Video Article

Eye Irritation Test (EIT) for Hazard Identification of Eye Irritating Chemicals using Reconstructed Human Cornea-like Epithelial (RhCE) Tissue Model

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Abstract

To comply with the Seventh Amendment to the EU Cosmetics Directive and EU REACH legislation, validated non-animal alternative methods for reliable and accurate assessment of ocular toxicity in man are needed. To address this need, we have developed an eye irritation test (EIT) which utilizes a three dimensional reconstructed human cornea-like epithelial (RhCE) tissue model that is based on normal human cells. The EIT is able to separate ocular irritants and corrosives (GHS Categories 1 and 2 combined) and those that do not require labeling (GHS No Category). The test utilizes two separate protocols, one designed for liquid chemicals and a second, similar protocol for solid test articles. The EIT prediction model uses a single exposure period (30 min for liquids, 6 hr for solids) and a single tissue viability cut-off (60.0% as determined by the MTT assay). Based on the results for 83 chemicals (44 liquids and 39 solids) EIT achieved 95.5/68.2/ and 81.8% sensitivity/specificity and accuracy (SS&A) for liquids, 100.0/68.4/ and 84.6% SS&A for solids, and 97.6/68.3/ and 83.1% for overall SS&A. The EIT will contribute significantly to classifying the ocular irritation potential of a wide range of liquid and solid chemicals without the use of animals to meet regulatory testing requirements. The EpiOcular EIT method was implemented in 2015 into the OECD Test Guidelines as TG 492.

Video Link

The video component of this article can be found at https://www.jove.com/video/52979/

Introduction

Consumer products such as cosmetics, detergents, and household cleaners include a variety of chemicals that may induce serious damage if they contact the eyes. Therefore, testing of these agents for eye irritation is required by the US and EU regulatory agencies to ensure consumer safety[1]. An assessment of the eye irritation potential of mixtures and formulations is also a requirement for complying with REACH (Registration, Evaluation, Authorization, and Restriction of Chemicals) legislation for the labeling of cosmetic ingredients under the EU Cosmetics Directive for transport of chemicals, and for the labeling of pesticides and household products[2]. Currently, regulatory agencies require ocular hazard assessment using the Globally Harmonized System of Classification and Labeling of Chemicals (GHS)[3]. GHS is mainly based on the Draize eye irritation test, the most widely used eye irritation assay in which foreign substances and mixtures are introduced directly into the conjunctival sac of the rabbit eye[4]. According to GHS classification, GHS Category 1 (ocular corrosives) refers to test chemicals that cause severe initial injury to the eye tissues or serious damage to the eye and vision which is not fully reversible within 21 days following exposure[3,5]. GHS Category 2 refers to test chemicals that produce significant changes in the eye that are fully reversible within 21 days of exposure. Test chemicals that are not corrosives or irritants are referred to as GHS No Category.

For more than 40 years, the Draize rabbit eye test has been criticized for its lack of reproducibility, overestimation of human responses, and the use of live animals[5-8]. These concerns have encouraged many proposals for refinement, reduction, and replacement of the *in vivo* test[9]. The need for validated non-animal alternatives was further strengthened by the adoption of the Seventh Amendment to the Cosmetics Directive, which banned the use of animals in the safety evaluation of cosmetic products (in 2005) and ingredients (in 2009)[2].

Since 1996, the reconstructed cornea-like tissue model has been widely used by the cosmetic industry to evaluate the irritation potential of raw materials, surfactant-based formulations, and compounded mixtures that are designed for use in, or in the vicinity of, the eye[10-13]. Use of the RhCE tissue model allows direct topical application of the test material onto the tissue surface in its native, undiluted form. In this way, non-water soluble formulations can be tested without diluting them with solvents. In response to EURL ECVAM's (European Union Reference Laboratory European Centre for the Validation of Alternative Methods) request for a widely applicable, straightforward, and economic method the Eye Irritation Test (EIT) which utilizes a single exposure time and is able to separate ocular irritants and corrosives from materials that do not require labeling was developed (**Figure 1**)[14]. Based on the results for 83 chemicals (44 liquids and 39 solids), the EIT achieved 95.5/68.2/ and 81.8% sensitivity/specificity and accuracy (SS&A) for liquids, 100.0/68.4/ and 84.6% SS&A for solids, and 97.6/68.3/ and 83.1% for overall SS&A.

In 2007, a multi-laboratory pre-validation study sponsored by Cosmetics Europe (formerly Colipa) under the auspices of the EURL ECVAM assessed the relevance and reliability of the EIT with the goal of bringing it to formal validation[15]. In this study, 298 independent trials were performed in seven independent laboratories. Study results demonstrated 99.7% agreement in prediction with low coefficients of variation across all participating laboratories[15]. As a result, in 2010 the EIT protocol entered a formal EURL ECVAM validation program. The validation study utilized 104 coded test chemicals, including individual substances and chemical mixtures, for which *in vivo* reference data (Draize eye irritation data) were available. Based on the success of this work, an OECD draft test guideline was submitted in 2014. It is anticipated that the EIT will contribute significantly to classification of ocular irritation potential of a wide range of materials according to the UN GHS classification and labeling system.

Protocol

1. Preparation of RhCE Tissues for Treatment – Day 0

1. Upon receipt of the commercial human cornea-like epithelial (RhCE) kit, check all kit components for integrity (for kit details see Standard Assay Kit Components (**Table 1**) and Equipment and Materials required to perform the EIT assay (**Table 2**). On the day of receipt, equilibrate tissues (in its 24-well shipping container) to RT for 15 min.

Amount	Reagent	Conditions	Source	Description	Expiration Date
1	Sealed 24-well plate of EpiOcular tissues (OCL-200)	2-8 °C	MatTek	Contains 24 tissues of cell culture inserts, package on agarose	72 hr
1 bottle, 200 ml	EpiOcular Assay Medium (OCL-200-ASY)	2-8 °C	MatTek	DMEM based medium	21 days
1 bottle, 100 ml	$Ca^{++}Mg^{++}$-Free Dulbecco's#PBS (DPBS)	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

Table 1: Standard Assay Kit Components.

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 °C)	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, No: FK 623)	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	Contains MTT-Thiazolyl Blue Tetrazolium Bromide reagent (Sigma #M-5655) and isopropanol extract.

Table 2: Equipment and materials required to perform the EIT.

2. Under sterile conditions, open the plastic bag containing the 24-well plate with the RhCE tissues and remove the sterile gauze. Inspect all tissues for air bubbles between the agarose gel and insert. Do not use cultures with air bubbles under the insert covering >50% of the insert area, defective tissues, or tissues which are completely covered with liquid.
3. Label the 6-well plates with the test article or control codes and exposure times. Aliquot 1.0 ml of Assay Medium (provided with the kit), pre-warmed to approximately 37 °C, into the wells of pre-labeled 6-well plates.
4. Use sterile forceps to remove each insert containing the RhCE tissue and place the insert in the labeled 6-well plate. During this step, remove any remaining shipping agarose that adheres to the outer sides of the insert by gentle blotting on sterile filter paper. Release any air bubbles trapped underneath the inserts.
5. Pre-incubate the RhCE tissues in the 6-well plates to standard culture conditions (SCC, humidified atmosphere with 5 ± 1% CO_2 at 37 ± 1 °C) for 1 hr.
6. After 1 hr, replace the Assay Medium with 1.0 ml of fresh Assay Medium prewarmed to 37 °C and incubate the RhCE tissues at SCC conditions (overnight = O/N) (16-24 hr).

2. Pre-treatment – Day 1

1. After the O/N incubation, apply 20 µl of Ca^{2+} Mg^{2+}-free-Dulbecco's Phosphate Buffered Saline (DPBS, provided) using an appropriate pipetting device. If the DPBS does not spread across the tissues, gently tap the insert on the plate to assure that the DPBS wets the entire tissue surface.
2. Incubate the RhCE tissues at SCC for 30 ± 2 min.
   Note: This step is necessary for tissue hydration and to mimic *in vivo* conditions.

3. Test Material Exposure Procedures

1. Apply each test article and controls to duplicate RhCE tissues (n = 2). The test article dosing procedure is different for liquids and solids. Topically apply 50 µl of liquid test articles using a pipette. The exposure time for liquids is 30 min. Apply 50 mg of solid test articles using a leveled spoonful (calibrated to hold 50 mg of sodium chloride). The exposure time for solids is 6 hr.
   Note: Liquids are defined as fluid substances (*e.g.*, liquids, gels, and creams) that can be applied utilizing a pipetting device. Solids are defined as non-fluid substances (*e.g.,* powders, resinous or waxy materials) that cannot be applied using a pipette.
   1. If the physical state of test articles is not easy to determine, place the vials with test article in a water bath for 15 min (37 °C). Follow EIT protocol for liquids for those test articles that liquefy at 37 °C.
   2. Use a positive displacement pipette for particularly viscous materials.

2. Dose the negative control and positive controls first and then dose the test articles.
   1. Apply 50 µl of the negative control (NC) and the positive control (PC) to the RhCE tissues using a standard pipette. The NC is sterile de-ionized water; the PC is methyl acetate (CAS# 79-20-9). Apply the NC and PC for 30 min when testing liquid test articles and for 6 hr when testing solid test articles.

4. Test Article Exposure – Day 1

1. For the treatment of liquid test articles, follow the timing schedule given in **Table 3**. Leave 1 min intervals between applications of each test article to ensure equal exposure for all tissues.
   1. After the 30 ± 2 min DPBS pre-treatment, topically apply 50 µl of NC and PC, and each liquid test article topically onto the RhCE tissues using an appropriate pipetting device.
   2. Apply 50 µl of the liquid test article directly onto the tissue to cover the upper surface. Cut off the narrow point of the pipette tip to widen the orifice for viscous materials. For very viscous materials, apply the test article to a dosing device (a flat headed cylinder of diameter

slightly less than the inner diameter of the tissue insert or a plastic pushpin), invert the dosing device and place it onto the tissue so that the test article evenly contacts the tissue surface.
3. If test article does not spread across the tissue, gently tap the insert to make sure that it spreads on the entire tissue surface. Mechanical spreading of the test articles (*e.g.*, with a pipette tip) is not recommended since it may damage the tissues.
4. Incubate the tissues at SCC for 30 ± 2 min.

2. For the treatment of solid test articles – Day 1, follow the timing schedule given in **Table 4**. Leave 2 min intervals between applications of each test article to ensure equal exposure for all tissues.
    1. After the 30 ± 2 min DPBS pre-treatment, apply 50 µl of NC and PC topically on the RhCE tissues using an appropriate pipetting device.
    2. For solid test article application, remove the inserts (n = 2) from the well and place them onto a sterile surface (*e.g.*, the lid of a multiwell plate) to avoid a test article spilling into the medium.
    3. Using a leveled spoon, topically apply approximately 50 mg of the test article onto the tissue surface; make sure that the surface of the tissue is completely covered by the test article. If the test article does not spread across the tissue, shake the insert gently from side to side to ensure that the tissue is completely covered by the test article. Mechanical spreading of the test articles (*e.g.*, with a pipette tip) is not recommended since it may damage the tissues.
        1. If needed, grind crystalline powders with a mortar and pestle to guarantee better contact between the test article and the tissue.
        2. Alternatively, place powders directly onto the tissue culture inside the insert by using a 1 ml syringe with its head cut off. Stuff powders into the syringe when the plunger is drawn back and then apply by pressing the plunger down.
        3. If the outer wall of the insert is contaminated *e.g.*, by powders, wipe the particles off with a sterile gauze.
    4. After dosing, return the tissues to the 6-well plates containing culture medium and incubate at SCC for 6 h ± 15 min.

5. Rinsing

1. Prepare a set of three clean beakers (150 ml capacity) per test article and fill each of them with 100 ml of DPBS. For each test article, utilize a different set of three beakers.
2. At the end of the 30 ± 2 min exposure for liquid materials or 6 hr ± 15 min exposure for solid materials, remove and discard the dosing device if it was used.
3. Lift the inserts containing the RhCE tissue out of the medium by grasping the upper edge of the plastic 'collar' with fine forceps. Use curved forceps to facilitate handling and decanting. Rinse the tissues two at a time by holding the duplicate inserts together by their collars using forceps. Be careful not to damage the tissues with the forceps.
4. Decant the test articles or controls from the tissue surface onto a clean absorbent material (paper towel, gauze, *etc.*)
5. Dip the inserts into the first beaker of DPBS, swirl in a circular motion in the DPBS for approximately 2 sec, lift the inserts so that they are mostly filled with DPBS, and decant the liquid back into the beaker. Repeat this process three times in the first beaker.
6. Rinse the inserts in the second and third beakers of DPBS three times each in the same manner.
7. Decant any liquid remaining in the insert onto the absorbent material. Rotate the insert to an approximate 45° angle (open end down) and touch the upper lip to the absorbent material.
   Note: If it is not possible to remove all of the visible test material, no further rinsing should be done to avoid tissue damage due to excessive handling.

6. Post-soak

1. After rinsing, immediately immerse tissues in 5 ml of Assay Medium previously warmed to RT in a pre-labeled 12-well plate.
2. Incubate the tissues for 12 ± 2 min for liquid materials or 25 ± 2 min for solid materials immersed at RT to facilitate removal of any residual test article.

7. Post-incubation

1. At the end of the Post-Soak immersion period, decant the Assay Medium from the tissues and blot the inserts onto an absorbent material.
2. Transfer the inserts into the pre-labeled 6-well plate containing 1 ml of warm Assay Medium.
    1. Incubate the tissues for 120 ± 15 min at SCC for liquid test materials.
    2. Incubate the tissues for 18 ± 0.25 hr at SCC for solid test materials.

8. MTT Viability Assay – Day 1 (Protocol for Liquids) and Day 2 (Protocol for Solids)

1. Perform the MTT assay after the Post-Incubation of 120 ± 15 min for liquids and 18 ± 0.25 hr for solids, respectively.
2. Prepare 1.0 mg/ml MTT solution and aliquot 0.3 ml of the solution into each well of a pre-labeled 24-well plate.
    1. Use the commercial MTT kit (**Table 5**):
    2. 2 hr before use, thaw the MTT concentrate at RT. Combine 2 ml of the MTT concentrate and 8 ml of the MTT diluent to produce 1.0 mg/ml MTT solution.
    3. Store the MTT solution at 4 °C in the dark until use. Do not store the MTT solution for more than 1 day.
3. At the end of the Post Incubation, remove each insert from the 6-well plate and gently blot on an absorbent material.
4. Place the inserts into the 24-well plate containing 0.3 ml of MTT solution. Release any air bubbles trapped underneath the inserts. Incubate the plate for 180 ± 10 min at SCC.

5. MTT extraction
    1. After 180 ± 10 min incubation in the MTT solution, remove each insert from the 24-well plate and blot the bottom of the insert on an absorbent material.
    2. For non-colorant liquid test articles (submerged extraction): Transfer the inserts into a pre-labeled 24-well plate containing 2.0 ml of an extractant solution (isopropanol) so that it submerges the insert.
    3. For solids and liquid colorants (non-submerged extraction to avoid contamination of the extractant solution): Transfer the inserts into a pre-labeled 6-well plate containing 1.0 ml of the extractant solution (isopropanol) so that it does not submerge the insert.
    Note: Perform the same non-submerged extraction for the corresponding negative and positive controls.

6. Seal the plates (*e.g.,* with parafilm between the plate cover and upper edge of the wells or with a standard plate sealer). Place the plates on an orbital plate shaker and shake for 2 to 3 hr at RT to extract the MTT.
    1. Alternatively, perform the extraction O/N at 2-8 °C in the dark without shaking.

7. For non-colorant liquid test articles (submerged extraction): At the end of the extraction period, decant the liquid from each insert back into the well and discard the inserts with the RhCE tissues.
    1. Mix the extract solution and transfer two 200 µl aliquots into the appropriate wells of a pre-labeled 96-well plate according to the plate configuration (**Figure 2**).

8. For solids and liquid colorants (non-submerged extraction): At the end of the extraction period, discard the tissues (make sure not to pierce the tissues).
    1. Add 1.0 ml of the extractant solution into each well of the 24-well plate containing the solution extracted from the tissues. Mix the extractant solution and transfer two 200 µl aliquots into the appropriate wells of a pre-labeled 96-well plate according to the plate configuration (**Figure 2**).

9. Determine the optical density (OD) of the extracted samples at a single wavelength between 550 and 590 nm (should be consistent within a laboratory) on a plate reader or a spectrophotometer.
    1. In case of turbid extract solutions caused by insoluble solids, centrifuge the solutions prior to measuring the OD (cool down the centrifuge to 4 °C to avoid evaporation). In case rinsing does not remove the test article (TA) and the TA interferes with MTT reduction, additional controls must be used. Please refer to a detailed SOP to correct for MTT reduction[16].
    2. In case a TA is shown to have, or to develop color which can interact with the MTT measurement, an additional test must be performed to determine the amount of color bound to, and subsequently extracted from the tissues. Please refer to a detailed SOP to correct for colored test articles[16].

9. Calculations for Tissue Viability Test (Table 6 and Figure 3)

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 (Blk corrected data).
    3. Calculate the mean value of the two aliquots for each tissue (= corrected OD).

2. Calculate the percent viability of each of the two replicate tissues for each control and test article relative to the average negative control (100% control).
    Viability (%) = [corrected OD treated tissues / corrected OD negative control] x 100%
3. Calculate the difference of the viability (the viability difference between two replicate tissues).
4. Calculate the mean test article viability (TA viability) and classify the test article according to the prediction model.

10. Prediction Model (Figure 3)

1. If the TA-treated tissue viability is >60.0 relative to NC-treated tissue viability, label the test article as non-irritant (NI) (GHS No Category).
2. If the TA-treated tissue viability is ≤ 60.0 relative to NC-treated tissue viability, label the test article as irritant (I) (GHS Categories 1 and 2).
    Note: The EIT test results are considered qualified if:
    - the EIT NC OD >0.8 and <2.5;
    - the EIT PC tissue viability (%, relative to NC) is ≤50.0%;
    - the difference between the two replicate tissues (NC, PC, and test article) is <20.0%.

Representative Results

Representative EIT results conducted with 10 test articles (TA) and negative and positive controls are presented in **Table 6 and Figure 3**. The mean OD = 1.31 for the NC corresponds to 100% tissue viability, therefore the PC (mean OD = 0.41) had relative tissue viability of 31.2%. When the EIT protocol was performed in 15 valid independent experiments in 7 laboratories using the liquid exposure protocol and in 8 independent valid experiments in 4 laboratories, using the solid exposure protocol, the average tissue viability for the PC using the liquid protocol was 36.4 ±4.0% and 32.3±6.4% for the solids protocol. In all cases, the positive control results were below the cut off value of 60.0%[15].

As shown in **Figure 3**, TA1, TA2, TA4, TA7, and TA8 had tissue viabilities >60.0% and therefore were classified as “NI”. TA3, TA5, TA6, TA9, and TA10 had tissue viabilities ≤60.0% and therefore were classified as “I”. The difference of tissue viability between duplicate tissues was <20.0% for all TAs with an exception of TA2. Therefore, results for all the test articles, with an exception of TA2, were considered “qualified” since they

met all the EIT acceptance criteria (Section 10.2). Because of high variability between duplicate tissues for TA2 in the initial experiment, a second experiment was necessary to obtain qualified EIT results.

The EIT test method as described herein utilizing the RhCE tissue model was used for the assessment of ocular irritation in several multilaboratory validation studies, including formal validation by EURL ECVAM/Cosmetics Europe[15,17-19]. In all of the studies, the EIT was shown to be reproducible and was able to correctly identify chemicals (both substances and mixtures) not requiring classification and labeling for eye irritation or serious eye damage according to UN GHS[15,17-19]. The EIT test method fulfilled the acceptance criteria of the Validation Management Group (VMG) for eye irritation for sensitivity, specificity, and overall accuracy and currently it is pending formal implementation as a partial replacement for *in vivo* rabbit Draize test[19].

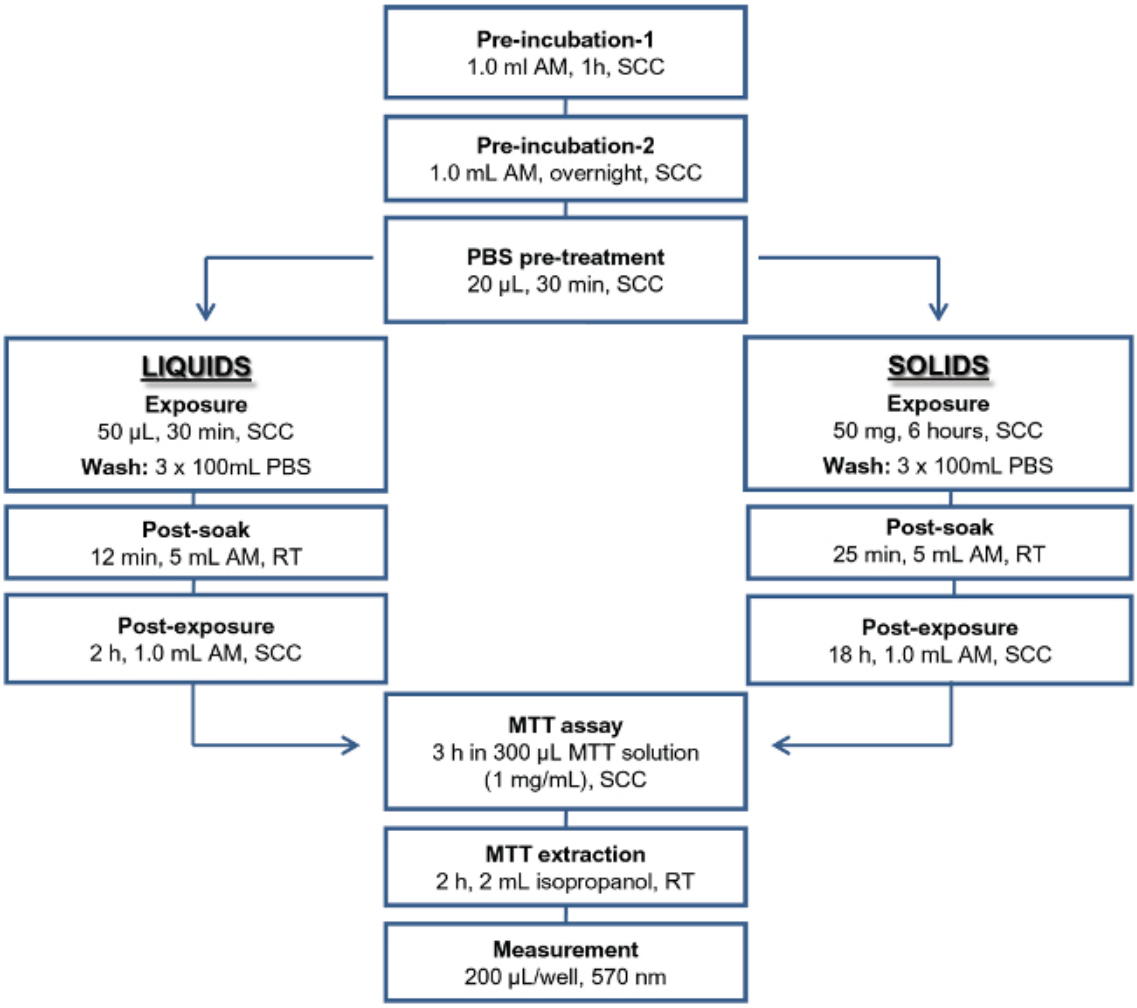

Figure 1: Outline of the EIT protocol for liquid and solid test articles. Abbreviations used: AM, assay medium; SCC, standard culture conditions; PBS, Dulbecco's Phosphate Buffered Saline; RT, room temperature.

	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	

Figure 2: The standardized 96-well plate configuration for the MTT tissue viability test. Two 200 µl aliquots are transferred to the appropriate wells of a pre-labeled 96-well plate. Abbreviations used: NC, negative control; PC, positive control; TA1-TA20, test articles 1-20; Blank, extractant solution. 96- MTT plate configuration is used with Excel spreadsheet designed to calculate RhCE tissue viability and EIT results.

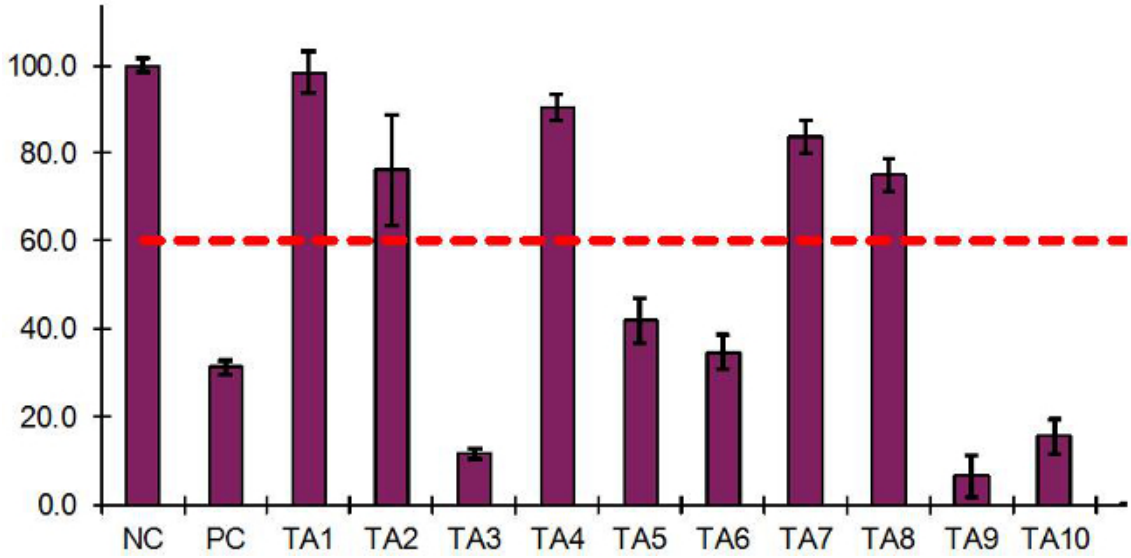

Figure 3: EIT results obtained for 10 test articles, NC and PC controls using the RhCE tissue model. The graph is generated from an Excel spreadsheet designed to present EIT results. Test chemicals which reduced tissue viability ≤ 60.0% relative to NC are classified as irritants ("I", TA3, TA5, TA6, TA9, and TA10) and test chemicals which had tissue viability >60.0% are classified as non-Irritants ("NI", TA1, TA2, TA4, TA7, and TA8).

Start time for the EIT protocol work steps (one day's work for one operator)							
Each line corresponds to one pair of tissues							
Order of steps:	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Liquids:	**PBS**	**TA Exposure**	**Post-Soak**	**Post-Incub.**	**MTT Reaction**	**MTT Extraction**	**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	

Table 3: Sample time schedule for testing of liquid test articles. Protocol steps including pre-wetting the tissues with DPBS, Application of Test Articles (TAs), Rinsing and Post-soak, Post-incubation period, MTT assay, Extraction of MTT, and Measurement of MTT OD are presented in columns. Times for the duplicate tissues are organized in rows. The entire assay testing 10 TAs and controls can be finished in one day.

Start time for the EIT protocol steps (2 days work for one operator)							
Each line corresponds to one pair of tissues							
Day 1					Day 2 (next day)		
Order of steps:	**1**	**2**	**3**	**4**	**5**	**6**	**7**

Solids:	PBS	TA Exposure	Post-Soak	Post-Incubaction	MTT Reaction	MTT Extraction	Measure
	30 min	6 hr	26 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	

Table 4: Sample time schedule for testing of solid test articles. Protocol steps including pre-wetting the tissues with DPBS, Application of Test Articles, Rinsing and Post-soak, Post-incubation period, MTT assay, Extraction of MTT, and Measurement of MTT OD are presented in columns. Times for the duplicate tissues are organized in rows. The entire assay testing 10 TAs and controls is performed over a two day period.

Amount	Reagent	Storage Conditions	Source	Description	Expiration Date
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

Table 5: MTT-100 Assay Kit Components.

Code N°	Tissue	Raw data		Blank corrected data		mean of OD	% of viability
	n	Aliq. 1	Aliq. 2	Aliq. 1	Aliq. 2		
NC	1	1.316	1.352	1.316	1.352	1.334	101.6
	2	1.277	1.309	1.277	1.309	1.293	98.4
PC	1	0.379	0.397	0.379	0.397	0.388	29.6
	2	0.419	0.442	0.419	0.442	0.431	32.8
TA1	1	1.213	1.244	1.213	1.244	1.229	93.5
	2	1.355	1.355	1.355	1.355	1.355	103.2
TA2	1	1.210	1.122	1.210	1.122	1.166	88.7
	2	0.828	0.837	0.828	0.837	0.833	63.4
TA3	1	0.167	0.168	0.167	0.168	0.167	12.7
	2	0.138	0.136	0.138	0.136	0.137	10.4
TA4	1	1.137	1.160	1.137	1.160	1.149	87.4
	2	1.262	1.191	1.262	1.191	1.227	93.4
TA5	1	0.610	0.621	0.610	0.621	0.616	46.9
	2	0.480	0.484	0.480	0.484	0.482	36.7
TA6	1	0.502	0.513	0.502	0.513	0.508	38.7
	2	0.396	0.407	0.396	0.407	0.402	30.6
TA7	1	1.048	1.050	1.048	1.050	1.049	79.9
	2	1.149	1.150	1.149	1.150	1.150	87.5

TA8	1	1.032	1.034	1.032	1.034	1.033	78.7
	2	0.941	0.935	0.941	0.935	0.938	71.4
TA9	1	0.022	0.022	0.022	0.022	0.022	1.7
	2	0.144	0.149	0.144	0.149	0.147	11.2
TA10	1	0.150	0.150	0.150	0.150	0.150	11.4
	2	0.254	0.255	0.254	0.255	0.255	19.4

	mean	Dif.	mean of	Dif.	Dif./2	Classification	
	of OD	of OD	viabilities [%]	of viabilities			
NC	1.314	0.041	100.0	3.12	1.56	NI	qualified
PC	0.410	0.043	31.2	3.23	1.62	I	qualified
TA1	1.292	0.127	98.3	9.63	4.81	NI	qualified
TA2	0.999	0.333	76.1	25.36	12.68	NI	D>20
TA3	0.152	0.030	11.6	2.32	1.16	I	qualified
TA4	1.188	0.078	90.4	5.94	2.97	NI	qualified
TA5	0.549	0.134	41.8	10.16	5.08	I	qualified
TA6	0.455	0.106	34.6	8.07	4.03	I	qualified
TA7	1.100	0.101	83.7	7.65	3.82	NI	qualified
TA8	0.986	0.095	75.0	7.23	3.62	NI	qualified
TA9	0.085	0.125	6.4	9.48	4.74	I	qualified
TA10	0.203	0.105	15.4	7.95	3.98	I	qualified

Table 6: EIT results obtained for 10 test articles, NC and PC controls. The tables are produced by an Excel spreadsheet designed to calculate tissue viability and EIT results. Test chemicals which reduced tissue viability ≤ 60.0% relative to NC are classified as irritants ("I", TA3, TA5, TA6, TA9, and TA10) and test chemicals that had tissue viability >60.0% are classified as non-Irritants ("NI", TA1, TA2, TA4, TA7, and TA8).

Discussion

We have presented the Eye Irritation Test (**Figure 1**) that was developed for the EpiOcular tissue model. The EIT is able to separate ocular irritants and corrosives (GHS Categories 1 and 2 combined) from materials that do not require labeling (GHS No Category) with high degree of sensitivity and specificity[17]. The EIT as presented herein does not discriminate between GHS Category 1 from Category 2 chemicals. The EIT was validated for classification and labeling of ocular irritation potential of a wide range of chemicals, including cosmetic and pharmaceutical ingredients. In conjunction with other *in vitro* tests, the EIT will serve as a replacement for the in vivo rabbit eye irritation test.

The EIT uses two similar but distinct protocols for liquid and solid materials, which vary in the length of exposure and post-exposure incubation periods (**Figure 1**). The endpoint used in the EIT is tissue viability, determined by the MTT assay, which has been previously used in validated human epithelial tissue models[20,21]. To perform this assay, no special equipment besides standard cell culture equipment is needed. Due to the high level of tissue-to-tissue reproducibility, n = 2 tissues instead of the usually recommended n = 3 are used. The ability to use n= 2 tissues is a critical aspect of the protocol, since it allows an experienced operator to process two tissues simultaneously, thereby minimizing the variability of the assay that can arise due to the different handling of individual tissues[14]. Also, by using n = 2 tissues per test article, the irritancy of 10 test substances of the same physical state (liquid or solid), along with the positive and negative controls, can be evaluated using one kit (24 tissues).

Other key points that ensure reliable classification of materials are specifications for the positive control substance (tissue viability ≤50.0%), reproducibility between duplicate tissues (difference <20.0%), and negative control OD readings (>0.8 and <2.5).

When performing the EIT test, it is important to adhere to the validated protocol and to the suggested dosing and rinsing schedules (**Tables 3** and **4**), since deviation from the protocol or changes in the incubation periods may result in altered outcome. Likewise, deviations from the 3 hr time for MTT incubation will result in different MTT readings and may affect assay result.

Occasionally, a test chemical may have optical or other properties which may interfere with MTT tissue viability assay or cause reduction of MTT. For instance, a test chemical may directly reduce MTT into blue-purple reaction product, or may be a colored substance that absorbs light in the same range as MTT formazan (~570 nm). However, these test chemicals will present a problem only, if at the time of the MTT assay, a sufficient amount of the material is still present on (or absorbed by) the tissue. To avoid this interference, extensive rinsing procedures are incorporated into the EIT protocol. If rinsing does not remove the TA and the TA interferes with MTT reduction, additional controls must be used to detect and correct for it. Briefly, if direct MTT reduction of the test chemical is suspected, 50 µl (or 50 mg for solids) of the chemical in question is incubated for 3 hr with working MTT solution at SCC (NC, 50 µl of sterile deionized water, should be run concurrently). If the MTT solution turns blue-purple, the test article is presumed to have reduced the MTT. In this case a functional check using freeze-killed tissue controls should be performed to evaluate whether the test material is binding to the tissue and leading to a false MTT reduction signal. If there is appreciable

MTT reduction in the TA-exposed, killed tissue control (relative to the amount in the untreated viable tissue), the mean tissue viability of the test article must be corrected by subtracting the mean viability of the killed control.

The EIT errs on the side of safety, as demonstrated by the low incidence of false negative classifications[14,15,18.] Importantly, none of the GHS Category 1 chemicals, which are corrosive to the eye and which represent the most serious ocular hazard, were classified as non-irritating in this assay[14,15,18,19]. Finally, one of the major advantages of the RhCE *in vitro* test method is the possibility of testing neat liquid and solid materials (which is not possible with two-dimensional, submerged cell cultures).

The EIT will contribute significantly in determining the ocular irritation potential of a wide range of materials according to the UN GHS classification and labeling system. The replacement of animals to determine ocular toxicity has been a goal of toxicological research for many years. The EIT test method has completed a formal validation study supported by EURL ECVAM in 2014 and the EpiOcular EIT was implemented into the OECD test Guidelines as OECD TG 492 in 2015.

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