Video Article

An *In Vitro* Skin Irritation Test (SIT) using the EpiDerm Reconstructed Human Epidermal (RHE) Model

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Abstract

The EpiDerm Skin Irritation test (EpiDerm SIT) was developed [1,2,3] and validated [4,5] for *in vitro* skin irritation testing of chemicals, including cosmetic and pharmaceutical ingredients. The EpiDerm SIT utilizes the 3D *in vitro* reconstructed human epidermal (RHE) model EpiDerm. The procedure described in this protocol allows for discrimination between irritants of GHS category 2 and non-irritants [6]. The test is performed over the course of a 4 day time period, consisting of pre-incubation, 60 minute exposure, 42 hour post-incubation and MTT viability assay. After tissue receipt and overnight pre-incubation (Day 0), tissues are topically exposed to the test chemicals (Day 1), which can be liquid, semi-solids, solid or wax. Three tissues are used for each test chemical, as well as for the positive control (5% aq. SDS solution), and a negative control (DPBS). Chemical exposure lasts for 60 minutes, 35 min of which the tissues are kept in an incubator at 37°C. The test substances are then removed from the tissue surface by an extensive washing procedure. The tissue inserts are blotted and transferred to fresh medium. After a 24 hr incubation period (Day 2), the medium is exchanged. The medium can be saved for further analysis of cytokines or other endpoints of interest. After the medium exchange, tissues are incubated for an additional 18 hours. At the end of the entire 42h post-incubation (day 3), the tissues are transferred into yellow MTT solution and incubated for 3 hours. The resultant purple-blue formazan salt, formed mainly by mitochondrial metabolism, is extracted for 2 hours using isopropanol. The optical density of the extracted formazan is determined using a spectrophotometer. A chemical is classified as an irritant if the tissue viability relative to the negative control treated tissues is reduced below 50%. This procedure can be used as full replacement of the in vivo rabbit skin irritation test for hazard identification and labeling of chemicals in line with EU regulations [7].

Video Link

The video component of this article can be found at https://www.jove.com/video/1366/

Protocol

I. Tissue conditioning - Day 0

1. Upon receipt of the EpiDerm EPI-200-SIT kit, check all the kit components for integrity (for kit details see Table of specific reagents and equipment).
2. For every three EpiDerm tissues, prepare one sterile 6-well plate pre-filled with 0.9 ml of assay medium.
3. Under sterile conditions, open the plastic bag containing the 24-well plate containing the EpiDerm tissues and remove the sterile gauze.
4. Use sterile forceps to remove each insert containing the EpiDerm tissue and place the insert in the empty 24-well plate. During this step, remove any remaining shipping agarose that adheres to the outer sides of the insert by gentle blotting on sterile filter paper.
5. Within the next 5 minutes, visually inspect the tissues. Do not use defective tissues or tissues which are completely covered with liquid.
6. Using a sterile cotton tip swab, carefully dry the surface of the tissues. Try not to touch the tissue directly - capillary effects are sufficient to remove the moisture from the tissue surface.
7. After drying the tissue surface, transfer three tissues to the top row of the 6-well plates pre-filled with 0.9 mL of assay medium. Release any air bubbles trapped underneath the inserts.
8. Place the 6-well plates containing the inserts into the incubator for a 60 ± 5 min pre-incubation at 37 ± 1° C, 5 ± 1% CO_2, 95 % RH(Relative Humidity).
9. At the end of the 60 ± 5 min pre-incubation period, transfer the inserts from the upper wells into the lower wells of the 6-well plate. Place the plates back into the incubator (37 ± 1° C, 5 ± 1% CO_2, 95 % RH) for an overnight pre-incubation (18 ± 3 hr).
10. Place the rest of the assay medium into the refrigerator (5 ± 3 °C). Following the overnight pre-incubation, the test chemicals can be applied to the EpiDerm tissues.
    Note: The pre-incubation procedure can be shortened in case of late tissue delivery (e.g. if tissues arrive on Wednesday instead of Tuesday).

II. Chemical exposure - Day 1

1. Prepare one 6-well plate per test chemical by filling the upper row only with 0.9 ml per well of assay medium.

2. Approximately 5 minutes before the planned exposure to chemicals, remove the 6-well plates containing the pre-conditioned tissues from the incubator.
3. Evaluate the surface of tissues and remove any moisture using sterile cotton tips.
4. Label all 6-well plate lids with the test material codes or names.
5. Dose the tissues with the test chemicals at 1 minute intervals to facilitate rinsing of the test chemicals after exposure. Keep the plates with dosed tissues in the sterile hood, until the last tissue is dosed.
6. To test liquid chemicals, dispense 30 µL of the solution directly atop the tissue and place the nylon mesh on the tissues surface. If necessary, gently position the mesh using the bulb headed glass Pasteur pipette. Do not press on the tissue surface.
7. For testing semisolids, use a positive displacement pipette to dispense 30 µL directly atop the tissue. If necessary spread the chemical with Pasteur pipette to cover the tissue surface as much as possible.
8. For solid materials, shortly before application of the test substance, moisten the tissue surface with 25 µL of sterile DPBS. This will improve contact of the tissue surface with the test chemical. Fill a 25 mg calibrated sharp application spoon (or any other appropriate tool) with approximately 25 mg of finely ground test material. Apply the solid test chemical to the tissue surface. If necessary, use a bulb headed Pasteur Pipette to empty the spoon completely. Gently shake the inserts to improve the spreading of the solid on the surface.
9. For test substances with waxy consistency, try to form a flat "cookie like" piece about 8 mm in diameter and place it atop the tissue, which was previously wetted with sterile DPBS. To improve the contact between test substance and tissue, weigh down the "cookie" with a stainless steel or plastic aid.
10. Apply DPBS as the negative control and 5% SDS as the positive control to the control tissues.
11. After dosing the last tissue, transfer all the plates for 35 ± 1 minutes to the humidified incubator (37 ± 1° C, 5 ± 1% CO_2, 95 % RH).
12. After the 35 ± 1 minute incubation at 37°C remove all the plates from the incubator. Place the plates into the sterile hood and wait until a period of 60 minutes chemical exposure is completed for the first dosed tissue.
13. Afterwards start the washing procedure using the one insert per minute time interval (to insure that the total exposure time for every insert is 60 minutes).
14. Use a wash bottle to rinse the tissues with sterile DPBS. Fill the tissue insert 15 times by using a constant stream of DPBS and empty it (Note: for tissues with a nylon mesh, wash the tissue surface 5 times and remove the mesh carefully with the pointed, sharp forceps. Afterwards, continue with the remaining 10 washes).
15. After the 15th rinse using the wash bottle, completely submerge the insert 3 times in 150 ml DPBS and shake to remove the remainder of the test material.
16. Finally, rinse the tissue insert once from the inside and once from the outside with sterile DPBS.
17. Remove any excess of DPBS from the tissue by gently shaking the insert, and blotting it on the sterile blotting paper.
18. Transfer the blotted tissue inserts to the new 6-well plate previously pre-filled with assay medium. All of these steps (14-18) must be done within 1 min for each insert.
19. After the last tissue is rinsed, carefully dry the surface of each tissue with a sterile cotton tipped swab.
20. Incubate the tissues in the incubator for the next 24 ± 2 hours (37 ± 1° C, 5 ± 1% CO_2, 95 % RH).
    Note: Each assay technician should not test more than 6 test substances including the negative and positive controls in a single testing run (set), to be able follow the protocol as described below

III. Medium exchange - Day 2

1. After the 24 ± 2 hour post-incubation, transfer the inserts into the lower part of the 6-well plate, pre-filled with 0.9 ml of media. Media from upper rows can be collected for analysis of the additional endpoints (cytokines, chemokines etc.). Analysis of the secondary endpoints is optional.
2. Continue with the post-incubation (37 ± 1° C, 5 ± 1% CO_2, 95 % RH) for another 18 ± 3 hours.

IV. MTT Viability Assay - Day 3

1. Label two 24-well plates with the chemical names or codes. One plate will serve for tissue incubation with MTT and the other for the extraction step.
2. Prepare the MTT solution by thawing the MTT concentrate (5 mg/ml) and diluting it with the MTT diluent. Final concentration of MTT solution is 1 mg/ml.
3. Pipette 300 µL of MTT solution into each well of the 24 well plate.
4. Remove the 6-well plates from incubator, blot the bottom of the inserts on a blotting paper, and transfer them into the 24-well plate pre-filled with MTT.
5. Place the 24-well plate in the incubator (37 ± 1° C, 5 ± 1% CO_2, 95 % RH) and incubate for 3 hours ± 5 min. Strictly adhere to the 3 hours ± 5 min incubation time to avoid deviation between different MTT readings.
6. When the MTT incubation is complete, use a suction pump to gently aspirate the MTT medium from all the wells.
7. Refill the wells with DPBS and aspirate again. Repeat this rinsing two more times and make sure that tissues are dry after the last aspiration.
8. After the washes transfer the inserts to a new 24-well plate. Immerse the inserts by gently pipetting 2 mL extractant solution (Isopropanol) into each insert. The level will rise above the upper edges of the insert, thus completely submerging the tissues.
9. Seal the 24-well plate (e.g. with Parafilm or using sealing bag) to inhibit extractant evaporation. Extract the MTT from the tissues for at least 2 hours at room temperature with gentle shaking on a plate shaker (~ 120 rpm). Overnight extraction without shaking can also be used.
10. After the extraction period is complete, pierce the inserts with an injection needle or bulb beaded Pasteur pipette and allow the extract to run into the well from which the insert was taken.
11. Discard the punctured insert and pipette the solution in the well up and down three times until it is homogenous.
12. For each tissue, transfer two 200 µL aliquots of the purple formazan solution into a 96-well flat bottom microtiter plate. Transfer the duplicates according to the fixed plate design given in the spreadsheet accompanying this video protocol. For blanks, use isopropanol.
13. Read the optical density (OD) of the MTT extracts in a 96-well plate spectrophotometer using a wavelength of 570 nm (540-580) without a reference filter.

14. Enter the results in the spreadsheet for automatic calculation of the results (Figure 1).
15. A test chemical is labeled 'irritant' (R38 or GHS category 2) if the percent tissue viability determined by MTT assay is <50% relative to the negative control.
    Note: MTT is toxic, so be sure to wear protective gloves when handling. Protect MTT and its solutions from light, since MTT is light sensitive. Use MTT solution within a few hours after preparation because it degrades over time.

V. Assay Quality Controls

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- **EPI-200-SIT Assay Acceptance Criterion 1: Negative Control:**
  The absolute OD of the negative control (NC) tissues (treated with sterile DPBS) in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures and under specific conditions of use. The assay meets the acceptance criterion if the mean OD_{570} of the NC tissues is ≥1.0 and ≤2.5.
- **EPI-200-SIT Assay Acceptance Criterion 2: Positive Control**
  A 5% SDS (in H_2O) solution is used as positive control (PC) and tested concurrently with the test chemicals. Concurrent means that the PC has to be tested in each assay, but not more than one PC is required per testing day. Viability of positive control should be within 95% confidence interval of the historical data. The assay meets the acceptance criterion if the mean viability of PC tissues expressed as % of the negative control tissues is 20%.
- **EPI-200-SIT Assay Acceptance Criterion 3: Standard Deviation (SD)**
  The skin irritancy is predicted from the mean viability determined on 3 single tissues and therefore the variability of tissue replicates should be acceptably low. The assay meets the acceptance criterion if the SD calculated from individual % tissue viabilities of the 3 identically treated replicates is <18%.

Representative Results

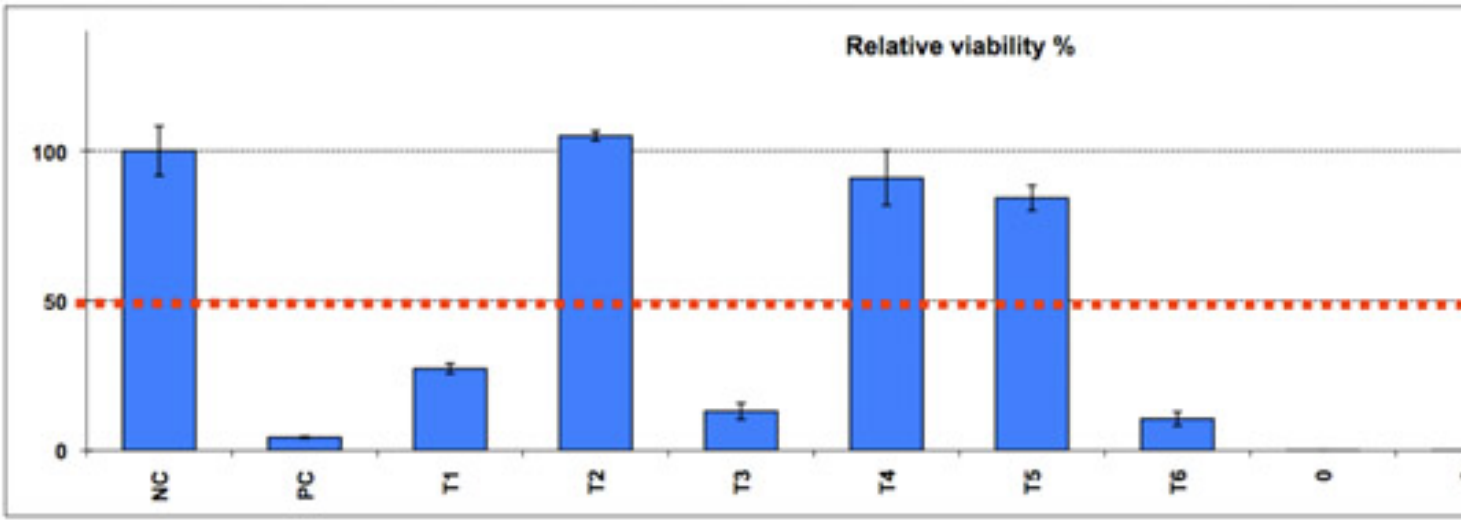
exp. no.:	EpiDerm SIT
tissue-lot no.:	11461
date:	20/03/2009
operator:	Kandarova

Blank	0.04
	0.04
	0.04
	0.04
	0.04
	0.04
Mean	0.0365

	mean of OD	SD of OD	mean of viabilities [%]	SD of viabilities	CV % [%]
NC	1.949	0.161	100.0	8.24	8.24
PC	0.085	0.007	4.4	0.34	7.82
T1	0.532	0.033	27.3	1.68	6.16
T2	2.045	0.031	104.9	1.58	1.50
T3	0.255	0.052	13.1	2.67	20.40
T4	1.772	0.177	90.9	9.09	10.00
T5	1.642	0.082	84.3	4.21	5.00
T6	0.206	0.046	10.6	2.34	22.09
0	0.000	0.002	0.0	0.10	-1135.78
0	-0.001	0.001	-0.1	0.03	-49.49
0	0.001	0.004	0.0	0.19	562.92
0	0.002	0.007	0.1	0.34	284.55

Classification

NC	NI	qualified
PC	I	qualified
T1	I	qualified
T2	NI	qualified
T3	I	qualified
T4	NI	qualified
T5	NI	qualified
T6	I	qualified
0	I	qualified
0	I	qualified
0	I	qualified
0	I	qualified


Relative viability %
100
50
0
NC
PC
T1
T2
T3
T4
T5
T6
0

Code N°	Tissue n	Raw data Aliq. 1	Aliq. 2	Blank corrected data Aliq. 1	Aliq. 2	mean of aliquotes	% of viability
NC	1	2.08	2.18	2.048	2.142	2.095	107.5
	2	1.8	1.83	1.761	1.793	1.777	91.2
	3	2.04	1.98	2.003	1.947	1.975	101.3
PC	1	0.12	0.13	0.082	0.096	0.089	4.5
	2	0.12	0.11	0.079	0.077	0.078	4.0
	3	0.13	0.13	0.089	0.091	0.090	4.6
T1	1	0.63	0.58	0.592	0.548	0.570	29.2
	2	0.55	0.56	0.511	0.522	0.516	26.5
	3	0.56	0.53	0.527	0.494	0.510	26.2
T2	1	2.02	2.08	1.980	2.039	2.009	103.1
	2	2.08	2.12	2.048	2.079	2.063	105.9
	3	2.08	2.11	2.048	2.076	2.062	105.8
T3	1	0.38	0.31	0.346	0.276	0.311	15.9
	2	0.29	0.28	0.256	0.242	0.249	12.8
	3	0.25	0.24	0.210	0.205	0.207	10.6
T4	1	1.68	1.7	1.646	1.664	1.655	84.9
	2	1.68	1.77	1.639	1.732	1.685	86.5
	3	2.02	2.01	1.979	1.973	1.976	101.4
T5	1	1.79	1.74	1.752	1.701	1.726	88.6
	2	1.68	1.67	1.639	1.638	1.638	84.1
	3	1.61	1.59	1.572	1.553	1.562	80.2
T6	1	0.31	0.27	0.269	0.233	0.251	12.9
	2	0.25	0.24	0.214	0.204	0.209	10.7
	3	0.2	0.2	0.161	0.159	0.160	8.2
0.000	1	0.04	0.04	-0.001	-0.001	-0.001	-0.1
	2	0.04	0.04	-0.001	-0.001	-0.001	-0.1
	3	0.04	0.04	-0.001	0.006	0.002	0.1
0.000	1	0.04	0.04	-0.001	-0.001	-0.001	-0.1
	2	0.04	0.04	-0.001	-0.001	-0.001	0.0
	3	0.04	0.04	-0.001	-0.001	-0.001	-0.1
0.000	1	0.05	0.04	0.012	-0.001	0.005	0.3
	2	0.04	0.04	-0.001	-0.001	-0.001	-0.1
	3	0.04	0.04	-0.001	-0.001	-0.001	-0.1
0.000	1	0.04	0.04	-0.001	-0.001	-0.001	-0.1
	2	0.04	0.04	-0.001	-0.001	-0.001	-0.1
	3	0.04	0.06	-0.001	0.022	0.010	0.5

Figure 1. Results obtained for 6 test articles, NC and PC control - Part of the automated spreadsheet for calculation of results. Test chemicals which reduced tissue viability below 50% referenced to NC are classified as irritants. The test is optimized to provide results that are sufficiently far from the classification cut-off (50% viability) thus increasing the robustness of the assay.

Standard EpiDerm SIT Assay Kit Components (MatTek Corporation, catalogue number (EPI-200-SIT))			
Amount	**Name of the component**	**Details**	**St**
1	Sealed 24-well plate containing EpiDerm tissues	Contains 24 tissues in cell culture inserts, packaged on agarose	re
100 ml	Assay Medium (EPI-100-NMM)	DMEM based medium	re
10 ml	1% Triton X-100 Solution (TC-TRI)	Skin irritant reference chemical Do not use in present method as positive control	re
100 ml	DPBS Rinse Solution (TC-PBS)	Used for rinsing the inserts	re
2	24-well plates (sterile)	Used for MTT viability assay	R
8	6-well plates (sterile)	Used for maintaining tissues during assay protocol	R
25 pieces	Nylon Mesh circles 8 mm diameter, 200 µm pore (EPI-MESH)	Used for spreading test chemicals	R

Standard MTT Assay Kit Components (MatTek Corporation, catalogue number (MTT-100))			
Amount	**Name of the component**	**Details**	**St**
2 ml	MTT concentrate 5 mg/ml (MTT-100-CON)	Frozen MTT concentrate	fr (-
8 ml	MTT diluent (MTT-100-DIL)	For diluting MTT concentrate prior to use in the MTT assay (DMEM based medium)	re
60 ml	Extraction Solution - Isopropanol (MTT-100-EXT)	For extraction of formazan crystals	ro

Other Reagents			
Name of the reagent	**Company**	**Catalogue number**	**C**
SDS-Sodium dodecyl sulfate; [151-21-3]	e.g SIGMA	L-4509, purity min. 98.5	5% us
MTT - Thiazolyl Blue Tetrazolium Bromide; [298-93-1]	e.g. SIGMA	M-5655, cell culture tested, purity min. 97.5 %	Fo M
Isopropanol p.a. [67-63-0]	e.g. Acros Organics	AC18413-0010	Fo M
DPBS-Sterile Dulbecco's Phosphate Buffered Saline, without Ca++ and Mg++	e.g. Invitrogen	14190136	Fo

Equipment	Comments
Laminar flow hood	For safe work under sterile conditions
Humidified incubator (37±1°C, 5±1%, CO_2, 95% relative humidity (RH))	For incubating tissues prior to and dur
Vacuum source/trap (optional)	For aspirating media and solutions
37±1°C water bath	For warming Media and MTT solution
Laboratory balance	For pipette verification and checking s
96-well plate photometer	For reading OD at 570 nm (acceptabl
Plate shaker	For extraction of formazan
Stop-watch	For timing of application of test materi
Sterile, blunt-edged forceps	For handling tissue inserts
500 ml plastic wash bottle	For rinsing tissue with DPBS
200 ml beaker	For collecting DPBS rinses
Mortar and pestle	For grinding granular solids

Co

Discussion

In this video, we have demonstrated the EpiDerm Skin Irritation test (EpiDerm SIT) developed and validated for *in vitro* skin irritation testing of chemicals, including cosmetic and pharmaceutical ingredients. When performing this method, it is important to work in aseptic conditions and to strictly follow the validated protocol, since deviation from the protocol may cause different outcomes. Some modifications of the assay are possible, however, changes to the protocol should be discussed directly with the authors.

The only limitation of this method is a possible interference of the test substance with the MTT endpoint. A colored test substance or one that directly reduces MTT (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the MTT endpoint. However, these test substance are a problem only if at the time of the MTT test (i.e. 42 hours after test substance exposure) sufficient amounts of the test substance are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic MTT reduction and the contribution by a colored test material or (false) direct MTT reduction by the test material can be quantified by a procedure described in detail in the assay Standard Operation Procedure provided by MatTek Corp.

Disclosures

The authors are employees of MatTek corporation which produce reagents and tools used in this article.

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