Evaluation of the eye hazard potential of iron chelate of lysine using the EpiOcular™ Cornea Epithelial model

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Evaluation of the eye hazard potential of iron chelate of lysine using the EpiOcular™ Cornea Epithelial model

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The report of this study was carried out by Wageningen Food & Biobased Research, funded by Metex Noovistago.
Experiments were carried out by Charles River Laboratories Den Bosch BV.

Wageningen Food & Biobased Research
Wageningen, October 2023

Confidential until October 2025

Report 2458
DOI: 10.18174/635399

WAGENINGEN
UNIVERSITY & RESEARCH

WFBR Project number: 6239235600
Version: final
Reviewer: Paulo de Boer
Approved by: Ben Langelaan
Report carried out by: Wageningen Food & Biobased Research
Funded by: Metex Noovistago
This report is confidential until: October 2025

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Contents

Summary

A new animal feed product is developed by Metex Noovistago (Metex). The product is an iron chelate of lysine. This product will be marketed in dry form.

The evaluation of a potential eye hazard, relevant to the safety considerations of workers while handling this product is an important aspect for Metex. With this motivation, a representative sample of the iron chelate of lysine was evaluated for the eye hazard potential using the EpiOcular™ Cornea Epithelial model.

The study design described in this report is based on the most recent OECD guidelines (OECD Guideline 492).

The maximum difference between the percentage of viability of two tissues treated identically was 9.3%, indicating that the test system functioned properly.

Based on the test results, the relative mean tissue viability, which is expressing the eye hazard potential, for the test item was found 3.0%, while positive control was 5.9%. These values are well below the 60% remaining viability that is considered the threshold for a reliable prediction of the items' classification in the EpiOcular™ test under the experimental conditions described in this report. Therefore, the iron chelate of lysine has to be considered as potentially irritant or corrosive to the eye.

Consequently, the iron chelate of lysine was further tested using the Bovine Corneal Opacity and Permeability test [13].

1 Introduction

A new animal feed product is developed by Metex Noovistago (Metex). This new product is an iron chelate of lysine. In this report, the above-described product will be referred as "iron chelate of lysine" or as "test item".

Workers' safety considerations relevant to handling this product are important aspects for Metex.
The objective of this study was to evaluate the eye hazard potential of iron chelate of lysine. The eye hazard potential of the iron chelate of lysine was determined using the EpiOcular™ Cornea Epithelial model.

The design of this study is based on the following study guideline:

- OECD Guideline 492: *Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*, (Adopted June 18, 2019)[1].

The EpiOcular™ tissue construct is a non-keratinized epithelium prepared from normal human keratinocytes. It models the cornea epithelium with progressively stratified, but not cornified cells. The test consists of application of the test item to the surface of the corneal epithelium construct for 6 hours. Afterwards, the tissues are transferred to a fresh medium and incubated for 18 hours at standard culture conditions, prior to determination of the cytotoxic (irritancy) effect [1-12].

Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at the end of the treatment.

The experimental part on the determination of the evaluation of the eye hazard potential of the iron chelate of lysine using the EpiOcular™ Cornea Epithelial model was outsourced to a third party – Charles River Laboratories Den Bosch BV - while results and conclusions were assembled by WFBR in the present report.

2 Methods

The test item, iron chelate of lysine, was delivered by Metex to Wageningen Food & Biobased Research (WFBR). WFBR stored the test item for 3 months at ambient conditions. 200g of the test item was placed in plastic bag and shipped to Charles-River Laboratories Den Bosch BV, a third party, which carried out the evaluation of the eye hazard potential using the EpiOcular™ Cornea Epithelial Model.
The experimental study began on 30th January 2023 and was completed on 03 February 2023.
The design of this study is based on the following study guideline:

OECD Guideline 492: *Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*, (Adopted June 18, 2019)[1].

As a test system, the EpiOcular™ (OCL-200-EIT MatTek Corporation, Lot: 38504) was used, a tissue construct which is non-keratinized epithelium (0.6 cm^2) prepared from normal human keratinocytes (MatTek). It models the cornea epithelium with progressively stratified, but not cornified cells. These cells are not transformed or transfected with genes to induce an extended life span in culture. The tissue construct is prepared in inserts with a porous membrane, through which the nutrients pass to the cells. A cell suspension is seeded into the insert in specialized medium. After an initial period of submerged culture, the medium is removed from the top of the tissue so that the epithelial surface is in direct contact with the air. This allows the test item to be directly applied to the epithelial surface in a fashion similar to how the corneal epithelium would be exposed *in vivo*.

The test was performed on a total of 2 tissues per test item together with a negative control (sterile Milli-Q water) and positive control (methyl acetate, as irritating agent).

A test material may interfere with the MTT endpoint if it is colored and/or it is able to directly reduce MTT. The cell viability measurement is affected only if the test material is present on the tissues when the MTT viability test is performed. As a result, the iron chelate of lysine was checked for possible color interference and direct MTT reduction before the study was started. For more details on those tests, the reader is referred to Annex 1.

3 Results

In this section the key points of the results acquired from the above-described experiments are provided. For the detailed description of the results, the reader is referred to the report of Charles River Laboratories Den Bosch BV provided in Annex 1.

3.1 Interference of the Test Material with the MTT Endpoint

The test material was checked for color interference in aqueous conditions. Addition of the test material to Milli-Q and isopropanol resulted after subtraction of the blank in an OD_{570} of 0.2457 and 0.0281, respectively. From this, it was concluded that the test material induced color interference.

In addition, because a color change was also observed in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) it was concluded that the test material interacted with the MTT endpoint.

As a result, in addition to the normal procedure, two freeze-killed tissues treated with test material and one freeze-killed negative control treated tissue were used for the cytotoxicity evaluation with MTT. The non-specific reduction of MTT by the test material was -0.98% of the negative control tissues. Since the non-specific reduction of MTT ≤ 0.0, there was no need to correct for interference of the test material.

Since the test material showed color interference in aqueous conditions, in addition to the normal procedure, two tissues were treated with test material. Instead of MTT solution these tissues were incubated with assay medium. The non-specific color of the test material was 0.91% of the negative control tissues. The OD of the treated tissues without MTT assay was subtracted from the ODs of the test material treated viable tissues with MTT assay.

Since the test material both interacted with MTT and showed color interference, in addition to the normal procedure, two freeze-killed tissues were treated with test material. Instead of MTT solution these tissues were incubated with assay medium. The non-specific color in killed tissues was 0.84% of the negative control tissues. The OD of the freeze-killed treated tissues without MTT assay was added to the ODs of the test material treated viable tissues with MTT assay to avoid double correction.

3.2 Main Assay

A test item is identified as not requiring classification and labelling according to UN GHS (No Category) if the mean percent tissue viability after exposure and post-exposure incubation is more than (>) 60%. The test chemical is identified as "no prediction can be made" if the mean percent tissue viability after exposure and postexposure incubation is less than or equal (≤) to 60%.

The mean absorption at 570 nm measured after treatment with the test item along with controls are presented in Table 3-1 reproduced from the detailed results annexed to this report.

Table 3-1 Mean absorption results of the iron chelate of lysine obtained from the EpiOcular™ Test.

	A (OD570)	B (OD570)	Mean (OD570)		SD
Negative control	1.346	1.477	1.411	±	0.093
Test material [(1)]	0.022	0.063	0.042	±	0.029
Positive control	0.131	0.034 [(2)]	0.083	±	0.068

OD = optical density

SD = standard deviation

[(1)] The test material values are corrected for the color / MTT interference.
[(2)] This value was outside the historical data range, but did meet all the acceptability criteria, therefore it did not impact the study results.

Duplicate exposures are indicated by A and B.

In this table the values are corrected for background absorption (0.044). Isopropanol was used to measure the background absorption.

Table 3-2 provides the mean tissue viability obtained after 6 hours ± 15 minutes treatment with the test item compared to the negative and positive control tissues and is reproduced from the detailed results annexed to this report.

Table 3-2 Mean tissue viability results of the iron chelate of lysine from the EpiOcular™ Test .

	Mean tissue viability (percentage of control)	Difference between two tissues (percentage)
Negative control	100	9.3
Test material	3.0	2.9
Positive control	5.9	6.8

Eye hazard potential is expressed as the remaining cell viability after exposure to the test item. The relative mean tissue viability obtained after 6 hours ± 15 minutes treatment with the test item compared to the negative control tissues was 3.0%. Since the mean relative tissue viability for the iron chelate of lysine was below 60% the test item is identified as "no prediction can be made".

The positive control had a mean cell viability after 6 hours ± 15 minutes exposure of 5.9%, indicating proper functioning of the positive control (*i.e.* a strong reduction of viability).
The absolute mean OD_{570} of the negative control tissues was within the laboratory historical control data range. The maximum difference between the percentage of viability of two tissues treated identically was 9.3%, indicating that the test system functioned properly.

Based on the above-described key results the iron chelate of lysine is identified as "no prediction can be made" regarding the classification according to the EpiOcular™ test under the experimental conditions described in this report. Therefore, the iron chelate of lysine has to be considered as potentially irritant or corrosive to the eye.

For a more detailed description of the results, we refer to the report of Charles River Laboratories Den Bosch BV provided in Annex 1.

4 Conclusions

The objective of this study was to evaluate the iron chelate of lysine for its potential eye hazard potential using the EpiOcular™ Cornea Epithelial model according to the most recent OECD guideline.

Eye hazard potential is expressed as the remaining cell viability after exposure to the iron chelate of lysine. The relative mean tissue viability obtained after 6 hours ± 15 minutes treatment with the test item compared to the negative control tissues was 3.0%, which is well below the 60% remaining cell viability that is considered the threshold for a compound to be considered not requiring classification and labelling. Therefore, the test chemical is classified as “no prediction can be made”, resulting in the conclusion that the iron chelate of lysine has to be considered as potentially irritant or corrosive to the eye.

Consequently, the iron chelate of lysine was further tested using the Bovine Corneal Opacity and Permeability test [13].

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Annex 1 Report Charles River

Final Report

Test Facility Study No. 20418214

Evaluation of the Eye Hazard Potential with Chélate-Iron Using the EpiOcular™ Cornea Epithelial Model

GLP

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TEST FACILITY:
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QUALITY ASSURANCE STATEMENT

This report was inspected by the Test Facility Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s). The reported method and procedures were found to describe those used and the report reflects the raw data. The Test Facility inspection program was conducted in accordance with Standard Operating Procedure. During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Test Facility Study No. 20418214

Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study				
	Final Study Plan	01-Feb-2023	01-Feb-2023	02-Feb-2023
	Report	12-Apr-2023	12-Apr-2023	12-Apr-2023
	Final Report	20-Sep-2023	20-Sep-2023	20-Sep-2023
Process				
	Genetic and In Vitro Toxicology	14-Nov-2022	24-Nov-2022	01-Dec-2022
		25-Jan-2023	25-Jan-2023	26-Jan-2023
	Test Item Handling			
	Exposure			
	Observations/Measurements			
	Specimen Handling			
	Test Item Receipt	19-Sep-2022	21-Sep-2022	21-Sep-2022
		13-Dec-2022	15-Dec-2022	20-Dec-2022
	Test Item Handling			

*TFM=Test Facility Management SD = Study Director

All electronic signatures appear at the end of this Report at finalization.

COMPLIANCE STATEMENT AND REPORT APPROVAL

The study was performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI) and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

This study was conducted in accordance with the procedures described herein. There were no deviations from the study plan and standard operating procedures. The report represents an accurate and complete record of the results obtained.

There were no deviations from the above regulations that affected the overall integrity of the study or the interpretation of the study results and conclusions.

All electronic signatures appear at the end of this Report at finalization.

1. RESPONSIBLE PERSONNEL

1.1. Test Facility

Role/Phase	Quality Assurance Unit	Name	Contact Information
Study Director	Charles River	Maysae Kallouchi, MSc	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: maysae.kallouchi@crl.com
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Test Facility QAU	Charles River	Lead QA	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: QADenBosch@crl.com

2. SUMMARY

The objective of this study was to evaluate the eye hazard potential of Chélate-Iron. For this purpose Chélate-Iron was topically applied on the Reconstructed Human EpiOcular™ Model.

The possible eye hazard potential of the test material was tested through topical application for 6 hours.

The study procedures described in this report were based on the most recent OECD guideline.

Batch Chélate-Iron of the test material was a dark brown powder. The test material (54.69 to 75.56 mg) was applied directly on top of the tissue for 6 hours ± 15 minutes.

After exposure the cornea epithelial construct was thoroughly rinsed to remove the test material and transferred to fresh medium for an immersion incubation. Afterwards, the tissues were transferred to fresh medium and incubated for 18 hours at standard culture conditions, prior to determination of the cytotoxic (irritancy) effect.

The positive control had a mean cell viability of 5.4% after 6 hours ± 15 minutes exposure. The absolute mean OD_{570} (optical density at 570 nm) of the negative control tissues was within the laboratory historical control data range. The difference between the percentage of viability of two tissues treated identically was less or equal to 6.8%, indicating that the test system functioned properly.

The test material did interact with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

In addition to the normal procedure, two freeze-killed tissues treated with test material and one freeze-killed negative control treated tissue were used for the cytotoxicity evaluation with MTT. The non-specific reduction of MTT by the test material was -0.92% of the negative control tissues. Since the non-specific reduction of MTT ≤ 0.0, there was no need to correct for interference of the test material

The test material showed color interference in aqueous conditions. In addition to the normal procedure, two tissues were treated with test material. Instead of MTT solution these tissues were incubated with assay medium. The non-specific color of the test material was 0.77% of the negative control tissues. The OD of the treated tissues without MTT assay was subtracted from the ODs of the test material treated viable tissues with MTT assay.

Furthermore, since the test material was identified as MTT reducer and caused color interference, the test material was applied to three killed tissue replicates which underwent the entire testing procedure but were incubated with assay medium instead of MTT solution during the MTT assay. The non-specific color in freeze-killed tissues by the test material was 0.71% of the negative control tissues.

Eye hazard potential is expressed as the remaining cell viability after exposure to the test material. The relative mean tissue viability obtained after 6 hours ± 15 minutes treatment with the test material compared to the negative control tissues was 2.7%. Since the mean relative tissue viability for the test material was below 60% after 6 hours ± 15 minutes treatment it is considered to be potentially irritant or corrosive to the eye.

In conclusion, the test material is identified as no prediction can be made regarding the classification in the EpiOcular™ test under the experimental conditions described in this report.

3. INTRODUCTION

The objective of this study was to evaluate the eye hazard potential of Chélate-Iron. For this purpose the test material was topically applied on the Reconstructed Human EpiOcular™ Model.

Background of the test system

The EpiOcular tissue construct is a nonkeratinized epithelium prepared from normal human keratinocytes. It models the cornea epithelium with progressively stratified, but not cornified cells. A cell suspension is seeded into the insert in specialized medium. After an initial period of submerged culture, the medium is removed from the top of the tissue so that the epithelial surface is in direct contact with the air. This allows the test material to be directly applied to the epithelial surface in a fashion similar to how the corneal epithelium would be exposed in vivo.

The test consists of application of the test material to the surface of the corneal epithelium construct for 6 hours. After exposure, the tissue construct is thoroughly rinsed to remove the test material and transferred to fresh medium for an immersion incubation. Afterwards, the tissues were transferred to fresh medium and incubated for 18 hours at standard culture conditions, prior todetermination of the cytotoxic (irritancy) effect.

Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at the end of the treatment.

The design of this study is based on the following study guideline:

- OECD Guideline 492: *Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*, (Adopted June 18, 2019).

The study plan is presented in Appendix 1.

Study Initiation Date:	30 Jan 2023
Experimental Start Date:	30 Jan 2023
Experimental Completion Date:	03 Feb 2023

4. MATERIALS AND METHODS

4.1. Test Materials

4.1.1. Test Material Characterization

The Sponsor provided to the Test Facility documentation of the identity, purity, composition and stability for the test material. The characterization of the test material was conducted in a sponsor or sponsor subcontractor quality environment.
The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test material, and this information is available to the appropriate regulatory agencies should it be requested.

4.1.2. Test Material

Identification:	Chélate-Iron
Batch (Lot) Number:	Chélate-Iron
Expiry date:	01 June 2025
Physical Description:	Dark brown powder
Purity/Composition:	Iron: 18%, lysine: 23%, Sulfates: 38%
Storage Conditions:	At room temperature

Additional information

Test Facility test material number:	500475/A
Purity/Composition correction factor:	No correction factor required
Test material handling:	No specific handling conditions required

4.1.3. Control Materials

4.1.3.1. Negative Control

Sterile Milli-Q water (Millipore Corp., Bedford, Mass., USA).

4.1.3.2. Positive Control

Methyl Acetate [CAS Number 79-20-9] (Mattek, Ashland MA, USA)

4.2. Reserve Samples

For each batch (lot) of test material, a reserve sample (about 0.5 gram) was collected and maintained under the appropriate storage conditions by the Test Facility.

4.3. Test Material Inventory and Disposition

The test material was received by the Testing Facility. Records of the storage, distribution, and disposition of the test materials was maintained.

4.4. Test System

Test System

EpiOcular™ (OCL-200-EIT MatTek Corporation, Lot: 38504 Kit F, Appendix 2)

The EpiOcular tissue construct is a non-keratinized epithelium (0.6 cm^2) prepared from normal human keratinocytes (MatTek). It models the cornea epithelium with progressively stratified, but not cornified cells. These cells are not transformed or transfected with genes to induce an extended life span in culture. The “tissue” is prepared in inserts with a porous

membrane through which the nutrients pass to the cells. A cell suspension is seeded into the insert in specialized medium. After an initial period of submerged culture, the medium is removed from the top of the tissue so that the epithelial surface is in direct contact with the air. This allows the test material to be directly applied to the epithelial surface in a fashion similar to how the corneal epithelium would be exposed in vivo.

Rationale

In the interest of sound science and animal welfare, a sequential testing strategy is recommended to minimize the need of in vivo testing. One of the validated in vitro eye irritation tests is the EpiOcular test, which is recommended in international guidelines and scientific publications (e.g. OECD).

Source

MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia.

4.5. Experimental Design

4.5.1. Test for the Interference of the Test Material with the MTT Endpoint

A test material may interfere with the MTT endpoint if it is colored and/or it is able to directly reduce MTT. The cell viability measurement is affected only if the test material is present on the tissues when the MTT viability test is performed.

4.5.1.1. Test for Color Interference by the Test Material

The test material was checked for possible color interference before the study was started. Some non-colored test materials may change into colored materials in aqueous conditions and thus stain the tissues during the exposure. To assess the color interference, approximately 50 mg of the test material or 50 µL sterile Milli-Q water as a negative control was added to 1.0 mL Milli-Q water. The mixture was incubated for at least 1 hour at 37.0 ± 1.0°C in the dark. After incubation approximately 0.6 mL of the mixture was centrifuged for 2 minutes at 14100 *g*. Furthermore, approximately 50 mg of the test material or 50 µL sterile Milli-Q water as a negative control was added to 2.0 mL isopropanol. The mixture was incubated for 2 - 3 hours at room temperature with gentle shaking.

At the end of the exposure time, the absorbance of the solutions was determined spectrophotometrically at 570 nm in duplicate with the TECAN Infinite® M200 Pro Plate Reader. Centrifugation was not considered necessary. If after subtraction of the negative control, the OD for the test material solution is >0.08, the test material is considered as possibly interacting with the MTT measurement.

4.5.1.2. Test for Reduction of MTT by the Test Material

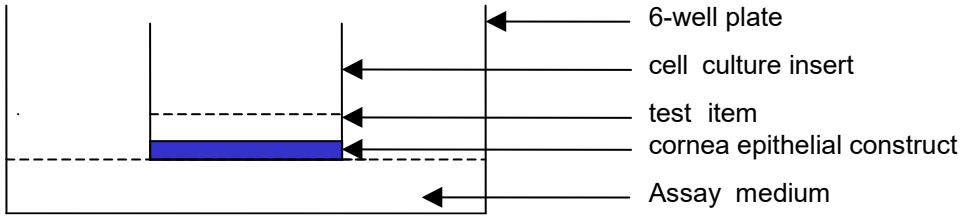
The test material was checked for possible direct MTT reduction before the study was started. To assess the ability of the test material to reduce MTT, approximately 50 mg of the test material was added to 1 mL MTT solution (1 mg/mL MTT in phosphate buffered saline). The mixture was incubated for approximately 3 hours at 37.0 ± 1.0°C in the dark. A negative control, 50 µL sterile Milli-Q water was tested concurrently. If the MTT solution color turned blue / purple or if a blue / purple precipitate was observed the test material interacts with MTT. Only test materials which bind to the tissue after rinsing can interact with MTT in the main assay.

4.5.2. Test System Set Up

On the day of receipt the tissues were equilibrated (in its 24-well shipping container) to room temperature. Subsequently, tissues were transferred to 6-well plates and incubated for 20 ± 4

hours at 37°C in 1.0 mL fresh pre-warmed Assay Medium. Assay Medium was supplied by MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia.

Figure 1
A Diagram of the Application



DMEM (Dulbecco's Modified Eagle's Medium)

Supplemented DMEM medium, serum-free supplied by MatTek Corporation.

MTT medium

MTT concentrate (5 mg/mL) diluted (1:5) with MTT diluent.

Freeze-killed tissues (Lot: 31797 Kit J, 28095 Kit R, Appendix 2)

Living epidermis was transferred to a freezer set to maintain -20°C, thawed, and then again transferred to a freezer freezer set to maintain -20°C. The freeze-killed epidermis was stored in a freezer set to maintain -20°C until use. Freeze-killed tissues were thawed by placing them at room temperature. Further use of killed tissues was similar to living tissues.

Environmental conditions

All incubations, were carried out in a controlled environment, in which optimal conditions were a humid atmosphere of 80 - 100% (actual range 44 - 100%), containing 5.0 ± 0.5% CO_2 in air in the dark at 37.0 ± 1.0°C (actual range 36.2 - 37.0°C). Temperature and humidity were continuously monitored throughout the experiment. The CO_2 percentage was monitored once on each working day. Temporary deviations from the temperature, humidity and CO_2 percentage may occur due to opening and closing of the incubator door. Based on laboratory historical data these deviations are considered not to affect the study integrity.

4.5.3. Test Material Preparation

No correction was made for the purity/composition of the test material.

The solid test material (54.69 to 75.56 mg) was applied directly on top of the skin tissue. The test material was spread to match the size of the tissue.

Any residual volumes were discarded.

4.5.4. Application/Treatment of the Test Material

The test was performed on a total of 2 tissues per test material together with a negative control and positive control.

Since the test material induced color interference in aqueous conditions, two tissues were treated with test material. Instead of MTT solution these tissues were incubated with DMEM. In addition, since the test material reacted with the MTT medium, two freeze-killed tissues were treated with test material and one freeze-killed non treated tissue were used per exposure time for the cytotoxicity evaluation with MTT. Furthermore, since the test material was

identified as MTT reducer and caused color interference, a third set of adapted controls was required. In this control, the test material was applied to two killed tissue replicates per exposure time which underwent the entire testing procedure but were incubated with assay medium instead of MTT solution during the MTT assay.

Before the assay was started the entire tissues were pre-wetted with 20 µL of $Ca^{2+}Mg^{2+}$-Free-DPBS. The tissues were incubated at standard culture conditions for minimal 30 minutes.

Two tissues were treated with 50 µL Milli-Q water (negative control) and 2 tissues with 50 µL Methyl Acetate (positive control) respectively.

At least 50 mg solid was added into the 6-well plates on top of the tissues. After the exposure period with the test material (6 hours ± 15 minutes at 37.0 ± 1.0°C), the tissues were thoroughly rinsed with Ca2+Mg2+-free D-PBS (brought to room temperature) to remove residual test material. After rinsing the cell culture inserts were each dried carefully and immediately transferred to and immersed in 5 mL of previously warmed Assay Medium (room temperature) in a pre-labeled 12-well plate for a 25 ± 2 minute immersion incubation at room temperature (Post-Soak). After the Post-Soak period cell culture inserts were each dried carefully and transferred to the 6-well plate containing 1.0 mL of warm Assay Medium and were incubated for 18 hours ± 15 minutes at 37°C.

4.5.5. Cell Viability Measurement

After incubation, cell culture inserts were dried carefully to remove excess medium. The cell culture inserts were transferred into a 24-wells plate prefilled with 0.3 mL MTT-medium (1.0 mg/mL). The tissues were incubated for 180 ± 10 minutes at 37°C.

After incubation with MTT-medium the tissues were placed on blotting paper to dry the tissues and then transferred to a pre-labeled 6-well plate containing 2 mL isopropanol in each well so that no isopropanol is flowing into the insert. Formazan was extracted with 2 mL isopropanol for 2 - 3 hours at room temperature with gentle shaking.

The amount of extracted formazan was determined spectrophotometrically at 570 nm in duplicate with the TECAN Infinite® M200 Pro Plate Reader.

Cell viability was calculated for each tissue as a percentage of the mean of the negative control tissues. Eye hazard potential of the test material was classified according to remaining cell viability following exposure of the test material.

5. ACCEPTABILITY CRITERIA

The in vitro eye irritation test is considered acceptable if it meets the following criteria:

a) The absolute mean OD_{570} of the two tissues of the negative control should reasonably be > 0.8 and < 2.5.

b) The mean relative tissue viability of the positive control should be <50% relative to the negative control.

c) The difference between the % tissue viabilities of the two identically treated replicates should be <20.

d) The non-specific MTT reduction should be ≤ 50% relative to the negative control OD.

e) The non-specific color of the test material should be ≤ 50% relative to the negative control OD.

All results presented in the tables of the report are calculated using values as per the raw data rounding procedure and may not be exactly reproduced from the individual data presented.

6. INTERPRETATION

The test chemical is identified as not requiring classification and labelling according to UN GHS (No Category) if the mean percent tissue viability after exposure and post-exposure incubation is more than (>) 60%. In this case no further testing in other test methods is required.

The test chemical is identified as "no prediction can be made" if the mean percent tissue viability after exposure and postexposure incubation is less than or equal (≤) to 60%.

7. ANALYSIS

7.1. Calculation of Cell Viability

Optical Density readings were transferred into Microsoft Excel to allow further calculations to be performed.

The corrected OD (OD_c) for each sample or control was calculated by subtracting the value of the blank mean (OD_{bl}) from each reading (OD_{raw}).

$OD_c = OD_{raw} - OD_{bl}$

The OD value representing 100% cell viability is the average OD of the negative controls (OD_{lt_u+MTT}).

The %Viability for each sample and positive control is calculated as follows:

$\%Viability = (OD_c/\text{mean } OD_{lt\_u+MTT}) * 100$

7.2. Coloring Test Materials

Nonspecific color in living tissues (NSC_{living}) was calculated. NSC_{living} is the mean OD of the treated living tissues without MTT reagent (OD_{lt_t-MTT}) expressed as percentage of the mean of the negative control tissues (OD_{lt_u+MTT}).

$\%NSC_{living} = [OD_{lt\_t-MTT} / OD_{lt\_u+MTT}] * 100$

True tissue viability is calculated as the difference between the OD obtained with the test material treated living tissues incubated with MTT medium (OD_{lt_t+MTT}) and the OD obtained with the test material treated living tissues incubated with medium without MTT (OD_{lt_t-MTT}), and subsequently divided by the OD of the negative control (OD_{lt_u+MTT}).

$OD = OD_{lt\_t+MTT} - OD_{lt-t-MTT}$

$\%Viability = (OD / \text{mean } OD_{lt\_u+MTT}) * 100.$

In case the $\%NSC_{living} \leq 0.0$, there is no need to correct for color interference of the test material.

7.3. MTT Interacting Test Materials

Nonspecific MTT reduction (NSMTT) was calculated. NSMTT is the difference between the mean OD of the untreated freeze-killed tissues (OD_{kt_u+MTT}) and test material treated freeze-killed tissues (OD_{kt_t+MTT}) expressed as percentage of the mean of the negative control tissues (OD_{lt_u+MTT}).

$\%NSMTT = [(OD_{kt\_t+MTT} - OD_{kt\_u+MTT})/ \text{mean } OD_{lt\_u+MTT}] * 100$

True tissue viability is calculated as the difference between the living test material treated tissues incubated with MTT medium (OD_{lt_t+MTT}) and the difference between OD_{kt_t+MTT} and OD_{kt_u+MTT}.

$OD = OD_{lt\_t+MTT} - (OD_{kt\_t+MTT} - OD_{kt\_u+MTT})$

$\%Viability = [OD / \text{mean } OD_{lt\_u+MTT}] * 100$

Since the %NSMTT ≤ 0.0, there is no need to correct for interference of the test material.

7.4. Coloring and MTT Interacting Test Materials

Nonspecific color in freeze-killed tissues (NSC_{killed}) was calculated. NSC_{killed} is the mean OD of the test material treated killed tissues without MTT reagent (OD_{kt_t-MTT}) expressed as percentage of the mean of the negative control tissues (OD_{lt_u+MTT}).

$\%NSC_{killed} = (OD_{kt\_t-MTT}) / OD_{lt\_u+MTT}) * 100$

True tissue viability is calculated as the OD obtained with the test material treated living tissues incubated with MTT medium (OD_{lt_t+MTT}), minus the OD obtained with the test material treated living tissues incubated with medium without MTT (OD_{lt_t-MTT}), minus the difference between the mean OD of the untreated freeze-killed tissues (OD_{kt_u+MTT}) and test material treated freeze-killed tissues (OD_{kt_t+MTT}) plus the mean OD of the test material treated killed tissues without MTT reagent (OD_{kt_t-MTT}), subsequently divided by the OD of the negative control (OD_{lt_u+MTT}).

$OD = OD_{lt\_t+MTT} - OD_{lt\_t-MTT} - (OD_{kt\_t+MTT} - OD_{kt\_u+MTT}) + OD_{kt\_t-MTT}$

$\%Viability = (OD / OD_{lt\_u+MTT}) * 100.$

8. COMPUTERIZED SYSTEMS

Critical computerized systems used in the study are listed below. All critical computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

Text Table 1
Computerized Systems

System name	Description of Data Collected and/or Analyzed
M-Files®	Reporting and collection of 21 CFR Part 11 compliant signature
REES Centron	Temperature and humidity (laboratory facilities) Data collection
Magellan Tracker	Optical Density Measurement

9. RETENTION AND DISPOSITION OF RECORDS

All study-specific raw data, documentation, study plan and final reports from this study were archived at the Test Facility at finalization of the report. At least 2 years after issue of the final report, the Sponsor will be contacted.

Electronic data generated by the Test Facility were archived as noted above, files stored on M-Files® (Study Plan and reporting files) were archived at the Charles River Laboratories facility location in Wilmington, Massachusetts, USA.

10. RESULTS

10.1. Interference of the Test Material with the MTT Endpoint

The test material was checked for color interference in aqueous conditions. Addition of the test material to Milli-Q and isopropanol resulted after subtraction of the blank in an OD of 0.2457 and 0.0281, respectively. Therefore it was concluded that the test material induced color interference.

In addition, because a color change was observed in the presence of MTT it was concluded that the test material interacted with the MTT endpoint.

Since the test material did interact with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), in addition to the normal procedure, two freeze-killed tissues treated with test material and one freeze-killed negative control treated tissues were used for the cytotoxicity evaluation with MTT. The non-specific reduction of MTT by the test material was -0.92% of the negative control tissues. Since the non-specific reduction of MTT $\leq$ 0.0, there was no need to correct for interference of the test material

The test material showed color interference in aqueous conditions therefor in addition to the normal procedure, two tissue were treated with test material. Instead of MTT solution these tissues were incubated with assay medium. The non-specific color of the test material was 0.77% of the negative control tissues. The OD of the treated tissues without MTT assay was subtracted from the ODs of the test material treated viable tissues with MTT assay.

Since the test material both interacted with MTT and showed color interference, in addition to the normal procedure, two freeze-killed tissues were treated with test material. Instead of MTT solution these tissues were incubated with assay medium. The non-specific color in killed tissues was 0.71% of the negative control tissues. The OD of the freeze-killed treated tissues without MTT assay was added to the ODs of the test material treated viable tissues with MTT assay to avoid double correction.

10.2. Main Assay

The mean absorption at 570 nm measured after treatment with the test material and controls are presented in Table 1.

The individual OD_{570} measurements are presented in Table 3.

Table 2 shows the mean tissue viability obtained after 6 hours ± 15 minutes treatment with the test material compared to the negative control tissues. Eye hazard potential is expressed as the remaining cell viability after exposure to the test material. The relative mean tissue viability obtained after 6 hours ± 15 minutes treatment with the test material compared to the negative control tissues was 2.7%. Since the mean relative tissue viability for the test material was below 60% it is considered to be potentially irritant or corrosive to the eye.

The positive control had a mean cell viability after 6 hours ± 15 minutes exposure of 5.4%. The absolute mean OD_{570} of the negative control tissues was within the laboratory historical control data range (See Table 4). The difference between the percentage of viability of two tissues treated identically was less or equal to 6.8%, indicating that the test system functioned properly.

11. CONCLUSION

In conclusion, Chélate-Iron is identified as no prediction can be made regarding the classification in the EpiOcular™ test under the experimental conditions described in this report.

12. REFERENCES

1. Test Submission Template (TST) for ECVAM submissions: Ocular Irritation Assay for Chemicals Using the EpiOcular™ Human Cell Construct. Revised submission September 11 (2008).
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TABLES

Table 1
Mean Absorption in the EpiOcular™ Test with Chélate-Iron

	A (OD570)	B (OD570)	Mean (OD570)		SD
Negative control	1.458	1.561	1.509	±	0.073
Test material	0.022[(1)]	0.063[(1)]	0.042	±	0.029
Positive control	0.129	0.034[(2)]	0.081	±	0.067

OD = optical density

SD = Standard deviation

[(1)] The test material values are corrected for the color / MTT interference.
[(2)] This value was outside the historical data range, but did meet all the acceptability citeria, therefore it did not impact the study results.

Duplicate exposures are indicated by A and B.

In this table the values are corrected for background absorption (0.044). Isopropanol was used to measure the background absorption.

Table 2
Mean Tissue Viability in the EpiOcular™ Test with Chélate-Iron

	Mean tissue viability (percentage of control)	Difference between two tissues (percentage)
Negative control	100	6.8
Test material	3	2.7
Positive control	5.4	6.3

Table 3
Individual OD Measurements at 570 Nm

	A (OD_{570})	B (OD_{570})
Negative control		
OD_{570} measurement 1	1.5103	1.6216
OD_{570} measurement 2	1.4954	1.5893
Test material on viable tissue		
OD_{570} measurement 1	0.0779	0.1195
OD_{570} measurement 2	0.0784	0.1189
Test material on viable tissue (no MTT)		
OD_{570} measurement 1	0.0575	0.0556
OD_{570} measurement 2	0.0577	0.0556
Non treated killed tissue		
OD_{570} measurement 1	0.0934	
OD_{570} measurement 2	0.0923	
Treated killed tissue		
OD_{570} measurement 1	0.0756	0.0827
OD_{570} measurement 2	0.0744	0.0831
Treated killed tissue (no MTT)		
OD_{570} measurement 1	0.0566	0.0566
OD_{570} measurement 2	0.0543	0.0554
Positive control		
OD_{570} measurement 1	0.1740	0.0792
OD_{570} measurement 2	0.1737	0.0784

OD = Optical density

Duplicate exposures are indicated by A and B.

Table 4
Historical Control Data for EpiOcular™ Studies

	Negative control (absorption; OD_{570})	**Positive control (absorption; OD_{570})**	**Positive control (viability; %)**
Range	0.648 – 2.190	0.035 – 0.720	5.80 – 44.55
Mean	1.599	0.373	23.54
SD	0.271	0.166	9.95
n	80	80	80

SD = Standard deviation
n = Number of observations

The above mentioned historical control data range of the controls were obtained by collecting all data over the period of November 2017 to November 2022.

Appendix 1
Study Plan

FINAL STUDY PLAN

Test Facility Study No. 20418214

Evaluation of the Eye Hazard Potential with Chélate-Iron Using the EpiOcular™ Cornea Epithelial Model

GLP

SPONSOR:
Wageningen Food & Biobased Research
P.O.Box 17,
6700 AA Wageningen
The Netherlands

TEST FACILITY:
Charles River Laboratories Den Bosch BV
Hambakenwetering 7
5231 DD 's-Hertogenbosch
The Netherlands

TABLE OF CONTENTS

1. OBJECTIVE

The objective of this study is to evaluate the eye hazard potential of Chélate-Iron. For this purpose the test material will be topically applied on the Reconstructed Human EpiOcular™ Model.

Background of the test system

The EpiOcular tissue construct is a nonkeratinized epithelium prepared from normal human keratinocytes. It models the cornea epithelium with progressively stratified, but not cornified cells. A cell suspension is seeded into the insert in specialized medium. After an initial period of submerged culture, the medium is removed from the top of the tissue so that the epithelial surface is in direct contact with the air. This allows the test material to be directly applied to the epithelial surface in a fashion similar to how the corneal epithelium would be exposed in vivo.

The test consists of application of the test material to the surface of the cornea epithelial construct for 6 hours. After exposure the cornea epithelial construct is thoroughly rinsed to remove the test material and transferred to fresh medium for an immersion incubation. After transfer to fresh medium an 18 hours incubation period determination of the cytotoxic (irritancy) effect is performed.

Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at the end of the treatment.

2. PROPOSED STUDY SCHEDULE

Proposed study dates are listed below. Actual dates will be included in the Final Report.

Experimental Start Date:	30 Jan 2023 (First date of study-specific data collection)
Experimental Completion Date:	12 Feb 2023 (Last date data are collected from the study)
Unaudited Draft Report:	05 Mar 2023

3. SPONSOR

Role	Name	Contact Information
Sponsor Representative Name	Theo Verkleij	Address as cited for Sponsor Tel: +31 317 481 096 E-mail: theo.verkleij@wur.nl
Study Monitor Name	Panagiotis Voudouris	Address as cited for Sponsor Tel: + 31 317 48 6758 E-mail: panagiotis.voudouris@wur.nl

4. RESPONSIBLE PERSONNEL

Role/Phase	Quality Assurance Unit	Name	Contact Information
Study Director	Charles River	Maysae Kallouchi, MSc	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: maysae.kallouchi@crl.com
Test Facility Management	Charles River	Beppy van de Waart, MSc, ERT	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: beppy.vandewaart@crl.com
Test Facility QAU	Charles River	Lead QA	Address as cited for Test Facility Tel: +31 73 640 6700 E-mail: QADenBosch@crl.com

5. TEST MATERIALS

5.1. Test Material Characterization

The Sponsor will provide to the Test Facility documentation of the identity, strength, purity, composition, and stability for the test material(s). A Certificate of Analysis or equivalent documentation may be provided for inclusion in the Final Report.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test material, and this information is available to the appropriate regulatory agencies should it be requested.

5.2. Test Material Identification

5.2.1. Test Material

Identification: Chélate-Iron

Batch (Lot) Number: Chélate-Iron

Expiry date: 01 June 2025

Physical Description: Dark brown powder

Purity/Composition: Iron: 18%, lysine: 23%, Sulfates: 38%

Storage Conditions: At room temperature

Additional information

Test Facility test material number: 500475/A

Purity/Composition correction factor: No correction factor required

Test material handling: No specific handling conditions required

5.2.2. Control Materials

5.2.2.1. Negative Control

Sterile Milli-Q water.

5.2.2.2. Positive Control

Methyl Acetate. Details on the supplier will be included in the final report.

5.3. Reserve Samples

For each batch (lot) of test material and if practically possible, a reserve sample will be collected and maintained under the appropriate storage conditions by the Test Facility.

5.4. Test Material Inventory and Disposition

Records of the receipt, distribution, storage, and disposition of test materials will be maintained.

5.5. Safety

The following safety instruction(s) apply to this study:

- Standard safety precautions specified in Charles River Den Bosch procedures

6. DOSE FORMULATION

Test material amounts may be pre-weighed for a part of- or even the entire study at the discretion of the study director when stored under the same conditions as prescribed for the bulk container.

7. TEST SYSTEM

Test System

EpiOcular™ (OCL-200-EIT MatTek Corporation)

The EpiOcular tissue construct is a nonkeratinized epithelium 0.6 cm^2 prepared from normal human keratinocytes (MatTek). It models the cornea epithelium with progressively stratified, but not cornified cells. These cells are not transformed or transfected with genes to induce an extended life span in culture. The “tissue” is prepared in inserts with a porous membrane through which the nutrients pass to the cells. A cell suspension is seeded into the insert in specialized medium. After an initial period of submerged culture, the medium is removed from the top of the tissue so that the epithelial surface is in direct contact with the air. This allows the test material to be directly applied to the epithelial surface in a fashion similar to how the corneal epithelium would be exposed in vivo.

Rationale

In the interest of sound science and animal welfare, a sequential testing strategy is recommended to minimize the need of in vivo testing. One of the validated in vitro eye irritation tests is the EpiOcular test, which is recommended in international guidelines and scientific publications (e.g. OECD).

Source

MatTek Corporation, Ashland MA, U.S.A, or MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia.

8. EXPERIMENTAL DESIGN

8.1. Test for the Interference of the Test Material with the MTT Endpoint

A test material may interfere with the MTT endpoint if it is colored and/or it is able to directly reduce MTT. The cell viability measurement is affected only if the test material is present on the tissues when the MTT viability test is performed.

8.1.1. Test for Color Interference by the Test Material

The test material will be checked for possible color interference before the study is started. Some non-colored test materials may change into colored materials in aqueous conditions and thus stain the tissues during the exposure. To assess the color interference, at least 50 mg of the test material or 50 µL Milli-Q water as a negative control will be added to 1.0 mL Milli-Q water. The mixture will be incubated for at least 1 hour at 37.0 ± 1.0°C in the dark. Furthermore, at least 50 mg of the test material or 50 µL Milli-Q water as a negative control will be added to 2.0 mL isopropanol. The mixture will be incubated for 2 – 3 hours at room temperature with gentle shaking.

At the end of the exposure time, the mixtures will be centrifuged for 2 min at 14,100 *g* if needed and the absorbance of the solutions will be determined spectrophotometrically at 570 nm in duplicate with the TECAN Infinite® M200 Pro Plate Reader.

If after subtraction of the negative control, the OD for the test material solution is >0.08, the test material is considered as possibly interacting with the MTT measurement. A functional test with living EpiOcular tissues will be performed to show that the test material will not bind to the tissue and leads to a false MTT reduction signal. In addition to the normal procedure, two tissue are treated with test substance. Instead of MTT solution these tissues will be incubated with Assay medium. This generates a non-specific color in living tissues (NSC_{living}) control.

8.1.2. Test for Reduction of MTT by the Test Material

The test material will be checked for possible direct MTT reduction before the study is started. To assess the ability of the test material to reduce MTT, at least 50 mg of the test material will be added to 1 mL MTT medium or 1 mg/mL MTT solution in phosphate buffered saline. The mixture will be incubated for approximately 3 hours at 37.0 ± 1.0°C in the dark. A negative control, 50 µL sterile Milli-Q water will be tested concurrently. If the MTT solution color will turn blue / purple or if a blue / purple precipitate will be observed the test material interacts with MTT.

Test materials which bind to the tissue after rinsing require additional steps. Therefore, a functional test with freeze-killed EpiOcular tissues will be performed to show that the test material will not bind to the tissue and leads to a false MTT reduction signal. In addition to

the normal procedure, two freeze-killed tissues treated with test substance and one untreated freeze-killed tissue will be used for the cytotoxicity evaluation with MTT.

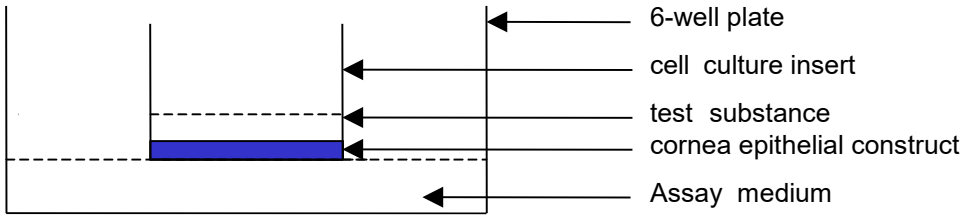
8.1.3. Color Interference and Reduction of MTT by the Test Material

Test materials that are identified as reducing MTT and causing color interference will require a third set of adapted controls. This is the non-specific color in killed tissues (NSC_{killed}) control. In this control, two freeze-killed tissues will be treated with test substance. Instead of MTT solution these tissues will be incubated with Assay medium.

8.2. Test System Set Up

On the day of receipt the tissues will be equilibrated (in its 24-well shipping container) to room temperature. Subsequently, tissues are transferred to 6-well plates and incubated for 20 ± 4 hours at 37°C in 1.0 mL fresh prewarmed Assay medium (Figure 1), optionally after 1 hour the medium will be refreshed. Assay medium is supplied by MatTek Corporation, Ashland MA, U.S.A.

Figure 1
A diagram of the application.



Freeze-killed tissues

Living epidermis was transferred to a freezer set to maintain -15 °C or lower, thawed, and then again transferred to a freezer set to maintain -15 °C or lower. The freeze-killed epidermis was stored in a freezer set to maintain -15 °C or lower until use. Freeze-killed tissues will be thawed by placing them at room temperature. Further use of killed tissues will be similar to living tissues.

MTT medium

MTT concentrate (5 mg/mL) diluted (1:5) with MTT diluent.

Environmental conditions

All incubations will be carried out in a humid atmosphere (80 - 100%) containing 5.0 ± 0.5% CO_2 in air in the dark at 37.0 ± 1.0°C. Temperature and humidity will be continuously monitored throughout the experiment. The CO_2 percentage will be monitored once on each working day. Temporary deviations from the temperature, humidity and CO_2 percentage may occur due to opening and closing of the incubator door. Any variation to these conditions will be evaluated and maintained in the raw data.

8.3. Test Material Preparation

No correction will be made for the purity/composition of the test material.

Since the test material is a solid, it will be crushed and ground in a mortar with pestle if this improves the consistency. The weighed (at least 50 mg) fine ground test material will be applied on top of cornea epithelial construct. If necessary the test material will be spread to match the size of the tissue. If the exact amount of test material cannot be determined due to the physical properties of the test material, an excessive amount of test material will be applied on top of the skin tissue.

Any residual volumes will be discarded unless otherwise requested by the Study Director.

8.4. Application/Treatment of the test material

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

Before the assay will be started the entire tissues will be pre-wetted with 20 µL of $Ca^{2+}Mg^{2+}$-Free-DPBS. The tissues will be incubated at standard culture conditions for minimal 30 minutes.

Two tissues are treated with 50 µL Milli-Q water (negative control) and 2 tissues with 50 µL Methyl Acetate (positive control) respectively.

At least 50 mg solid will be added into the 6-well plates on top of the tissues. After the exposure period with the test material (6 hours ± 15 minutes at 37.0 ± 1.0°C), the tissues are thoroughly rinsed with $Ca^{2+}Mg^{2+}$-free D-PBS to remove residual test material. If necessary cotton wool swabs will be used to remove any remaining test material.

After rinsing the cell culture inserts are each dried carefully and immediately transferred to and immersed in 5 mL of Assay Medium in a pre-labeled 12-well plate for a 25 ± 2 minutes immersion incubation at room temperature (Post-Soak). After the Post-Soak period cell culture inserts are each dried carefully and transferred to the 6-well plate containing 1.0 mL of warm Assay Medium and are incubated for 18 hours ± 15 minutes at 37.0 ± 1.0°C.

8.5. Cell Viability Measurement

After incubation, cell culture inserts are dried carefully to remove excess medium. The cell culture inserts are transferred into a 24-wells plate prefilled with 0.3 mL MTT-medium (1.0 mg/mL). The tissues are incubated for 180 ± 10 minutes at 37°C.

After incubation with MTT-medium the tissues are placed on blotting paper to dry the tissues and then transferred to a pre-labeled 6-well plate containing 2 mL isopropanol in each well so that no isopropanol is flowing into the insert. Formazan will be extracted with 2 mL isopropanol for 2 - 3 hours at room temperature with gentle shaking or refrigerated overnight in the dark.

The amount of extracted formazan will be determined spectrophotometrically at 570 nm in duplicate with the TECAN Infinite® M200 Pro Plate Reader.

9. ACCEPTABILITY CRITERIA

The in vitro eye irritation test is considered acceptable if it meets the following criteria:

a) The absolute mean OD_{570} of the two tissues of the negative control should reasonably be > 0.8 and < 2.5.

b) The mean relative tissue viability of the positive control should be <50% relative to the negative control.

c) The difference between the % tissue viabilities of the two identically treated replicates should be <20.

In case of color interference and/or MTT interacting test materials:

a) The $\%NSC_{living}$ should be ≤ 50% relative to the negative control OD.

b) The non-specific MTT reduction should be ≤ 50% relative to the negative control OD.

Actual values will be reported and evaluated by the Study Director in the final report. If (one of) the acceptability criteria are not met and the Study Director decides that this has a critical effect on the outcome of study, the test will be rejected and repeated.

10. INTERPRETATION

The test chemical is identified as not requiring classification and labelling according to UN GHS (No Category) if the mean percent tissue viability after exposure and post-exposure incubation is more than (>) 60%. In this case no further testing in other test methods is required.

The test chemical is identified as “no prediction can be made” if the mean percent tissue viability after exposure and postexposure incubation is less than or equal (≤) to 60%.

11. ANALYSIS

11.1. Calculation of Cell Viability

Optical Density readings will be transferred into Microsoft Excel to allow further calculations to be performed.

The corrected OD (OD_c) for each sample or control will be calculated by subtracting the value of the blank mean (OD_{bl}) from each reading (OD_{raw}).

$OD_c = OD_{raw} - OD_{bl}$

The OD value representing 100% cell viability is the average OD of the negative controls (OD_{lt_u+MTT}).

The %Viability for each sample and positive control is calculated as follows:

$\%Viability = (OD_c/\text{mean } OD_{lt\_u+MTT}) * 100$

11.2. Coloring Test Materials

Nonspecific color in living tissues (NSC_{living}) will be calculated. NSC_{living} is the mean OD of the treated living tissues without MTT reagent (OD_{lt_t-MTT}) expressed as percentage of the mean of the negative control tissues (OD_{lt_u+MTT}).

$\%NSC_{living} = [OD_{lt\_t\text{-}MTT} / OD_{lt\_u+MTT}] * 100$

True tissue viability is calculated as the difference between the OD obtained with the test material treated living tissues incubated with MTT medium (OD_{lt_t+MTT}) and the OD obtained with the test material treated living tissues incubated with medium without MTT ($OD_{lt\_t\text{-}MTT}$), and subsequently divided by the OD of the negative control (OD_{lt_u+MTT}).

$OD = OD_{lt\_t+MTT} - OD_{lt\text{-}t\text{-}MTT}$

$\%Viability = (OD / \text{mean } OD_{lt\_u+MTT}) * 100.$

In case the $\%NSC_{living} \leq 0.0$, there is no need to correct for color interference of the test material.

11.3. MTT Interacting Test Materials

Nonspecific MTT reduction (NSMTT) will be calculated. NSMTT is the difference between the mean OD of the untreated freeze-killed tissues (OD_{kt_u+MTT}) and test material treated freeze-killed tissues (OD_{kt_t+MTT}) expressed as percentage of the mean of the negative control tissues (OD_{lt_u+MTT}).

$\%NSMTT = [(OD_{kt\_t+MTT} - OD_{kt\_u+MTT})/ \text{mean } OD_{lt\_u+MTT}] * 100$

True tissue viability is calculated as the difference between the living test material treated tissues incubated with MTT medium (OD_{lt_t+MTT}) and the difference between OD_{kt_t+MTT} and OD_{kt_u+MTT}.

$OD= OD_{lt\_t+MTT} - (OD_{kt\_t+MTT}\text{-}OD_{kt\_u+MTT})$

$\%Viability = [OD/ \text{mean } OD_{lt\_u+MTT}] * 100$

In case the $\%NSMTT \leq 0.0$, there is no need to correct for interference of the test material.

11.4. Coloring and MTT Interacting Test Materials

Nonspecific color in freeze-killed tissues (NSC_{killed}) will be calculated. NSC_{killed} is the mean OD of the test material treated killed tissues without MTT reagent ($OD_{kt\_t\text{-}MTT}$) expressed as percentage of the mean of the negative control tissues (OD_{lt_u+MTT}).

$\%NSC_{killed} = (OD_{kt\_t\text{-}MTT}) / OD_{lt\_u+MTT}) * 100$

True tissue viability is calculated as the OD obtained with the test material treated living tissues incubated with MTT medium (OD_{lt_t+MTT}), minus the OD obtained with the test material treated living tissues incubated with medium without MTT ($OD_{lt\_t\text{-}MTT}$), minus the difference between the mean OD of the untreated freeze-killed tissues (OD_{kt_u+MTT}) and test material treated freeze-killed tissues (OD_{kt_t+MTT}) plus the mean OD of the test material treated killed tissues without MTT reagent ($OD_{kt\_t\text{-}MTT}$), subsequently divided by the OD of the negative control (OD_{lt_u+MTT}).

$OD = OD_{lt\_t+MTT} - OD_{lt\_t\text{-}MTT} - (OD_{kt\_t+MTT}\text{-}OD_{kt\_u+MTT}) + OD_{kt\_t\text{-}MTT}$

$\%Viability = (OD / OD_{lt\_u+MTT}) * 100.$

12. COMPUTERIZED SYSTEMS

The following computerized systems may be used in the study. The actual computerized systems used will be specified in the Final Report.

Computerized Systems

System Name	Description of Data Collected and/or Analyzed
M-Files®	Reporting and collection of 21 CFR Part 11 compliant signature
REES Centron	Temperature and humidity (laboratory facilities) Data collection
Magellan Tracker	Optical Density Measurement

Data for parameters not required by Study Plan, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by Study Plan and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

13. REGULATORY COMPLIANCE

The study will be performed in accordance with the OECD Principles of Good Laboratory Practice as accepted by Regulatory Authorities throughout the European Union, United States of America (FDA and EPA), Japan (MHLW, MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

14. QUALITY ASSURANCE

14.1. Test Facility

The Test Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the Study Plan, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

15. AMENDMENTS AND DEVIATIONS

Changes to the approved Study Plan shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary Study Plan changes in advance with the Sponsor. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

16. RETENTION AND DISPOSITION OF RECORDS

All applicable study-specific raw data, electronic data, documentation, Study plan and Final Report will be archived at finalization of the report. All materials generated by Charles River from this study will be transferred to a Charles River archive. At least 2 years after issue of the Final Report, the Sponsor will be contacted.

Records to be maintained will include, but will not be limited to, documentation and data for the following:

- Study plan, Study Plan amendments, and deviations
- Study schedule
- Study-related correspondence
- Test system receipt
- Test material receipt, identification and preparation
- Measurements and observations

17. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft Report. The Final Report will be provided in Adobe Acrobat PDF format (hyperlinked and searchable). The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Test Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation.

Reports should be finalized within 6 months of issue of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft issue, the report will be finalized by the Test Facility unless other arrangements are made by the Sponsor.

18. JUSTIFICATIONS AND GUIDELINES

18.1. Guidelines for Study

The design of this study was in compliance with the study objective, the overall product development strategy for the Test Material, and the following study design guideline:

- OECD Guideline 492: *Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*, (Adopted June 18, 2019).

19. REFERENCES

1. Test Submission Template (TST) for ECVAM submissions: Ocular Irritation Assay for Chemicals Using the EpiOcular™ Human Cell Construct. Revised submission September 11 (2008).
2. Ocular Irritation REACH Protocol (DRAFT), MatTek Corporation (workbook).
3. M.V. Berridge, A.S. Tan, K.D. McCoy, R. Wang. The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. Biochemica 4, 14-19 (1996).
4. J.V. Jester, et al.. Extent of initial corneal injury as a basis for alternative eye irritation tests. Toxicology In Vitro 15, 115-30 (2001).

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8. L. Scott, et al.. A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom up and Top-down approaches. Toxicology in vitro 24, 1-9 (2010).

9. Y. Kaluzhny, et al.. Development of the EpiOcularTM Eye Irritation Test (EpiOcular-EIT) for Hazard Identification and Labeling of Eye Irritating Chemicals in Response to the Requirements of the Cosmetics Directive and REACH Legislation. ATLA 39, 1-26, (2011).

10. S.N. Kolle, H. Kandarova, B. Wareing, B. van Ravenzwaay and R. Landsiedel. In-house validation of the EpiOcular™ eye irritation test and its combination with the bovine corneal opacity and permeability test for the assessment of ocular irritation. ATLA 39, 365–387 (2011).

11. U. Pfannenbecker, et al.. Cosmetics Europe multi-laboratory pre-validation of the EpiOcular™ reconstituted human tissue test method for the prediction of eye irritation. Toxicol In Vitro 27, 619-626 (2013).

TEST FACILITY APPROVAL

All electronic signatures appear at the end of the document upon finalization.

SPONSOR APPROVAL

The Study Plan was approved by the Sponsor by e-mail on the date designated below. The correspondence giving approval will be archived, as appropriate with other Sponsor communications.

18 Jan 2023
Date of Sponsor Approval

ATTACHMENT A

Distribution List

Electronic copies will be supplied unless otherwise specified below.

Version	Recipient
Original	Study Director
1 Copy	Sponsor Representative / Study Monitor
1 Copy	QAU / Management

SIGNATURE(S) FOR DOCUMENT: 20418214 - 500475 20418214 Wageningen University Epiocular Final Study Plan

TFM Approval-GLP:	I approve the Study Director identified in this document and management's responsibility to the study as defined by the relevant GLP.
Name:	**Westerink, Walter**
Westerink, Walter	30-Jan-2023 10:58:48 (UTC+00:00)
Electronically Signed in M-Files	Timestamp

Study Director Approval:	I approve this document.
Name:	**Kallouchi, Maysae**
Kallouchi, Maysae	30-Jan-2023 11:34:08 (UTC+00:00)
Electronically Signed in M-Files	Timestamp

Appendix 2
Technical Data, Safety Sheet and Certificate of Analysis of the Reconstructed Human EpiOcular™ Model

MATTEK
A BICO COMPANY

Certificate of Analysis

Product: EpiOcular™ Tissue

Lot Number: **38504**

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

Description: Reconstructed skin tissue 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 test method given. The product resulted in "no detection" for the following potential biological contaminants determined by the stated test method:

Keratinocytes:

HIV-1 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]	1.511 ± 0.147	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]	16.22 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: **IS**
Date: **1/31/23**

Nelson Rivas
Quality Assurance Department
Document Control Manager

January 31, 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 eyeware and appropriate disposal procedures is strongly recommended.

MatTek Corporation www.mattek.com support@mattek.com
MatTek Headquarters 200 Homer Avenue, Ashland, MA - USA +1-508-881-6771
MatTek Europe Mlynské Nivy 73, Bratislava - Slovakia +421-2-3260-7401

Dead-tissue

Certificate of Analysis

MatTek CORPORATION

Product: EpiOcular™ Tissue

Lot Number: 31797

Part#: OCL-200, OCL-212
Description: Reconstructed skin tissue 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 test method given. The product resulted in "no detection" for the following potential biological contaminants determined by the stated test method:

Keratinocytes:

HIV-1 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]	1.49 ± 0.057	**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]	20.43 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: TT
Date: 7/21/21

Nelson Rivas
Quality Assurance Associate

July 21, 2021
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 eyeware and appropriate disposal procedures is strongly recommended.

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200 Homer Avenue, Ashland, MA - USA
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information@mattek.com

Certificate of Analysis

MatTek IN VITRO LIFE SCIENCE LABORATORIES

Product: EpiOcular™ Tissue

Lot Number: 28095

Part#: OCL-200, OCL-212
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, were performed according to the test method given. The product resulted in "no detection" for the following potential biological contaminants determined by the stated test method:

Keratinocytes:

HIV-1 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 and quality

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]	1.567 ± 0.021	**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]	21.63 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: MP
Date: 9/15/21

Nelson Rivas
Quality Assurance Associate

September 15, 2021
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 eyeware and appropriate disposal procedures are strongly recommended.

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SIGNATURE(S) FOR DOCUMENT: 20418214 Genetic Toxicology Final Report 500475 Wageningen Food & Biobased Research EpiOcular

QA Approval: I approve the Quality Assurance Statement for this report.

Name: **Sonnenberg-Menkveld, Annemarije**

Sonnenberg-Menkveld, Annemarije

27-Sep-2023 14:18:46 (UTC+00:00)

Electronically Signed in M-Files Timestamp

Study Director Approval: I approve this Report.

Name: **Kallouchi, Maysae**

Kallouchi, Maysae

27-Sep-2023 20:20:31 (UTC+00:00)

Electronically Signed in M-Files Timestamp

To explore the potential of nature to improve the quality of life

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Report 2458

The mission of Wageningen University and Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 5,000 employees and 10,000 students, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.