EpiIntestinal™ Small Intestine Tissue Model (SMI-100) Use Protocol

I. Storage of Tissues (SMI-100)

a) **Storage:** Upon receipt of the EpiIntestinal Tissue Model, place the sealed 24-well plate containing the EpiIntestinal tissues at room temperature and the maintenance medium into the refrigerator (2-8°C). Transfer the tissues to the incubator as described in Section II, Preparation of EpiIntestinal, preferably on the day of arrival. Storage conditions for components of EpiIntestinal are summarized below:

Part #	Description	Storage Conditions	Shelf Life
SMI-100	EpiIntestinal Tissues	Room Temperature	96 hours*
SMI-100-MM	Maintenance Medium	Refrigerate (2-8°C)	2 weeks
MTT-100-DIL	MTT Diluent	Refrigerate (2-8°C)	2 months**
MTT-100-CON	MTT Concentrate	Freezer (-20 ±5°C)	2 months**
MTT-100-EXT	MTT Extractant	Room Temperature	1 year**

Notes: **Refers to storage time at room temperature in unopened package from Monday at 3:00 PM (i.e. 96 hours would be Friday at 3:00 PM).*

***MTT-100 kits must be ordered separately.*

Before performing any tests or experiments, the EpiIntestinal Tissue Model needs to be pre-equilibrated overnight. It is strongly recommended to proceed with Section II, Preparation of EpiIntestinal, immediately after receiving the tissue model.

II. Preparation of EpiIntestinal

a) **Prepare HNG-TOP-12 plate:** Per kit of 24 SMI-100 tissues, pre-warm 125 mL of SMI-100-MM medium in a 37°C water bath for 10 minutes. Under sterile conditions, open the HNG-TOP-12 and remove together the regular lid and the hanging-top lid (**Figure 1A**) from the bottom of the 12-well plate (**Figure 1B**). Pipette 5.0 mL of the pre-warmed SMI-100-MM medium into each well of the 12-well plate and replace the hanging-top lid on top of the bottom plate. Label the plates indicating the test material and the dosing time to be used**.**

b) Transfer tissues: Under sterile conditions, open the package containing the tissue samples and using sterile forceps, transfer the inserts from the agarose package into the hanging-top lid (**Figure 1C**). Care should be taken to remove all agarose sticking to the outside of the cell culture inserts containing the tissue samples. Pipette 100 µL of the pre-warmed SMI-100-MM medium (provided) onto the apical surface of each tissue (**Figure 2).**

c) Pre-equilibration: Place the regular lid over the hanging-top lid (**Figure 1D**) and return the fully assembled HNG-TOP-12 plate containing the EpiIntestinal samples into a humidified incubator overnight at 37°C, 5% CO_2. This allows the tissues to recover from the stress of shipping **(Figure 2)**. *Note: Any air bubbles trapped underneath the cell culture insert should be released (tilt the cell culture insert using a sterile forceps) so that adequate nutrients are supplied to the EpiIntestinal tissues.*

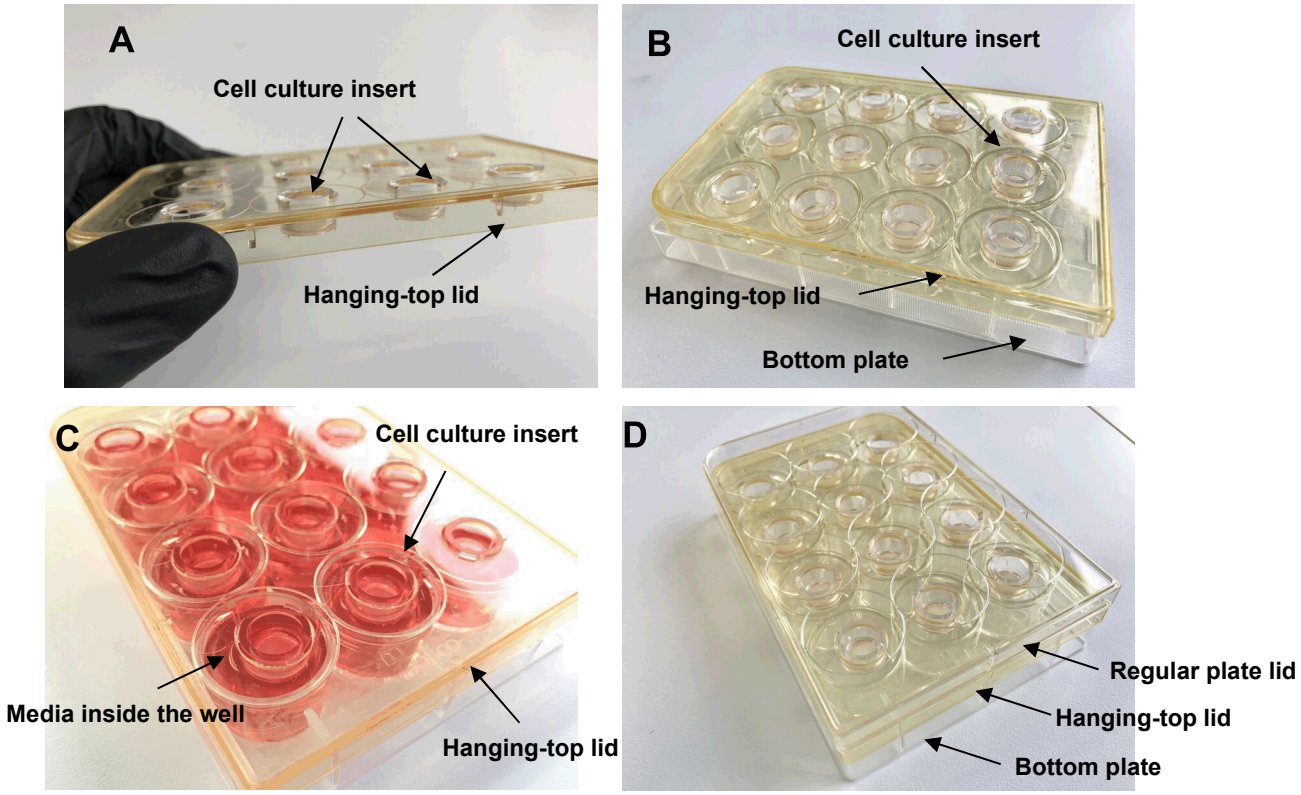

Figure 1: (**A**) Cell culture inserts (8.8 mm ID) in the hanging-top lid; (**B**) Hanging-top lid with inserts on top of the bottom plate without media; (**C**) Hanging-top lid on top of the bottom plate containing media; (**D**) Fully assembled HNG-TOP-12.

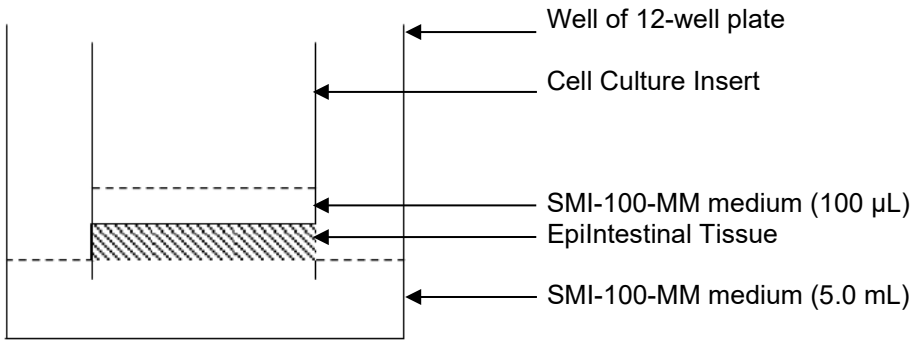

Figure 2: Pre-equilibration of EpiIntestinal tissue (overnight at 37°C, 95% rH, 5% CO_2).

III. Dosing

a) Replace medium: Following the overnight incubation, aliquot 125 mL of SMI-100-MM (per kit of 24 SMI-100 tissues) using sterile technique. Pre-warm the aliquoted media for 10 min in a 37°C water bath. Aspirate off the SMI-100-MM media contained in the 12 well plates and replace with 5.0 mL per well of pre-warmed, fresh SMI-100-MM medium. *Note: Any air bubbles trapped underneath the cell culture insert should be released, as previously described.* The tissues are now ready for dosing / experimentation.

b) Treatment conditions: To test the toxicity or efficacy of drug formulations or other test materials, apply the test article directly to the apical tissue surface using a positive displacement pipette. For short-term exposure (≤24 hrs), apply 50 µL of test material. For longer-term exposures (>24 hrs), apply 50 µL of test article as needed and re-feed tissues with 5.0 mL of fresh SMI-100-MM every other day (see next section and Table 1 to follow). For drug permeation studies, see Protocol # MK-24-007-0104. *Note: If DMSO is used to help solubilize the drug, the final DMSO concentration applied to the tissue should be ≤ 1.0%.*

Test materials (e.g. cytokines, growth factors, hormones, etc.) can also be added to basal compartment of the bottom 12-well plate containing the SMI-100-MM to simulate systemic exposure to the tissue. Alternatively, bacteria, viruses, or other pathogens can be applied topically to the tissue surface (i.e. the intestinal lumen) to model intestinal infection. The surface area of the SMI-100 tissues is 0.6 cm^2 **(Figure 3)**.

Note: Antibiotics (gentamicin) and antimycotics (Amphotericin-B) are utilized in the culture of the tissue model and are normally included in the maintenance media provided. However, tissues and media free of these agents are available upon request. Please contact a MatTek technical representative to discuss your experimental requirements.

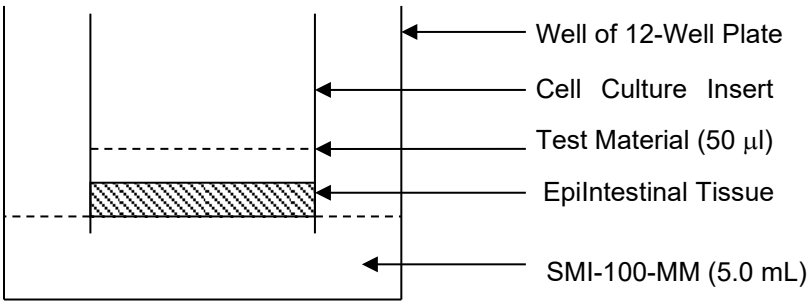

Figure 3: Dosing Configuration

c) Extended culture (>6 days): Chronic exposure experiments with EpiIntestinal are possible provided that the SMI-100-MM medium is replaced (every other day) with 5.0 mL of fresh, pre-warmed medium and the apical surface of the tissue is rinsed (every 7 days), as follows:

1. Aspirate the basolateral medium and add 5.0 mL of fresh, pre-warmed SMI-100-MM into each well of the 12-well plates containing the tissue culture inserts.
2. If any medium remains on the apical surface, grasp the insert firmly with sterile forceps and decant the apical medium.
3. Topically add 100 µL of the pre-warmed SMI-100-MM medium into each cell culture insert.
4. Repeat steps 1-3 every Monday, Wednesday, Friday, and Saturday.
5. On Day 6 (and every 7 days thereafter), gently pipet 200 µL of PBS (pH 6.8) onto the apical tissue surface. Grasp the insert with sterile forceps and rock it back and forth and then decant. Repeat this procedure 2X to remove any cell debris sloughing off the apical tissue surface. Then proceed with the medium change as described in steps 1-3. Tissues should be fed every Monday, Wednesday, Friday, and Saturday and the apical tissue surface should be rinsed every Monday **(Table 1).**

d) Inclusion of controls: It is important to include negative, positive, and benchmark controls to compare the effects of the various test conditions. N=3 tissues are recommended for controls.

Day	Replace Media	Apical Rinse (PBS pH6.8)*	Note
0 (Tues)	+		Receipt of tissue, Post shipment recovery
1 (Wed)	+		Replace medium/apply dose
3 (Fri)	+		*
4 (Sat)	+		*
6 (Mon)	+	+	*
8 (Wed)	+		*
10 (Fri)	+		*
11 (Sat)	+		*
13 (Mon)	+	+	*
15….	…	….	Maintain culture as needed

Table 1: Schedule for extended culture experiment using EpiIntestinal model.

*Notes:

a) The timing of test article re-application and analysis / endpoint measurements depend on the specific experiment design. Using proper sterile technique, TEER can be measured repeatedly on the same tissues (e.g. at 24 or 48-hour intervals). The apical medium, as well as any topically applied compounds, should be replenished after each TEER measurement.

b) Adjust the PBS rinse solution (provided) to pH 6.8. Alternatively, order part # DPBS-6.8.

III. MTT tissue viability assay

a) **Apply dose:** Add 50 μL of the test article into the cell culture insert atop the EpiIntestinal tissue. If systemic exposure is desired, add an appropriate amount of test article to the culture medium underneath the cell culture insert. Negative controls (no dose, or diluent for diluted samples) should be treated in an identical manner to the dosed inserts.

b) **Exposure time:** Return the 12-well plates containing the dosed EpiIntestinal samples to the incubator for the desired time periods. For unknowns, initial exposure times of 15 min, 1 hr, and 2 hrs are recommended. If the MTT-100 kit is ordered, a positive control, 0.3% Triton X-100, is included.

c) **Prepare MTT solution:** Approximately 1 hour prior to the end of the first dosing period, prepare the MTT solution. Thaw the MTT concentrate and dilute it with the MTT diluent (provided in the MatTek MTT toxicology kit, Part # MTT-100). Spin down (300 x g for 5 minutes) the MTT solution to remove any precipitate present. Store the remaining MTT solution in the dark at 4°C for the later time points. *Note*: To get optimal results, the MTT solution should not be stored for more than 1 day.

d) **Prepare MTT plate:** 15 minutes before the first dosing period is complete, prepare a 24-well plate (provided) w/ 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 cytokine, inflammatory mediator, or LDH release measurements will be made.

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

f) **Media for inflammatory mediator analysis:** Save the media from the 12-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 -20°C; samples to be assayed for PGE-2 should be stored under nitrogen and frozen.

g) **MTT loading:** Return the EpiIntestinal samples in the 24-well plate to the incubator for 3 hours ± *5 minutes*. See **Figure 4.** *Note: Deviations from the 3 hour time for MTT incubation will result in different readings.*

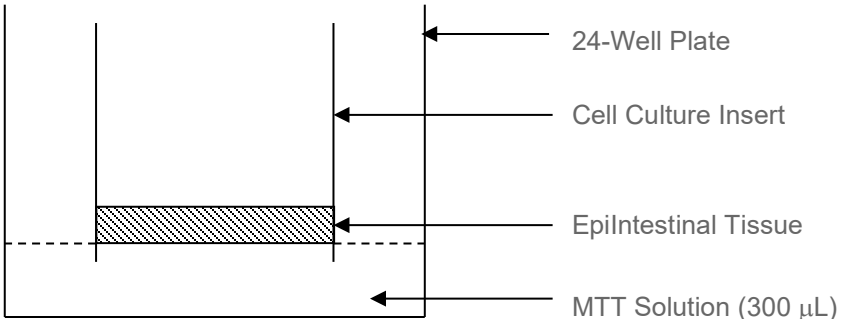

Figure 4: Incubation in the MTT solution (37°C, 95% rH, 3 hours)

h) **Reduction of MTT by test article**: 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 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.

A negative control, 100 µL 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. If the TA reduces MTT, a false viability measurement may be obtained. Please contact MatTek technical assistance for further guidance.

MTT 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. Place the inserts into the pre-labeled 24-well extraction plate.

b) **Add extractant:** Immerse the cell culture inserts in 2.0 mL of the extractant solution to completely immerse the EpiIntestinal tissue. See **Figure 5**. 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.

c) **Extraction conditions:** Place the extraction plate with its top in place into a sealable plastic bag (e.g. Ziplock) to minimize evaporation. Allow the extraction to proceed in the dark for 2 hours on an orbital shaker or overnight (without shaking) at room temperature. Protect the plate from light using aluminum foil. If shaking is used, shaking should be vigorous enough for mixing within the wells, but not too vigorous such that liquid will leave the wells. Allow the extractions to proceed until all samples have been extracted for at least 2 hours (with shaking) or overnight (without shaking) so that all MTT readings can be made at the same time. If evaporation of solvent is prevented, extraction times beyond 2 hours 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 back into 24-well plate:** After the extraction 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.

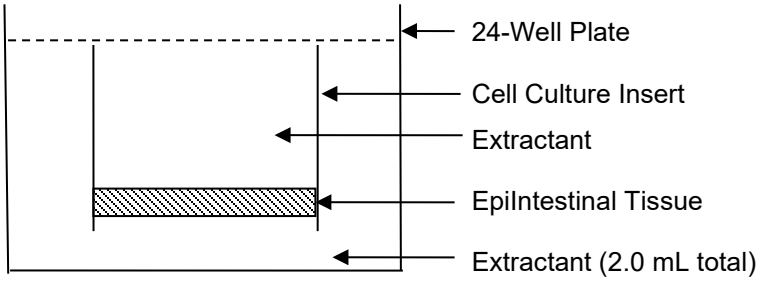

Figure 5: Extraction of MTT (In the dark, at room temperature for 2 hr w/ shaking or overnight w/o shaking

Construction of Dose Response Curve

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

b) **Transfer to 96-well plate**: Pipet 200 µL of the mixed extraction into a 96-well microtiter plate. *Note: If a 96-well plate reader is not available, any visible spectrophotometer can be used to determine the optical density (as follows).*

c) **Measure optical density**: Determine the optical density of the extracted samples at 570 nm using 200 µL of the extractant as a blank. *Note: 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:

% 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 ET-50 value. *Note: An excel spreadsheet is available from MatTek technical service for calculation of ET-50.*

f) **Chronic exposure:** Extended, chronic exposure times can be used so long as the tissues are fed with 5.0 mL of fresh medium every 48 hrs as per section III.c. Such experiments will require additional maintenance medium (Part #: SMI-100-MM). Please contact MatTek technical personnel for assistance.

V. Choice of assay procedures: Please contact your MatTek technical service representative for any additional protocols required. The effect of the test materials or treatments to the tissue can be monitored using a broad variety of endpoints and assay methods, including:

Barrier function: Use transepithelial electrical resistance (TEER) measurements to probe the status of tight junction within the tissue. The EVOM Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL) is recommended *(Protocol, MK-24-007-0084)*.

Gene expression: Place tissues in an RNA stabilization reagent (e.g. RNAlater), harvest RNA, and analyze gene expression using RT-PCR or q-PCR *(Protocol, MK-24-007-0065)*.

Protein expression: Place tissues in a lysis buffer, purify and quantify protein, and analyze using Western blot analysis *(Protocol # MK-24-007-0098)*.

Cytokine release: Save the apical or basolateral medium (beneath the tissue) and analyze secreted cytokines using commercially available ELISA or BioPlex kits.

Tissue viability: Measure the tissue viability using the MTT assay *(See Section IV above)*.

Histology and Immunostaining: Fix, embed, section, and H&E stain the tissues to observe the tissue histology or perform immunohistochemical staining (IHC) to observe specific protein expression in the tissues*. Histology or IHC analysis is offered as a Service by MatTek (Protocol OH-24-007-0001).*

Drug permeation: To model the permeability of drugs through the small intestine tissue, please request the EpiIntestinal Tissue Drug Permeation Protocol (*Protocol MK-24-007-0104).*

MatTek can also provide Contract Services to analyze the effects of your test materials.

VI . Materials Provided

EpiIntestinal™ (Part No. SMI-100)

Quantity	Description	Part No.
24	EpiIntestinal tissue-model samples	SMI-100
2	HNG-TOP-12 plates (sterile)	HNG-TOP-12
2	24-well plates (sterile)	MW-15-003-0028
1	PBS rinse solution, 100 mL	TC-PBS
1	Maintenance medium, 250 mL	SMI-100-MM
1	SMI-100 Use Protocol	MK-24-007-0130

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 Materials

Quantity	Description	Part No.
1	Maintenance medium (for extended culture), 250 mL	SMI-100-MM
1	PBS rinse solution, pH 6.8, 125 mL	DPBS-6.8

VIII. Alternative Tissues

Quantity	Description	Part No.
24 tissues	SMI-100 cultured without antibiotics	SMI-100-ABF
24 tissues	Full thickness small intestinal tissue (enterocytes and fibroblasts)	SMI-200-FT
96 tissues	SMI-100 cultured in high throughput, 96-well plate	SMI-196
96 tissues	SMI-200-FT culture in high throughput, 96-well plate	SMI-296-FT