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Protocol

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

For the prediction of acute ocular irritation of chemicals:

Identification of chemicals not requiring classification and labeling

for eye irritation or serious eye damage

For use with MatTek Corporation's Reconstructed Human EpiOcular™ Model

This protocol was approved as the new OECD TG 492 at the 2015 meeting of the OECD Working Group of the National Coordinators for the Test Guidelines Programme.

The updated test guideline can be found at: OECD/OCDE (2019), Test No. 492: Reconstructed human Cornea-like Epithelium (RhCE) test method for identifying chemicals not requiring classification and labeling for eye irritation or serious eye damage, Section 4, OECD Publishing, Paris. DOI: https://doi.org/10.1787/9789264242548-en

Performing the EpiOcular-EIT as outlined fulfills criteria set forth in OECD TG492

A detailed video demonstrating use of this protocol is available via the Journal of Visualized Experiments (JoVE): https://www.mattek.com/application/eye-irritation-test-oecd-492

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A. PROTOCOL OVERVIEW

The EpiOcular™ Eye Irritation Test (EIT) predicts chemicals not requiring classification and labeling for eye irritation or serious eye damage by measurement of its cytotoxic effect on the EpiOcular™ cornea-like epithelial model.

A1. Objectives and Applications

Type of Testing:
Replacement

Level of Toxicity Assessment:
Hazard

Purpose of Testing:
Classification and labeling of chemicals concerning their eye irritation potential. More specifically, this test identifies chemicals not requiring classification and labeling for eye irritation or serious eye damage in accordance with UN GHS (**1** and **2**)

Context of Use:
Regulatory purposes as a test within a test battery/integrated testing strategy (replacement of the Draize Eye Irritation Test according to Method B.5 of Annex V to Directive 67/548/EEC or OECD TG 405). Approved as new OECD TG 492.

Applicability Domain:
Full spectrum chemicals (substances and mixtures), no differentiation between cat.1 and cat.2

Additional Information:
This test method and the prediction model have been developed by MatTek Corporation and was introduced to and used in pre-validation and validation projects of the Eye Irritation Project Team of COLIPA (European Cosmetics Association) (**3-5**). The original workbook and the first SOP version were produced by the Institute for In Vitro Sciences, Inc. (IIVS) under contract to Mary Kay Inc. (**6**, **7**).

A2. Basis of the Method

In a series of studies conducted in the late 1990s the cellular basis for cornea damage induced by a wide range of chemistries (**8, 9**) were examined. These studies used confocal microscopy and traditional histology to measure the depth of injury, the cell involved and the eventual recovery (or not) of the cornea. Their conclusions provide the basis for current approaches to the development of *in vitro* assays for the prediction of eye irritation. They observed that initial depth of injury to the cornea was highly predictive of the overall degree and duration of the eye irritation (**10, 11**). Eye irritation includes more than just the corneal response. The conjunctiva and iris are also scored in the standard rabbit eye irritation assay. However, the cornea damage is central to chronic loss of visual acuity or frank blindness so changes in this tissue are of particular concern.

The EpiOcular tissue construct is a non-keratinized epithelium 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.

The ability to expose the tissue topically is essential to model the same kind of progressive injury expected in vivo. It also allows both solid and liquid test materials to be applied directly to the tissue.

In the standard *in vivo* rabbit assay, damage to the corneal epithelium is measured indirectly by changes in corneal clarity (increased opacity) as water is able to enter the hydroscopic stroma and makes it swell. The viability of the cells within the tissue construct is measured through the continued production of ATP through oxidative phosphorylation. Certain vital dyes are able to capture some of the electrons from this process and are themselves reduced (**12**). One such dye is MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide) and it is used to measure the continued redox cycling of the population of cells within each tissue construct. Viability of the tissue is determined by the amount of dye reduced within a specific period (in this case three hours) under conditions where the amount of dye is not limiting and the cells are provided with glucose to maintain metabolic activity. The relative viability of the test article-treated tissue is measured by comparing the amount of dye reduced by the treated tissue with the amount of dye reduced by the negative control-treated tissues. The oxidized dye does not absorb in the same spectral region as the reduced dye so it does not interfere with the measurements. One does need to account for possible chemical reduction of the dye by the test material and provide the appropriate controls. A pre-screen for direct reduction is used to identify such test materials.

The EpiOcular™ EIT was pre-validated in a multicenter international study at 7 different laboratories (**5**). Pre-validation studies served to optimize protocols and refine a prediction model to insure high degree of sensitivity and specificity (**4**). A prospective validation study of the in vitro EpiOcular-EIT for the detection of chemical induced serious eye damage/eye irritation was conducted by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and Cosmetics Europe - The Personal Care Association (**13**).

Based on the depth of injury model, the EpiOcular EIT is intended to differentiate those materials that are non-irritants (do not require a warning label in the European chemical classification systems) from those that require labeling as either GHS 1 or 2. That is, EpiOcular EIT identifies materials that induce no damage or damage limited to the corneal epithelium from those that damage/destroy the epithelium and continue damage into the stroma. The current assay is not intended to differentiate between GHS class 1 and GHS class 2 or R36 and R41 (degree of stromal damage) (**13**). As proposed by the ECVAM workshop of February 2005, this differentiation is left to a second portion of a testing tier (**14, 15**).

A.3 Experimental description

Endpoint & Endpoint Detection:
Relative tissue viability by photometrical measurement of formazan production by enzymatic reduction of MTT (MTT assay)

Endpoint Value:
Relative tissue viability [%]

Test System:
Reconstructed Human Cornea-like Epithelium (NHCE), EpiOcular™ (MatTek Corporation, MatTek IVLSL)

Basic Procedure:
The EpiOcular EIT is based on the depth of injury model of Maurer and Jester (**8, 9, 10**). In this assay, the test article is applied to the surface of the cornea epithelial construct for a fixed period, removed, and the tissue allowed to express the resulting damage. Liquids and solids are treated with different exposure and post-exposure incubations. Two construct tissues are used for each treatment and control group. Relative tissue viability is determined against the negative control-treated constructs by the reduction of the vital dye MTT (3-[4,5 - dimethylthiazol-2-yl] - 2,5 - diphenyltetrazolium bromide). A concurrent positive control is used with each assay (**4**).

Note: Detailed video demonstrating the protocol is available via Journal of Visualized Experiments https://www.mattek.com/application/eye-irritation-test-oecd-492 ***(21)***

A.4. Data Analysis/ Prediction Model

This prediction model is applicable to all chemicals and can be used for the classification of all classes of chemicals. For each test chemical, the mean optical density (OD) of two treated tissues is determined and expressed as relative percentage of viability of the negative control. Tissues treated with eye **irritants** (GHS class 1 or 2) have relative viability of ≤ 60.0% while treatment with **non-irritants (No Category)** show viability >60.0%.

*Note: OECD/OCDR TG No. 492 adopted the following classification in 2019: If the test article-treated tissue viability is > 60.0% relative to negative control-treated tissue viability, the test article is not requiring classification and labeling (**No Category**) and no further testing is requires. If the test article-treated tissue viability is ≤ 60.0% relative to negative control-treated tissue viability, then **no prediction can be made** for the test article and further testing is required (**13**).*

A.5. Test Compounds & Results Summary

In the method development and pre-validation, a total of 94 chemicals (20 solids and 74 liquids) of various chemical classes were tested. Among the liquids, 15 test samples were dilutions of chemicals. During the EURL ECVAM/Cosmetic Europe validation study, 113 coded test articles (53 liquids and 60 solids, substances and chemical mixtures), for which quality assured in vivo reference data (Draize eye irritation data) are available, were tested. The testing in the participating laboratories was conducted in compliance to GLP principles.

A.6. Modifications of the Method

This protocol, which discriminates only between non-irritant and irritant chemicals, is different to the time-to-toxicity protocol originally developed by Avon and also used by Colgate-Palmolive that measures the exposure time required to reduce tissue viability to 50% of the control treated tissue, which allows discrimination between irritation sub-classes depending on the specific prediction model used (**16, 17**).

A7. Discussion

The Draize rabbit eye irritation test (**18, 19**) has been the globally accepted regulatory method for assessing eye irritation potential of chemicals. This test has been criticized for animal welfare reasons since many years. These discussions have led to a ban of *in vivo* eye irritation tests using animals on ingredients for cosmetic products within the European Union after March 2009.

The EpiOcular EIT can be used to differentiate irritant chemicals which have to be classified as R36 or R41 according to the former EU classification based on the Dangerous Substances Directive (DSD) or as Cat 1 or Cat 2 according to UN GHS classification and the new EU classification based on the Classification, Labeling and Packaging (CLP) Regulation (implementing UN GHS in the EU) from those chemicals which have not to be classified. Together with an adjunct test which can differentiate between R36 vs. R41 or GHS/CLP Cat 1 vs. GHS/CLP Cat 2 (like the validated BCOP and ICE tests) this EpiOcular EIT can be used for the classification of chemicals without using animals (**14, 20**).

To perform this assay, no special equipment besides usual cell culture equipment is needed.

To establish the method in a naïve laboratory, training at an experienced laboratory of two days is recommended. Also, testing of a set of reference chemicals is advisable (**13**).

For the testing of 10 chemicals, one EpiOcular™ EIT kit with 24 tissues is required.

Note: Deviations from this SOP may cause different outcomes.

A.8.Status

This procedure was validated by EURL-ECVAM and Cosmetics Europe and adopted by the OECD in 2015 (Test Guideline 492) to be used for identifying chemicals not requiring classification and labeling for serious eye damage/eye irritation according to UN GHS and contribute as part of a testing strategy to the full replacement of the in vivo Draize eye irritation test.

A.9. Health & Safety Issues

Although the cells used for the EpiOcular™ tissues are screened and are negative for HIV, hepatitis B and hepatitis C the tissues should be handled at biosafety level 2. It is therefore recommended to wear gloves during all experiments with the tissues and all kit components. All test chemicals should be handled according to the recommendations in the material safety data sheets. Unknown or coded test materials with no risk and safety information provided must be handled as if they were irritating and toxic. Work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear gloves, protect eyes and face).

The chemicals should be stored according to the recommendations.

Due to the long post-incubation period at least for solids, it is necessary to perform the test under aseptic conditions in the microbiologically safety cabinet (laminar flow hood).

A.10. Contacts

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B. TECHNICAL DESCRIPTION

B.1. Cell / Test system

The EpiOcular™ human cell construct (MatTek Corporation, MatTek IVLSL) is used in the assay. The use of EpiOcular™ cultures offers features appropriate to model ocular irritation: (1) the model is composed of stratified human keratinocytes in a three-dimensional structure, and (2) test materials can be applied topically to the model so that water insoluble materials may be tested. The toxicity of the test article (and thus the ocular irritation potential) is evaluated by the relative viability of the treated tissues relative to the negative control-treated tissues. Viability is determined by the MTT assay. Data are presented in the form of relative survival (relative MTT conversion).

The protocol was designed by MatTek Corporation for testing 10 test articles plus one negative and one positive control article in one kit of EpiOcular (24 OCL-200 tissues). A prediction model was developed by *MatTek* Corporation to determine the *ocular irritation classification* as follows:

> *If the test article-treated tissue viability is > 60.0% relative to negative control-treated tissue viability, the test article is labeled* ***non-irritant.***
>
> *If the test article-treated tissue viability is ≤ 60.0% relative to negative control-treated tissue viability, the test article is labeled* ***irritan****t.*

OECD/OCDE TG No. 492 adopted the following classification in 2019:

> *If the test article-treated tissue viability is > 60.0% relative to negative control-treated tissue viability, the test article does not require classification and labeling (****No Category****) and no further testing is required.*
>
> *If the test article-treated tissue viability is ≤ 60.0% relative to negative control-treated tissue viability,* ***no prediction can be made*** *for the test article (further testing is required).*

NOTE: *This protocol defines two specific exposure and post-exposure procedures, depending on the physical nature of the test article.*

Protocol for Liquid Test Articles: Liquid test articles are tested by applying 50 µL of test article topically on cultures for 30 minutes, followed by a 12-minute post-treatment immersion, and a 120-minute post-treatment incubation, prior to the MTT endpoint.

Protocol for Solid Test Articles: Solid test articles are tested by applying a leveled spoonful (designed to hold approximately 50 mg) of test article topically on cultures for 6 hours, followed by a 25-minute post-treatment immersion, and 18-hour post-treatment incubation, prior to the MTT endpoint.

A separate Method Documentation Sheet (MDS) is suggested for recording the materials and procedures used to perform this study.

B.2 Materials Provided in OCL-200-EIT kit

B.2.1 EpiOcular™ EIT Kit Components

EpiOcular-EIT kits are shipped from Boston and Bratislava each Monday. Upon receipt of the EpiOcular tissues follow directions as indicated in B.5.4.

B.2.1.1 Standard Assay Kit Components (part # OCL-200-EIT)

Amount	Reagent	Conditions	Source	Description	Shelf Life
1	Sealed 24-well plate of EpiOcular™ tissues (OCL-200)	2-8°C	MatTek	Contains 24 tissues of cell culture inserts, package on agarose	96 hours
1 bottle, 200 mL	EpiOcular™ Assay Medium (OCL-200-ASY)	2-8ºC	MatTek	DMEM based medium	3 weeks
1 bottle, 100 mL	$Ca^{++}Mg^{++}$-Free D-PBS	RT	Sigma-Aldrich, D5652, or equiv.	Used for rinsing inserts	1 year
4	6-Well Plates	RT	Falcon	Used for maintaining tissues during assay protocol	NA
2	12-Well Plates	RT	Falcon	Used during assay protocol	NA
2	24-Well Plates	RT	Falcon	Used to perform MTT assay	NA
1 vial, 0.5 mL	Methyl Acetate (CAS#79-20-9)	RT	Sigma-Aldrich, Cat# 186325	Used as PC in the assay	1 month

B.2.1.2 MTT-100 Assay Kit Components (MTT-100 must be ordered separately)

Amount	Reagent	Conditions	Source	Description	Shelf Life
1 vial, 2 mL	MTT Concentrate (MTT-100-CON)	Protected from light (-20ºC)	MatTek	Frozen MTT concentrate	2 months
1 vial, 8 mL	MTT diluent	2-8ºC	MatTek	For diluting MTT concentrate prior to use in the MTT assay	2 months
1 bottle, 60 mL	Isopropanol (CAS #67-63-0)	RT	Sigma-Aldrich	extractant solution	NA

The individual shelf life of the reagents has to be respected.

B.3. Equipment and materials (not provided with OCL-200-EIT kit)

Equipment/ Material	Needed for
Humidified incubator (37±1°C, 5±1% CO_2, 90±10% humidity)	Incubating tissues prior to and during assays
Laminar flow hood	Safe work under sterile conditions
Vacuum pump (optional)	Aspirating medium and solutions
Plate-reader photometer (for 96-well plates)	Reading OD
Plate shaker	Extraction of formazan
Sterile, blunt-edged forceps	Handling tissue inserts
Stop-watches	Timing of application of test materials and other timed steps in the protocol
Water bath (37°±1)	Warming media and MTT solution
Mortar and pestle	Grinding granular solids
Positive displacement pipette (50 µL)	Application of viscous and semi-solid materials and suspensions
Adjustable pipettes (200 µL – 2 mL)	Application of liquid materials, assay medium and MTT
Pre-sterilized tips (200 µL and 20 µL), Rainin Cat#HR-200F and HR-20F (or equivalent)	Application of liquid materials, assay medium and MTT
Wide orifice pre-sterilized tips (250 µL), Rainin Cat#HR-250WS (or equivalent)	Application of viscous and semi-solid materials and suspensions
8 oz/220 mL specimen containers, Falcon Cat# 3540200 (or equivalent)	Rinsing tissues
Sterile single-use syringes (e.g. 1 mL tuberkulin syringe Omnifix-F, B. Braun Melsungen AG, cat. No. 9161406V)	Delivery of ~50 mg solid materials (optional)
Ted Pella micro spatula/spoon, Ted Pella Inc., Cat# 13504 (or equivalent, sharp spoon or bone curette, e.g. Aesculap, Part #: FK 623R)	Delivery of ~50 mg solid materials
Ca^{++} and Mg^{++} free Dulbecco's phosphate buffered saline ($Ca^{++}Mg^{++}$Free-DPBS): Sigma-Aldrich, Cat# D5652 (or equivalent)	Rinsing tissues during assay
Sterile deionized water, tissue culture grade (quality biological or equivalent)	Use as NC
96-well flat bottom plates, Falcon (or equivalent)	For reading OD
Cotton tip swaps (sterile)	For drying the tissue surface (optional)
Adhesive tape or Parafilm	Covering plates during formazan extraction
MTT-100 assay kit – MUST BE ORDERED SEPARATELY	Contains MTT-Thiazolyl Blue Tetrazolium Bromide reagent (Sigma #M-5655) and isopropanol extract.

B.4. Preparations for the test
B.4.1 Preparation of MTT Solution

MTT solution and MTT diluent together with the extractant solution are ordered as MTT test kit (MTT-100, MatTek). For one OCL-200-EIT kit (24 tissues) one MTT-100 kit is needed.

Thaw the MTT concentrate (MTT-100-CON) and dilute the concentrate with the MTT diluent (MTT-100-DIL). Once diluted, store the MTT solution in the dark at 5±3°C for use on the same day (do not store overnight since MTT will degrade with time). After the MTT solution is pipetted into 24 well plates, it should not be stored longer than one hour at 37°C.

If the MTT-100 is not used, proceed according to the following example: Per 24 tissues, dissolve 12.5 mg MTT (e.g. Sigma # M5655) in 2.5 mL PBS and thoroughly mix this stock-solution. After filtration (using a sterile 0.45 µm filter), add 2 mL of the stock-solution to 8 mL MTT-Assay Medium (final concentration: 1 mg MTT / mL medium). Keep the MTT medium in the dark.

Safety precaution: MTT is toxic (Risk phrases: H315, H319, H335, H341). Wear protective gloves during manipulation with MTT and its solutions!

B.4.2 Phosphate Buffered Saline (PBS)

If powdered salts are used, prepare the PBS solution (without Ca^{++} and Mg^{++}) according to the preparation instructions of the supplier. Before use, the PBS solution should be sterilized by autoclaving or sterile filtration.

B.4.3 Test Compounds

Test chemicals are administered neat by topical application onto the construct.
Testing of dilutions is also possible and can be addressed.

B.4.4 Positive Control

Neat Methyl Acetate (CAS#79-20-9) is used as positive control (PC).

B.4.5 Negative Control

Sterile deionized water is used as negative control (NC).

B.4.6 Microtiter plates

Prior to use, each plate (6, 12, and 24-well) and its cover should be uniquely identified in permanent marker by a plate number, the test article number, and the test phase.

B.5. METHOD
B.5.1 Test System Procurement

The EpiOcular™ tissues must be ordered from MatTek 2-3 weeks prior to scheduled test. Upon receipt of the EpiOcular EIT test kit; the solutions should be stored as indicated by the manufacturer. The tissues are stored at 2-8ºC until use.

B.5.2 Assessment of Direct Test Article Reduction by MTT

Test articles may have the ability to directly reduce MTT and to form a blue/purple reaction product which could have an impact on the quantitative MTT measurement. Therefore, it is necessary to assess this ability for each

test article prior to conducting any assays with viable tissues. For this purpose, a 1 mg/mL MTT solution (in DMEM) is prepared as described in Section B.4.1. 50 µL (liquid test articles, see Section B.5.6.1.) or approximately 50 mg (solid test articles, see Section B.5.6.2.) is added to 1 mL of the MTT solution in a 6-well plate and the mixture is incubated in the dark at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air (standard culture conditions, SCC) for approximately three hours. A negative control (50 µL of sterile deionized water) should be run concurrently. If the MTT solution colour turns blue/purple, the test article is presumed to have reduced the MTT. Water insoluble test materials may show direct reduction (darkening) only at the interface between the test article and the medium.

In cases where the test article is shown to reduce MTT, a functional check using freeze-killed tissue controls (killed controls = KC) should be performed in at least one definitive assay to evaluate whether the test material is not binding to the tissue and leading to a false MTT reduction signal (see Section B.5.8 Method, Killed Controls for Assessment of Residual Test Article Reduction of MTT). Blue, dark purple and black test articles should also be tested on killed controls because it may not be possible to assess their potential to directly reduce MTT.

It is recommended that the results are documented by taking photos.

B.5.3 Assessment of Colored or Staining Materials

Colored test articles or test articles which become colored after application to the tissues may interfere with the quantitative photometric MTT measurement if the colorant binds to the tissue and is extracted together with MTT. Therefore, each test article must be checked for its colorant properties.

In this context, chemicals which absorb light and appear red, yellow, green, and blue should be considered as intrinsic colorants. Also test articles which look black may absorb light and should be considered as colorants. Blue, dark purple and black test articles may be directly tested on colorant controls without further tests because it is obvious that they can interfere with the blue MTT product. Such test articles should also be tested on killed controls because it may not be possible to assess their potential to directly reduce MTT.

a) All intrinsically colored test articles (e.g. red, yellow, green colorants) have to be tested for their ability to significantly absorb light at the wavelength used for the MTT determination. The maximum amount of each test article (50 µL for liquids and approximately 50 mg for solids) is added to 2 mL of isopropanol (the same amount as used for MTT extraction), incubated in 6-well plates, and placed on an orbital plate shaker for 2 to 3 hours at room temperature. Two 200 µL aliquots of isopropanol solutions and of pure isopropanol are transferred to a 96-well plate and the absorbance is measured with a plate reader at the MTT measurement wavelength. Materials not completely soluble in isopropanol should be centrifuged if necessary (e.g. in 1 mL Eppendorf tubes for 15-30 s at 16,000 g) and 200 µL aliquots of the supernatant should be used for measurement. After subtraction of the OD for isopropanol, if the OD of the test article solution is > 0.08 (approximately 5% of the mean viability of the negative control) the material has to be considered as possibly interacting with the MTT measurements and an additional test on colorant controls has to be performed (see Section B.5.9.).

b) For non-colored test articles additional tests have to be performed to assess if they become colorants after contact with water or isopropanol. For this purpose, 50 µL (liquid test articles, see B.5.6.1.) or approximately 50 mg (solid test articles, see Section B.5.6.2.) of each test article is added to 1.0 mL of water in a 6-well plate and the mixture is incubated in the dark at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air (standard culture conditions) for at least one hour. Furthermore, approximately 50 µL of liquids or 50 mg of solids is added to 2 mL isopropanol and are incubated in 6-well plates for 2 to 3 hours at room temperature.

If the test article becomes colored either in water or isopropanol, it has to be considered as possibly interacting with the MTT measurement and an additional test on colorant controls has to be performed (see Section B.5.9.).

B.5.4 Preparation of EpiOcular Tissues for Treatment

1) On the day of receipt, normally on Tuesday, the tissues should be equilibrated (in its 24-well shipping container) to room temperature for about 15 minutes.

2) An appropriate volume of EpiOcular™ Assay Medium should be equilibrated to room temperature (20-25°C). One (1) mL of Assay Medium is aliquoted into the appropriate wells of pre-labeled 6-well plates. The 6-well plates should be labeled with the test article or control codes and exposure times.

3) Each 24-well shipping container is removed from its plastic bag under sterile conditions and its surface disinfected by wiping with 70% isopropanol- or ethanol-soaked tissue paper. The sterile gauze is removed (see Fig. 1, left) and each tissue should be inspected for air bubbles between the agarose gel and insert (see Fig. 1, right). Cultures with air bubbles under the insert covering greater than 50% of the insert area should not be used. Any unusual observation should be noted separately. The tissues should be carefully removed from the 24-well shipping containers using sterile forceps. Any agarose adhering to the inserts should be removed by gentle blotting on sterile filter paper or gauze. The insert is then transferred aseptically into the 6-well plates and pre-incubated at standard culture conditions for one hour in the Assay Medium. After one hour, the Assay Medium should be replaced by 1 mL of fresh Assay Medium at 37°C and the EpiOcular™ tissues should be incubated at standard culture conditions overnight (16 - 24 hours).

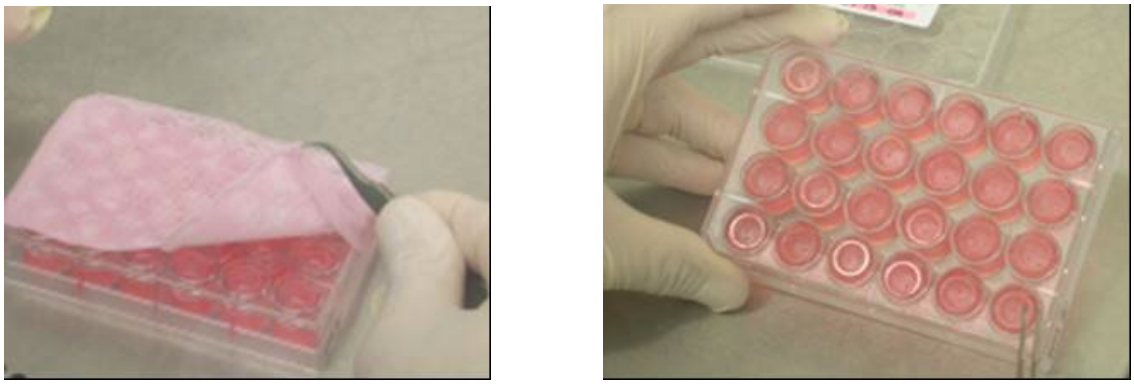

Figure 1. Removal of the gauze and visual inspection of the EpiOcular tissues.

Alternatively, if the tissues arrive in the laboratory late Tuesday afternoon so that it is not possible to perform the 1-hour pre-incubation, the EpiOcular™ tissues should be incubated at standard culture conditions overnight (16 - 24 hours) without medium exchange.

In case the delivery of the tissues is delayed until Wednesday morning, the transfer of the EpiOcular™ tissues, followed by the same procedure with pre-incubation as described above for normal receipt, should be started at an appropriate time so that the experiments can begin 16 - 24 hours later on Thursday.

The time of receipt and the pre-incubation procedure should be documented in the Method Documentation Sheet.

B.5.5 Controls

The **negative control** (NC) is sterile deionized water. For the liquid protocol, 50 µL of the negative control is applied to the tissues exactly as for the liquid test articles for a 30 ± 2-minute exposure. For the solid protocol, 50 µL of the negative control is applied to the tissues (as described for the liquid test articles) for a 6 hr ± 15-minute exposure.

The **positive control** (PC) is methyl acetate. For the liquid protocol, 50 µL of the positive control is applied to the tissues exactly as for the liquid test articles for a 30 ± 2-minute exposure. For the solid protocol, 50 µL of the positive control is applied to the tissues (as described for the liquid test articles) for a 6 hr ± 15-minute exposure.

Both, negative and positive controls are tested concurrently with the test articles in every run performed by a single technician.

B.5.6 Test Material Exposure Procedures

Each test article and control is tested by treating two tissues (n=2). The test article dosing procedure is different for liquids and solids, as described later. Generally, the negative control and positive control is dosed first, followed by the test articles.

Liquids are fluid, pipetteable substances, such as liquids, gels, and creams.
Solids are non-pipetteable substances like powders, resinous or waxy materials etc.

If the physical state of test articles is not easy to determine, follow these considerations: Test chemicals that can be pipetted at 37°C or lower temperatures (using a positive displacement pipette, if needed) should be treated as liquids. Viscous, waxy, resinous and gel-like test chemicals with unclear physical state should be incubated at 37±1°C for 15±1 minutes before deciding which treatment protocol to use. If such test chemicals become pipettable after this incubation period, they should be treated as liquids and should be applied to the tissues directly from the water bath (at 37±1°C), otherwise they should be treated as solids.

B.5.6.1 Testing of liquid test articles

1) **Pre-Treatment:** After the overnight incubation, the tissues are pre-wetted with 20 µL of $Ca^{++}Mg^{++}$Free-DPBS. If the $Ca^{++}Mg^{++}$Free-DPBS does not spread across the tissues, the plate may be tapped to assure that the entire tissue surface is wetted. The tissues are incubated at standard culture conditions for 30 ± 2 minutes.
2) ***Test article exposure:*** After the 30 ± 2 minute $Ca^{++}Mg^{++}$Free-DPBS pre-treatment, each liquid test and control article is tested by applying **50 µL** topically on the EpiOcular™ tissues. The tissues are incubated at standard culture conditions for **30 ± 2 minutes**.

Dosing details: 50 µL of the test article is applied directly on the tissue to cover the upper surface (see Fig. 2, left). The narrow points of the pipette tips may be cut off to widen the orifice for viscous materials. If positive displacement pipettes (see Fig. 2, right) are used for particularly viscous materials, the test article may first be transferred to a syringe to aid in filling the pipette. The pipette tip of the positive displacement pipette should be inserted into the dispensing tip of the syringe so that the material can be loaded into the displacement tip under pressure. Simultaneously, the syringe plunger is depressed as the pipette piston is drawn upwards. If air bubbles appear in the pipette tip, the test article should be removed (expelled) and the process repeated until the tip is filled without air bubbles. For very viscous liquids a dosing device (a flat headed cylinder of slightly less diameter than the inner diameter of the tissue insert or a plastic pushpin) may be placed over the test article to assure even spreading, if required.

Figure 2: Application of normal liquids with a standard pipette (left) and viscous liquids with a positive displacement pipette (right).

3) **Rinsing:** At the end of the 30 ± 2 minutes treatment time, the test articles are removed by extensively rinsing the tissues with $Ca^{++}Mg^{++}$-free D-PBS (brought to room temperature), as described in detail below. If a dosing device is used, it should be removed and discarded. Three clean beakers (glass or plastic with minimal 150 mL capacity), containing a minimum of 100 mL each of $Ca^{++}Mg^{++}$-free D-PBS should be used per test article (8 oz/220 mL disposable specimen containers from Falcon, Cat# 354020 are suggested). Each test article utilizes a different set of three beakers. The inserts containing the tissue be lifted out of the medium by grasping the upper edge of the plastic "collar" with fine forceps. Use of curved forceps facilitates handling and decanting. To assure throughput, the tissues should be rinsed two at a time by holding replicate inserts together by their collars using forceps. Be careful not to damage the tissues by the forceps. The test or control articles should be decanted from the tissue surface onto a clean absorbent material (paper towel, gauze, etc.) and the cultures dipped into the first beaker of D-PBS, swirled in a circular motion in the liquid for approximately 2 seconds (see Fig. 3, left), lifted out so that the inserts are mostly filled with D-PBS, and the liquid will be decanted back into the container. This process should be performed two additional times in the first beaker. The culture should then be rinsed in the second and third beakers of D-PBS three times each in the same fashion. Finally, any remaining liquid should be decanted onto the absorbent material. Decanting is most efficiently performed by rotating the insert to approximately a 45° angle (open end down, see Fig. 3, right) and touching the upper lip to the absorbent material (to break the surface tension).

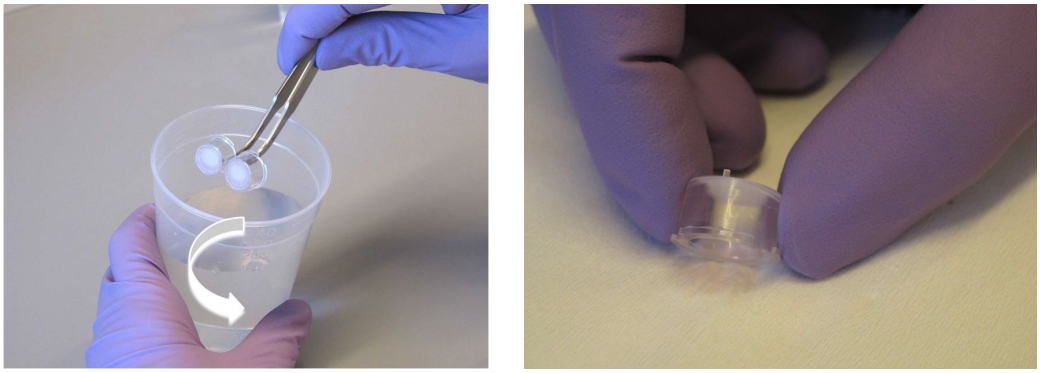

Figure 3: Rinsing of two inserts (left) and decanting (right).

Note: If it is not possible to remove all of the visible test material, this should be noted in the notebook and under remarks in the spreadsheet. No further rinsing should be done. It is recommended to document any rinsing problems by taking photos, if possible.

4) **Post-Soak:** After rinsing, the tissues are immediately transferred to and immersed in 5 mL of previously warmed Assay Medium (room temperature) in a pre-labeled 12-well plate for a **12 ± 2-minute** immersion incubation (Post-Soak) at room temperature. This incubation in Assay Medium is intended to remove any test article absorbed into the tissue.

5) **Post-incubation:** At the end of the Post-Soak immersion, each insert is removed from the Assay Medium, the medium is decanted off the tissue, and the insert is blotted on absorbent material, and transferred to the appropriate well of the pre-labeled 6-well plate containing 1 mL of warm Assay Medium. The tissues are incubated for **120 ± 15 minutes** at standard culture conditions (Post-treatment Incubation).

B.5.6.2 Testing of solid test articles

1) **Pre-Treatment:** After the overnight incubation, the tissues are pre-wetted with 20 µL of $Ca^{++}Mg^{++}$Free-DPBS. If the $Ca^{++}Mg^{++}$Free-DPBS does not spread across the tissues, the plate may be tapped to assure that the entire tissue surface is wetted. The tissues should be incubated at standard culture conditions for 30 ± 2 minutes.

2) **Test article exposure:** After the 30 ± 2-minute $Ca^{++}Mg^{++}$Free-DPBS pre-treatment, the negative and positive controls are tested by applying **50 µL** topically on the EpiOcular™ tissues. Each solid test article is tested by applying one leveled spoonful of the test article or by the means of an alternative tool (approximately 50 mg)

topically on the EpiOcular™ tissues. To avoid that test article is spilled into the medium under the tissue inserts, the tissue insert should be removed from the medium, placed onto a sterile surface (e.g. the lid of a microtiter plate) and dosed by pouring the solid test article onto the tissue surface so that the surface of the tissue is completely covered by the test article. The tissue should be placed back into the culture medium after dosing and incubated at standard culture conditions for **6 hr ± 15 minutes**. If the outer wall of the insert is contaminated by e.g. powders it may be necessary to wipe the particles off with cellulose tissue. Crystalline powders should be ground with a mortar and pestle to guarantee better contact to the tissue, if needed. Powders can be placed directly onto the culture at approximately 50 mg / culture by the means of a 1 mL syringe with head cut off (see Fig. 4, left). Powders are stuffed in the syringe when the plunger is drawn back and then applied by pressing the plunger down. With the help of the scale on the syringe, the amount of test article can be applied more reproducible after having weighed the specific chemical (see Fig. 4, right).

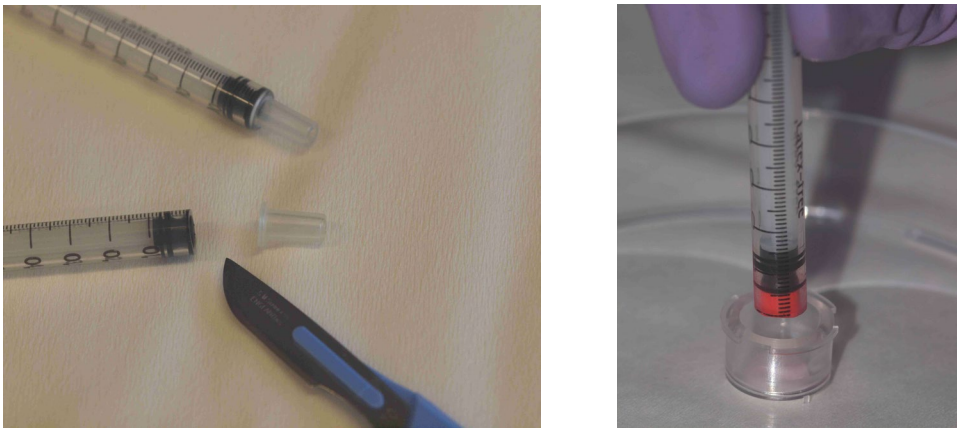

Figure 4: Syringe with head cut off (left) and application of a powder with the stuffed syringe (right).

As an alternative, powders can be placed by measuring one leveled spoonful of the test article (using a Ted Pella micro spatula/spoon, Cat# 13504, designed to measure approximately 50 mg) and applying the solids directly onto the tissue surface (see Fig. 5).

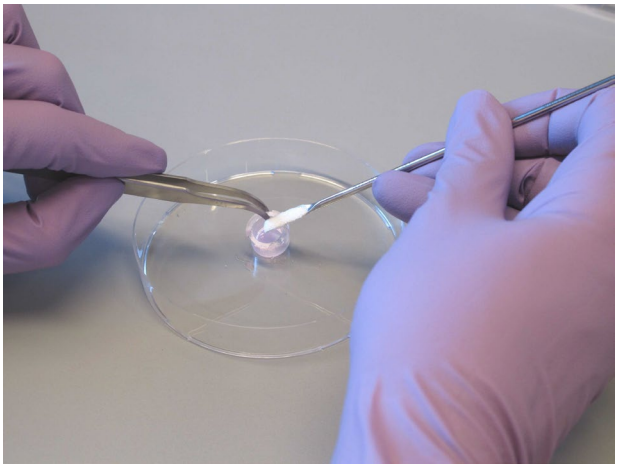

Figure 5: Application of solids with Ted Pella spatula.

For testing solids, at the time of dosing, the tissue insert will be removed from the medium, placed onto a sterile surface (e.g. the lid of a microtiter plate) and dosed by pouring the solid test article onto the tissue surface. **Shake insert gently from side to side to ensure that tissue is completely covered by the test article.** The tissue will

be placed back into the culture medium after dosing. The goal of this procedure is to eliminate the chance that test article is spilled into the medium under the tissue inserts.

3) **Rinsing:** At the end of the 6 hr ± 15 minutes treatment time, the test articles should be removed by extensively rinsing the tissues with $Ca^{++}Mg^{++}$-free D-PBS brought to room temperature, according to the same procedure as described in B.5.6.1 for liquid test articles.

   NOTE: *If it is not possible to remove all of the visible test material, this should be noted under remarks in the spreadsheet. It is recommended to document any rinsing problems by taking photos, if possible.*

4) **Post-Soak:** After rinsing, the tissues are 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 (Post-Soak) at room temperature. This incubation in Assay Medium is intended to remove any test article absorbed into the tissue.

5) **Post-incubation:** At the end of the Post-Soak immersion, each insert is removed from the Assay Medium, the medium is decanted off the tissue, and the insert is blotted on absorbent material and transferred to the appropriate well of the pre-labeled 6-well plate containing 1 mL of warm Assay Medium. The tissues should be incubated for **18 ± 0.25 hours** at standard culture conditions (Post-treatment Incubation).

B.5.7 MTT Assay

After the Post-treatment Incubation of 120 ± 15 minutes for liquids and 18 ± 0.25 hours for solids, respectively, the MTT assay is performed.

1) A 1.0 mg/mL MTT solution is prepared as described in B.4. “Preparations”: 300 µL of the MTT solution is added to each designated well of a pre-labeled 24-well plate.

2) At the end of the Post-treatment Incubation, each insert is removed from the 6-well plate and gently blotted on absorbent material. The tissues are placed into the 24-well plate containing 0.3 mL of MTT solution. Once all the tissues are placed into the 24-well plate, the plate is incubated for **180 ± 10 minutes** at Standard Culture Conditions.

3) **For liquids (except colorants)**, each insert is removed from the 24-well plate after 180 ± 10 minutes, the bottom of the insert is blotted on absorbent material, and then transferred to a pre-labeled 24-well plate containing 2.0 mL of isopropanol in each designated well so that isopropanol is flowing into the insert on the tissue surface. The plates are sealed with parafilm (between the plate cover and upper edge of the wells) or a standard plate sealer and are either stored overnight at 2-8°C in the dark or immediately extracted. To extract the MTT, the plates are placed on an orbital plate shaker and shaken for 2 to 3 hours at room temperature. At the end of the extraction period, the tissue is pierced and the liquid within each insert is decanted into the well from which it was taken. The extract solution is mixed and **two 200 µL aliquots** are transferred to the appropriate wells of a pre-labeled 96-well plate(s) according to the plate configuration given below (see Fig. 6).

4) **For solids and liquid colorants,** a different procedure should be used by extracting only from beneath since they might remain on the tissue and could contaminate the isopropanol extraction solution. Inserts are removed from the 24-well plate after 180 ± 10 minutes; the bottom of the insert is blotted on absorbent material, and then transferred to a pre-labeled 6-well plate containing 1 mL isopropanol in each well so that no isopropanol is flowing into the insert. The plates are handled as described above except that at the end of the extraction period, the tissues should **not** be pierced. The corresponding negative, positive, and colorant controls have to be treated identically without piercing. For this procedure it is necessary to seal the plates particularly thorough since a higher evaporation rate has to be expected due to the larger surface of wells in 6-well plates.

   At the end of the non-submerged extraction inserts and tissues are discarded without piercing and 1 mL of isopropanol is added into each well. The extract solution is mixed and **two 200 µL aliquots** are transferred to the appropriate wells of a pre-labeled 96-well plate(s) according to the plate configuration given below (see Fig. 6).

	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	

Figure 6: Standard plate configuration for viability tests

In case of turbid extract solutions, caused by insoluble solids which have passed the insert membrane and which may lead to increased OD values, the solutions should be centrifuged (e.g. in 1 mL Eppendorf tubes for 15-30 sec at 16,000 x **g** and 200 µL aliquots should be taken from the supernatant. 200 µL of isopropanol is added to the wells designated as blanks. The absorbance at 570 nm (OD_{570} or simply OD) of each well is measured with a Molecular Devices Vmax (or similar) plate reader. Since the absorption spectrum of the extracted dye builds a plateau (see Fig. 7) with the maximum at approximately 570 nm, a range of 550 to 590 nm is acceptable but should be consistent within a laboratory's data set. No reference wavelength measurement should be used.

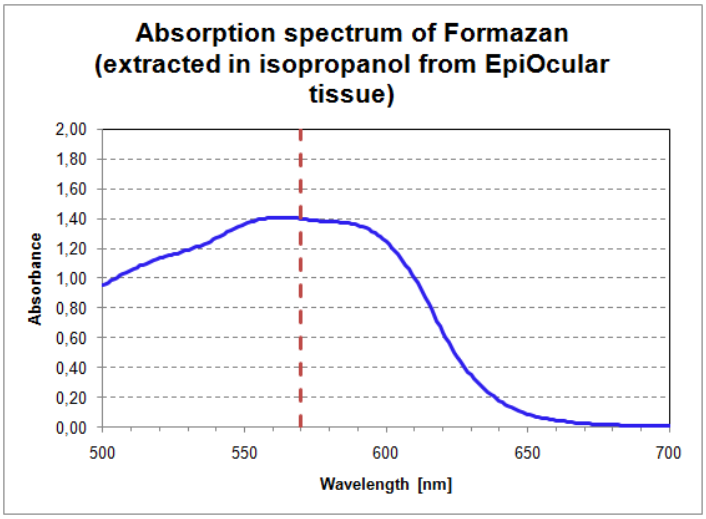

Figure 7: Absorption spectrum of Formazan

Since the absorbance value of the negative control can be already ~2.0, the additional absorbance of colorants and/or MTT reduction can lead to even higher absorbance values than 2.0. Therefore, the range of linearity of the photometer used may be exceeded. In this case the easiest way to avoid irregular viability results calculated from absorbance values above a linearity limit is to repeat the measurement of the entire experiment by measuring 100 µL aliquots (instead of 200 µL) in the same way as described above. All samples of the same test should be treated identically.

Note: *The range of linearity for the photometer used can be assessed by measuring a calibration curve of e.g. Trypan Blue solution. Prepare a 0.04% Trypan Blue solution (e.g. Trypan Blue 0.4%, Sigma T-8151, diluted 1:10 v/v with water). From this stock solution transfer different volumes from 50 µL up to 800 µL to the corresponding wells of a 24-well plate and fill these volumes up with water to 1 mL (concentration range from 50 to 800 µL/mL*

with an increment of 50 µL/mL). Shake the plate and transfer two 200 µL aliquots of each dilution to a 96-well microtiter plate. Use two 200 µL aliquots of water as blanks. Measure the absorbance of these dilutions at 570 nm with the plate reader used for the MTT assay, subtract the blank and draw a graph of the results (concentrations on the x-axis and absorbance on the y-axis). The linearity is considered as sufficient if the R^2 value of the OD measurement is > 0.999 (example see Fig. 8). It is recommended to repeat the linearity check every three months.

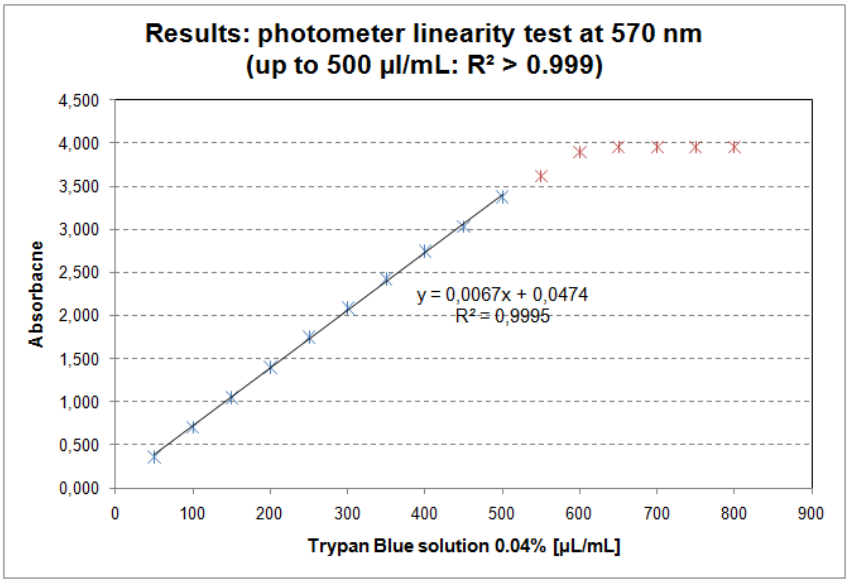

Figure 8: Example result of the photometer linearity test

B.5.8 Killed Controls for Assessment of Residual Test Article Reduction of MTT

In cases where the test article is shown to reduce MTT, only test articles that remain bound to the tissue after rinsing, resulting in a false MTT reduction signal, present a problem. To demonstrate that residual test article is not acting to directly reduce the MTT, a functional check on killed controls (KC) is performed in the definitive assay to show that the test material is not binding to the tissue and leading to a false MTT reduction signal.

To determine whether residual test article is acting to directly reduce the MTT, two freeze-killed control tissues per test article are used. Freeze killed tissues are prepared by placing untreated EpiOcular™ constructs (in a 24 well plate) in the –20°C freezer overnight, thawing to room temperature, and then refreezing (two freeze-thaw cycles). Once frozen, the tissue may be stored indefinitely in the freezer.

To test for residual test article reduction, two killed tissues are treated with the test article in the normal fashion. All assay procedures are performed as for the viable tissue. A killed control treated with sterile deionized water (negative killed control) is tested in parallel since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue.

If little or no MTT reduction is observed in the test article-treated killed control, the MTT reduction observed in the test article-treated viable tissue may be ascribed to the viable cells. If there is appreciable MTT reduction in the treated killed control (relative to the amount in the treated viable tissue), additional steps must be taken to account for the chemical reduction, or the test article may be considered un-testable in this system. The OD values of the killed controls are analyzed as described in B.7. “Data Analysis / Calculation of Results”.

- *If the direct reduction of MTT by the test article is > 60% of the viable negative control, the results obtained should be taken with caution as this is the cut-off used in EpiOcular™ EIT to distinguish classified from not classified chemicals.*

- *It may not be necessary to perform a test on killed controls for those chemicals classified as irritants due to a viability being lower than the cut-off of 60% because any MTT reduction would not change the classification for such chemicals.*
- *If the uncorrected ODs of the tissue extracts obtained with the test article fall outside of the linear range of the spectrophotometer, the test article may be considered as not testable.*

B.5.9 Colorant Controls for Assessment of Colored or Staining Test Articles

In cases where a test article has shown to have or to develop relevant color which can interact with the MTT measurement (for criteria and screening tests to be performed see Section B.5.3), an additional test has to be performed to determine the amount of color bound to and extracted from the tissues. For this purpose, each colored test article is applied to two additional tissues, the colorant controls (CC), and is treated in the same way as described for liquids and solids in Section B.5.6.1. and Section B.5.6.2., respectively. In contrast to the normal viability test, **no MTT assay** is performed. The bound color is extracted and the absorbance of the isopropanol extracts is measured identically as in the MTT assay for colored test articles (according to Section B.5.7., as described for the MTT assay with 2 mL extraction solution in 6-well plates without piercing the tissue, and beginning from step 2 but starting with a 180 ± 10 min incubation in medium instead of MTT solution). The amount of extracted color will be subtracted from the results of the viability assay according to Section B.7. “Data Analysis / Calculation of Results”.

- *If the colorant control result is > 60% of the viable negative control, the results obtained with standard OD measurement should be taken with caution as this is the cut-off used in EpiOcular™ EIT to distinguish classified from not classified chemicals.*
- *It may not be necessary to perform a test on colorant controls for those chemicals classified as irritants due to a viability being lower than the cut-off of 60% because any color interference would not change the classification for such chemicals.*
- *If tissues are stained by a colorant and remain stained even after isopropanol extraction, this should be documented in the remarks and by taking photographs.*
- *If the uncorrected ODs of the tissue extracts obtained with the test article fall outside of the linear range of the spectrophotometer, the test article may be considered as not testable using standard OD measurement.*

If tissues are stained by a colorant and remained stained even after isopropanol extraction, this should be documented in the remarks and by taking photographs.

B.5.10 Non-Specific Killed Control for Assessment of Colored and Direct MTT Test Articles

In cases where the test article was identified as producing both color interference and direct MTT reduction, a third set of controls will need to be performed. This is usually the case with darkly colored test chemicals absorbing light in the range of 570±30 nm (e.g., blue, purple, black) because their intrinsic color impedes the assessment of their capacity to directly reduce MTT. This force by default the use of both controls, KC and CC, which can lead to double correction for color interference. In NSKC, the test chemical is applied on two killed tissue replicates, which undergo the entire testing procedure but are incubated with medium instead of MTT solution during the MTT incubation step. NSKC should be performed concurrently to the KC control and with the same tissue batch. To calculate true tissue viability the percent non-specific color obtained with NSKC will be added to relative tissue viability (Section B.7.5).

Note: *For colorants, it may be necessary to determine the amount of the MTT product using an HPLC/UPLC-spectrophotometry, since the HPLC/UPLC system allows for the separation of the MTT formazan from the chemical before its quantification (**22**).*

Another method to overcome problems with colorants is to measure the absorption spectra of the isopropanol extracts of the viability assay and the colorant control test. In case of high absorbance differences of dyes between tissues of the viability and the colorant control test, the absorbance spectra may indicate the impact of

bound color more exactly as the colorant controls measured at a single wavelength. Please contact MatTek Technical Assistance for more help in this area.

B.6. ACCEPTANCE CRITERIA

Acceptance Criteria for Test Results

The results are acceptable if:
1) The negative control OD > 0.8 and < 2.8,
2) The mean relative viability of the positive control is:
    a) 30-minute exposure: below 50% of control viability
    b) 6 hr exposure: below 50% of control viability
3) The difference of viability between the two relating tissues of a single chemical is < 20% in the same run (for positive and negative control tissues and tissues of single chemicals). This applies also to the killed controls (single chemicals and negative killed control) and the colorant controls which are calculated as percent values related to the viability of the relating negative control.

B.7. DATA ANALYSIS / CALCULATION OF RESULTS

The measured OD values are processed according to the following rules and calculations (Sections B.7.1-B.7.5). Excel spreadsheets are provided which are used for the calculation of the results. The Excel file provided by MatTek Corporation contains two spreadsheets named IMPORT and SPREAD (Fig. 9 and 10). For details of the additional spreadsheets see Annex 2.

B.7.1 General Calculations

1) Calculate the mean OD value of the blank control wells (OD_{Blk}) for each experiment.
2) Subtract OD_{Blk} from each OD value of the same experiment (blank corrected values).
3) Calculate the mean value of the two aliquots for each tissue (= corrected test article OD).
4) Calculate the mean value of the two relating tissues for each control and test article (= corrected mean OD). For further calculations only the corrected mean negative control OD value is needed and in case of the killed controls the negative killed control OD value (OD_{NC_KC}).
5) The corrected OD value of the negative control corresponds to 100% viability.

Corrected negative control OD = Negative control OD - OD_{Blk} = 100% Viability

B.7.2 Calculations for Viability Tests only

1) Calculate the percent viability of each of the two relating tissues for each control and test article relative to the negative control (100% control).

$$\textit{Viability [\%]} = \frac{\textit{corrected test article OD}}{\textit{corrected mean negative control OD}} \times 100$$

2) Calculate the difference of the viability. If the difference is >20%, the test is considered as non-qualified.

3) Calculate the mean test article viability (TA viability) and classify the test article according to the prediction model.

B.7.3 Calculations for Viability plus Killed Control Tests

1) Calculate the OD values of the killed control experiment as described in B.7.1. Step 1 to 3 = corrected OD test article KC.

2) Subtract the mean negative control killed control value (OD_{NC_KC}) from the test article-treated killed controls to determine the individual killed control values for the test articles (OD_{KC}).

OD killed control = Corrected OD test article KC - OD_{NC_KC}

The net OD killed control represents the amount of reduced MTT due to direct reduction by test article residues.

3) Transfer the killed control OD for the two relating tissues to a percentage value relative to the viability scale (killed control "viability") by comparison to the corrected viability negative control OD of the relating experiment in which the test article viability test is performed.

$$\text{Killed control "viability" [\%]} = \frac{\text{corrected test article OD}}{\text{corrected mean negative control OD}} \times 100$$

4) Calculate the difference of the viability of the two tissues. If the difference is >20%, the killed control test is considered as non-qualified.
5) Calculate the mean viability for the killed control and subtract this mean killed control "viability" value (KC "viability") from the relating mean viability of the same test article (TA viability) to determine the killed control corrected viability (KC corrected viability).

KC corrected viability = TA viability – KC "viability"

6) Classify the test article regarding the **killed control corrected viability** according to the prediction model.

B.7.4 Calculations for Viability plus Colorant Control Tests

1) Calculate the OD values of the colorant control test as described in B.7.1. Points 1 to 4.
2) Transfer the colorant control OD for the two relating tissues to a percentage value relative to the viability scale (colorant control "viability") by comparison to the corrected viability negative control of the same experiment.

$$\text{Colorant control "viability" [\%]} = \frac{\text{OD colorant control}}{\text{corrected mean negative control OD}} \times 100$$

3) Calculate the difference of the viability of the two tissues. If the difference is >20%, the colorant control test is considered as non-qualified.
4) Calculate the mean viability for the colorant control and subtract this mean colorant control "viability" value (CC "viability") from the relating mean viability of the same test article (TA viability) to determine the colorant control corrected viability (CC corrected viability).

CC corrected viability = TA viability – CC "Viability"

5) Classify the test article regarding the **colorant control corrected viability** according to the prediction model.

B.7.5 Calculations for Viability plus Killed Control, Colorant Control, and Non-Specific Killed Control Tests

1) Calculate the OD values as well as the test article viability (TA viability), the killed control "viability" (KC "viability"), the colorant control "viability" (CC "viability"), and the non-specific killed control "viability" (NSKC "viability") values of a specific test article according to the rules given in B.7.2., B.7.3. and B.7.4.

2) Subtract the KC "viability" and the CC "viability" from the TA viability, then add NSKC "viability to determine the killed, colorant, and non-specific killed control corrected viability (KC+CC+NSKC corrected viability).

KC+ CC + NSKC corrected viability = TA viability –KC "viability – CC "viability" + NSCK "viability"

3) Classify the test article regarding the killed, colorant, and non-specific killed control corrected viability according to the prediction model.

Correspondend Informations of MDS	
Exp. no.:	OCL-EIT
Tissue-lot no.:	XWZXWZ
Date:	XX-YY-ZZ
Operator:	YZ

Negative control	**NC**		
Positive control	**PC**		
Test Chemical No. 1	TA1	Test Chemical No. 11	TA11
Test Chemical No. 2	TA2	Test Chemical No. 12	TA12
Test Chemical No. 3	TA3	Test Chemical No. 13	TA13
Test Chemical No. 4	TA4	Test Chemical No. 14	TA14
Test Chemical No. 5	TA5	Test Chemical No. 15	TA15
Test Chemical No. 6	TA6	Test Chemical No. 16	TA16
Test Chemical No. 7	TA7	Test Chemical No. 17	TA17
Test Chemical No. 8	TA8	Test Chemical No. 18	TA18
Test Chemical No. 9	TA9	Test Chemical No. 19	TA19
Test Chemical No. 10	TA10	Test Chemical No. 20	TA20

FIXED DESIGN OF 96 WELL PLATE

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	Tissue1
B	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	
C	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	Tissue2
D	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	
E	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BLANK	BLANK	Tissue1
F	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BLANK	BLANK	
G	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BLANK	BLANK	Tissue2
H	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BLANK	BLANK	

IMPORT:

PLATE 1

	1	2	3	4	5	6	7	8	9	10	11	12	
A	1.668	0.403	0.502	1.624	0.198	0.499	0.202	0.402	1.373	1.521	1.618	0.368	Tissue1
B	1.698	0.407	0.501	1.609	0.196	0.498	0.201	0.409	1.361	1.406	1.607	0.404	
C	1.769	0.315	0.710	1.642	0.299	0.107	0.179	0.456	1.451	1.423	1.700	0.534	Tissue2
D	1.747	0.311	0.536	1.729	0.299	0.106	0.197	0.494	1.444	1.438	1.620	0.380	
E	1.768	0.203	0.502	1.540	0.198	0.599	0.402	0.502	1.273	1.521	0.037	0.038	Tissue1
F	1.698	0.207	0.501	1.390	0.196	0.698	0.401	0.609	1.261	1.506	0.037	0.038	
G	1.690	0.215	0.710	1.420	0.209	0.767	0.179	0.616	1.351	1.423	0.037	0.038	Tissue2
H	1.547	0.211	0.536	1.429	0.199	0.696	0.197	0.704	1.410	1.438	0.037	0.038	

REMARKS

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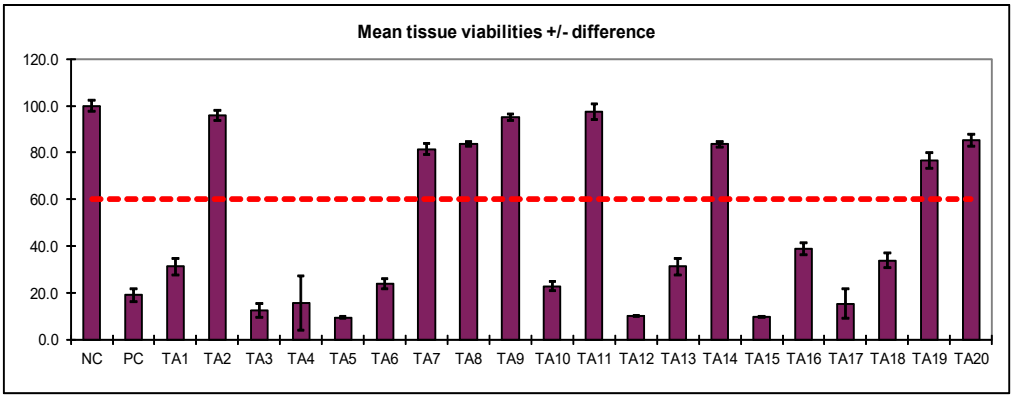
Figure 9: Example of the "IMPORT" spreadsheet of EpiOcular-EIT Excel file

RESULTS

exp. no.:	OCL-EIT
tissue-lot no.:	XWZXWZ
date:	XX-YY-ZZ
operator:	YZ

Blank	0.037
	0.037
	0.037
	0.037
	0.038
	0.038
	0.038
	0.038
Mean	0.0375



Code N°	Tissue	Raw data		Blank corrected data		mean of OD	% of viability
	n	Aliq. 1	Aliq. 2	Aliq. 1	Aliq. 2		
NC	1	1.668	1.698	1.631	1.661	1.646	97.8
	2	1.769	1.747	1.732	1.710	1.721	102.2
PC	1	0.403	0.407	0.366	0.370	0.368	21.8
	2	0.315	0.311	0.278	0.274	0.276	16.4
TA1	1	0.502	0.501	0.465	0.464	0.464	27.6
	2	0.71	0.536	0.673	0.499	0.586	34.8
TA2	1	1.624	1.609	1.587	1.572	1.579	93.8
	2	1.642	1.729	1.605	1.692	1.648	97.9
TA3	1	0.198	0.196	0.161	0.159	0.160	9.5
	2	0.299	0.299	0.262	0.262	0.262	15.5
TA4	1	0.499	0.498	0.462	0.461	0.461	27.4
	2	0.107	0.106	0.070	0.069	0.069	4.1
TA5	1	0.202	0.201	0.165	0.164	0.164	9.7
	2	0.179	0.197	0.142	0.160	0.151	8.9
TA6	1	0.402	0.409	0.365	0.372	0.368	21.9
	2	0.456	0.494	0.419	0.457	0.438	26.0
TA7	1	1.373	1.361	1.336	1.324	1.330	79.0
	2	1.451	1.444	1.414	1.407	1.410	83.8
TA8	1	1.521	1.406	1.484	1.369	1.426	84.7
	2	1.423	1.438	1.386	1.401	1.393	82.8
TA9	1	1.62	1.607	1.581	1.570	1.575	93.6
	2	1.7	1.62	1.663	1.583	1.623	96.4
TA10	1	0.368	0.404	0.331	0.367	0.349	20.7
	2	0.534	0.38	0.497	0.343	0.420	24.9
TA11	1	1.768	1.698	1.731	1.661	1.696	100.7
	2	1.69	1.547	1.653	1.510	1.581	93.9
TA12	1	0.203	0.207	0.166	0.170	0.168	10.0
	2	0.215	0.211	0.178	0.174	0.176	10.4
TA13	1	0.502	0.501	0.465	0.464	0.464	27.6
	2	0.71	0.536	0.673	0.499	0.586	34.8
TA14	1	1.54	1.39	1.503	1.353	1.428	84.8
	2	1.42	1.429	1.383	1.392	1.387	82.4
TA15	1	0.198	0.196	0.161	0.159	0.160	9.5
	2	0.209	0.199	0.172	0.162	0.167	9.9
TA16	1	0.599	0.698	0.562	0.661	0.611	36.3
	2	0.767	0.696	0.730	0.659	0.694	41.2
TA17	1	0.402	0.401	0.365	0.364	0.364	21.6
	2	0.179	0.197	0.142	0.160	0.151	8.9
TA18	1	0.502	0.609	0.465	0.572	0.518	30.8
	2	0.616	0.704	0.579	0.667	0.623	37.0
TA19	1	1.273	1.261	1.236	1.224	1.230	73.1
	2	1.351	1.41	1.314	1.373	1.343	79.8

	mean of OD	Dif. of OD	mean of viabilities [%	Dif. of viabilities	Dif./2	Classification	
NC	1.683	0.075	100.0	4.46	2.23	NI	qualified
PC	0.322	0.092	19.1	5.47	2.73	I	qualified
TA1	0.525	0.122	31.2	7.22	3.61	I	qualified
TA2	1.614	0.069	95.9	4.10	2.05	NI	qualified
TA3	0.211	0.102	12.5	6.06	3.03	I	qualified
TA4	0.265	0.392	15.7	23.29	11.65	I	D>20
TA5	0.157	0.014	9.3	0.80	0.40	I	qualified
TA6	0.403	0.070	23.9	4.13	2.06	I	qualified
TA7	1.370	0.081	81.4	4.78	2.39	NI	qualified
TA8	1.410	0.033	83.7	1.96	0.98	NI	qualified
TA9	1.599	0.048	95.0	2.82	1.41	NI	qualified
TA10	0.384	0.071	22.8	4.22	2.11	I	qualified
TA11	1.638	0.115	97.3	6.80	3.40	NI	qualified
TA12	0.172	0.008	10.2	0.48	0.24	I	qualified
TA13	0.525	0.122	31.2	7.22	3.61	I	qualified
TA14	1.407	0.040	83.6	2.41	1.20	NI	qualified
TA15	0.163	0.007	9.7	0.42	0.21	I	qualified
TA16	0.653	0.083	38.8	4.93	2.47	I	qualified
TA17	0.257	0.214	15.3	12.69	6.34	I	qualified
TA18	0.570	0.105	33.9	6.21	3.10	I	qualified
TA19	1.286	0.114	76.4	6.74	3.37	NI	qualified
TA20	1.435	0.083	85.2	4.93	2.47	NI	qualified

Figure 10: Example of the “SPREAD” spreadsheet of EpiOcular-EIT Excel file

B.8. PREDICTION MODEL

A prediction model was developed by *MatTek* Corporation to determine the *ocular irritation classification* as follows:

If the test article-treated tissue viability is > 60.0% relative to negative control-treated tissue viability, the test article is labeled ***Non-irritant****.*

If the test article-treated tissue viability is ≤ 60.0% relative to negative control-treated tissue viability, the test article is labeled ***Irritant****.*

In 2019, OECD/OCDE TG No. 492 adopted the following classification:

If the test article-treated tissue viability is > 60.0% relative to negative control-treated tissue viability, the test article is not requiring classification and labeling ***(No Category),*** *no further testing is required.*

If the test article-treated tissue viability is ≤ 60.0% relative to negative control-treated tissue viability, ***No prediction can be made*** *for the test article (further testing is required).*

***In vitro* result**	***In vivo* prediction**	
	OCL-200-EIT classification ***(2015, 2017, 2019 updates)***	**OECD TG No. 492** ***(adopted and updated in 2019)***
mean tissue viability ≤ 60.0%	Irritant (I)	No prediction can be made (further information will be required for classification according to IATA guidance)
mean tissue viability > 60.0%	Non-irritant (NI)	No Category (not requiring classification and labeling according to UN GHS)

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B.10. ANNEXES

Annex 1: Example Time Schedules for Liquids and Solids

Annex 2: Test for Interference of Chemicals with MTT Endpoint and Correction Procedures

Annex 3: Excel Spreadsheets Explanations for Data Input and Calculations

Annex 4: Method Documentation Sheets (MDS)

Annex 5: Characterization of Test Substances

ANNEX 1A: SAMPLE TIME SCHEDULE FOR TESTING OF LIQUIDS

Example: Test schedule for liquids + controls

Start time of the different work steps (daywork for one operator)

Each line corresponds to one pair of tissues

1 to 7 = order of work steps

Each line corresponds to one pair of tissues

	1	2	3	4	5	6	7
Liquids:	**PBS**	**Treatment**	**Post-Soak**	**Post-Incub.**	**MTT**	**Extract.**	**Measure**
	30 min	*30 min*	*12 min*	*120 min*	*180 min*	*120 min*	*OD*
NC	9:00	9:30	10:00	10:12	12:12	15:12	after
PC	9:01	9:31	10:01	10:13	12:13	15:13	17:30
TA-1	9:02	9:32	10:02	10:14	12:14	15:14	
TA-2	9:03	9:33	10:03	10:15	12:15	15:15	
TA-3	9:04	9:34	10:04	10:16	12:16	15:16	
TA-4	9:05	9:35	10:05	10:17	12:17	15:17	
TA-5	9:06	9:36	10:06	10:18	12:18	15:18	
TA-6	9:07	9:37	10:07	10:19	12:19	15:19	
TA-7	9:08	9:38	10:08	10:20	12:20	15:20	
TA-8	9:09	9:39	10:09	10:21	12:21	15:21	
TA-9	9:10	9:40	10:10	10:22	12:22	15:22	
TA-10	9:11	9:41	10:11	10:23	12:23	15:23	

ANNEX 1B: SAMPLE TIME SCHEDULE FOR TESTING OF SOLIDS

Example: Test schedule for solids + controls

Start time of the different work steps (daywork for one operator)

Each line corresponds to one pair of tissues

1 to 7 = order of work steps

1st day **2nd day**

work steps	1	2	3	4	5	6	7
Solids:					next day		
	PBS	**Treatment**	**Post-Soak**	**Post-Incub.**	**MTT**	**Extract.**	**Measure**
	30 min	*6 hr*	*25 min*	*18 hr*	*180 min*	*120 min*	*OD*
NC	9:00	9:30	15:30	15:56	9:56	12:56	after
PC	9:02	9:32	15:32	15:58	9:58	12:58	15:30
TA-1	9:04	9:34	15:34	16:00	10:00	13:00	
TA-2	9:06	9:36	15:36	16:02	10:02	13:02	
TA-3	9:08	9:38	15:38	16:04	10:04	13:04	
TA-4	9:10	9:40	15:40	16:06	10:06	13:06	
TA-5	9:12	9:42	15:42	16:08	10:08	13:08	
TA-6	9:14	9:44	15:44	16:10	10:10	13:10	
TA-7	9:16	9:46	15:46	16:12	10:12	13:12	
TA-8	9:18	9:48	15:48	16:14	10:14	13:14	
TA-9	9:20	9:50	15:50	16:16	10:16	13:16	
TA-10	9:22	9:52	15:52	16:18	10:18	13:18	

ANNEX 2: TEST FOR INTERFERENCE OF CHEMICALS WITH MTT ENDPOINT AND CORRECTION PROCEDURES

Possible interactions between test chemicals and test system

	Direct MTT Interaction (Section B.5.2)	Intrinsically Colored Materials (Section B.5.3.a)	Materials that Develop Color (Section B.5.3.b)	Test Conditions
Case 1	-	-	-	A
Case 2	-	-	+	B
Case 3	-	+	-	B
Case 4	-	+	+	B
Case 5	+	-	-	C
Case 6	+	-	+	C
Case 7	+	+	-	B+C+D
Case 8	+	+	+	B+C+D

Test Conditions:

A – Perform all steps according to the basic SOP. Correction of results using additional controls is not needed.

B – Perform the basic SOP and correction of results with viable tissues (colorant controls, see Section B.5.9).

C – Perform the basic SOP and correction of results with frozen tissues (killed controls, see Section B.5.8).

B+C+D - Perform the basic SOP and correction of results with viable tissue (**B,** colorant controls, see Section B.5.9), with frozen tissue (**C,** killed controls, see Section B.5.8), and extra set of frozen tissues (**D,** non-specific killed control, see Section B.5.10). In rare cases where the test article was identified as producing both color interference and direct MTT reduction, a third set of controls is required to eliminate double correction for color interference.

Annex 3: Excel Spreadsheets - Explanations for Data Input and Calculations

"Import" Sheet:

The 96-well microtiter plate has to be configured according to the plate configuration indicated in this sheet (different for Viability/CC Test and Killed Control Test!). The raw data (OD values) have to be copied into the marked cells. The sheet has space for 20 test articles

Plate configuration: **Viability/CC Test**

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	Tissue 1
B	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	
C	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	Tissue 2
D	NC	PC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	
E	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	Tissue 1
F	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	
G	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	Tissue 2
H	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	

NC = negative control; PC = positive control; TA1 ... TA20 = test article 1 ... 20; BL = blank

PLATE

	1	2	3	4	5	6	7	8	9	10	11	12	
A	1,913	0,897	0,765	1,292	1,671	0,168	0,046	0,046	0,046	0,046	0,046	0,047	Tissue 1
B	1,877	0,872	0,748	1,290	1,620	0,162	0,046	0,046	0,048	0,046	0,046	0,046	
C	1,950	0,799	0,702	1,697	1,888	0,071	0,046	0,046	0,046	0,046	0,046	0,046	Tissue 2
D	1,958	0,772	0,689	1,694	1,822	0,071	0,046	0,047	0,046	0,046	0,046	0,046	
E	0,765	1,292	1,671	0,168	0,046	0,046	0,046	0,046	0,046	0,047	0,042	0,041	Tissue 1
F	0,748	1,290	1,620	0,162	0,046	0,046	0,048	0,046	0,046	0,046	0,041	0,041	
G	0,702	1,697	1,888	0,071	0,046	0,046	0,046	0,046	0,046	0,046	0,041	0,041	Tissue 2
H	0,689	1,694	1,822	0,071	0,046	0,047	0,046	0,046	0,046	0,046	0,041	0,042	

For Killed Control Tests no Positive Control will be tested and therefore the wells for test articles TA1 to TA 10 are switched one column to the left. Wells A12 to D12 are kept empty.

Plate configuration: **Killed Control Test**

	1	2	3	4	5	6	7	8	9	10	11	12	
A	NC_KC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	empty	Tissue 1
B	NC_KC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	empty	
C	NC_KC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	empty	Tissue 2
D	NC_KC	TA1	TA2	TA3	TA4	TA5	TA6	TA7	TA8	TA9	TA10	empty	
E	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	Tissue 1
F	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	
G	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	Tissue 2
H	TA11	TA12	TA13	TA14	TA15	TA16	TA17	TA18	TA19	TA20	BL	BL	

NC_KC = negative killed control; TA1 ... TA20 = test article 1 ... 20; BL = blank

Excel Spreadsheets Explanations for Data Input and Calculations (continued)

"Data" Sheet:

- The spreadsheet controls if the negative control and positive control fulfill the acceptance criteria.

  Non-qualified run: OD NC < 0.8 or > 2.8
  Non-qualified run: PC > 50%

- If a test article does not fulfill the acceptance criterion regarding the difference of viability (D<20), this will be highlighted in red. Otherwise the test is qualified.

	mean of OD	Dif. of OD	mean of viabilities [%	Dif. of viabilities	Dif./2	Classification	
NC	1.683	0.075	100.0	4.46	2.23	NI	qualified
PC	0.322	0.092	19.1	5.47	2.73	I	qualified
TA1	0.525	0.122	31.2	7.22	3.61	I	qualified
TA2	1.614	0.069	95.9	4.10	2.05	NI	qualified
TA3	0.211	0.102	12.5	6.06	3.03	I	qualified
TA4	0.265	0.392	15.7	23.29	11.65	I	D>20

Note: *This Excel file has been produced by using Microsoft Office Excel 2007. If a different version of Excel is being used, the data may be presented differently than described here (e.g. highlighting 'D>20' in red in the Calculation Sheet, column 'Qualified?").*

Note: A set of spreadsheets (Figure 9 and 10) is available from MatTek Corporation (Ashland, MA, US) and MatTek In Vitro Life Science Laboratories (Bratislava, Slovakia).

Annex 4: Method Documentation Sheet (MDS)

IDENTIFICATION AND PHYSICAL DESCRIPTION OF TEST ARTICLES USED IN THE EXPERIMENT

Study Number _____

Study Director _____

Performed by: _____

Corresponding spreadsheet file name _____

	Test Article ID	Secondary Code (if applicable)	Physical Description (form; colour; consistency)	Other Remarks
1	NC		Water (Colorless Liquid)	
2	PC		Methyl Acetate (Colorless Liquid)	
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				

ID/Date

DEVICES VERIFICATION

Name of the controlled Device	Identification Number	Parameter 1 (✓)		Parameter 2 (✓)		Parameter 2 (✓)		ID / Date
Incubator		Temperature <37±1°C>		CO_2 <5±1%>		water in reservoir		
Refrigerator		Temperature < 5°C ± 3°C >						
Water bath		Temperature <37±1°C>						

PIPET VERIFICATION

A calibration check was performed on all micropipettors used in this assay on the day of use. Each micropipettor that was used was calibrated on at least one volume that was used in the assay on that given day by measuring the weight of the targeted volume of water. The micropipettor was used to deliver the targeted volume of water into a weigh boat using an analytical scale (ID#_____). The weight of the water must be within 1% of the expected weight for the volume pipetted. This procedure is intended to verify the performance of each micropipettor on the day of use, and is not intended to replace a formal micropipettor calibration program.

Table 3: Pipette verification

Pipet Size	Serial Nuber	Volume (µL)	Weight (mg)	Next Calibration Date	Initials & Date

EpiOcular Kit Components Verification

The EpiOcular ™ kit was received on _____
Upon receipt of the MatTek Assay Kit, the components and reagents were stored as indicated. The MatTek EpiOcular ™ constructs and culture media were stored at 2-8°C until use.

EpiOcular (OCL-200_2.0) Lot no.:	Production date:
Assay medium (OCL-200-ASY) Lot no.:	Expiration date:
MTT concentrate (MTT-100-CON); 2 ml Lot no.:	Expiration date:
MTT diluent (MTT-100-DIL); 8 ml Lot no.:	Expiration date:
MTT extractant (MTT-100-EXT), 60 ml Lot no.:	Expiration date:
DPBS (TC-PBS); 100 ml Lot no:.	Expiration date:
Methyl Acetate (TC-MA); 0,5 ml Lot no:	Expiration date:
Position of Ice-packs: (direct contact of the ice-packs with the tissues must be avoided)	
Other remarks	

ID/ Date:

DOSING PROCEDURE

Tick (✓) in the columns the type of application (pipette, spoon, spatula…)

P: pasty; **V**: viscous; **W**: waxy; **ST**: sticky; **C**: creamy

Code	Liquid/ Solid (L/S)	Application (50 mg / 50 µl)					Remarks
		Pipette (✓)	Spoon (✓)	Syringe (✓)	Spatula (✓)	Pin (✓)	

ID/ Date:

EXPERIMENTAL SCHEDULE

DOSING TIMETABLE FOR LIQUID TEST ARTICLES (INCLUDING COLORANT CONTROLS)

Test Article	Pre-incubation		Main Experiment				MTT assay		
	Initial 60±5 min	Over-night 16-24 h	D-PBS Addition (20 µL) 30±2 min	Treatment (50 µL) 30±2 min	Treatment Term. / Post Soak Initiation 12±2 min	Post Soak Term. / Post Inc. Start 120±15 min	Post Inc Term. / MTT Addition 180 ± 10 min	MTT Termi-nation	Remarks
Negative Control									
Positive Control									

EXPERIMENTAL SCHEDULE

DOSING TIMETABLE FOR LIQUID TEST ARTICLES (KILLED CONTROL TISSUES)

Test Article	Pre-incubation		Main Experiment				MTT assay		
	Initial 60±5 min	Over-night 16-24 h	D-PBS Addition (20 µL) 30±2 min	Treatment (50 µL) 30±2 min	Treatment Term. / Post Soak Initiation 12±2 min	Post Soak Term. / Post Inc. Start 120±15 min	Post Inc Term. / MTT Addition 180 ± 10 min	MTT Termi-nation	Remarks
Negative Control									
Positive Control									

EXPERIMENTAL SCHEDULE

DOSING TIMETABLE FOR SOLID TEST ARTICLES (INCLUDING COLORANT CONTROLS)

Test Article	Pre-incubation		Main Experiment				MTT assay		
	Initial 60 ±5 min	**Over-night** 16-24 h	**D-PBS Addition** (20 µL) 30±2 min	**Treatment** (50 mg) 6 ± 0.25 h	**Treatment Term. / Post Soak Initiation** 25 ± 5 min	**Post Soak Term. / Post Inc. Start** 18 ± 0.25 h	**Post Inc Term. / MTT Addition** 180 ± 10 min	**MTT Termi-nation**	**Remarks**
Negative Control									
Positive Control									

EXPERIMENTAL SCHEDULE

DOSING TIMETABLE FOR SOLID TEST ARTICLES (KILLED CONTROL TISSUES)

Test Article	Pre-incubation		Main Experiment				MTT assay		
	Initial 60 ±5 min	**Over-night** 16-24 h	**D-PBS Addition** (20 µL) 30±2 min	**Treatment** (50 mg) 6 ± 0.25 h	**Treatment Term. / Post Soak Initiation** 25 ± 5 min	**Post Soak Term. / Post Inc. Start** 18 ± 0.25 h	**Post Inc Term. / MTT Addition** 180 ± 10 min	**MTT Termi-nation**	**Remarks**
Negative Control									
Positive Control									

ANNEX 5: CHARACTERIZATION OF TEST SUBSTANCES

Characterization of test substances used in the study should be filled in after conduction the initial assessment of direct test article reduction of MTT and assessment of colored or staining materials. Every test substance should be described and evaluated with regard of MTT reduction ability, color, and reaction with water. Special storage requirements, safety warnings should be recorded here.

Code	Physical state (S/L)	Colour	MTT Reaction environment - follow section B.5.2 of the SOP (visual evaluation +/-)	Color reaction in isopropanol – follow section B.5.3 of the SOP (Read OD's at 570nm)	Color reaction in aqueous environment – follow section B.5.3 of the SOP (visual evaluation)	Killed Control Testing needed? (x = yes)	Colorant Control Testing needed? (x = yes)	Special storage requirements
PC	L	colourless	-		-			
NC	L	colourless	-		-			

Characterization of test substances (continued)

Code	(S/L)	Colour	MTT	Color (IP)	Color (Aqua)	KC	CC	Storage

ID/Date:

SOLID SUBSTANCES ONLY EVALUATION OF WEIGHT APPLIED BY DOSING TOOLS FOR SOLIDS

Code	Weight of substance (when applied by 50 mg spoon, spatula or syringe) in mg			Grinding (yes / no)	Indicate the dosing device used SP-spoon SPA-spatula SY-syringe	Remarks
	1.	2.	3.			

ID/Date: