MatTek CORPORATION

Protocol

Ocular Irritation: Dilution Method

For Use with EpiOcular™ Tissue Model (OCL-200)

Overview: This protocol utilizes the MTT tissue viability assay to determine exposure time for a test article to reduce the viability to 50% of control tissues (ET-50). Based on the ET-50, an approximation of the Draize Rabbit Eye Score (MMAS) can be computed. This protocol is applicable to water-soluble materials with a specific gravity of ≥ 0.95 and requires an initial dilution of the test article to 20% in water. For non-water soluble materials or for exposing test articles undiluted, the "Ocular Irritation Protocol – Neat Method" should be used.

I. Storage of EpiOcular (OCL-200) and MTT Kit (MTT-100) Components

a) **Storage:** Upon receipt of the EpiOcular Tissue Model, place the sealed 24-well plate containing the EpiOcular tissue samples and the assay medium into the refrigerator (2-8^{o}C). If you have ordered the MTT toxicology kit (part # MTT-100) or the MTT diluent solution (part # MTT-100-DIL), place the vial containing MTT concentrate in the freezer (-20±5^{o}C) and the MTT diluent in the refrigerator. Storage conditions are summarized in the following table.

Part #	Description	Conditions	Shelf Life
OCL-200	EpiOcular samples	Refrigerate (2-8^{o}C)	72 hours*
OCL-200-ASY	Assay Medium	Refrigerate (2-8^{o}C)	7 days
MTT-100-DIL	MTT diluent	Refrigerate (2-8^{o}C)	2 months*
MTT-100-CON	MTT concentrate	Freezer (-20±5^{o}C)	2 months*

*Note: *Refers to storage time @ 2-8^{o}C in unopened package.*
***MTT kits must be ordered separately.*

II. Preparation of EpiOcular

a) **Pre-warm media:** Pre-warm the MatTek assay medium (provided) to 37^{o}C. Using sterile technique, pipet 0.9 ml of the assay medium into each well of the sterile 6-well plates (provided). Label the 6-well plates indicating the test material and the dosing time to be used.

b) **Transfer EpiOcular samples:** 1 hour before dosing is to begin, remove the package containing the tissue samples from the refrigerator. Under sterile conditions, open the package and using sterile forceps, remove the inserts containing the tissues from the agarose of the package while the package is still at refrigerator temperature. Transfer the inserts into the 6-well plates containing the pre-warmed assay medium. *Note: Removal of the inserts while cold will minimize the difficulty of separating the inserts from the agarose and will minimize the amount of agarose that adheres to the inserts. Care should be taken to remove all agarose sticking to the outside of the cell culture inserts containing the tissue samples.*

c) **Partial kit testing:** If all 24 tissues are not needed on day 1 of testing, carefully open the plastic bag containing the 24-well plate/EpiOcular tissues and remove the tissues for day 1 testing under sterile conditions as per the previous step. Return the cover to the 24-well plate containing the remaining tissues and put the 24-well plate back in the original bag without sealing it (a new plastic bag, which can be later sealed, can also be used). Place the 24-well plate in the open plastic bag into the incubator at 37^{o}C and 5% CO_2. Allow the atmosphere within the bag to re-equilibrate with 5% CO_2 for 10 minutes. Prior to removing the bag from the incubator, re-seal the bag using tape so that the 5% CO_2 atmosphere will be maintained. Return the sealed bag to the refrigerator (2-8^{o}C) where it can be stored for an additional 24 hours.

d) **Incubate:** Place the 6-well plates containing the EpiOcular samples into a humidified 37°C, 5% CO_2 incubator for 1 hour prior to dosing.

III. Dosing

a) **Density determination:** In order to use results from EpiOcular to predict an approximate Draize rabbit eye score, it is necessary to dilute to 20% all materials with a density of ≥ 0.95. The density is easily determined by pipetting 100 µl of the material into a beaker which has been tared on an analytical balance and dividing the weight by the weight of 100 µl of ultrapure H_2O pipetted using the same pipette. The material and the ultrapure H_2O should be equilibrated to room temperature.

b) **Dilution of test material:** If the density is ≥ 0.95 g/ml, dilute the material to 20% by making a 4:1 dilution in ultrapure H_2O. If the materials density is < 0.95 g/ml, apply the material neat. Viscous materials should be pipetted with a positive displacement pipette. If the material is not water soluble, the OCL-200 Neat Method Protocol should be used.

c) **Exposure times:** An initial time range finding exposure time of 16 minutes dosed in triplicate or duplicate samples of EpiOcular is recommended for neat or diluted test materials. Duplicate tissues can also be used once a user has experience using the EpiOcular tissue protocols.

d) **Negative controls:** 3 (or 2 if running the assay using duplicate cultures) inserts are dosed with 100 µL of deionized or ultrapure water to serve as a negative control. All manipulations with these negative controls should be identical to those inserts which are exposed to test materials. Exposure time for the negative controls is 60 minutes.

e) **Replace assay medium:** Following the 1 hour incubation, aspirate off the assay media contained within the 6-well plates and replace with 0.9 ml (per well) of pre-warmed, fresh assay media. *Note: Any air bubbles trapped underneath the Cell culture insert should be released (tilt the Cell culture insert with a sterile forceps) so that adequate nutrients are supplied to the EpiOcular samples during the dosing period.*

f) **Apply dose:** Pipet 100 µl of test material into the Cell culture insert atop the EpiOcular sample. Do not add the test material to the assay medium in the well. Negative controls should be treated in an identical manner to the dosed inserts. See Figure 1.

g) **Non-pipettable materials:** For materials that cannot be pipetted, applicator pins should be used to provide a reproducible even means of application. For solids, a 1:1 slurry/paste of material and ultrapure H_2O is made and 200 mg of the slurry is applied using the applicator pin. *Note: Different solid/H_2O ratios can be used to improve the consistency of the slurry/paste; however, the amount of slurry applied to the applicator pin should be adjusted so that 100 mg of the original material are applied.*

h) **Exposure time:** Return the 6-well plates containing the dosed EpiOcular samples to the incubator for the desired time period (16 minutes for initial testing with unknown material).

i) **Prepare MTT solution:** Prior to the end of the first dosing period, prepare the MTT solution. If you are using the MatTek MTT toxicology kit (Part # MTT-100), thaw the MTT concentrate and dilute with the MTT diluent (provided). If you are making your own MTT solution, use 1 mg/ml MTT diluted in DMEM. Spin down (300 g for 5 minutes) or filter the MTT solution to remove any precipitate. Store the remaining MTT solution in the dark at 2-8°C for the later time points. *Note: To obtain optimal results, MTT solutions should not be stored for more than 1 day since MTT will degrade with time.*

j) **Prepare MTT plate:** Prepare a 24-well plate (provided) with MTT solution by pipetting 300 µl of the MTT solution into the appropriate number of wells of the 24-well plate to accommodate all the inserts for the time period which is ending. Label the 24-well plate top to indicate to which wells the samples will be transferred. Label the second 24-well plate in an identical manner for later use in the extraction step. Also, label vials in which media samples will be stored if LDH or inflammatory mediator release measurements are to be made.

k) **Reduction of MTT by Test Article:** To insure that the MTT reaction is accurately measuring the tissue viability, it is necessary to determine whether the test article (TA) can directly reduce MTT. A 1.0 mg/ml MTT solution is prepared as above and 100 µl or 100 mg of the TA diluted to 20% in water are added to 1 ml of the MTT solution. This mixture is incubated in the dark at room temperature for 60 minutes. A negative control, 100 µl of ultrapure water, is tested concurrently. If the MTT solution color turns blue/purple, the TA has reduced the MTT; the absence of darkening indicates that the TA does not directly reduce MTT.

If the TA reduces MTT, a false viability measurement is obtained only if the TA remains bound to the tissue after rinsing. To test for residual TA induced MTT reduction, a single killed tissue (part # OCL-200-Frozen) is treated with the TA in the normal fashion. The incubation should be the longest incubation used for that TA. Rinsing and MTT exposure should be performed normally. An untreated killed control will be 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 TA treated killed control, the MTT reduction observed in the TA 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 TA may be considered untestable with MTT. If the direct reduction of MTT by the TA is less than 30% of the negative control value, the net OD of the treated killed control may be subtracted from the new ODs of the live TA-treated tissues. If the direct reduction by the TA is greater than 30% of the negative control value, please contact MatTek technical assistance for further guidance.

l) **Removal of test material:** After exposure of the EpiOcular samples to the test materials is complete, discard any liquid remaining a top the EpiOcular tissues. Next submerge each insert individually to fill it with PBS and then decant. Repeat this procedure 3 times or as many times as necessary to remove any residual test material. Following the 3 rinses submerge the cell culture insert containing tissue in 5.0 ml of assay media for 10 minutes at 37°C and 5% CO_2. See Figure 2. This final soak removes any residual absorbed test material and is crucial to obtaining accurate, repeatable dose response curves.

m) **Transfer samples to MTT plate:** After the 10 minute soak, decant and shake off the assay medium prior to placing the EpiOcular sample in the MTT containing 24-well plate. When the Cell culture inserts have been transferred to the MTT plate, make sure that no air bubbles are trapped underneath the Cell culture insert.

n) **Media for inflammatory mediator analysis:** Save the assay media from the 6-well plates in the labeled vials for subsequent LDH, PGE-2, IL-1α or other inflammatory mediator/cytokine analysis. Samples for LDH should be tested immediately or stored at 2-8°C for a maximum of 24 hours; samples for IL-1α should be stored at (2-8°C) for a maximum of 7 days; samples to be assayed for PGE-2 should be stored under nitrogen and frozen.

o) **MTT loading:** Return the EpiOcular samples in the 24-well plate to the incubator for 3 hours. See Figure 3. Note: Deviations from the 3 hour time for MTT incubation will result in different MTT readings and thus the 3 hour MTT incubation time should be adhered to very strictly.

IV. Extraction

a) **Transfer tissue samples to extraction plate:** After the 3 hour MTT incubation period is complete, remove each insert individually and gently rinse with PBS to remove any residual MTT solution. Remove excess PBS by gently shaking the insert and then blotting the bottom with a Kimwipe. Finally, place the inserts into the pre-labeled 24 well extraction plate.

b) **Add extractant:** Immerse the inserts using 2.0 ml of extractant solution per well, completely covering the EpiOcular sample. See Figure 4. Cover the extraction plate to reduce evaporation of extractant. *Note: If the test article is colored and does not completely rinse off, pipet 1.0 ml into the well so that the MTT is extracted through the bottom of the tissue culture insert. After extraction is complete, remove the insert and add an additional 1.0 ml of extractant to bring the total volume to 2.0 ml.*

c) **Extraction conditions:** Place the extraction plate with its top in place into a sealable plastic bag (e.g. Zip-lock) to minimize extractant evaporation. Allow the extraction to proceed for 2 hours at room temperature (RT) on an orbital shaker or overnight (without shaking) at RT in the dark. Protect the plate from light while shaking using aluminum foil. Shaking should be vigorous enough for some mixing within the wells, but not too vigorous such that liquid will leave the wells. *Note: We recommend allowing the extractions to proceed until all samples have been extracted for 2 hours (with shaking) or overnight (without shaking) so that all MTT readings can be made at once.* As long as evaporation of solvent is prevented, extraction times beyond these times will not affect MTT readings. *Note: If you are using your own reagents, the extractant should* ***not*** *contain acid (e.g. hydrochloric acid).*

d) **Decant extractant into 24-well plate:** After the extraction period is complete, decant the liquid within each insert back into the well from which it was taken (i.e. mix the solution with the extractant in the well). The inserts can be discarded.

V. Determine additional dosing points and ET-50

a) **Mix extractant solutions:** Pipet the extractant solution up and down at least 3 times to insure that the extraction solutions are well mixed.

b) **Transfer to 96-well plate:** Pipet 200 µl of the mixed extraction solution into a 96 well microtiter plate. Note: if a 96-well plate reader is not available, a spectrophotometer can be used to determine optical density of the extractant solution.

c) **Measure optical density:** Determine the optical density of the extracted samples at a single wavelength between 540 and 570 nm using 200 µl of the extractant as a blank. *Note: Subtracting out a background reading for all samples at 650 nm improves the quality of the data.*

d) **Calculate % viability:** Determine the % viability at each of the dosed concentrations using the following formula:

% viability = 100 x [OD (sample)/OD (negative control)]

e) **Choose additional time exposures:** Based on the initial determination of percent viability after a 16 minute exposure to the unknown test material, use the following table to determine additional dosing times so that a dose response curve can be generated:

Viability after 16 min. exposure	Additional Time Points
> 90%	64 minutes, 256 minutes
< 90% but > 30%	4 minutes, 64 minutes
< 30%	1 minute, 4 minutes

Repeat the dosing/MTT procedure using the 2 additional time points given in the above table. Include an additional set of negative controls if the additional time exposures are done on the following day. Each timed exposure is dosed using duplicate EpiOcular samples (Note: Time points above are equally spaced on a semi-log plot – other time points can be used, however the time points above will increase the likelihood of obtaining the most accurate ET-50 determination).

f) **Determine ET-50:** Using a semi-log scale, plot the % viability (linear y axis) versus the dosing time (log x axis). By interpolation, the time at which the % viability has dropped to 50% is considered the ET-50 value. See Figure 5. *Notes: a) If the viability is less than 50% after a 1 minute exposure, the ET-50 is set to 1.0 minute; b) If the viability exceeds 50% after a 256 minute exposure, the ET-50 is set to 256 minutes; c) Interpolation for the ET-50 is best done mathematically.*

g) **Positive control:** 0.3% Triton X-100 is provided with the kit. The typical ET-50 falls between 15 and 45 minutes. See Figure 5. Recommended dosing times are 15 and 45 minutes along with 4 minutes if enough tissue samples are available. Alternatively, MatTek tests every lot of EpiOcular with 0.3% Triton X-100 – these data are available by Wednesday each week.

VI. Correlation of In Vitro and In Vivo Results

a) **Benchmark ET-50 values and groupings:** Provided that the density determination/dilution procedure in III. a-b has been adhered to, as a general guideline, the following equation can be used to estimate the rabbit Draize eye score:

*Draize (*MMAS*) = -4.74+101.7/ (ET-50)^0.5* where the ET_{50} is expressed as $1 \leq ET_{50} \leq 256$ minutes.

Based on the literature (Kay, J.H. and Calandra, J.C., "Interpretation of eye irritation tests", **J. Soc. Cosmetic Chem**., 13, 281-289 (1962), the ocular irritancy can be categorized into the following groups based on the Draize score as given in Table 1.

A number of control materials have been tested using this protocol as given on the MatTek website at: http://www.mattek.com/pages/products/epiocular.

Table 1: Irritation Category Based on Draize Score and ET-50

Draize Score	Irritancy Classification	Example	EpiOcular ET-50 (min)
0-15	Non-irritating, Minimal	PEG-75 Lanolin, Tween 20	>256-26.5
15.1-25	Mild	3% Sodium dodecyl sulfate	<26.5-11.7
25.1-50	Moderate	5% Triton X-100	<11.7-3.45
50.1-110	Severe, Extreme	5% Benzalkonium chloride	<3.45

Note: Depending on the specific type of materials, the equation and the irritancy classifications may need to be adjusted. If your materials do not fit this correlation, MatTek scientists are always willing to assist you in developing a correlation which is accurate for your materials.

b) **Developing your own correlation:** Depending on the type of materials, it may be necessary to modify the above equation or develop your own in vitro/in vivo correlation. Choose materials which cover a broad range of known animal or human ocular irritation responses so that all unknowns will fall within the range of the correlation.

VII. Materials Provided

EpiOcular (Part No. OCL-200)

Quantity	Description	Part No.
24	EpiOcular tissue samples	OCL-200
4	6-well plates (sterile)	MW-15-003-0027
2	12-well plates (sterile	MW-15-003-0030
2	24-well plates (sterile)	MW-15-003-0028*
1	PBS rinse solution, 100 ml	TC-PBS
1	Assay medium, 200 ml	OCL-200-ASY
1	0.3% Triton X-100 solution, 10 ml	TC-TRI-0.3%
1	OCL-200 protocol	MK-24-007-0002

* Shipped only if MTT-100 is ordered.

VIII. Optional Materials

MTT Assay Kit (Part No. MTT-100)

Quantity	Description	Part No.
1	MTT diluent solution, 8 ml	MTT-100-DIL
1	Extractant solution, 60 ml	MTT-100-EXT
1	MTT concentrate (5:1), 2 ml	MTT-100-CON

Additional Materials

Quantity	Description	Part No.
24	Applicator pins	EPI-PIN-24

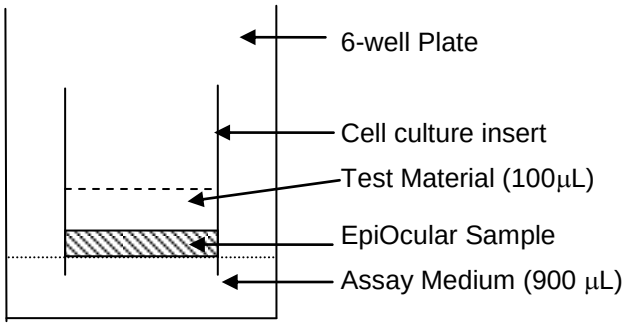

Figure 1: Dosing Configuration (37°C, 95% rH, 5% CO_2)

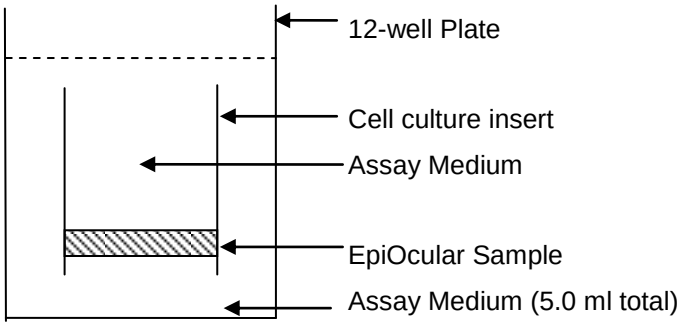

Figure 2: Configuration for Post Soak (37°C, 10 min.)

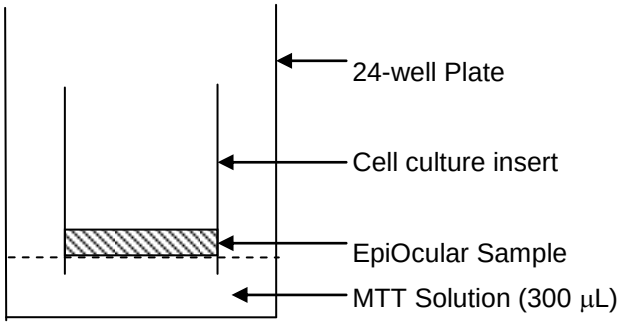

Figure 3: Incubation in the MTT solution (37°C, 95% rH, 3 hours)

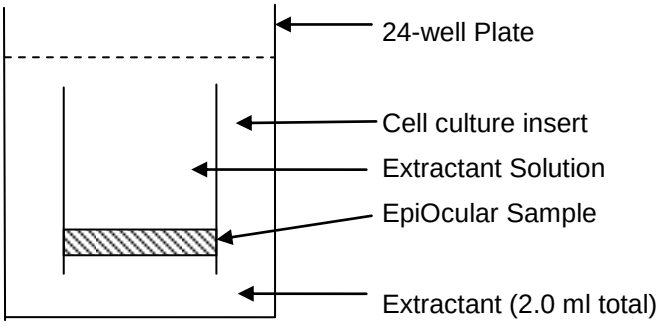

Figure 4: Extraction Configuration (room temp., in the dark)

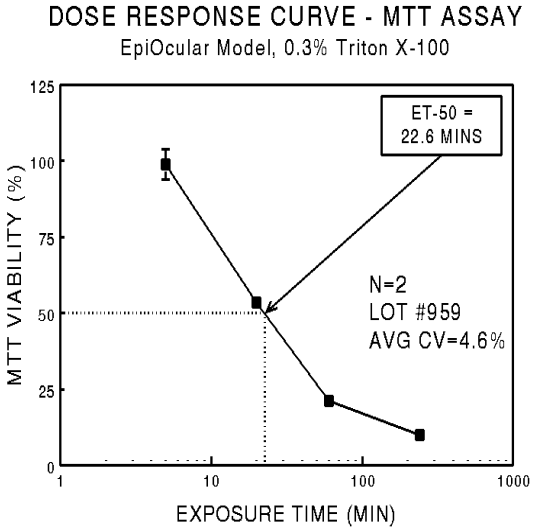

Figure 5: Typical dose response curve for EpiOcular tissues following exposure to the positive control, 0.3% Triton X-100.