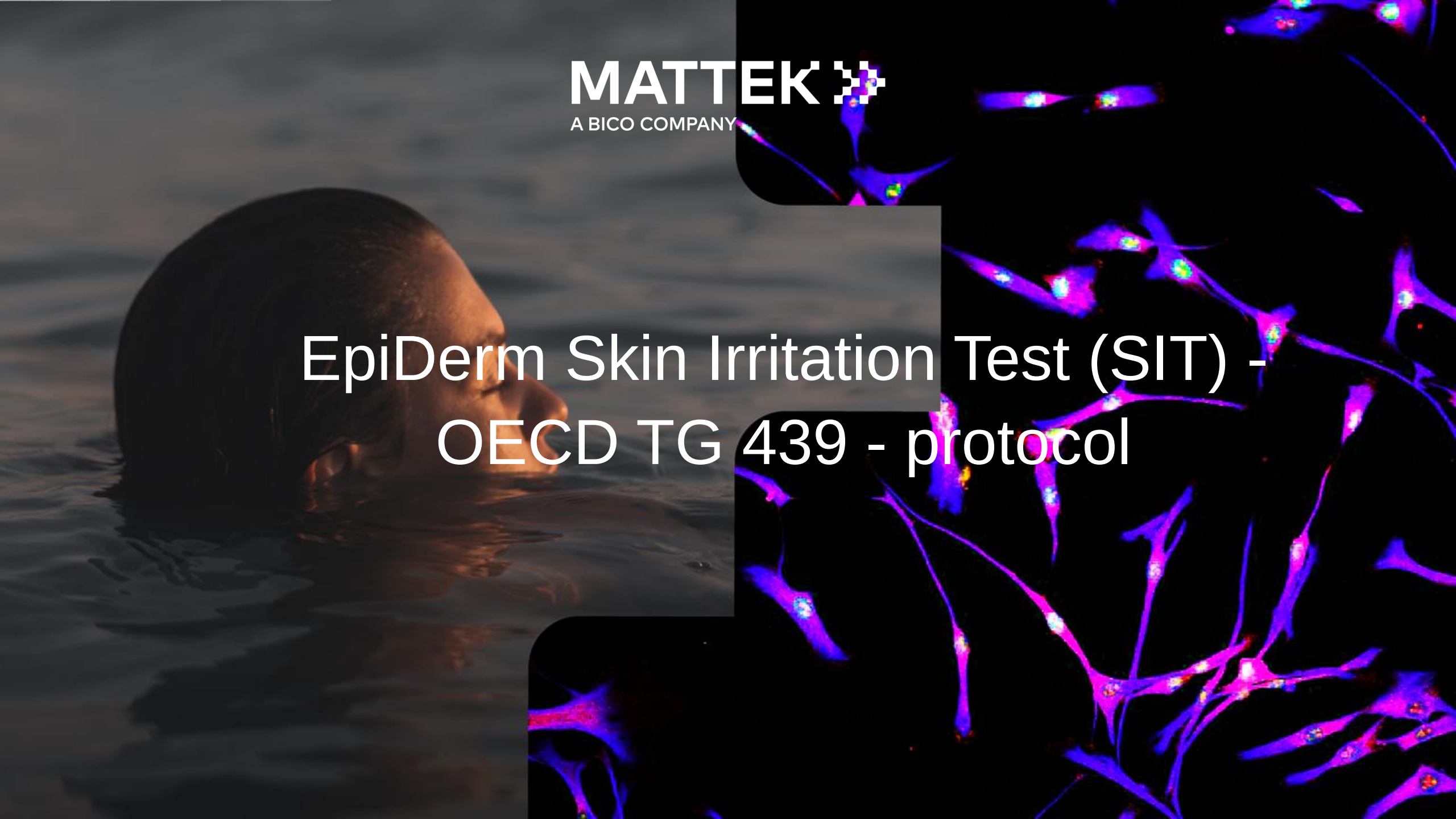

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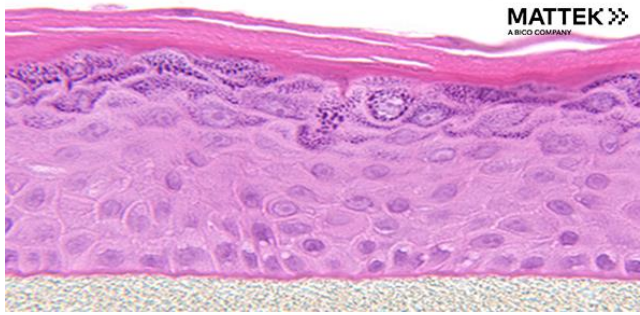
EpiDerm Skin Irritation Test (SIT) - OECD TG 439 - protocol

EpiDerm EPI-200-SIT kit (tissue size: 0,63 cm^2)



Tissues are supplied as kit containing:

- 24 tissue inserts on transport agar
- NMM culture medium (EPI-100-NMM)
- Positive control (5% SDS)
- 6 well and 24 well plates
- DBPS (100 ml)
- mesh

MTT - assay kit (ready-to-use kit)

- MTT concentrate (5 mg/ml) - 2ml
- MTT diluent (culture medium) - 8 ml
- MTT extractant (isopropanol) - 60 ml

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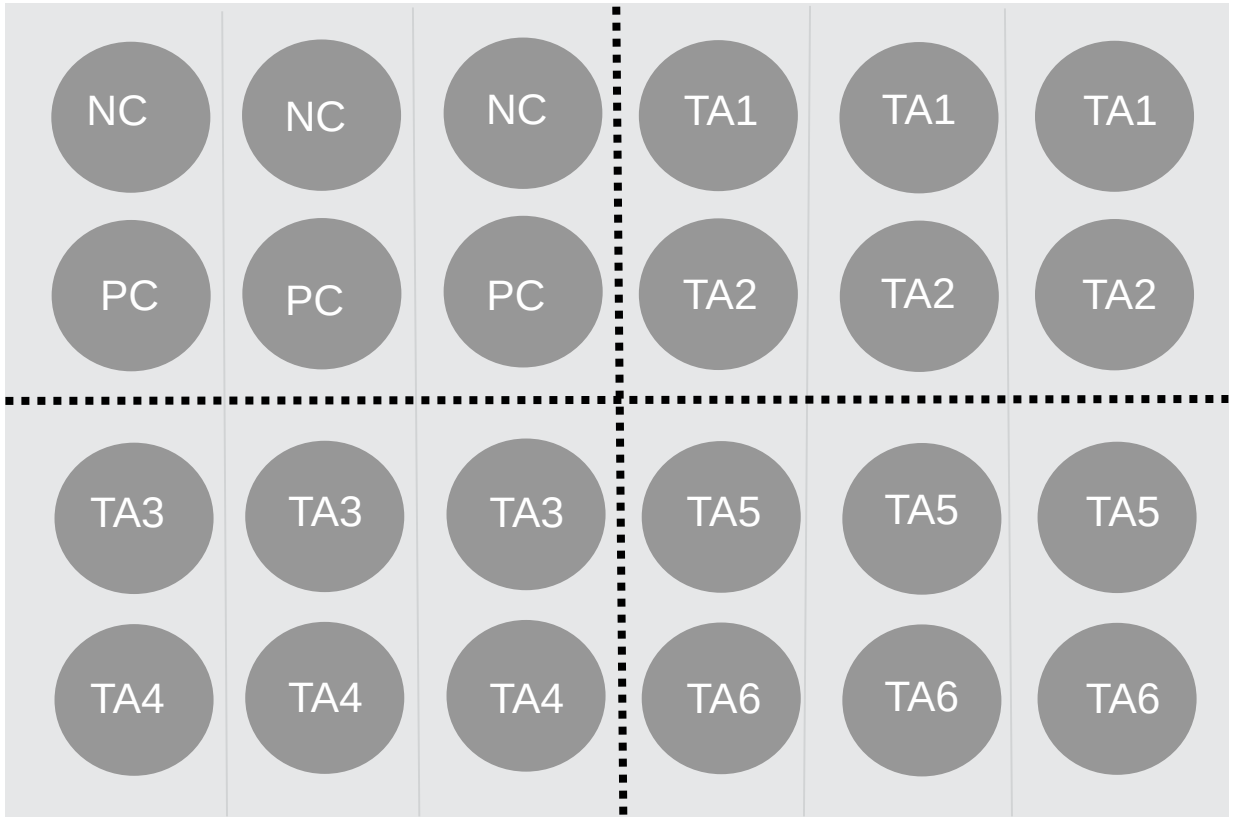
Experimental design

The test is performed on a total of 3 tissues per test material, 3 tissues for negative control, and 3 tissues for positive control.



24 tissues = 6 Test chemicals/articles (TC or TA) + Negative (NC) and Positive control (PC)

1. Pre-incubation

Before opening the EPI-200 kit, prefill all wells of eight 6-well plates with 0.9 mL NMM medium.
Use the following plate design:

negative control	negative control	negative control

plate A

positive control	positive control	positive control

plate B

test chemical 1	test chemical 1	test chemical 1

plate C

test chemical 2	test chemical 2	test chemical 2

plate D

test chemical 3	test chemical 3	test chemical 3

plate E

test chemical 4	test chemical 4	test chemical 4

plate F

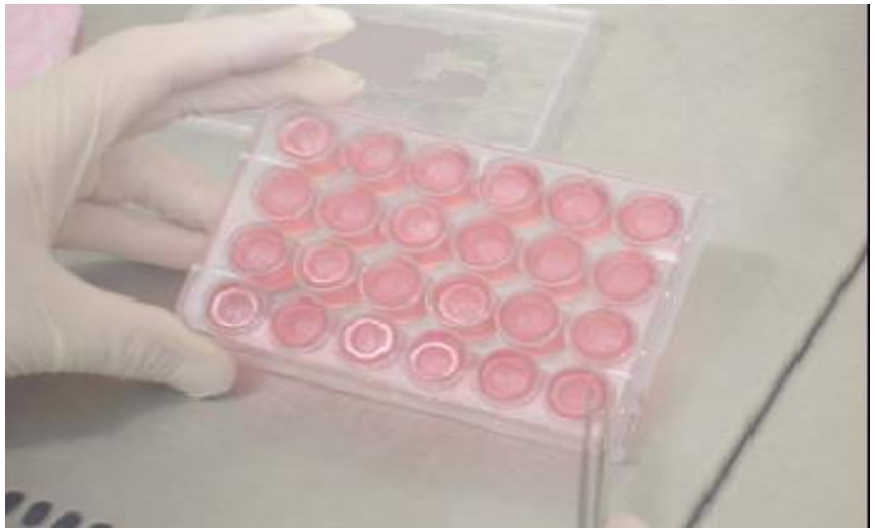
test chemical 5	test chemical 5	test chemical 5

plate G

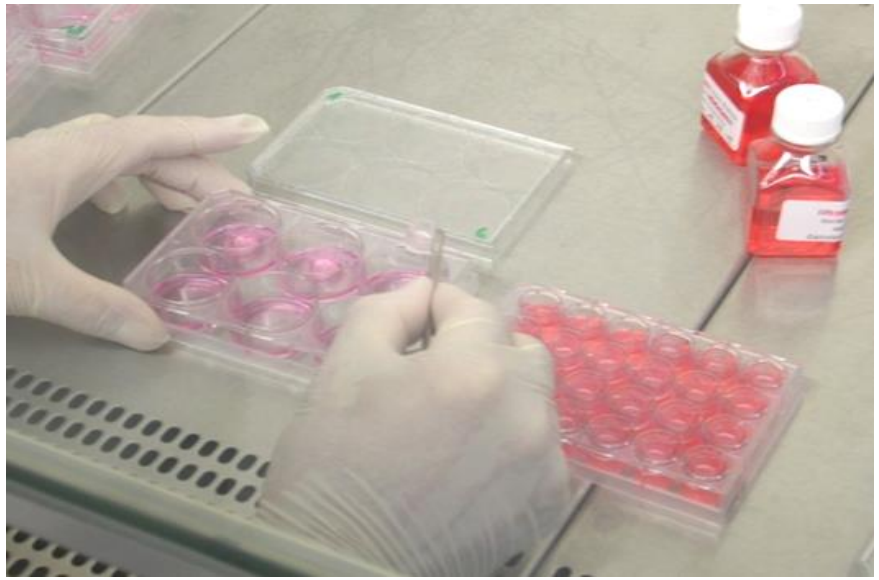
test chemical 6	test chemical 6	test chemical 6

plate H

1. Pre-incubation

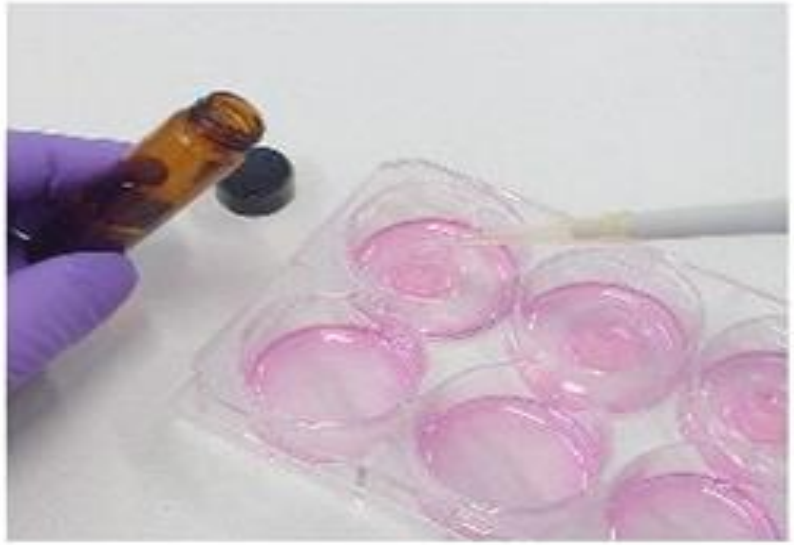


On day of receipt of the EpiDerm™ kit (usually Tuesday), tissues are transferred into 6-well plates (upper row) pre-filled with NMM medium (0,9 ml/well) and are conditioned 1 hour in the incubator (37°C, 5 % CO_2, humidified atmosphere).



After 1h, tissues are transferred into fresh NMM medium (lower row, 0,9 ml/well) and are further incubated overnight in the incubator (37°C, 5 % CO_2, humidified atmosphere).

2. Application of test chemicals



The test is performed on a total of 3 tissues per test material, 3 tissue for negative control, and 3 tissues for positive control.

Negative control: DPBS

Positive control: 5% SDS

DOSE

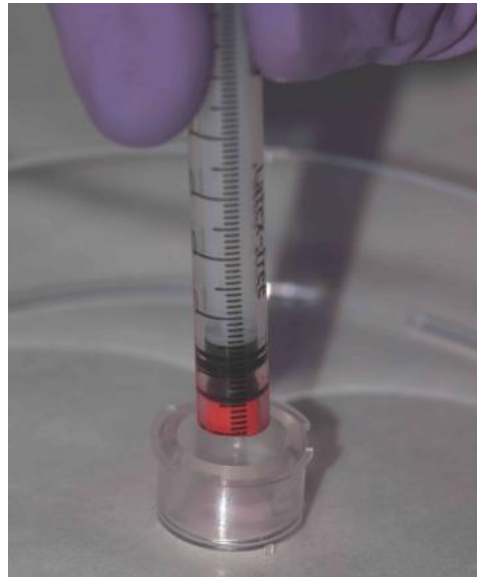
solids ~ 25 mg solid +25 µL H_2O
liquids - 30 µl (<u>undiluted</u> test material)

APPLICATION

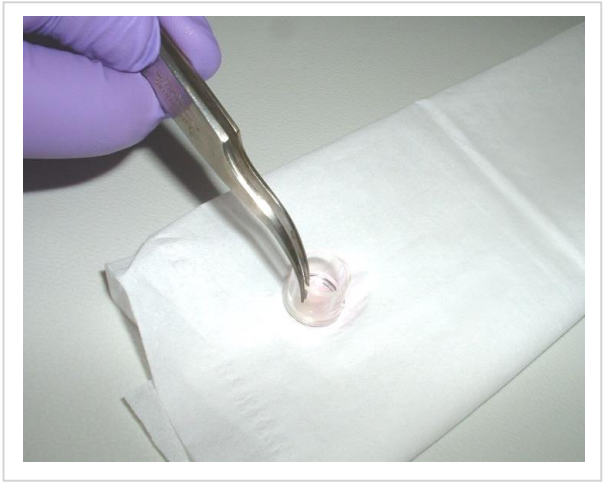
solids: spoon application
liquids: pipetting + mesh
viscous liquids: positive displacement pipette
soaps: stainless pin

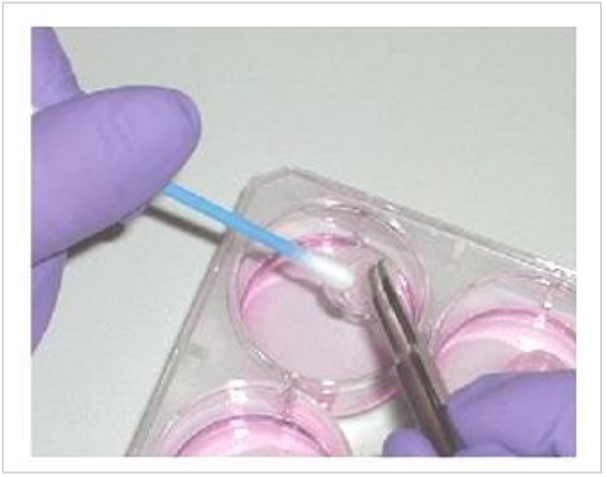
EXPOSURE TIME

60 min, out of which the dosed tissues are kept in the incubator for 35 min



3. Washing procedure





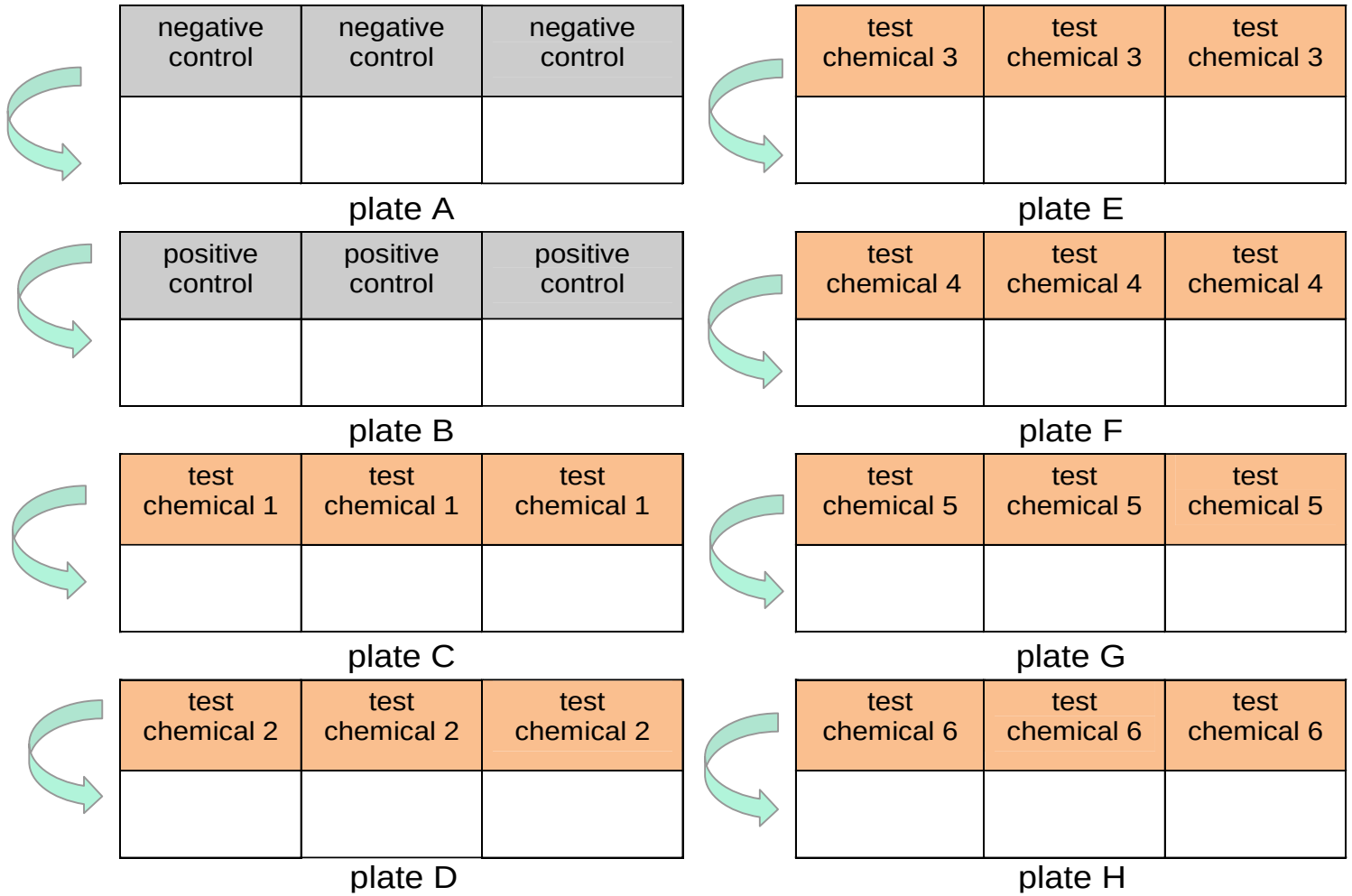
After 1 h exposure to the test chemical, the tissues are washed with phosphate buffered saline (DPBS) to remove residual test material.

Rinsed and blotted inserts are transferred into new 6-well plates (upper row) prefilled with 0.9 mL of NMM medium (use the same plate design as mentioned before)

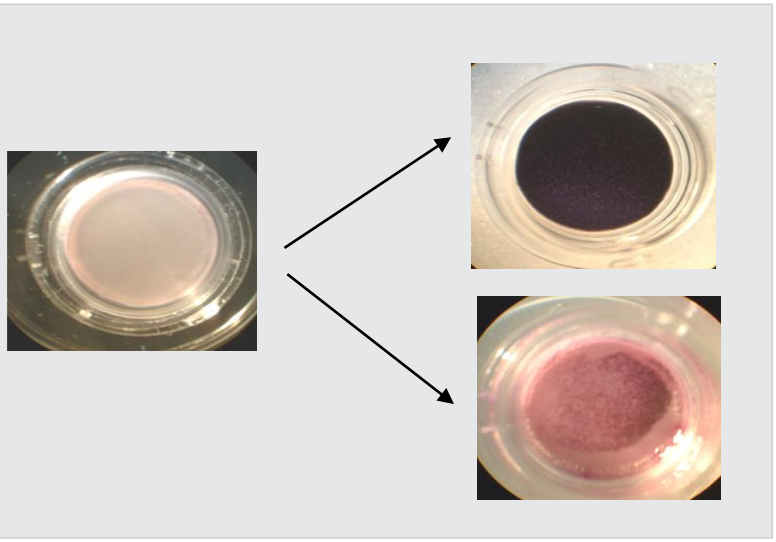
Afterwards, the tissue surface should be dried with cotton tip.
All tissues are post-incubated for 24 hours.

4. Post-incubation

After 24 hours of post-incubation, transfer all inserts from upper row into lower row of 6-well plate prefilled with NMM medium (0.9 ml/well) and incubate further for 18 hours (5% CO2, 37°C, 95% RH).

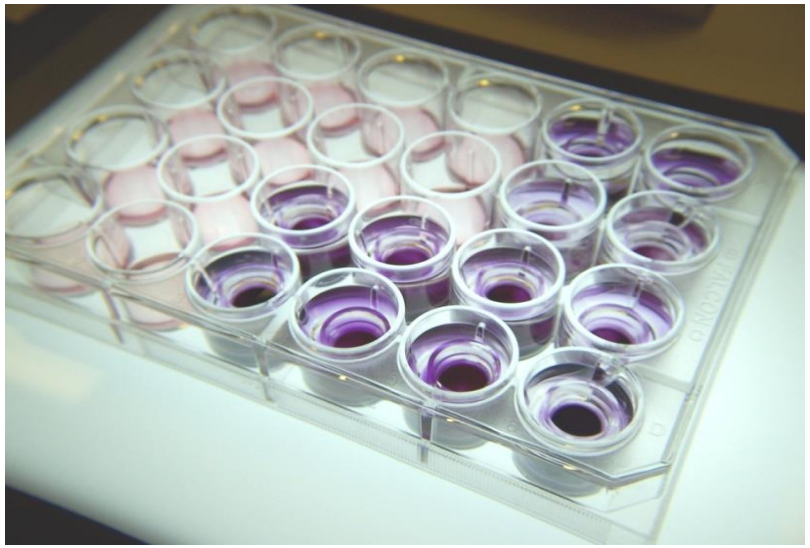


5. MTT assay and isopropanol extraction



After completion of post-exposure (42h), tissues are transferred into 24 well plates prefilled with 0,3 mL MTT medium. Tissue are incubated with MTT for 3 h (37ºC, 5% CO_2, humidified atmosphere) protected from light.

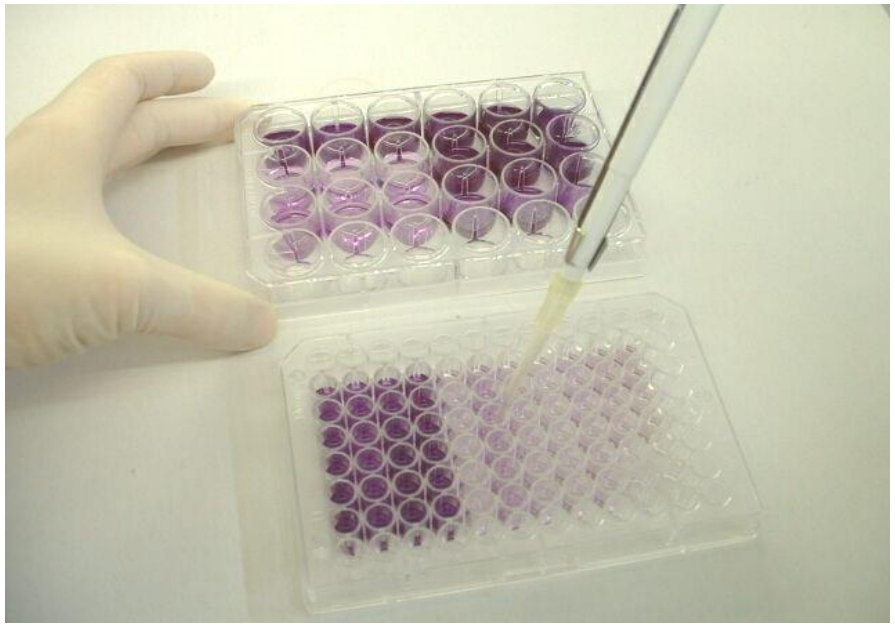
Viable cells/tissues will convert yellow MTT into purple/blue formazan product. Non-viable skin models will remain unstained.



After incubation with MTT is completed, tissues are washed twice with PBS, inserts are transferred into new 24 well plate and formazan is extracted with 2 mL isopropanol (2 hours or over night).

Plated should be sealed (e.g. with parafilm) to avoid evaporation of isopropanol.

After the extraction period is completed, inserts are pierced with an injection needle. Extract will run into the well from which the insert was taken. Afterwards, the insert can be discarded. Plates are placed on a shaker for 15 minutes until solution is homogeneous.



Per each tissue 3 × 200µL aliquots of the blue formazan solution are transferred into a 96-well flat
bottom microtiter plate

For the measurement in a 96 well plate, **use exactly the plate design given in the spreadsheet for calculation**

6. Measurement and viability calculation

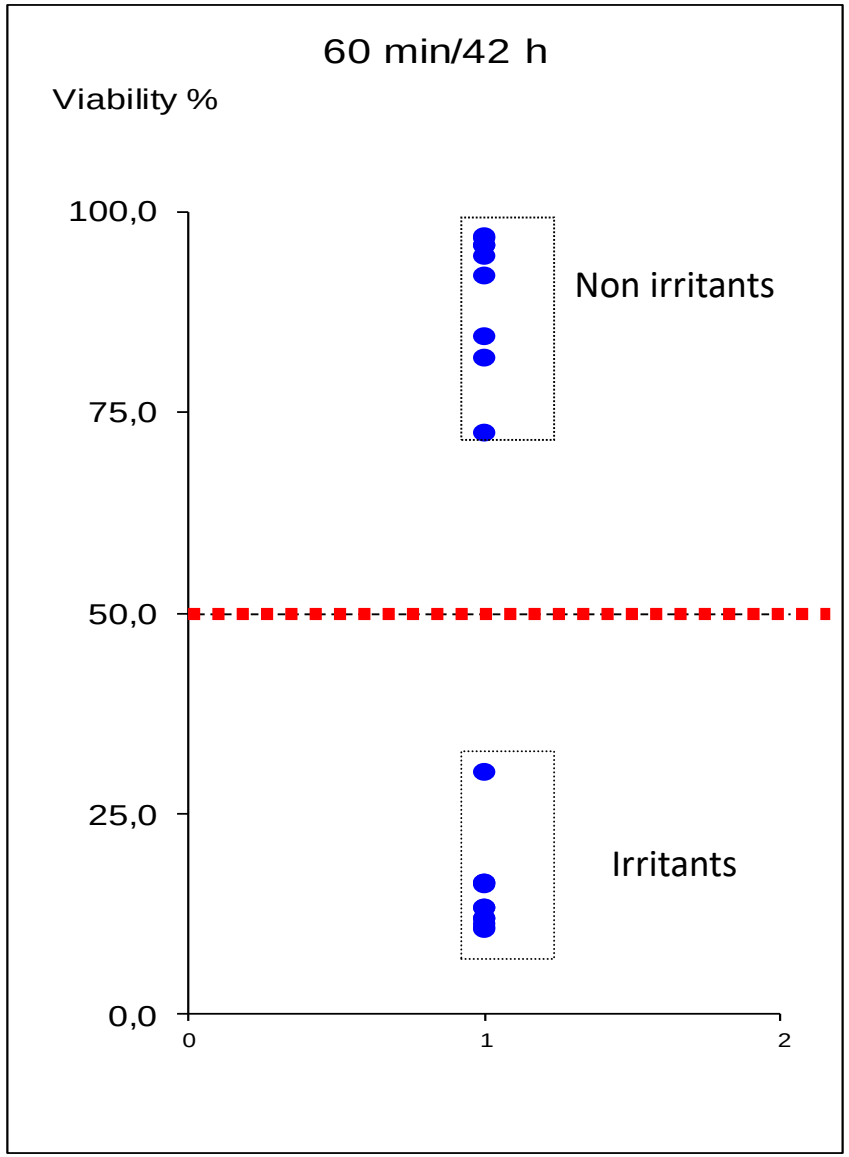
Fixed plate design

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	
B	NC	PC	B048	B073	B106	B203	B229	B243	B257	B288	B342	B439	Tissue1
C	NC	PC	B048	B073	B106	B203	B229	B243	B257	B288	B342	B439	
D	NC	PC	B048	B073	B106	B203	B229	B243	B257	B288	B342	B439	Tissue2
E	NC	PC	B048	B073	B106	B203	B229	B243	B257	B288	B342	B439	
F	NC	PC	B048	B073	B106	B203	B229	B243	B257	B288	B342	B439	Tissue3
G	NC	PC	B048	B073	B106	B203	B229	B243	B257	B288	B342	B439	
H	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	

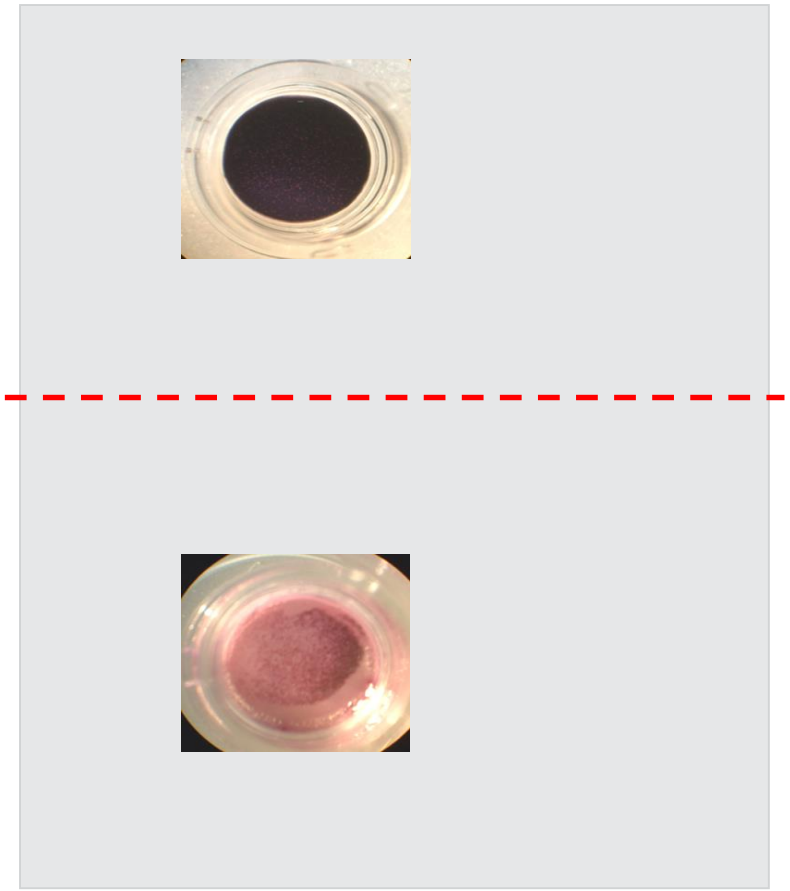
Read optical density (OD) in a plate spectrophotometer at **570 nm**, **without reference filter**.

The OD of the formazan can be read at in a range of **540 nm – 570nm**.

Prediction model



Chemical is predicted as Irritating if the viability after 42h of post-incubation is reduced below 50% compared to viability of NC.



EpiDerm Skin Irritation Test Spreadsheet – import sheet

EpiDerm Skin Irritation Test

(EPI-200-SIT)

Exp. no.:	1
Tissue-lot no.:	1234
Date:	13-Oct-07
Operator:	XY, ZQ, YA

Negative control	NC (DPBS)
Positive control	PC (5% SDS)
Test Article No. 1	C1
Test Article No. 2	C2
Test Article No. 3	C3
Test Article No. 4	C4
Test Article No. 5	C5
Test Article No. 6	C6
Test Article No. 7	C7
Test Article No. 8	C8
Test Article No. 9	C9
Test Article No. 10	C10

FIXED DESIGN OF 96 WELL PLATE

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	empty	empty	empty	empty	empty	empty	
B	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	Tissue1
C	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	
D	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	Tissue2
E	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	
F	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	Tissue3
G	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	
H	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	empty	

IMPORT:

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0,037	0,038	0,037	0,038	0,037	0,037	0,037	0,037	0,037	0,039	0,037	0,036	
B	1,868	0,203	0,502	1,754	0,198	0,199	0,202	0,202	1,873	1,921	1,818	0,368	Tissue1
C	1,898	0,207	0,501	1,739	0,196	0,198	0,201	0,209	1,861	1,906	1,807	0,404	
D	1,969	0,215	0,710	1,742	0,209	0,267	0,179	0,216	1,951	1,923	1,970	0,534	Tissue2
E	1,947	0,211	0,536	1,829	0,199	0,196	0,197	0,204	1,944	1,938	1,862	0,380	
F	1,842	0,210	0,737	1,767	0,199	0,262	0,173	0,210	1,971	1,350	0,900	0,531	Tissue3
G	1,862	0,213	0,747	1,799	0,200	0,268	0,174	0,208	2,043	1,340	0,900	0,541	
H	0,037	0,036	0,036	0,037	0,037	0,037	0,037	0,037	0,037	0,036	0,037	0,038	

REMARKS

Date:

Performed by:

MK-10-023-0001

Rev. Jul-15-2021

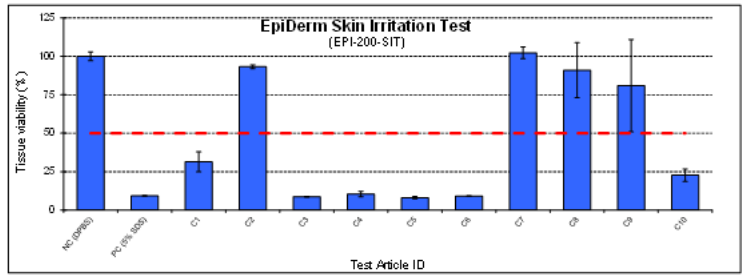
EpiDerm Skin Irritation Test Spreadsheet – calculations

EpiDerm Skin Irritation Test
(EPI-200-SIT)

RESULTS

Exp. no.:	1
Tissue lot no.:	1234
Date:	13-Oct-07
Operator:	XY, ZQ, YA

Blank	0.037
	0.038
	0.037
	0.036
	0.037
	0.037
Mean	0.037



Test Article ID	Tissue #	Raw data Aliq. 1	Raw data Aliq. 2	Blank corrected data Aliq. 1	Blank corrected data Aliq. 2	mean of aliquots	% of viability
NC (DPBS)	1	1.888	1.898	1.851	1.861	1.846	99.2
	2	1.969	1.947	1.932	1.910	1.921	103.2
	3	1.842	1.862	1.805	1.825	1.815	97.5
PC (5% SDS)	1	0.203	0.207	0.166	0.170	0.168	9.0
	2	0.215	0.211	0.178	0.174	0.176	9.4
	3	0.210	0.213	0.173	0.176	0.174	9.4
C1	1	0.502	0.501	0.465	0.464	0.464	25.0
	2	0.710	0.536	0.673	0.499	0.586	31.5
	3	0.737	0.747	0.700	0.710	0.705	37.9
C2	1	1.754	1.739	1.717	1.702	1.709	91.9
	2	1.742	1.829	1.705	1.792	1.748	94.0
	3	1.767	1.799	1.730	1.762	1.746	93.8
C3	1	0.198	0.196	0.161	0.159	0.160	8.6
	2	0.209	0.199	0.172	0.162	0.167	9.0
	3	0.199	0.200	0.162	0.163	0.162	8.7
C4	1	0.198	0.198	0.162	0.161	0.161	8.7
	2	0.267	0.196	0.230	0.159	0.194	10.4
	3	0.262	0.268	0.225	0.231	0.228	12.2
C5	1	0.202	0.201	0.165	0.164	0.164	8.8
	2	0.179	0.197	0.142	0.160	0.151	8.1
	3	0.173	0.174	0.136	0.137	0.136	7.3
C6	1	0.202	0.209	0.165	0.172	0.168	9.0
	2	0.216	0.204	0.179	0.167	0.173	9.3
	3	0.210	0.208	0.173	0.171	0.172	9.2
C7	1	1.873	1.861	1.836	1.824	1.830	98.4
	2	1.951	1.944	1.914	1.907	1.910	102.7
	3	1.971	2.043	1.934	2.006	1.970	105.9
C8	1	1.921	1.906	1.884	1.869	1.876	100.9
	2	1.929	1.928	1.892	1.891	1.892	101.8
	3	1.350	1.340	1.313	1.303	1.308	70.3
C9	1	1.818	1.807	1.781	1.770	1.775	95.4
	2	1.970	1.862	1.933	1.825	1.879	101.0
	3	0.900	0.900	0.863	0.863	0.863	46.4
C10	1	0.368	0.404	0.331	0.367	0.349	18.7
	2	0.534	0.480	0.497	0.443	0.470	25.2
	3	0.531	0.541	0.494	0.504	0.499	26.8

Test Article ID	mean (OD)	STD (OD)	mean viability [%]	STD of viability	CV [%]
NC (DPBS)	1.880	0.065	100.0	2.9	2.9
PC (5% SDS)	0.173	0.004	9.3	0.2	2.5
C1	0.585	0.120	31.4	6.5	20.6
C2	1.734	0.022	93.2	1.2	1.3
C3	0.163	0.004	8.8	0.2	2.2
C4	0.194	0.033	10.4	1.8	17.1
C5	0.150	0.014	8.1	0.8	9.3
C6	0.171	0.005	9.2	0.1	1.6
C7	1.903	0.070	102.3	3.8	3.7
C8	1.892	0.335	91.0	17.9	19.7
C9	1.506	0.559	80.9	30.1	37.1
C10	0.422	0.075	22.7	4.0	17.8

Classification

Test Article ID	Prediction	Qualified Assay
NC (DPBS)	NI	Qualified
PC (5% SDS)	I	Qualified
C1	I	Qualified
C2	NI	Qualified
C3	I	Qualified
C4	I	Qualified
C5	I	Qualified
C6	I	Qualified
C7	NI	Qualified
C8	NI	Qualified
C9	NI	Non-qualified
C10	I	Qualified

Date:

Performed by:

Page 2

MK-10-023-0001

Rev. Jul-15-2021

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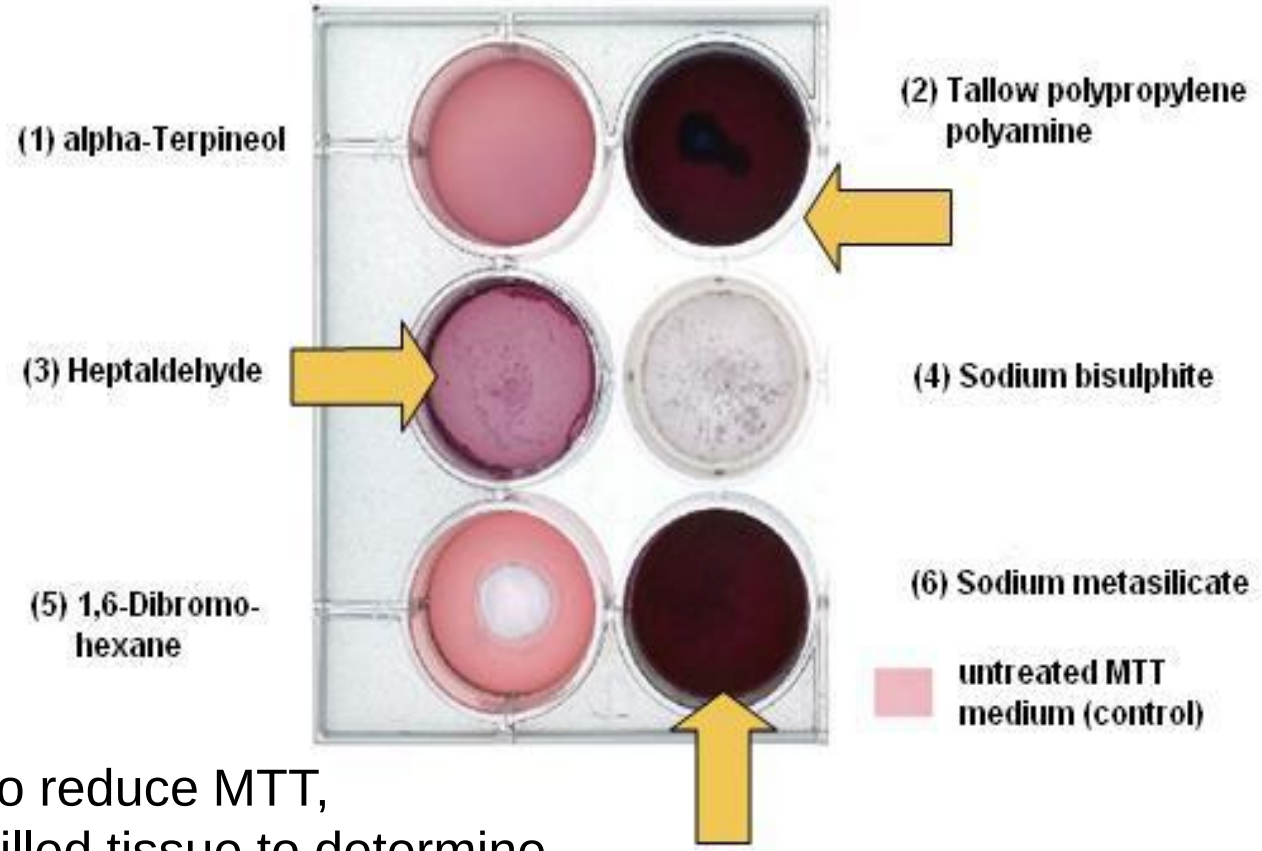
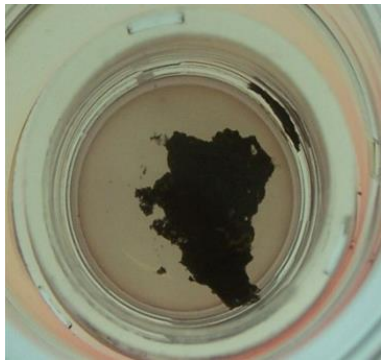
Direct MTT reducers

Step 1:
determine ability of test substance
to directly reduce MTT.



Add 25 mg or 30 µl into 1 ml MTT medium
incubate 3 hours (37°C, 5% CO_2 , 95 % humidity, dark). Visual scoring is sufficient in this step.

Step 2: if chemical shows significant ability to reduce MTT, perform the skin irritation test using freeze-killed tissue to determine the amount of residual chemical bound to the tissue.



Perform all protocol's steps on freeze-killed tissues.
Subtract the value of optical density (OD) of non-viable tissues
(where the MTT is reduced only by residuals of chemical)
from OD obtained with viable tissues to get the "real" (mitochondrial) conversion of MTT.

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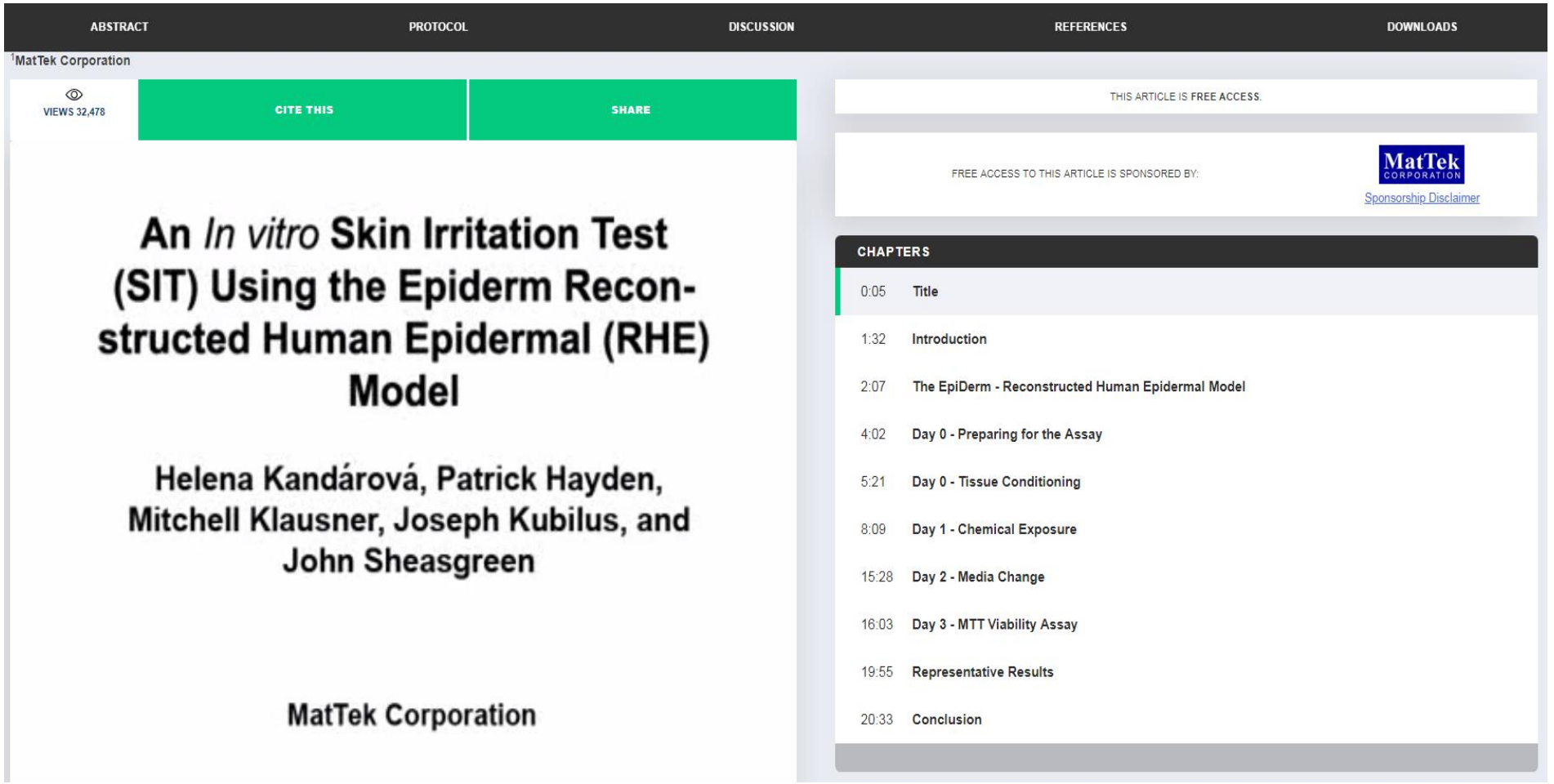
JoVE video-protocol EpiDerm SIT (free access)

https://www.jove.com/video/1366/an-vitro-skin-irritation-test-sit-using-epiderm-reconstructed-human

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