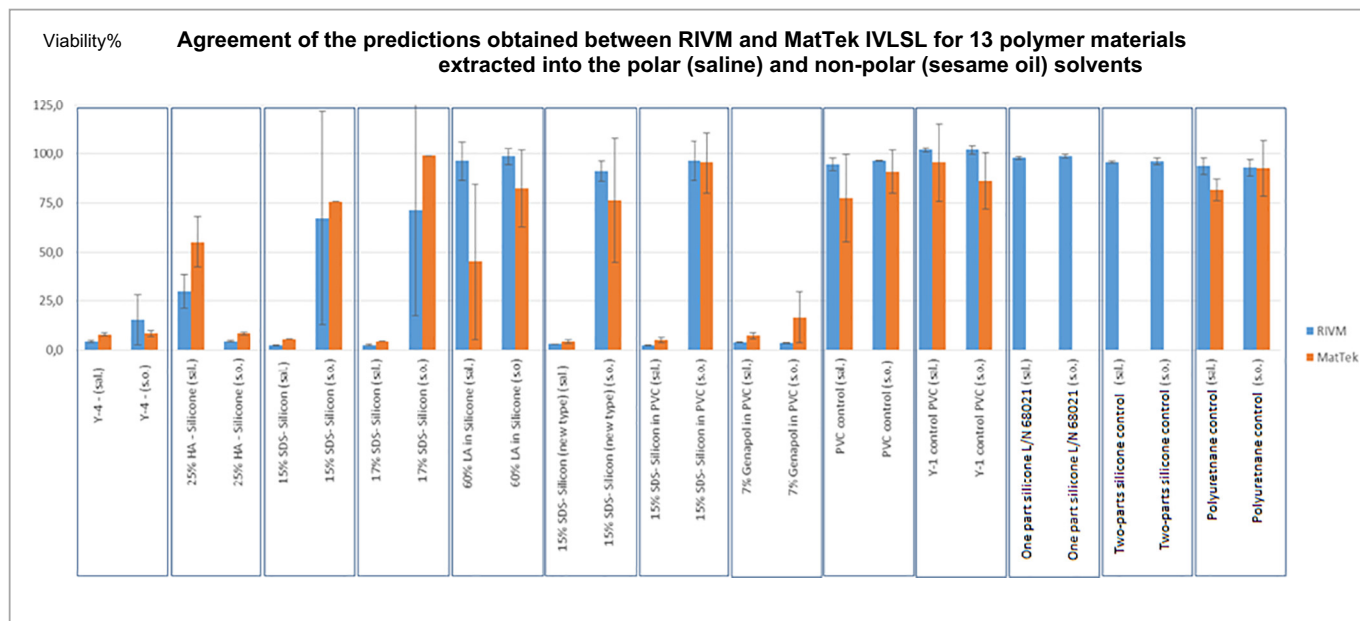
Fig. 5. Agreement of predictions obtained between RIVM and MatTek IVLSL. Each sample of the medical device polymer was extracted with the polar solvent (saline (sal.)) and the non-polar solvent (sesame oil (SO)). A material was classified as irritating if tissue viability in any of the solvent extracts fell below 50%. Due to the insufficient amount of the material, one part silicone L/N 60821 and Two-parts silicone control samples were tested only at RIVM.

protocol to laboratories that wished to participate in the round robin study. After discussions between the round robin management team and the laboratories, an agreement was reached to conduct a two-phase training program designed to assure optimal transfer of the test protocol into their facilities. In total, 17 laboratories were trained over a period of almost three years by MatTek Corporation and MatTek IVLSL in the US and Europe, and with help of Kurabo Industries, Ltd. in Japan (see Table 3).

Results of the Phase I Training, conducted by trainer with three benchmark chemicals, are shown in Fig. 7. The order of laboratories in the Fig. 7 graphs is randomized to protect their identity.

The Phase I Training was conducted for most of the laboratories using a 4-h exposure time because: (1) at 18 and 24 h, all the test materials provided relatively low viabilities (usually below 25–30%) and SOP deviations may not be revealed with such significant cell death, and (2) it was more efficient for the training laboratories to work
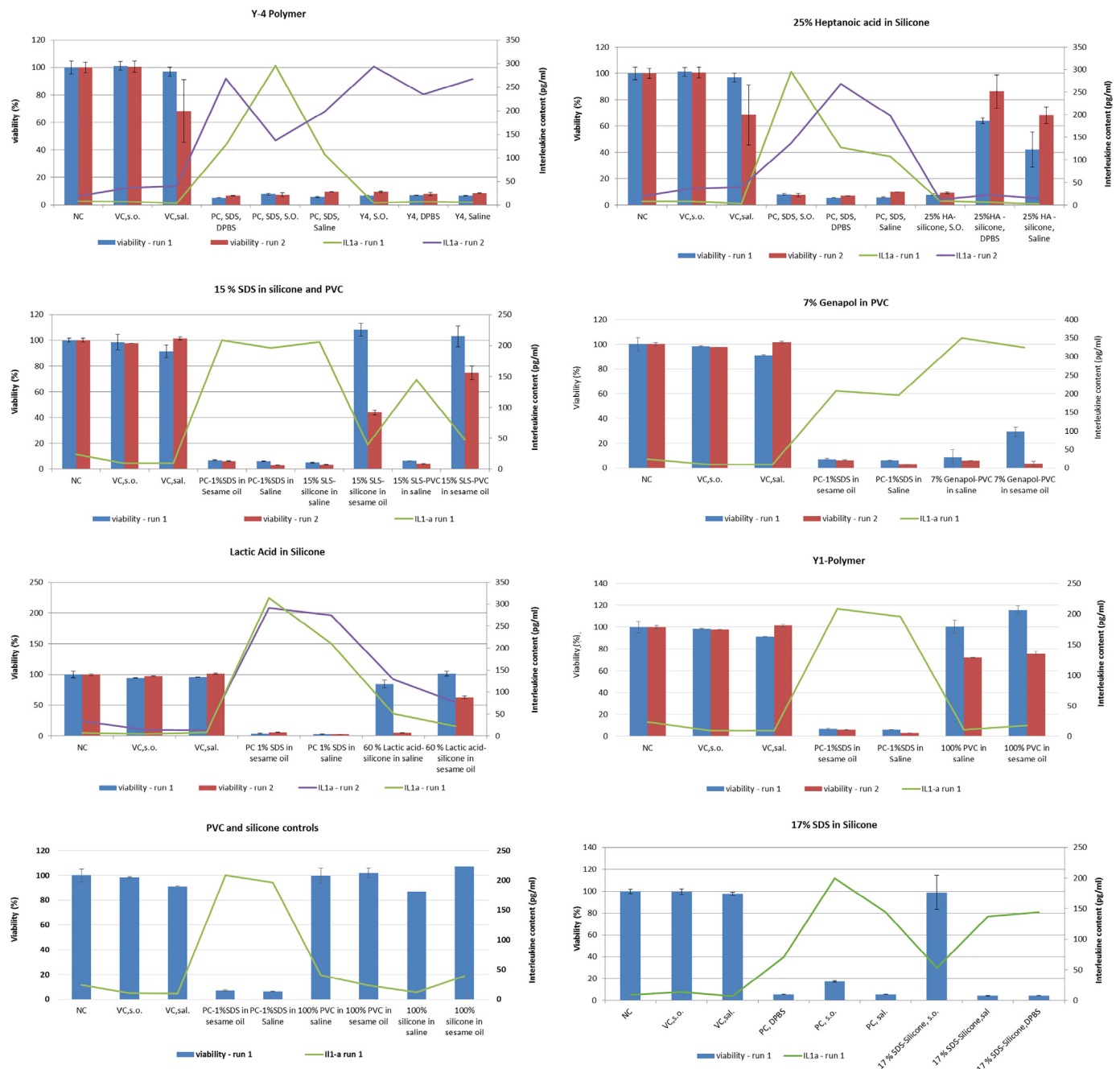
Fig. 6. Viability and cytokine release profiles obtained from testing of polymers and controls with 18 h exposure time. Bars represent tissue viability (%), while lines are representing interleukin release in pg/mL measured by human IL-1α Quantikine kits from R&D Systems. PC stands for positive control 1% SDS in DPBS, saline or sesame oil (SO).

with the shorter exposure time when undergoing on-site training. Four laboratories that joined the project shortly before the round robin began requested 18-h exposure training. Those are highlighted with yellow in Fig. 7.

As can be seen from the data presented, all laboratories achieved expected values for vehicle and positive controls (1% of SDS in polar and non-polar solvents). Increased variability has been observed for LA 4% and some false predictions for HA 2%. These results were however expected in an initial training session with the shortest exposure time.

Results of the Phase II Training, which was a proficiency test, are summarised in Fig. 8. Most of the laboratories conducted a full set of testing as requested by MatTek (i.e. with exposure times at 4, 18 and 24 h). Some of the laboratories, however, decided to conduct only 18 or 24 h exposure tests because these longer time-points had been selected for the upcoming round robin study for EpiDerm (18 h) and SkinEthic

tissue models (24 h).

All testing laboratories participating in Phase II Training fully qualified to join the round robin study except one (indicated in yellow). This laboratory provided highly elevated saline controls and did not predict one of the positive controls correctly (1% SDS in sesame oil). Instead of running 18-h exposures, two laboratories conduced 4- or 24-h exposures (data were not included into Fig. 8).

Fourteen out of seventeen laboratories qualified fully for the round robin study based on their data from the training Phases I and II. In addition, two laboratories that conducted either 4- or 24-h exposure studies during Phase II instead of the 18-h study, were also regarded as sufficiently trained and qualified based on additional studies conducted in their laboratories. Thus only one out of the 17 laboratories did not provide satisfactory results in Phase II. This laboratory produced highly elevated saline vehicle control results with mean tissue viability of
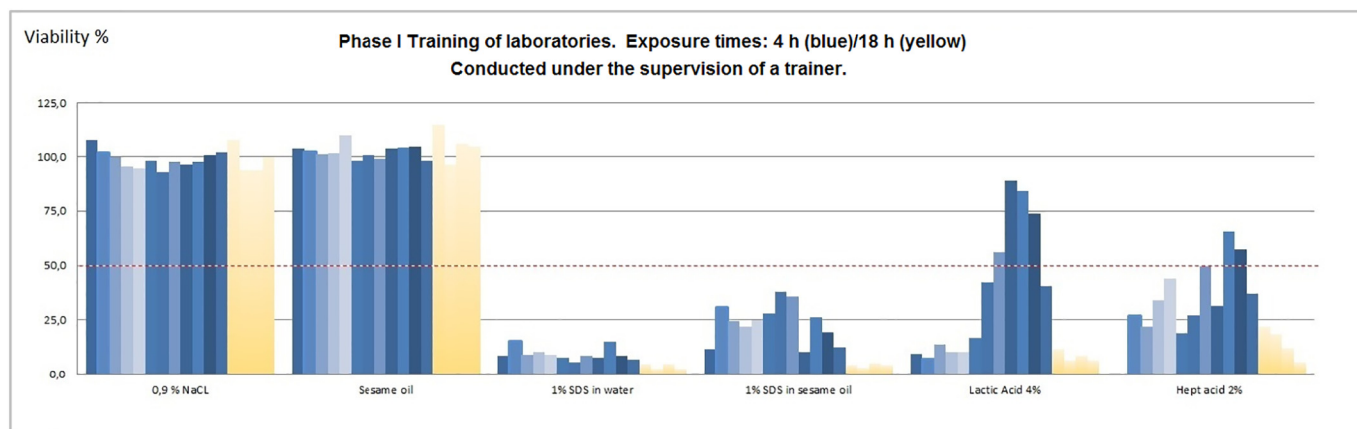
Fig. 7. Results obtained by the 16 test laboratories conducting Phase I training.The laboratories attended a practical training session (see Table 3) during which they were trained using the 4-h exposure time so that the training could be performed in one day. Four laboratories that joined the project shortly before the round robin began asked for training with the 18-h exposure. Those four laboratories are located at the end of the testing data series and are highlighted in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

153% and untreated control (UTC) of 173% (data for UTC not shown in Fig. 8). Moreover, it did not predict one of the positive controls correctly (1% SDS in sesame oil, viability 63%).

Overall, the fully qualified laboratories correctly classified the positive controls and benchmark chemicals. Negative controls with 18-h exposures provided an optical density (OD) of 1.787 ± 0.19. Vehicle controls were highly stable and comparable to the DPBS treated control. Tissues treated with saline provided on average viability in the range of 101.9 ± 6.9% and Sesame oil treated tissues provided viability 102.2 ± 6.7%. Untreated controls provided OD 2.077 ± 0.217 resulting into the viability of 117 ± 15.6%.

Positive controls: 1% SDS in water and 1% SDS in SO resulted in mean viabilities of 4.0 ± 1.4% and 4.7 ± 2.5%, respectively. Benchmark chemicals: 4% lactic acid with 18-h exposure conditions produced an average viability of 8.7 ± 4.2% and heptanoic acid 14.8 ± 11.5%. Data from the non-qualified laboratory were not included in above mentioned calculations.

4. Discussion and conclusions

The rabbit skin irritation test was used for decades as a well-established standard for testing the irritation potential of chemicals and products. In the 1980s it was adopted and modified by the medical device industry for two types of exposure: cutaneous (topical) and intracutaneous (injection). Both of these methods are used in the safety

assessment of medical devices and may cause pain and suffering to the test animals. It has been reported that this test overpredicts the irritation potential of chemicals due to differences between rabbit and human skin. In addition, it may not be sufficiently sensitive to predict some irritation responses seen with medical devices (e.g. surgical sutures) in patients.

Casas et al. (2013) suggested the use of the reconstructed human tissue model EpiDerm as a possible alternative test method for assessing the dermal irritancy potential of medical devices. Results of their study indicated that the EpiDerm RhE tissue model can detect the presence of strong skin irritants at low concentrations when spiked in medical device polymer extracts and recommended that a validation study be conducted with medical devices to confirm their findings.

In preparation for the validation study, it was necessary to formalize the SOP and to find appropriate controls and benchmark materials that could be tested during the training of participating laboratories and also in the validation study. As a first step, benchmark materials from Casas et al. (2013) were re-tested in several test designs and concentrations in order to optimize the exposure time of the assay without compromising sensitivity and at the same time to assure optimal conditions for the negative, positive and vehicle controls.

In the experiments of Casas et al. (2013), 24- and 48-h exposures were used. As reported in the paper, despite not seeing significant viability decrease in vehicle and negative controls, the extended exposure times led to the unwanted production and release of IL-1α into
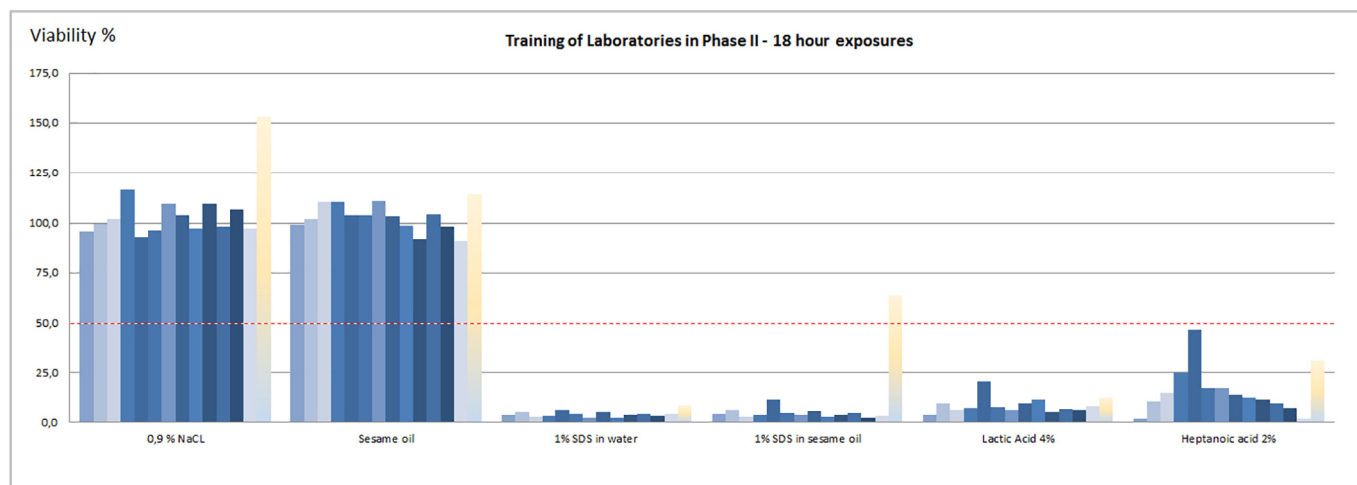

Fig. 8. Results obtained by the 14 test laboratories, who conducted Phase II training with 18 h exposure conditions over period of three years.

the basal media. In order to improve the results, we evaluated shorter exposure times of 4 and 18 h in addition to 24 h, which was set as the maximum exposure time based on the experience of Casas et al. (2013) and others.

Four-hour exposures, which has been initially suggested along with the 18- and 24-h exposures, did not prove to be a reliable time-point for an unambiguous prediction of the irritating potential. However, it was thought that it may improve the knowledge about the irritating potency of the material tested. Twenty-four-hour exposures provided almost identical results to 18-h exposures, but such long exposures negatively impacted the ODs of vehicle controls, and in a few cases, detachment of the tissue treated with the PCs from supporting membrane was noted. Eighteen-hour exposures provided similar tissue viabilities as those obtained at 24 h (see Figs. 2 and 4), however, it improved the ODs of the controls and provided significantly lower IL-1α levels in controls as those seen in Casas et al., 2013 (Fig. 6). With the 18-h exposure time, negative and vehicle controls were consistently below 40 pg/mL. Therefore, the optimal exposure time-point for EpiDerm tissue model was set to 18 h.

During our pilot tests, we also evaluated the impact of extraction conditions (24- vs. 72-h) on the test results. As demonstrated with Polymer Y-4, and confirmed with several other test materials (e.g. one-part silicone containing 25% HA (other testing data not shown)), extraction times of 24 vs. 72 h did not cause any differences in prediction (see Fig. 4). No significant differences were observed between extractions into the saline and DPBS. However, taking into the account the precautionary principle, the extraction time was set in the optimized SOP to 72 h.

After completing the optimisation of the SOP in collaboration with the prevalidation study management team, the first training sessions were organized. At the same time, transferability and interlaboratory exercises were conducted between RIVM and MatTek IVLSL in order to challenge the new SOP with materials that would be suitable for the upcoming validation. Since there were few, if any, positive (irritating) materials available on the market, dedicated specialized materials were synthetized by Medtronic and Arthrex (see Coleman et al., 2017, this issue) and provided for the interlaboratory testing purposes. In total 13 materials were assessed by the two laboratories, some in 2 or 3 independent runs (Table 4). MatTek also conducted IL-1α analysis for the selected materials in order to evaluate whether IL-1α may bring additional value to the predictions based on the cytotoxicity.

As can be seen from Fig. 5, the agreement in the predictions between the two laboratories was very good, with the exception of one-part silicone with 60% of powdered LA. This was most likely due to the highly inhomogeneous nature of the polymer sample, as well as the fact that the original sample of the active substance (solidly embedded into the silicone matrix) contained only 58–52% of powdered LA. The rest was calcium 2-hydroxypropanoate (calcium lactate) that may neutralize the activity of LA. It is also used as antacid in medicine and food industry (Galactic Powder 60. Galactic Inc. Milwaukee, Wisconsin, USA).

Interleukin analysis, conducted by MatTek, generally correlated with the viabilities as observed in the study of Casas et al. (2013). It was able to improve prediction only for the one-part silicone 60% LA material, providing in both runs, and in both extracting solutions, significantly higher IL-1α than in the vehicle controls (see Fig. 6). For Y-4 polymer, the first IL-1α run resulted in a negative prediction, while the second run yielded a highly positive prediction despite the fact that viabilities in both runs were comparably low. We hypothesize that this is likely due to the first run's fast cytotoxic effect, which prevented synthesis and release of cytokine IL-1α by the keratinocytes. These findings confirm experience and conclusions from previous validation studies, that IL-1α may be useful parameter in gathering additional data supporting the classification, but cannot be used as a stand-alone parameter for prediction, since it could result into false negative outcomes. The study conducted between MatTek IVLSL and RIVM with 13

polymers was presented in June 2015 at the ISO Technical Committee 194 meeting in Lund, Sweden. The results demonstrated that the new protocol was completely transferable and sufficiently sensitive, and thus ready to be evaluated in round robin validation study.

In parallel with the interlaboratory testing between MatTek and RIVM in Europe, MatTek scientists in the USA and Europe, along with Kurabo scientists in Japan, trained 17 laboratories in conducting the new irritation assay with RhE tissues. These training sessions were conducted over a time period of three years, between 2013 and 2016 (for details see Table 3). All laboratories participated in the training sessions on a voluntary basis and conducted additional follow-up testing after returning from the MatTek sessions. The data summarised in Fig. 8 demonstrates the proficiency of the testing laboratories. The high technical competence of the laboratories participating in this project, plus the robustness of the methodology had thus been demonstrated before the beginning of the validation study. Only one of the laboratories did not qualify fully during the Phase II Training. The laboratories who passed both training sessions were invited to join the round robin validation study that began in the second half of 2016, the results which are described in de Jong et al., 2017 (this TIV issue).

5. Conclusions

We have optimized and pre-validated the EpiDerm™ Skin irritation test (EPI-200-SIT-MD) for medical devices to detect the presence of skin irritants at low levels in medical device extracts. This test is able to detect low irritation potential of chemicals extracted from medical devices into the polar and non-polar solvents. Predictive capacity and reproducibility has been demonstrated in the inter-laboratory trial between MatTek IVLSL and RIVM between 2014 and 2015. 17 laboratories from Europe, USA and Asia participated in the 2 phased trainings to become qualified for the upcoming validation study. This assay, when successfully validated, can be used as a highly sensitive replacement for the rabbit cutaneous and intra-cutaneous irritation tests required by ISO 10993-10, 2010.

Transparency document

The http://dx.doi.org/10.1016/j.tiv.2018.02.007 associated with this article can be found, in online version.

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