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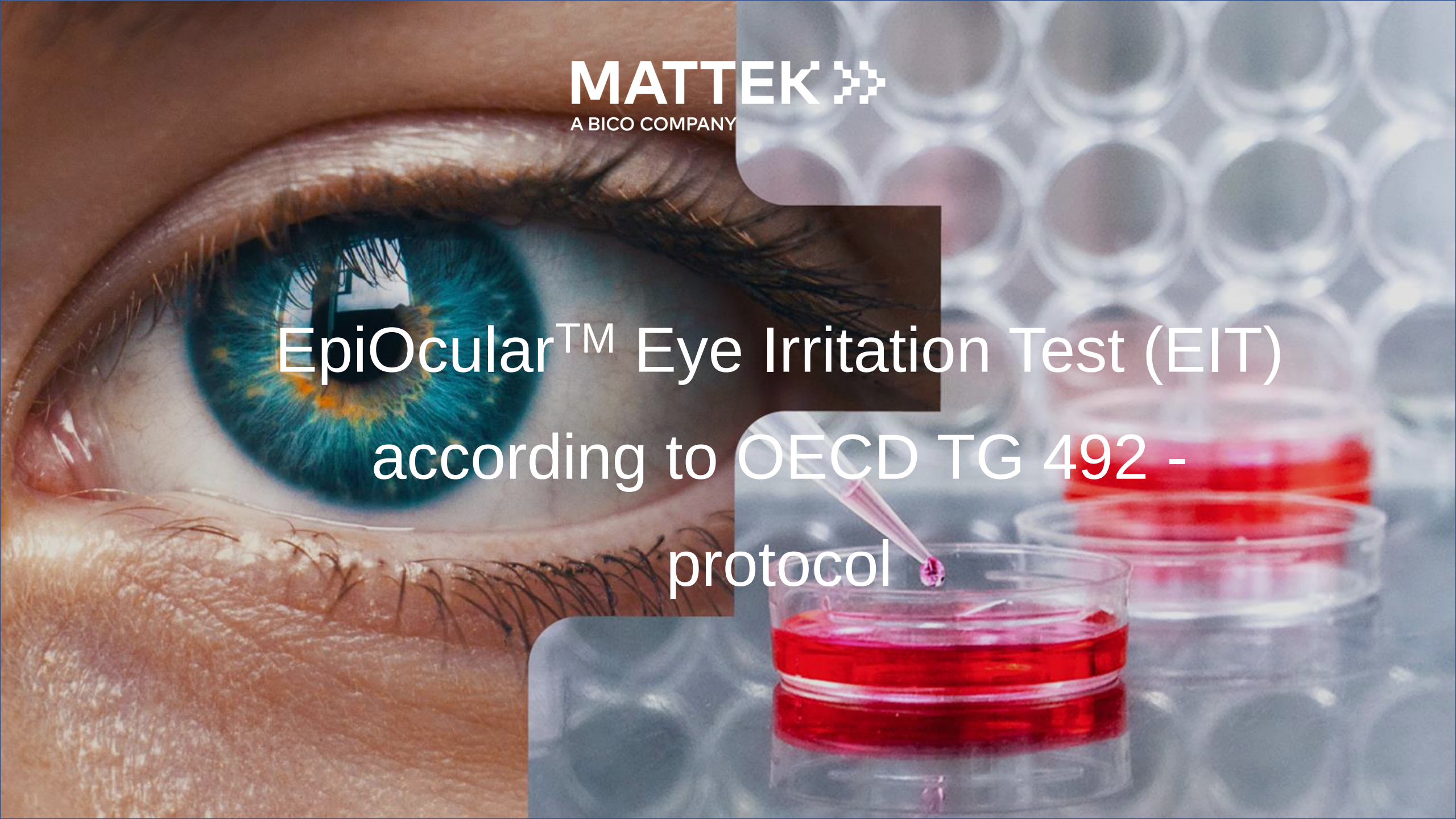


EpiOcular™ Eye Irritation Test (EIT) according to OECD TG 492 - protocol

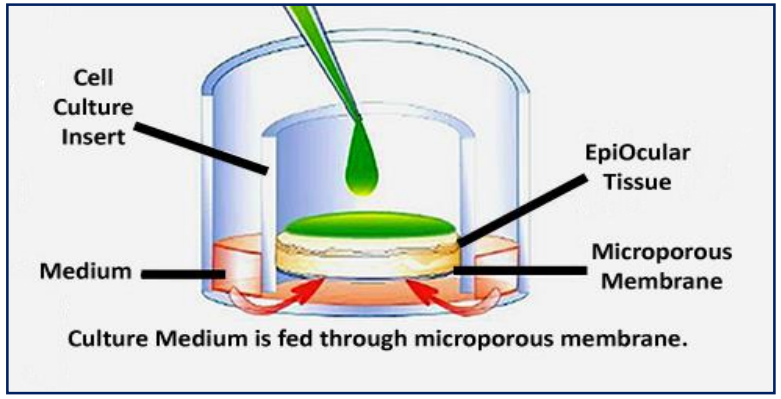
EpiOcular™ EIT kit (OCL-200-EIT kit)

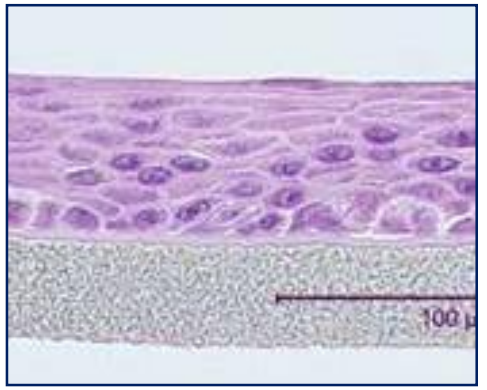
Tissues are supplied as kit containing:

24 tissue inserts on transport agar,
ASY culture medium (OCL-200-ASY),
PC (methyl acetate)
6 well, 12 well and 24 well plates

MTT - assay kit (MTT-100 kit):

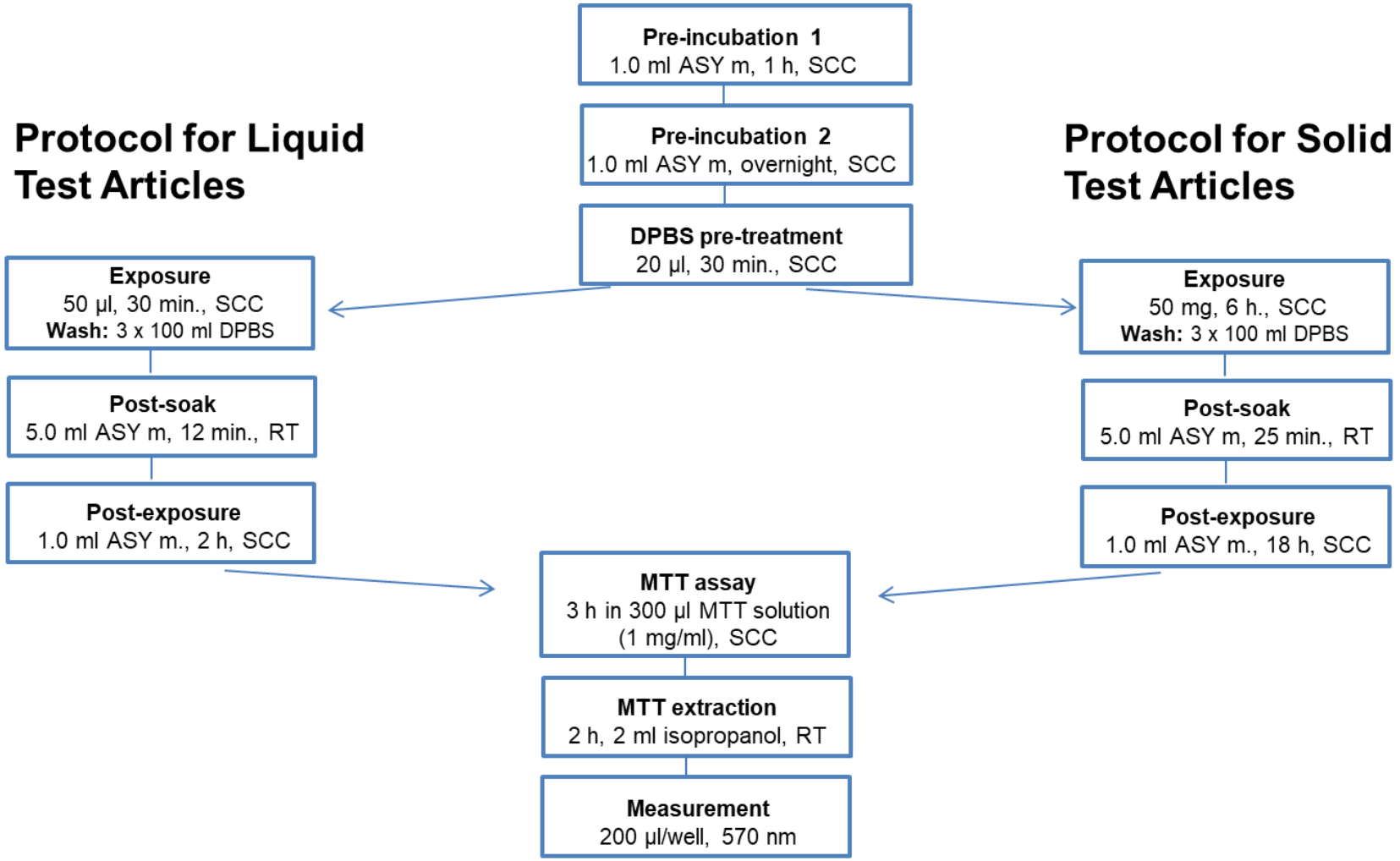
MTT concentrate (5 mg/ml) - 2ml
MTT diluent (culture medium) - 8 ml
MTT extractant (isopropanol) - 60 ml





Histology cross-section

Experimental design



Experimental design

The test is performed on a total of 2 tissues per test material, 2 tissues for negative control (NC), and 2 tissues for positive control (PC).

24 tissues = 10 Test chemicals (TC) + Negative (NC) and Positive control (PC)

The test is performed on a total of 2 tissues per test material, 2 tissues for negative control, and 2 tissues for positive control.

12 tissues = 4 Test articles (TA) - liquids + Negative control (NC) and Positive control (PC)
12 tissues = 4 Test articles (TA) - solids + Negative control (NC) and Positive control (PC)

1. Pre-incubation

Before opening the OCL-200 kit, prefill all wells of four 6-well plates with 1.0 mL ASY (OCL-200-ASY) medium. Use the following plate design:

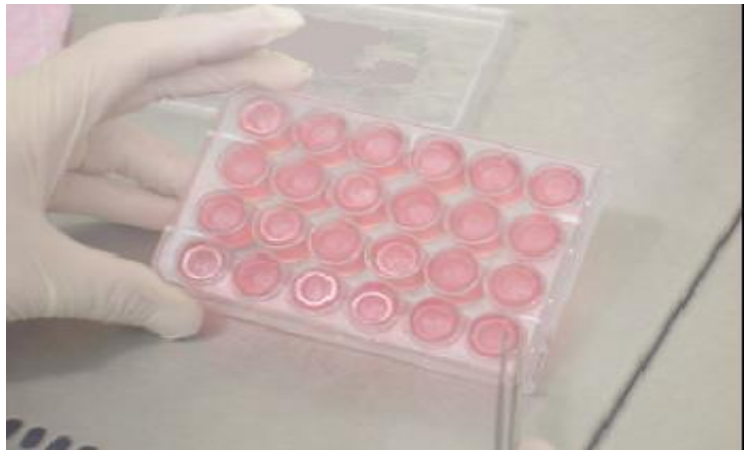
Negative control	Positive control	Test article 1
Negative control	Positive control	Test article 1

Test article 5	Test article 6	Test article 7
Test article 5	Test article 6	Test article 7

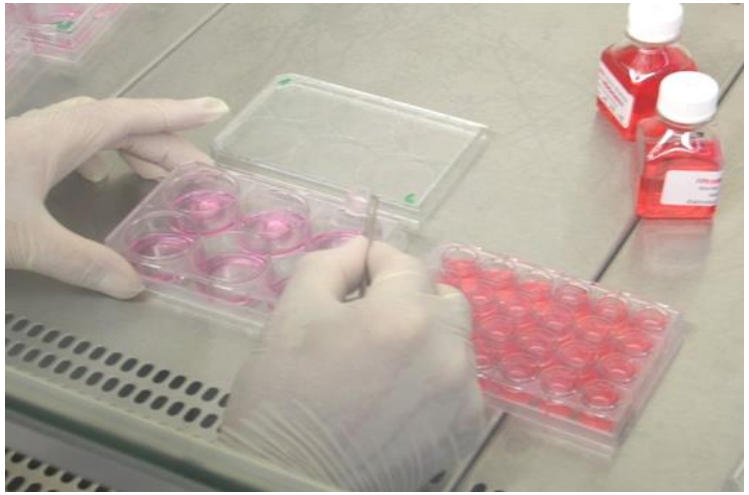
Test article 2	Test article 3	Test article 4
Test article 2	Test article 3	Test article 4

Test article 8	Test article 9	Test article 10
Test article 8	Test article 9	Test article 10

1. Pre-incubation



On day of receipt of the EpiOcular™ kit (usually Tuesday), tissues are transferred into 6-well plates pre-filled with ASY medium (OCL-200-ASY) (1.0 ml/well) and are conditioned 1 hour in the incubator (37°C, 5 % CO_2, humidified atmosphere).

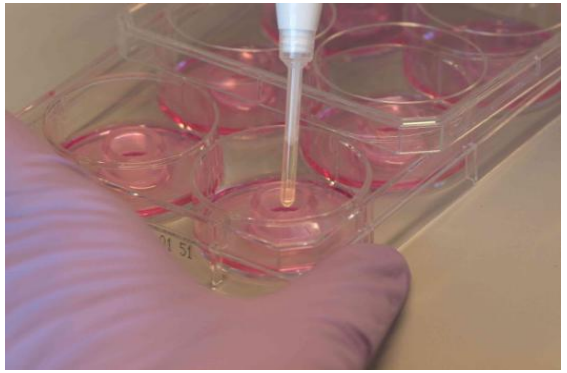


After 1h, the ASY medium (OCL-200-ASY) is exchanged for the fresh one to fresh ASY medium (1.0 ml/well) and are further incubated overnight in the incubator (37°C, 5 % CO_2, humidified atmosphere).

2. DPBS pre-treatment

After the overnight incubation, the tissues are pre-wetted with 20 µl of DPBS and are incubated at standard culture conditions for **30 ± 2 min.**

3. Application of liquid test articles



The test is performed on a total of 2 tissues per test material, 2 tissue for negative control, and 2 tissues for positive control.

Negative control: sterile ultrapure H2O

Positive control: methyl acetate

DOSE

liquids - 50 µl (undiluted test material)

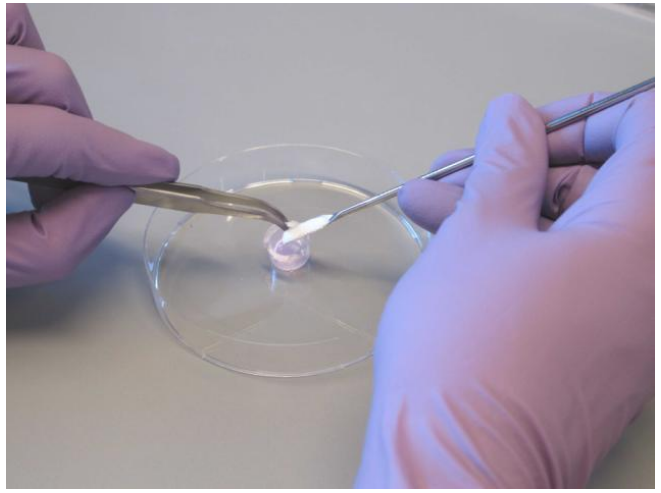
APPLICATION

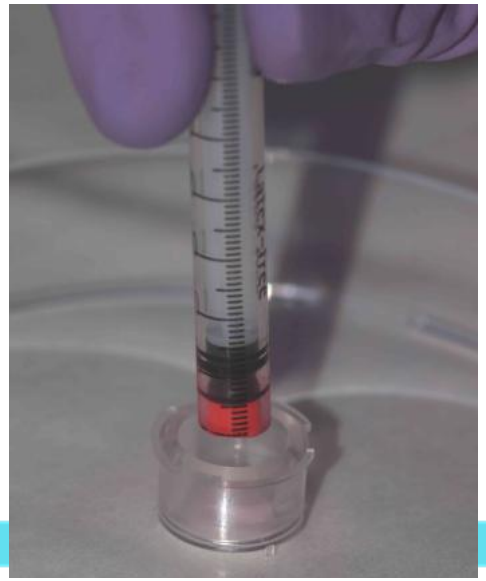
liquids: pipetting
viscous liquids: positive displacement pipette

EXPOSURE TIME

30 ± 2 min

4. Application of solid test articles





The test is performed on a total of 2 tissues per test material, 2 tissue for negative control, and 2 tissues for positive control.

Negative control: sterile ultrapure H2O

Positive control: methyl acetate

DOSE

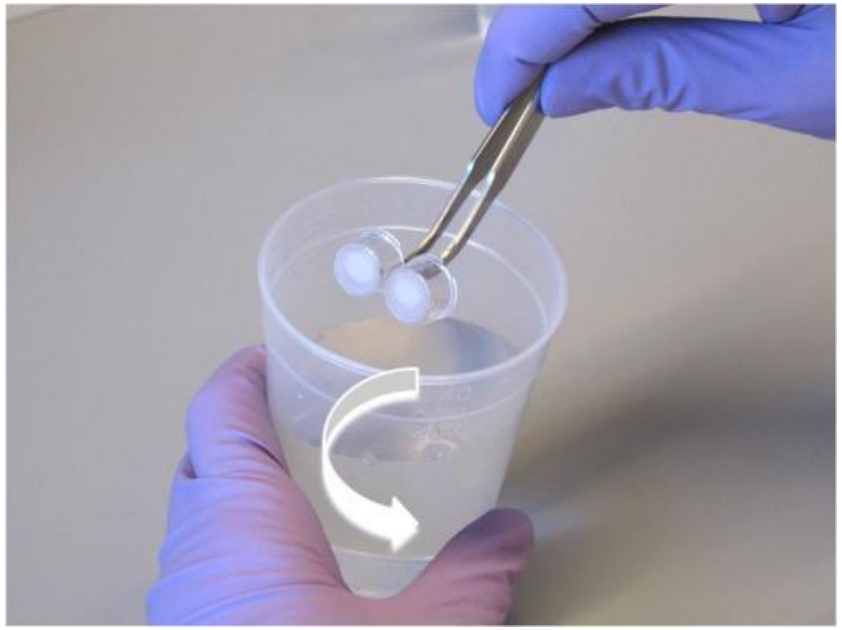
NC and PC - 50 µl
solids - 50 mg

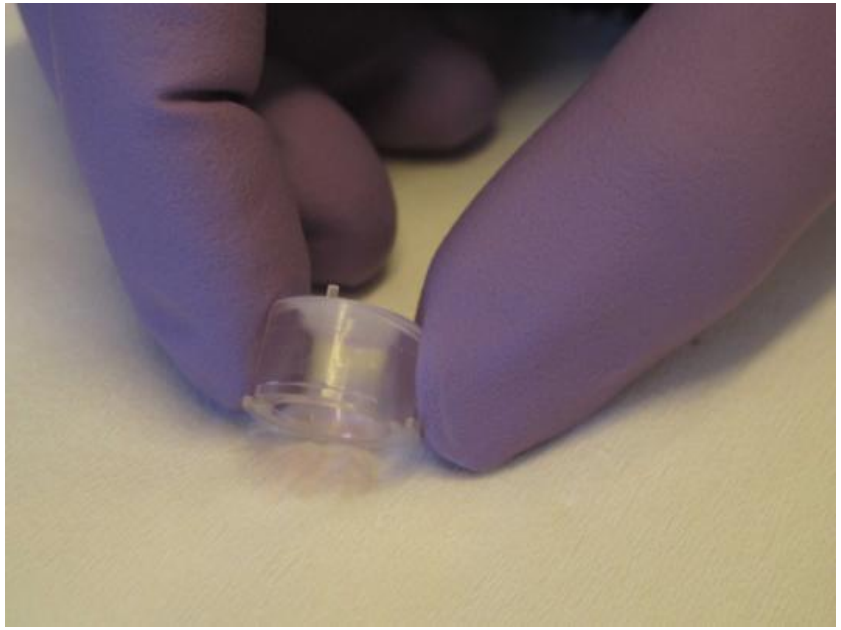
APPLICATION

solids: spoon or syringe application

EXPOSURE TIME
6 h ± 15 min

5. Washing procedure





After **30 min ± 2 min** exposure to the **liquid** test articles or after **6 h ± 15 min** exposure to the **solid** test articles, the tissues are washed 3 times with phosphate buffered saline (DPBS) to remove residual test material.

6. Post-soak and post-exposure

Rinsed and blotted inserts are transferred and immersed in 5 ml of ASY medium in a pre-labeled 12-well plates prefilled with 5 mL of ASY medium for post-soak for **12 min ± 2 min** for **liquids** and **25 min ± 2 min** for **solids.**

Afterwards, the medium is decanted off the tissue, the insert is blotted on the absorbent material, and transferred to the appropriate well of the pre-labelled 6-well plate containing 1 ml of ASY medium. The tissues are incubated for **120 min ± 15 min** for **liquids** and **18 h ± 0.25 h** for **solids** at standard culture conditions.

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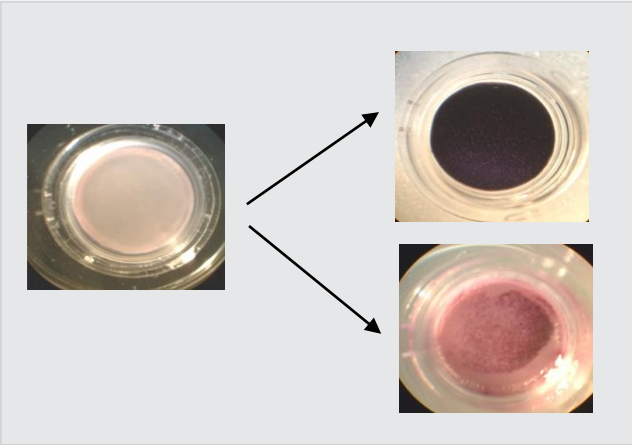
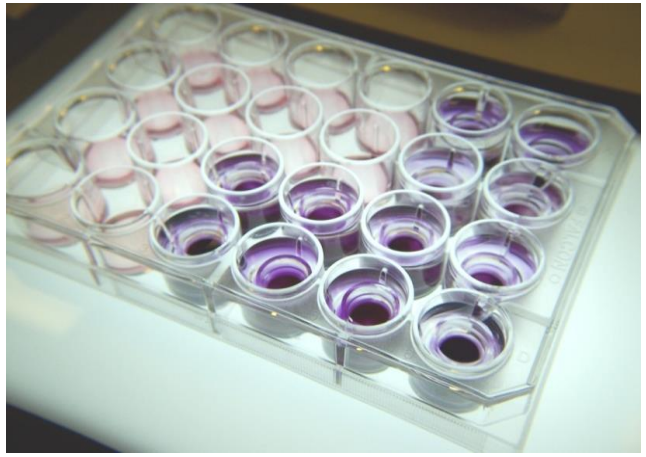
7. MTT assay and isopropanol extraction





After termination of post-exposure (**120 min ± 15 min** for **liquids** and **18 h ± 0.25 h** for **solids**), tissues are transferred into 24 well plates prefilled with 0.3 mL MTT medium. Tissue are incubated with MTT for **3 h ± 10 min** (37ºC, 5% CO_2, humidified atmosphere) protected from light.

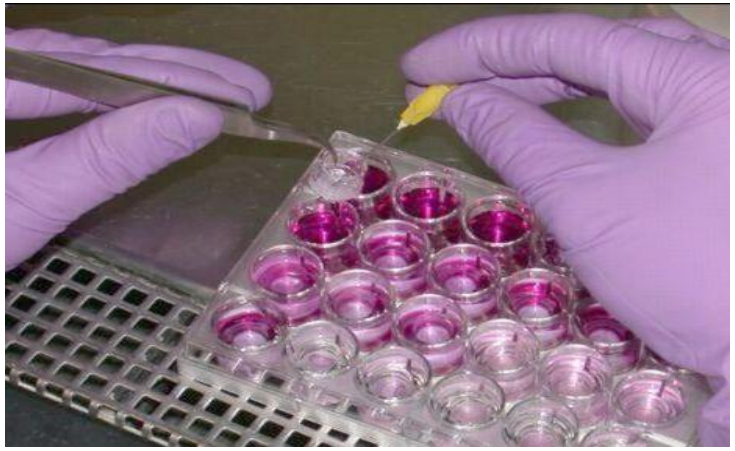
Viable cells/tissues will convert yellow MTT into purple/blue formazan product. Non-viable skin models will remain unstained.

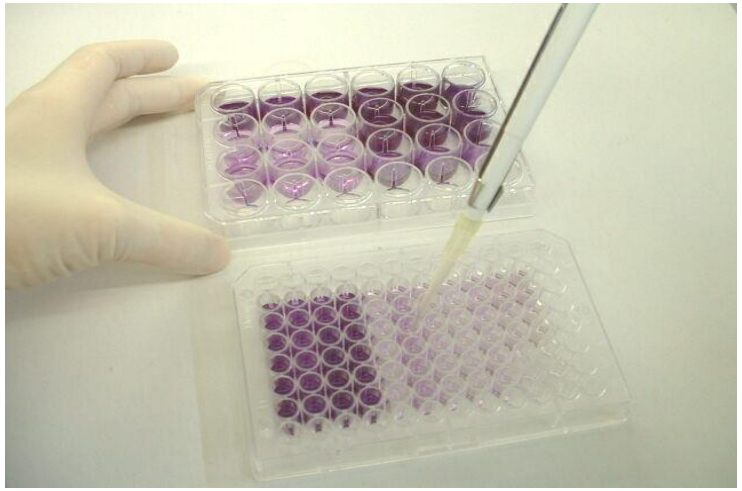
For liquids: After incubation with MTT is completed, tissues are transferred into new 24 -well plate and formazan is extracted with 2 mL isopropanol (2 h at room temperature or overnight at 4°C).

For solids: After incubation with MTT is completed, tissues are transferred into 6-well plate containing 2 ml of isopropanol so that no isopropanol is floating in the insert. (2 h at room temperature or overnight at 4°C).

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

8. Preparation of 96 well plate for OD measurement





After the extraction period is completed, inserts are pierced with an injection needle in the case of **liquids.**

For **solids**, the tissues should not be pierced.

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 2 × 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**.

9. Measurement

Fixed plate design

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	Tissue1
B	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	
C	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	Tissue2
D	NC	PC	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	
E	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	BLANK	BLANK	Tissue1
F	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	BLANK	BLANK	
G	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	BLANK	BLANK	Tissue2
H	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	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 **550 nm – 590nm**.

Acceptability ranges for NC OD values in EpiOcular EIT **are > 0.8 and < 2.8.**

EpiOcular Eye Irritation Test Spreadsheet - import

EpiOcular - Eye Irritation Test
(OCL-200-EIT)

Correspondend informations of MDS	
Exp. no.:	V16-5263
Tissue-lot no.:	23499 Kit F
Date:	27-Oct-16
Operator:	SN LV

Negative control	NC (DI H2O)
Positive control	PC (methyl acetate)
Test Chemical No. 1	1234
Test Chemical No. 2	2345
Test Chemical No. 3	1234
Test Chemical No. 4	2345
Test Chemical No. 5	1234
Test Chemical No. 6	2345
Test Chemical No. 7	1234
Test Chemical No. 8	2345
Test Chemical No. 9	2345
Test Chemical No. 10	2356
Test Chemical No. 11	
Test Chemical No. 12	
Test Chemical No. 13	
Test Chemical No. 14	
Test Chemical No. 15	
Test Chemical No. 16	
Test Chemical No. 17	
Test Chemical No. 18	
Test Chemical No. 19	
Test Chemical No. 20	
	7777
	8888
	9999
	8877
	7777
	8888
	9999
	8877
	6666
	3232

FIXED DESIGN OF 96 WELL PLATE

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NC	PC	1234	2345	1234	2345	1234	2345	1234	2345	2345	2356	Tissue1
B	NC	PC	1234	2345	1234	2345	1234	2345	1234	2345	2345	2356	
C	NC	PC	1234	2345	1234	2345	1234	2345	1234	2345	2345	2356	Tissue2
D	NC	PC	1234	2345	1234	2345	1234	2345	1234	2345	2345	2356	
E	7777	8888	9999	8877	7777	8888	9999	8877	6666	3232	BLANK	BLANK	Tissue1
F	7777	8888	9999	8877	7777	8888	9999	8877	6666	3232	BLANK	BLANK	
G	7777	8888	9999	8877	7777	8888	9999	8877	6666	3232	BLANK	BLANK	Tissue2
H	7777	8888	9999	8877	7777	8888	9999	8877	6666	3232	BLANK	BLANK	

IMPORT:

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2,142	0,840	1,906	1,853	1,480	1,787	1,812	0,510	1,576	1,523	1,150	1,457	Tissue1
B	2,147	0,820	1,912	1,854	1,536	1,776	1,817	0,490	1,582	1,524	1,206	1,446	
C	2,262	0,646	1,876	1,757	1,932	2,018	1,952	0,318	1,546	1,427	1,602	1,686	Tissue2
D	2,243	0,658	1,821	1,730	1,851	2,039	1,913	0,328	1,491	1,400	1,521	1,709	
E	0,109	0,119	0,101	1,754	0,198	0,309	0,319	0,301	1,954	0,511	0,037	0,036	Tissue1
F	0,109	0,114	0,104	1,739	0,209	0,309	0,314	0,304	1,939	0,512	0,036	0,037	
G	0,223	0,333	0,232	1,866	0,223	0,423	0,411	0,411	1,866	0,423	0,036	0,036	Tissue2
H	0,224	0,334	0,233	1,566	0,288	0,424	0,423	0,423	1,766	0,278	0,036	0,037	

REMARKS

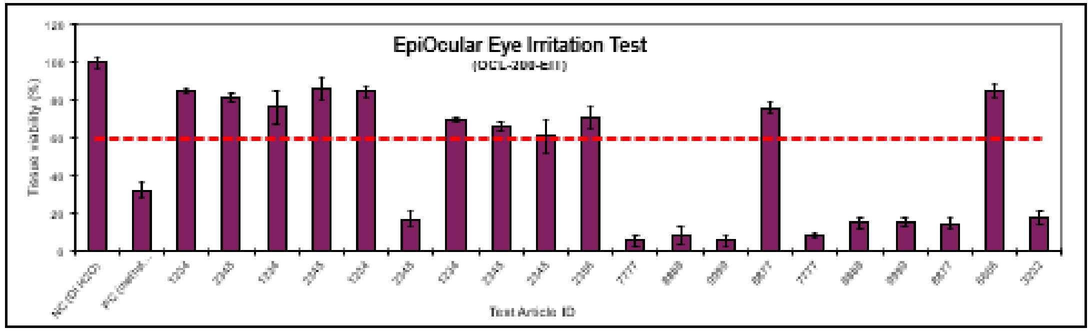
EpiOcular Eye Irritation Test Spreadsheet - calculation

EpiOcular - Eye Irritation Test
(OCL-200-EIT)

RESULTS

exp. no.:	V16-5363
tissue-lot no.:	23499 Kit F
date:	27 10 16
operator:	SN LV

Blank	0,037
	0,036
	0,036
	0,038
	0,038
	0,037
	0,036
	0,037
Mean	0,0368

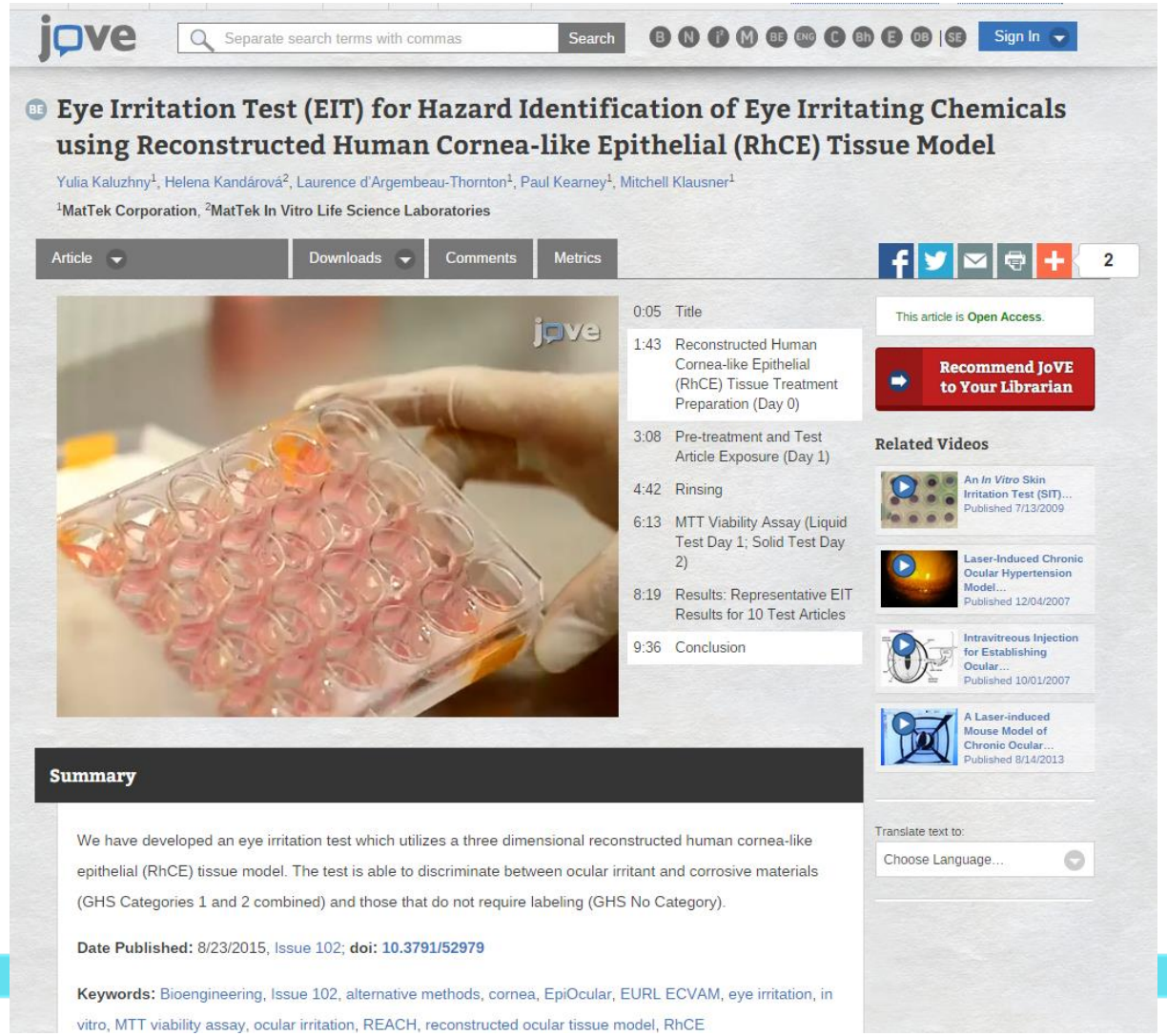


Test Article ID	Tissue #	Raw data		Blank corrected data		mean of OD	% of viability
		Alq. 1	Alq. 2	Alq. 1	Alq. 2		
NC (DI H2O)	1	2.142	2.147	2.105	2.110	2.108	97.3
	2	2.282	2.243	2.245	2.206	2.226	102.7
PC (methyl acetate)	1	0.840	0.820	0.803	0.783	0.793	36.6
	2	0.648	0.658	0.611	0.621	0.616	28.4
1234	1	1.906	1.912	1.869	1.875	1.872	86.4
	2	1.876	1.821	1.839	1.784	1.812	83.6
2345	1	1.853	1.854	1.816	1.817	1.817	83.8
	2	1.757	1.730	1.720	1.693	1.707	78.8
1234	1	1.480	1.536	1.443	1.499	1.471	67.9
	2	1.932	1.851	1.895	1.814	1.855	85.6
2345	1	1.787	1.776	1.750	1.739	1.745	80.5
	2	2.018	2.039	1.981	2.002	1.992	91.9
1234	1	1.812	1.817	1.775	1.780	1.778	82.0
	2	1.952	1.913	1.915	1.876	1.896	87.5
2345	1	0.510	0.490	0.473	0.453	0.463	21.4
	2	0.318	0.328	0.281	0.291	0.286	13.2
1234	1	1.576	1.582	1.539	1.545	1.542	71.2
	2	1.546	1.491	1.509	1.454	1.482	68.4
2345	1	1.523	1.524	1.486	1.487	1.487	68.6
	2	1.427	1.400	1.390	1.363	1.377	63.5
2345	1	1.150	1.206	1.113	1.169	1.141	52.7
	2	1.602	1.521	1.565	1.484	1.525	70.4
2356	1	1.457	1.446	1.420	1.409	1.415	65.3
	2	1.688	1.709	1.651	1.672	1.662	76.7
7777	1	0.109	0.109	0.072	0.072	0.072	3.3
	2	0.223	0.224	0.186	0.187	0.187	8.6
8888	1	0.119	0.114	0.082	0.077	0.080	3.7
	2	0.333	0.334	0.296	0.297	0.297	13.7
9999	1	0.101	0.104	0.064	0.067	0.066	3.0
	2	0.232	0.233	0.195	0.196	0.196	9.0
8877	1	1.754	1.739	1.717	1.702	1.710	78.9
	2	1.666	1.566	1.629	1.529	1.579	72.9
7777	1	0.198	0.209	0.161	0.172	0.167	7.7
	2	0.223	0.288	0.186	0.251	0.219	10.1
8888	1	0.309	0.309	0.272	0.272	0.272	12.6
	2	0.423	0.424	0.386	0.387	0.387	17.9
9999	1	0.319	0.314	0.282	0.277	0.280	12.9
	2	0.411	0.423	0.374	0.386	0.380	17.5
8877	1	0.301	0.304	0.264	0.267	0.266	12.3
	2	0.411	0.423	0.374	0.386	0.380	17.5
6666	1	1.954	1.939	1.917	1.902	1.910	88.1
	2	1.866	1.766	1.829	1.729	1.779	82.1
3232	1	0.511	0.512	0.474	0.475	0.475	21.9
	2	0.423	0.278	0.386	0.241	0.314	14.5

Test Article ID	mean OD	Diff. of OD	mean viability (%)	Diff. of viability	Diff/2	Classification	
NC (DI H2O)	2.167	0.118	100.0	5.45	2.72	NI	qualified
PC (methyl acetate)	0.705	0.177	32.5	8.17	4.08	I	qualified
1234	1.842	0.061	85.0	2.79	1.40	NI	qualified
2345	1.762	0.110	81.3	5.08	2.54	NI	qualified
1234	1.663	0.384	76.7	17.70	8.85	NI	qualified
2345	1.868	0.247	86.2	11.40	5.70	NI	qualified
1234	1.837	0.118	84.8	5.45	2.72	NI	qualified
2345	0.375	0.177	17.3	8.17	4.08	I	qualified
1234	1.512	0.061	69.8	2.79	1.40	NI	qualified
2345	1.432	0.110	66.1	5.08	2.54	NI	qualified
2345	1.333	0.384	61.5	17.70	8.85	NI	qualified
2356	1.538	0.247	71.0	11.40	5.70	NI	qualified
7777	0.129	0.115	6.0	5.28	2.64	I	qualified
8888	0.188	0.217	8.7	10.02	5.01	I	qualified
9999	0.131	0.130	6.0	6.00	3.00	I	qualified
8877	1.644	0.131	75.9	6.02	3.01	NI	qualified
7777	0.193	0.052	8.9	2.40	1.20	I	qualified
8888	0.329	0.115	15.2	5.28	2.64	I	qualified
9999	0.330	0.101	15.2	4.64	2.32	I	qualified
8877	0.323	0.115	14.9	5.28	2.64	I	qualified
6666	1.844	0.131	85.1	6.02	3.01	NI	qualified
3232	0.394	0.161	18.2	7.43	3.72	I	qualified

JoVE video-protocol EpiOcular EIT (free access)

http://www.jove.com/video/52979/eye-irritation-test-eit-for-hazard-identification-eye-irritating



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Thank you very much for your attention!
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REVOLUTION
Let´s start with the practical part!