In vitro evaluation of the eye irritation potential on corneal epithelium (according to OECD 492)

Study N°	CAAD1546/23-02
Study Protocol code	REL/CA3672/2023/TOX
Sponsor	Astonishing Developments Ltd. 2030 Union St Suite 206 94123 San Francisco
Analyzed substance	**RevivLash Lash & Brow Stimulating Serum** **Batch: 230202A**
Date	**November 10th, 2023**

The results reported here in do exclusively refer to the tested sample

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Note:

The results of the test in this report refer only to the tested product/s and to the particular experimental conditions here employed. This report cannot be partially duplicated without the preliminary written approval of the experimenters

1 PART ONE: GENERAL INFORMATION

1.1 Sponsor

Astonishing Developments Ltd.

1.2 Tested Sample

Sample	Internal code	Study Number	Description
RevivLash Lash & Brow Stimulating Serum Batch: 230202A	CA1245/23-02	CAAD1546/23-02	Gel Serum

1.3 Controls

Controls	Supplier	Batch
Methyl acetate Positive control (PC)	MatTek	071123ECC
Phosphate Buffer(PBS) Negative control (NC)	MatTek	050923RAHA

1.4 Assay

Evaluation of eye irritation on 3D in vitro reconstituted human corneal epithelium (Mattek EpiOcular™) through the evaluation of:

- Cytotoxicity by MTT test

1.5 Entrusted laboratory

ABICH Inc
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Study Dates

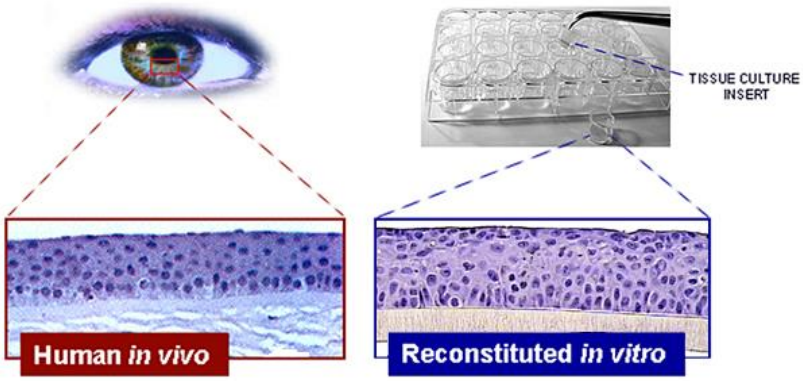
Start: 24/10/2023
End: 26/10/2023

2 PART TWO: STUDY DESIGN

2.1 Purpose of the test:

The purpose of this test is to evaluate the eye irritation potential of the tested sample, according to the method described in OECD 492. This method uses an artificial human corneal epithelial model (EpiOcular™ EIT) to assess the eye irritation of chemical substances and to properly label them according to the results.

The test is based on the evaluation of cell survival after the exposure to the substance through MTT assay and by comparison with epithelium treated with phosphate buffer only (negative control). The MTT method is a colorimetric assay that allows the determination of the percentage of cells alive within an in vitro cultured tissue. This assay is based on the ability of the mitochondrial succinate dehydrogenase enzyme to metabolise the nitro blue tetrazolium salt, giving a coloured compound that can be measured by spectrophotometer reading.



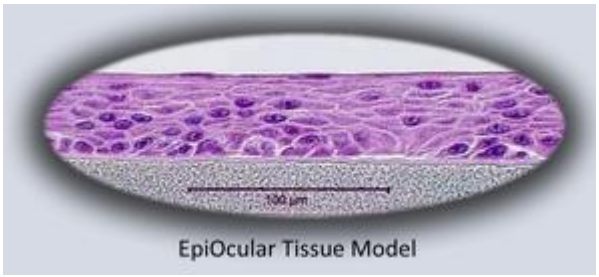
2.2 Assay procedures

The test has been carried out according to the method described in OECD 492 and in the MatTek validated protocol (2).

2.2.1 Cell model

The in vitro test systems employed consist of:

In vitro reconstituted human corneal epithelium.



This system consists of normal, human-derived epidermal keratinocytes which have been cultured to form a stratified, squamous epithelium similar to that found in the cornea. Cultured on specially prepared cell culture inserts using serum-free culture medium, the cells differentiate to form a multi-layered structure which closely parallels the corneal epithelium. It was purchased from MatTek (Batch 38672).

2.2.2 Treatment and Exposure

The sample has been tested as follows: Pure

Phosphate buffer alone has been used as negative control while methyl acetate was used as positive control.

50µl of the product and controls have been applied on each epithelium unit in three replicas. The exposition has been carried out for 30 minutes at 37°C. At the end of the exposure period, the product was removed with multiple washes with PBS and the units were then incubated at 37°C for 2 hours. At the end of the exposure, the MTT assay was performed to evaluate the cell survival on the epithelium units.

2.2.3 MTT cell viability assay

Epithelium units are treated with 1mg/ml MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) for 3 h at 37°C. The solution is then removed and replaced with 1.5 ml/well of isopropanol, with further 2 h incubation at room temperature under medium speed shaking. 2 aliquots of every sample are transferred to a 96 well plate for the reading. The absorbance is read at the wavelength of 550-570 nm with a colorimeter (TECAN model Sunrise remote) equipped with a microplate reader. The absorbance values are corrected by subtracting the reading of the blanks (diluent only).

2.2.4 Expression of results

The results are expressed in terms of viability:

% of cell viability = [mean OD(550-570 nm) test product / mean OD(550-570 nm) negative control] x 100

2.3 Acceptance criteria of method

Negative control (NC): The mean OD value of the 3 replica has to be >1.0 and <2.6,
The standard deviation has to be ≤ 18%

Positive control (PC): The viability mean (expressed as % of the NC) has to be ≤ 60%,
The standard deviation has to be ≤ 18%

Samples: The standard deviation has to be ≤ 18%

2.4 Results interpretation:

Mean cell viability is a predictive method of the ocular irritant potential of the sample.
In the following table, criteria for in vitro interpretation are reported:

Criteria for in vitro interpretation	Classification
Mean tissue viability ≤ 60%	No prediction can be made*
Mean tissue viability > 60%	Not irritant

*According to OECD 492: Guideline for the testing of chemicals. Edition 2017 (Section44. Interpretation of results and Prediction model)

3 PART THREE: RESULTS AND CONCLUSIONS

3.1 Assay validity requirements

	Value	Limits	Result
NC: mean OD from 3 replica	2.1	>1.0 e < 2.6	Complies
NC: % stand. Dev.	6.2%	≤ 18%	Complies
PC: % mean viability	33.2%	<60%	Complies
PC: % stand. Dev.	3.3%	≤ 18%	Complies

Acceptance criteria of the assay (see § 2.3) comply, hence the assay is valid.

3.2 Results

	% mean cell viability (SD) 30 min treatment 2 hours incubation	Classification
RevivLash Lash & Brow Stimulating Serum Batch: 230202A Pure	92.15% (SD: 1.43%)	Not Irritant

*SD : Standard Deviation

3.3 Conclusions:

Based on the results here shown, the sample**: RevivLash Lash & Brow Stimulating Serum, Batch: 230202A** tested in normal use conditions

Is **NOT IRRITANT** for the human corneal epithelium.

Study Director/Chemist
Nina Duru

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4 PART FOUR: ARCHIVING

The study protocol, the raw data and the final report will be kept at Abich Inc laboratory: 5160, Décarie Boulevard, suite 330, Montréal (Québec) H3X 2H9 – Canada, for a minimum period of 5 years from the issue of the final report.

The control sample of the test substance and eventual specific reference material will be kept for 3 months, unless the customer provides a specific request.

The Customer, upon drafting a suitable contract, may request either the extension of the conservation of all or part of the materials for a further period or their restitution.

QA STATEMENT

The collected data derived from this study is managed according to internal procedures following the GLP directive and is verified by the QA manager who checks the different parts of this study (comparison between raw data and recorded data, laboratory books and files, protocol and report) according to the quality plan of ABICH Inc laboratory (internal audits, periodical calibration status of the instruments if they are involved in the test).

Quality Assurance Supervisor

Amal Elmaoui

Amal Elma

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Amal Elma

REFERENCES

1. OECD 492 GUIDELINE FOR THE TESTING OF CHEMICALS Reconstructed Human Cornea-Like Epithelium (RhCE) Test Method Fo Identifyng Chemicals Not Requiring Classification And Labelling For Eye Irritation Or Serious Eye Damage. 28 July 2015.
2. Mattek Corporation. EpiOcular Eye irritation test (OCL-200-EIT) for the prediction of acute ocular irritation chemicals. For use with MatTek Corporation's Reconstructed Human EpiOcula Model. 7 July 2014

ANNEX 1: RAW DATA

Instrument: SUNRISE; Serial number: 1412005062; XFLUOR4 Version: V 4.51

Date of measurement: 26/10/2023

Time: 12:00

Measurement mode: Absorbance

Measurement wavelength: 570 nm

Read mode: Accuracy

Raw data measurement

<>	1	2	3	4	5	6	7	8	9
Blank	-0.001	0.000	-0.001	-0.001	-0.002	-0.001	-0.001	-0.001	-0.001
Negative Control (NC)	1.963	1.975	1.982	2.155	2.153	2.156	2.127	2.133	2.157
Methyl acetate (PC)	0.745	0.622	0.542	0.691	0.938	0.567	0.735	0.692	0.727
Sample : RevivLash Lash & Brow Stimulating Serum, Batch: 230202A	1.885	1.967	1.791	1.926	1.925	1.917	1.825	1.814	1.773

ANNEX 2: CoA

MATTEK
A BICO COMPANY

Certificate of Analysis

Product: EpiOcular™ Tissue

Lot Number: 38672

Part#: OCL-200, OCL-212, OCL-200-EIT, OCL-212-EIT

Description: Reconstructed ocular tissue model containing normal human keratinocytes. This product is for research use only. Not for use in animals, humans or diagnostic purposes.

I. Cell source

All cells used to produce EpiOcular™ are purchased or derived from tissue obtained by MatTek Corporation from accredited institutions. In all cases, consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the cells or derivatives of the tissue for research purposes.

Keratinocyte Strain: 4F1188

II. Analysis for potential biological contaminants

The cells used to produce EpiOcular™ tissue are screened for potential biological contaminants. Tests performed for each of the potential biological contaminant listed in the analysis that follows, where performed according to the last method given. The product resulted in "no detection" for the following potential biological contaminants determined by the stated last method:

Keratinocytes:

HIV-1 virus – Oligonucleotide-directed amplification	Not detected
HIV-2 virus – Oligonucleotide-directed amplification	Not detected
Hepatitis B virus – Oligonucleotide- directed amplification	Not detected
Hepatitis C virus – Oligonucleotide- directed amplification	Not detected
Bacteria, yeast, and other fungi – long term antibiotic, antimycotic free culture	Not detected

III. Analysis for tissue functionality

Test	Specification	Acceptance criteria	Result and QA Statement	
Tissue viability	MTT QC assay, 1 hour, n=3	OD (540-570 nm) [1.1-3.0]	1.729 ± 0.031	Pass
Barrier function	ET-50 assay, 100 µl 0.3% Triton X-100, 3 time-points, n=2, MTT assay	ET-50 [12.2-37.5 min]	14.13 min	Pass
Sterility	Long term antibiotic and antimycotic free culture	No contamination	Sterile	Pass

Tissue viability and the barrier function tests are within the acceptable ranges and indicate appropriate formation of the mucosal barrier and a viable basal cell layer.

Initials: EC
Date: 10/25/23

Nelson Rivas
Quality Assurance Department
Document Control Manager

October 25, 2023
Date

CAUTION: Whereas all information above is believed to be accurate and correct, no absolute guarantee that human derived material is non-infectious can be made or is implied by this certificate of analysis. All tissues should be treated as potential pathogens. The use of protective clothing and eyewear and appropriate disposal procedures is strongly recommended.

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