MatTek LIFE SCIENCES

Protocol

MTT Effective Time 50 (ET-50)

For use with EpiDerm Skin Model (EPI-200)

I. Storage of EpiDerm (EPI-200) and MTT Kit (MTT-100)

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

Part #	Description	Conditions	Shelf Life
EPI-200	EpiDerm cultures	Refrigerate (2-8°C)	96 hours*
EPI-100-ASY	Assay Medium	Refrigerate (2-8°C)	7 days
EPI-100-MM	Maintenance Medium	Refrigerate (2-8°C)	7 days
MTT-100-DIL	MTT diluent	Refrigerate (2-8°C)	2 months**
MTT-100-CON	MTT concentrate	Freeze (-20 ±5°C)	2 months**

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

II. Preparation of EpiDerm

a) **Pre-warm media:** Pre-warm the MatTek assay medium (provided) to 37°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 EpiDerm 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/EpiDerm tissues and remove the tissues for day 1 testing under sterile conditions. 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°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°C) where it can be stored for an additional 24 hours.
d) **Incubate:** Place the 6-well plates containing the EpiDerm samples into a humidified 37°C, 5% CO_2 incubator for 1 hour prior to dosing. *Note: Some laboratories have found that an overnight incubation ("pre-equilibration") is preferable, especially if cytokine release is to be measured. This allows the tissues to more fully recover from the stress of shipping. In any case, a standardized pre-equilibration time should be used.*

e) **Reduction of MTT by test articles:** *To ensure 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 neat TA is added to 1 mL of the MTT solution. This mixture is incubated in the dark at room temperature for 60 minutes.*
f) A negative control, 100µL of ultrapure water, is tested concurrently. If the MTT solution color turns blue/purple (or if a blue/purple precipitate is observed), the TA has reduced the MTT; the absence of darkening indicates that the TA does not directly reduce MTT.
g) 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 # EPI-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.

III. Dosing

a) **Exposure times:** Initial time range finding exposure times of 2, 5, and 18 hours dosed in duplicate samples of EpiDerm are recommended for neat test materials or for test materials to be tested at their final end use concentration.
b) **Negative controls:** If a neat test material is to be dosed, 2 inserts are left not dosed to serve as a negative control. It is sufficient to use the median dose time point for the negative controls (e.g. if test materials are exposed for 2, 5, and 18 hours, use 5 hours for the negative control).
c) **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 EpiDerm samples during the dosing period.*
d) **Apply dose (liquids):** Add 100 µL of test material into the cell culture insert atop the EpiDerm sample. Do not add the test material to the assay medium in the well. Negative controls (no dose, or diluent for diluted samples) should be treated in an identical manner to the dosed inserts. See **Figure 1**.

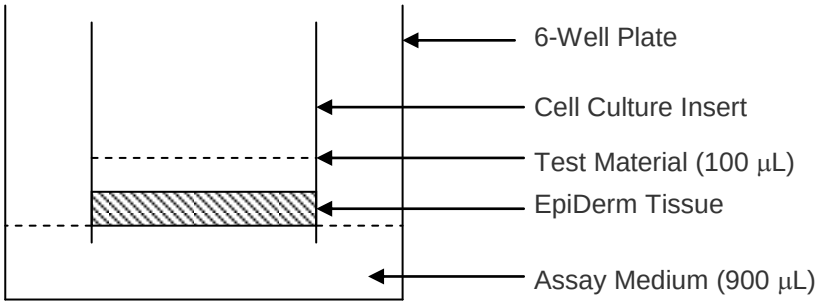

Figure 1: Dosing Configuration

e) **Apply dose (solids):** Crush and grind test material using a mortar with pestle. Shortly before application of the solid substance, moisten the tissue surface with 25 µL of sterile DPBS to improve contact of the tissue surface with the test chemical. Fill sharp application spoon with 100 mg finely ground test material. Level the

spoon by gently scratching the excess material away with an appropriate aid, avoiding compression ("packing") of the test material. Gently apply the 100 mg of the material to surface of the moistened tissue.

f) **Exposure time:** Return the 6-well plates containing the dosed EpiDerm samples to the incubator for the desired time periods.

g) **Prepare MTT solution:** Approximately 1 hour 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) the MTT solution to remove any precipitate present. Store the remaining MTT solution in the dark at 2-8°C for the later time points. *Note: To get optimal results, MTT solutions should not be stored for more than 1 day since MTT will degrade with time.*

h) **Prepare MTT plate:** 15 minutes before each dosing period is complete, prepare a 24-well plate (provided) with MTT solution. Pipet 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.

i) **Transfer samples to MTT plate:** After exposure of the EpiDerm samples to the test material(s) is complete, decant any liquid remaining atop the EpiDerm tissues. Remove each insert individually and gently rinse with PBS (provided) to remove any residual test material. Repeat this rinse a second time. Shake off excess liquid prior to placing the EpiDerm sample in the MTT containing 24-well plate making sure that no air bubbles are trapped underneath the cell culture insert.

j) **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 and IL-1α samples should be stored at 2-8°C; samples to be assayed for PGE-2 should be stored under nitrogen and frozen.

k) **MTT loading:** Return the EpiDerm samples in the 24-well plate to the incubator for 3 hours. See **Figure 2**. Note: Deviations from the 3 hour time for MTT incubation will result in different MTT readings thus the 3 hour MTT incubation time should be strictly adhered to.

l) **Positive control:** 1.0% Triton X-100 is provided with the kit. The typical ET-50 falls between 3.7 and 7.0 hours. See **Figure 4**. Recommended dosing times are 3 and 7 hours. Alternatively, MatTek tests every lot of EpiDerm with 1.0% Triton X-100 – these data are available by late Wednesday each week.

IV. Extraction

a) **Transfer samples to extraction plate:** After the 3 hour MTT incubation period is complete, remove each insert individually and gently blot the bottom with a KimWipe. Finally, place the inserts into the pre-labeled 24-well extraction plate.

b) **Add extractant:** Immerse the cell culture inserts with 2.0 mL of the extractant solution per well to completely cover the EpiDerm sample. See **Figure 3**. 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 of extractant into each 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.

Extraction conditions: Place the extraction plate with its top in place into a sealable plastic bag (e.g. Ziplock) 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 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). We recommend using only MatTek provided products (e.g. MTT-100).*

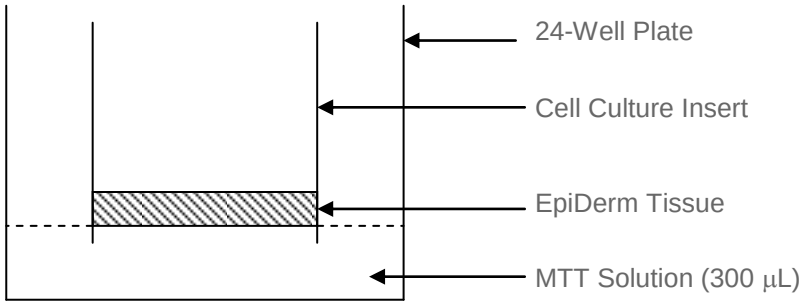

Figure 2: Incubation in MTT solution (37 °C, 95% rH, 3 hrs)

c) **Decant extractant in 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.

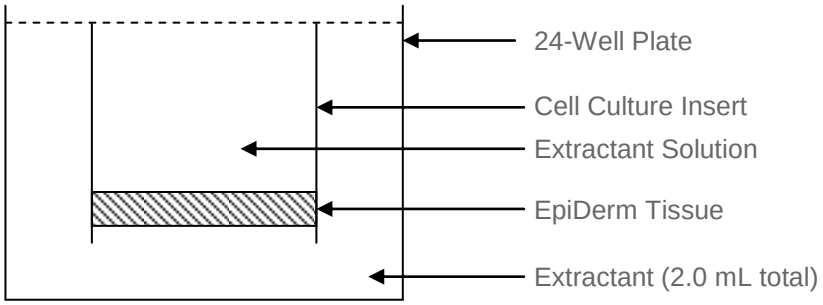

Figure 3: Extraction Configuration

V. Construction of Dose Response Curve

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, any visible 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 570 nm using 200 µL of extractant as a blank. *Note: Subtracting out a background reading for all samples at 650 nm improves the quality of the data; in addition, wavelengths between 540-570 nm can be used equally as well.*

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

$$\% \text{ viability} = 100 \times [OD(\text{sample})/OD(\text{negative control})]$$

e) **Construct dose response curve:** 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 "rough" ET-50 value. See **Figure 4**.

f) **Choose new times (if necessary):** Based on the time range finding plot, a more precise ET-50 can be obtained by choosing 3-4 dose times centered around the "rough" ET-50 value. Typically, times differing by 1-4 hours work nicely.

g) **Determination of "true" ET-50:** Repeat the procedure outlined above using a 3-4 different exposure times of the neat or end use concentration test material to determine a more precise ET-50 value. From the resulting dose response curve, a more precise ET-50 value can be determined as was done to determine the "rough" ET-50 value.

VI. Correlation of *In vitro* and *In vivo* Results

a) **Benchmark ET-50 values and groupings:** As a general guideline, the following groupings can be used in assigning expected *in vivo* irritancy responses based on the ET-50 results obtained using EPI-200:

ET-50 (hrs)	Expected *In vivo* Irritancy	Example
< 0.5	strong/severe, possible corrosive Conc.	Nitric acid
0.5-4	moderate	1% Sodium Dodecyl Sulfate
4-12	moderate to mild 1%	Triton X-100
12-24	very mild	Baby shampoo
24	non-irritating	10% Tween 20

Note: Depending on the specific type of materials being tested, these groupings may need to be adjusted. MatTek scientists are always willing to assist you in developing a correlation that is accurate for your materials.

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

c) **If *in vivo* and *in vitro* results do not correlate:** If *in vivo* and *in vitro* results do not appear to correlate, it is likely that your materials have different mechanisms by which they are causing *in vivo* irritation. Use of (an) additional *in vitro* endpoint(s) may be required. Again, MatTek scientists will be glad to assist you.

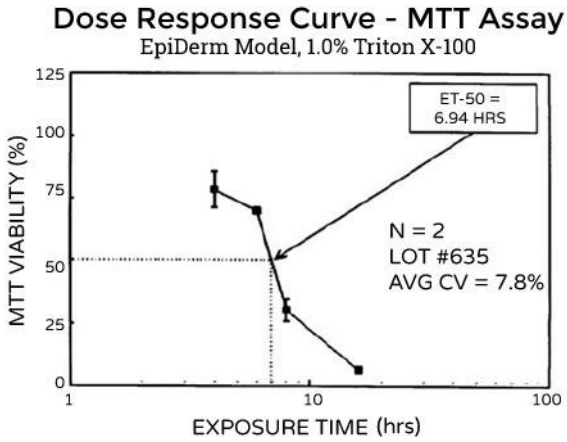

Figure 4: Graphical Depiction of ET-50 Determination - Typical Dose Response Curve for EpiDerm (EPI-200) Positive Control, 1.0% Triton X-100.

VII. Materials Provided

EpiDerm® (Part No. EPI-200)

Quantity	Description	Part No.
24	EpiDerm skin-model samples	EPI-200
4	6-well plates (sterile)	MW-15-003-0027
2	24-well plates (sterile)	MW-15-003-0028
1	PBS rinse solution, 100 mL	TC-PBS
1	Assay medium, 50 mL	EPI-100-ASY
1	1% Triton X-100 solution, 10 mL	TC-TRI-1.0%*
1	EPI-200 protocol	MK-24-007-0001

**Shipped only if MTT-100 has been 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.
1	Serum Free Maintenance Medium, 250 mL	EPI-100-NMM-3
12	H & E histology processing, EpiDerm (12 tissue minimum)	EPI-HIS
12	Unstained, deparaffinized slides (must be ordered w/ EPI-HIS)	EPI-HIS-UNST
1	12-well plate with hanging-top (2 plates needed/kit of EPI-200)	HNG-TOP-12
48	Stainless steel washers	EPI-WSHR
1	Culture Stand (24 stands needed/kit of EPI-200)	MEL-STND
6	EpiDerm frozen tissues (minimum order: 6 tissues)	EPI-200-FRZN-EA