MatTek LIFE SCIENCES

Protocol

MTT EFFECTIVE TIME-50 (ET-50) PROTOCOL For use with EpiOral™ (ORL-200) or EpiGingival (GIN-100) Tissue Models

I. Storage of Tissue (ORL-200 or GIN-100) and MTT Kit (MTT-100)

a) **Storage**: Upon receipt of the EpiOral or EpiGingival Tissue Model, place the sealed 24-well plate containing the tissues 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
ORL-200	EpiOral cultures	Refrigerate (2-8°C)	72 hours*
ORL-200-ASY	Assay Medium	Refrigerate (2-8°C)	7 days
ORL-200-MM	Maintenance Medium	Refrigerate (2-8°C)	7 days
GIN-100	EpiGingival cultures	Refrigerate (2-8°C)	72 hours*
GIN-100-ASY	Assay Medium	Refrigerate (2-8°C)	7 days
GIN-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: *Note: As early as possible after receipt, preferably on the same day they are received, the tissue should be returned to culture at 37°C and 5% CO_2, as described in Section II.

II. Preparation of EpiOral

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 tissues:** 1 hour before dosing is to begin, remove the 24-well plate containing the tissues from the refrigerator. Under sterile conditions using sterile forceps, transfer the cell culture inserts into the 6-well plates containing the pre-warmed assay medium. Note: Care should be taken to remove all adherent agarose sticking to the outside of the cell culture inserts.

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/ 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, e.g. a ZiplockTM bag, 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 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 tissue 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 will 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.

III. Dosing

a) **Exposure times:** Initial exposure times of 20, 60, and 120 minutes dosed in duplicate EpiOral tissues are recommended. Longer times should be used for very mild materials (e.g. 1, 4, and 18 hrs). For EpiGingival, time range finding dose times of 1, 4, and 18 hours are recommended. *Note: For experiments in excess of 24 hours, the use of MatTek 12-well plates with a hanging top (2 X HNG-TOP-12) or culture stands (part # MEL-STND) and 5.0 mL of media underneath the cultures are required to maintain good tissue morphology and function. Refer to MatTek protocol, MK-24-007-0026 "Extended Culture Times: Use of Hanging Top Plates, Culture Stands, and Washers" which describes the use of Hanging Top plates and Culture Stands in more detail.*

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 20, 60, and 240 minutes, use 60 minutes 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 tissue samples during the dosing period.

d) **Apply dose:** We recommend diluting all test articles 1:1 in ultrapure H_2O. Topically apply 40 µL of the 1:1 diluted solution into the cell culture insert atop the EpiOral sample; for EpiGingival, use 100 µL doses of the undiluted test article atop the tissue. Do not add the test material to the assay medium in the well unless you want to model systemic exposure. Negative controls (no dose, or diluent for diluted samples) should be treated in an identical manner to the dosed tissues. See **Figure 1.**

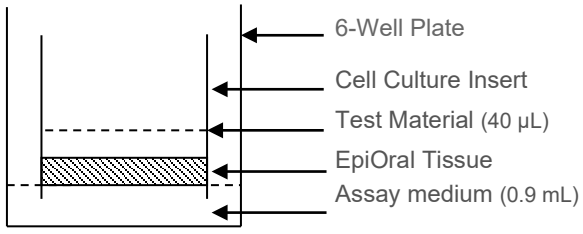

Figure 1a: EpiOral Dosing Conditions (40 µL dose diluted 1:1, 37ºC, 95% rH, 5% CO_2)

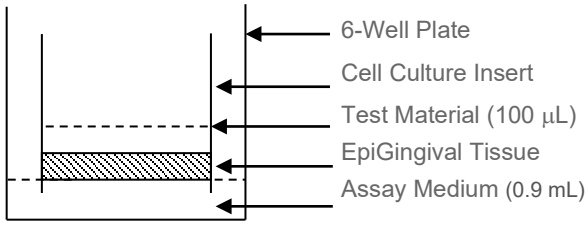

Figure 1b: EpiGingival Dosing Conditions (100 µL dose undiluted, 37ºC, 95% rH, 5% CO_2)

e) **Exposure times:** Return the 6-well plates containing the dosed tissues to the incubator for the desired time periods.

f) **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. 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.

g) **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.

h) **Transfer samples to MTT plate:** After exposure of the tissue samples to the test material(s) is complete, decant any liquid remaining within the cell culture insert. 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 insert in the MTT containing 24-well plate making sure that no air bubbles are trapped underneath the cell culture insert.

i) **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, IL-1β 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. See: Klausner, et. al, "Organotypic human oral tissue models for toxicological studies," Toxicology In Vitro, 21, 938-949, 2007. IL-1α and IL-1β release from the ORL-200 tissue was used to predict oral mucosal irritation.

j) **MTT loading:** Return the tissue 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.

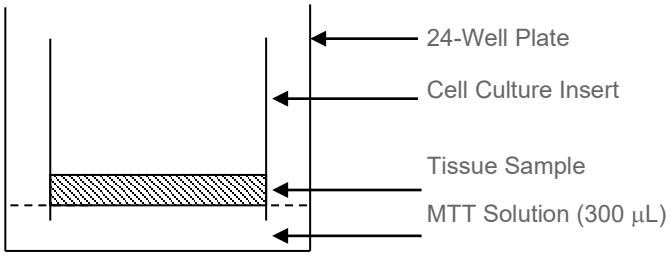

Figure 2: Incubation in the MTT Solution (37°C, 95% rH, 3 hrs, 5% CO_2

k) **Positive control:** 1.0% Triton X-100 is provided with the kit. For EpiOral, the acceptance criteria is 34.8 < ET-50 < 105.8 minutes; for EpiGingival, the acceptance criteria is 5.5 < ET-50 < 10.4 hrs. **See Figure 4.** Recommended positive control exposure times for EpiOral are 30 and 120 minutes; recommended dosing times for EpiGingival are 5.0 and 10.5 hrs. Alternatively, MatTek tests every lot of tissue with 1.0% Triton X-100 – these data are available by Thursday morning each week. Note: For positive control testing, 100 µl of undiluted 1% Triton X-100 (as supplied) should be applied.

IV. Extraction

e) **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.

f) **Add extractant:** Immerse the cell culture inserts with 2.0 mL of the extractant solution per well to completely cover the cell culture insert. 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.

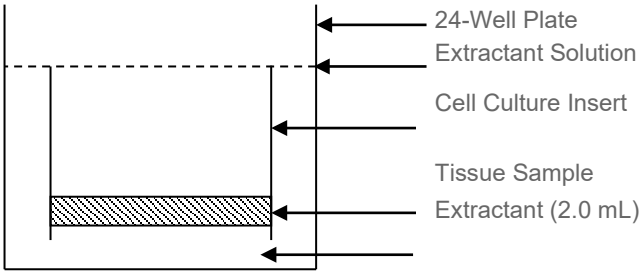

Figure 3: Extraction Configuration (room temperature in dark)

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. Construction of Dose Response Curve

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

e) **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.

f) **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 can be used equally as well.

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) **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, additional time points may be necessary or desirable. Please contact MatTek personnel for assistance.

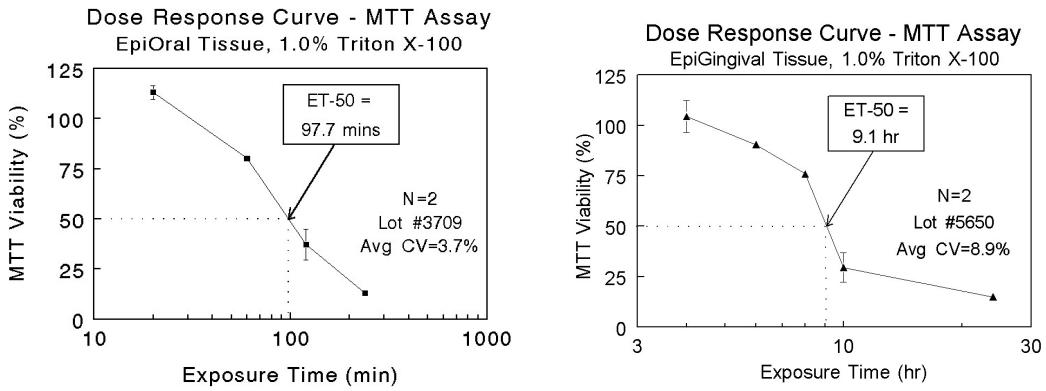

Figure 4: Graphical depiction of ET-50 determination -Typical dose response curves for the: A) EpiOral (ORL-200) and B) EpiGingival (GIN-100) tissue models. Positive Control = 1.0%Triton X-100. Dose = 100 µL

VI. Correlation of In Vitro and In Vivo Results

a) **Benchmark ET-50 values:** The effect of SDS in the concentration range commonly found in dentifrice materials on the ET-50 of the ORL-200 and GIN-100 tissue is shown in **Table 1**.

Initial SDS Conc. (%)	ET-50 (min)	
	ORL-200*	GIN-100**
0.75	>240	179
1.5	79	116
2.25	41	88
3.0	36	---
* ORL-200: solution diluted 1:1. Dose = 40 µL ** GIN-100: neat solution. Dose = 100 µL		

Table 1: Effect of sodium dodecyl sulfate (SDS) on the MTT ET-50 of ORL-200 and GIN-100 tissues at concentrations typically found in toothpastes. For ORL-200, SDS samples were diluted 1:1 in ultrapure water.

b) **Use of benchmark materials of known in vivo irritancy:** It is always best to compare your assay results to those of benchmark materials. A benchmark could be a material that is recognized as an industry leader or one for which clinical or customer responses are well known. By comparing the test results to those of the benchmark, one can gauge how the irritancy level of the test material will compare to that of the benchmark. MatTek scientists will be glad to assist you in picking appropriate benchmarks.

VII. Materials Provided

EpiOral (Part No. ORL-200)

Quantity	Description	Part No.
24	EpiOral tissues	ORL-200
4	6-well plates (sterile)	MW-15-003-0027
2	24-well plates (sterile)	MW-15-003-0028
1	PBS rinse solution, 125 mL	TC-PBS
1	Assay medium, 60 mL	ORL-200-ASY
1	1% Triton X-100 solution, 10 mL	TC-TRI-1%
1	MTT protocol	MK-24-007-0003

EpiOral (Part No. GIN-100)

Quantity	Description	Part No.
24	EpiGingival tissues	GIN-100
4	6-well plates (sterile)	MW-15-003-0027
2	24-well plates (sterile)	MW-15-003-0028
1	PBS rinse solution, 125 mL	TC-PBS
1	Assay medium, 60 mL	GIN-100-ASY
1	1% Triton X-100 solution, 10 mL	TC-TRI-1%
1	MTT protocol	MK-24-007-0003

VII. 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 Components

Quantity	Description	Part No.
1	12-well plates with hanging top (sterile) (2 HNG-TOP-12 needed / ORL-200 or GIN-100 kit)	HNG-TOP-12
1	Culture Stands (24 stands needed / ORL-200 or GIN-100 kit)	MEL-STND