EpiKidney™ KID-100 Use Protocol

I. Storage of EpiKidney™ (KID-100) and MTT Kit (MTT-100)

a) **Shipping:** EpiKidney™ (KID-100) is shipped on Mondays from MatTek Headquarters (US). Delivery to US locations is on Tuesdays and to Europe, Japan, and Korea on Wednesdays.
b) **Storage:** Upon receipt, store the sealed plate containing the EpiKidney tissues at room temperature (20-25°C) until use. Store the anitibiotic and antimycotic free medium (Part# KID-100-MM-AFAB) in the refrigerator (2-8°C). Storage conditions are summarized below.

Part #	Description	Conditions	Shelf Life
KID-100	EpiKidney™ cultures	Room temp.(20-25°C)	72 hours [1]
KID-100-MM-AFAB	Maintenance medium	Refrigerate (2-8°C)	14 days

Notes:
[1] Refers to storage time at room temperature in the unopened package from the time of shipment.

II. Preparation of EpiKidney™

a) **Receipt of tissues**: As soon as possible after receipt, preferably on the same day they are received, the EpiKidney tissues should be returned to culture, as per the following steps.
b) **Prepare HNG-TOP-12 plate:** For each kit of KID-100 tissues, pre-warm 125 mL of KID-100-MM-AFAB 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 1**) from the 12-well plate (**Figure 1B**). Pipette 5.0 mL of the pre-warmed KID-100-MM-AFAB medium into each well 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**.**
c) **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 KID-100-MM-AFAB medium (provided) onto the apical surface of each tissue (**Figure 2).**
d) **Pre-equilibration**: Place the regular lid over the hanging-top lid (**Figure 1D**) and transfer the fully assembled HNG-TOP-12 plate containing the EpiKidney samples into an incubator to equilibrate overnight at 37±1°C, 90±10% RH, 5±1% CO_2. This pre-equilibration allows the tissues to recover from the stress of shipping. *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 EpiKidney tissues.*
e) **Change medium:** At the end of the equilibration period, aspirate the medium from each well and replace with 5.0 mL of fresh, pre-warmed medium. In addition, decant any KID-100-MM-AFAB remaining on the apical tissue surface. Tissues are now ready for the experiment.

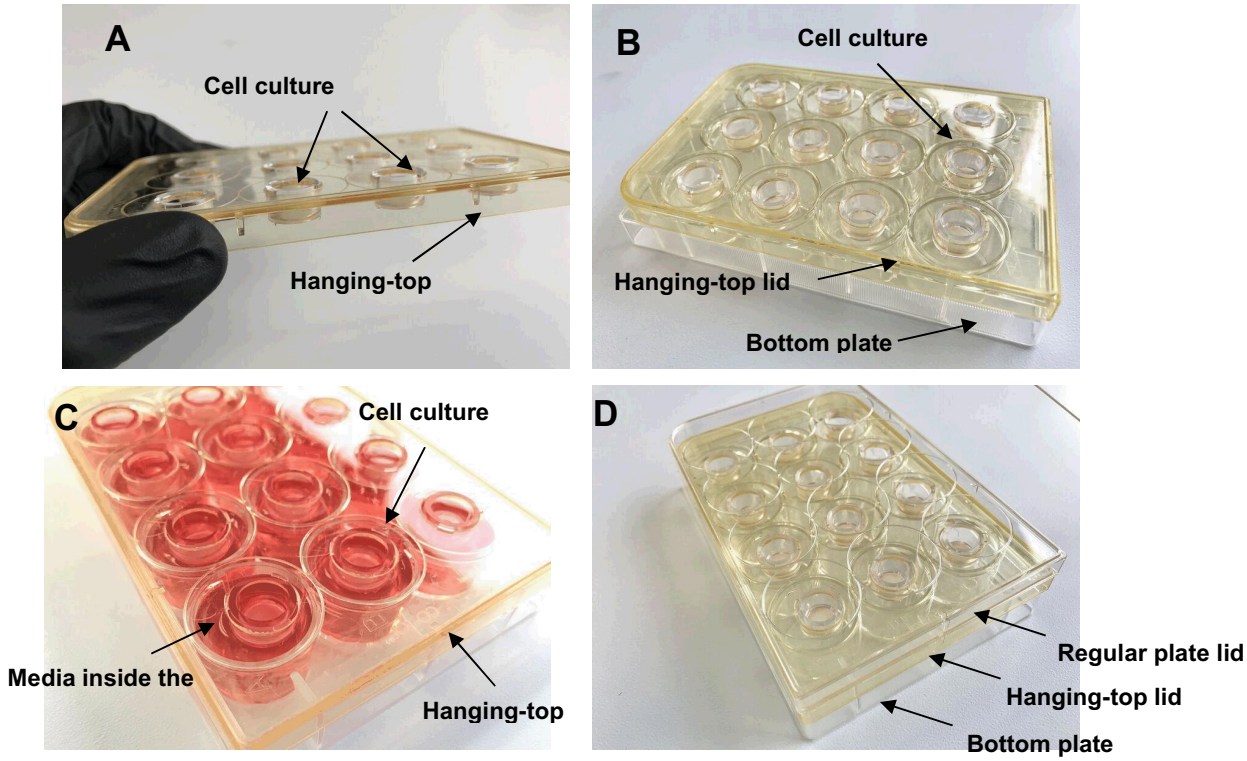

Figure 1: (**A**) Cell culture inserts (8.8 mm ID) in the hanging-top lid; (**B**) Hanging-top lid with inserts on top of the bottom 12-well plate without media; (**C**) Hanging-top lid on top of the bottom 12-well plate containing media; (**D**) Fully assembled HNG-TOP-12.

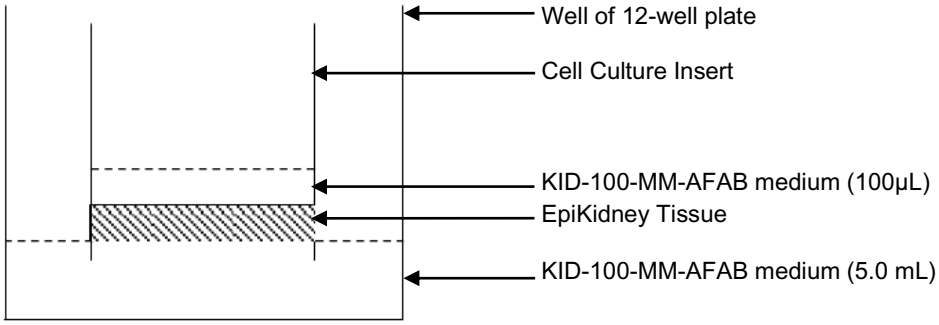

Figure 2: Pre-equilibration of EpiKidney tissue (overnight at 37°C, 95% rH, 5% CO_2).

III. Exposure of Test Materials

a) To test the toxicity or efficacy of drug formulations or other test materials, apply 100 µL of the test article diluted in KID-100-MM-AFAB directly to the apical tissue surface using a positive displacement pipette. For longer-term exposures (>24 hrs), apply 100 µL of test article diluted in KID-100-MM-AFAB and re-feed tissues with 5.0 mL of fresh KID-100-MM-AFAB every two days (see next section and Table 1 to follow).
b) Test materials (e.g. cytokines, growth factors, hormones, etc.) can also be added to basal compartment of the bottom 12-well plate containing the KIDI-100-MM-AFAB to simulate systemic exposure to the tissue. Alternatively,

bacteria, viruses, or other pathogens can be applied topically to the tissue surface (i.e. the tubule lumen) to model kidney infection. The surface area of the KID-100 tissues is 0.6 cm^2 (**Figure 3**).

Note: Antibiotics (penicillin/streptomycin) are utilized in the culture of the tissue model and are but are not included in the maintenance media provided (KID-100-MM-AFAB). However, antibiotic and/or antimycotic containing media is available upon request. Please contact a MatTek technical representative to discuss your experimental requirements.

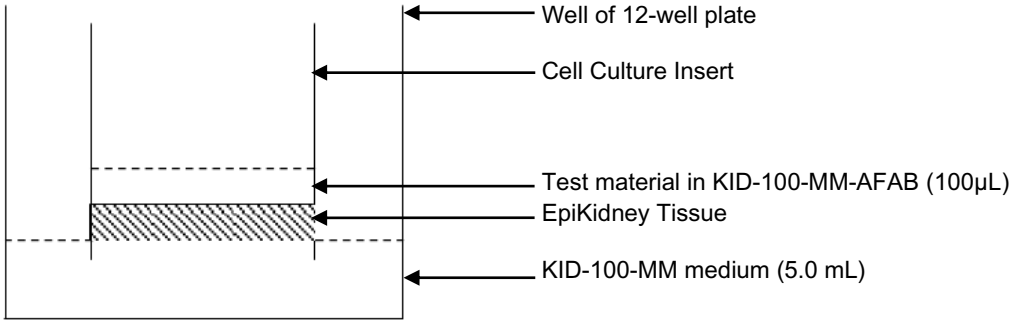

Figure 3: Dosing configuration

c) **Extended culture (>6 days)**: Chronic exposure experiments with EpiKidney are possible provided that the KID-100-MM-AFAB medium is replaced every two days with 5.0 mL of fresh, pre-warmed medium and the apical surface of the tissue is fed/dosed with 100 µL of KID-100-MM-AFAB:

1. Aspirate the basolateral medium and add 5.0 mL of fresh, pre-warmed KID-100-MM-AFAB 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 KID-100-MM-AFAB medium into each cell culture insert.
4. Repeat steps 1-3 every Monday, Wednesday, Friday, and Saturday. **See 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	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 EpiKidney model

***Note**: 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.

IV. Analytical Methods

The effect of the test materials for drug candidates can be monitored using a number of different assay methods including:

a) **Barrier function:** Transepithelial Electrical Resistance (TEER) is a non-invasive method that can be used to assess the barrier integrity of the tissue. See Section V. below.
b) **Tissue viability:** Drug induced kidney toxicity can be monitored by measuring Lactate Dehydrogenase (LDH) secretion or using the MTT viability assay. See Section VI below.
c) **Cytokine release:** Culture media can be saved and analyzed for cytokine secretion using ELISA or multiplex (e.g. BioPlex) kits.
d) **Gene expression:** RNA can be isolated from the tissues for gene expression analysis using RT-PCR or q-PCR. See MatTek protocol MK-24-007-0065, *Isolation of Total RNA from MatTek Tissue Models*. Tissues can be stored in an RNA stabilization solution (e.g. RNALater) until isolation.
e) **Protein expression:** Total protein can be extracted from the tissues for Western blot analysis See MatTek protocol MK-24-007-0098, *Preparing Protein Lysate for Western Blot*.
f) **Histology:** Tissues can be fixed and sectioned for histological or immunohistochemical analysis. See MatTek protocol OH-24-007-0001*, Histology Sample Preparation Procedure*. Note: Histology and immunohistochemistry services are offered by MatTek. See Technical Reference #1003 for typical KID-100 tissue morphology.

V. Transepithelial Electrical Resistance (TEER)

TEER measurements are a convenient non-destructive indicator of barrier function and tight junction development within the EpiKidney tissues.

Required Equipment:
a) EVOM2™ or EVOM3™ Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL)
b) Endohm-12 Tissue Resistance Measurement Chamber or STX100 electrode (World Precision Instruments)

Required Materials:
a) Potassium chloride (KCl, 100 mM)
b) Forceps – sterile
c) Blank inserts (Part #: MILCEL-MTK-PTFE or MILCEL-ECM-MTK-PTFE – *must be ordered separately*)

Procedure:
Note: Consult the EVOM Epithelial Voltohmmeter and Endohm Tissue Resistance Measurement Chamber manuals for additional information and instructions.

a) If culturing of tissues is to be continued after measurement, the Endohm Tissue Resistance Measurement Chamber must be sterilized prior to measurement (see Endohm Tissue Resistance Measurement Chamber manual). Likewise, all calibration and measurement operations must be performed in a tissue culture hood utilizing sterile technique and sterile buffers.
b) Connect the Endohm Tissue Resistance Measurement Chamber to the instrument with the electrode leads. Plug the RJ-11 plug at the end of the wire into the input jack. The longer electrode lead wire should attach to the bottom portion of the chamber for the correct polarity. If the EndOhm chamber has been stored dry, fill the chamber with enough KCl to immerse the top electrode. Equilibrate the electrodes for about 20 minutes with the power on prior to use.
c) With a small amount of KCl in the Endohm Tissue Resistance Measurement Chamber (1.5 mL for Endohm-12), place a blank insert (Part # MILCEL-MTK-PTFE) into the chamber. Add enough KCl to the top surface of

the insert to completely cover the membrane surface to a depth of 4-5 mm (approximately 250 µL) and adjust the top electrode so that it is close to, but not making contact with, the top surface of the insert membrane. Background resistance reading for the blank inserts should be <25 Ω. The instrument is now ready for tissue measurements.

d) Dispense 1.0 mL of room temperature KCl into the well(s) of a standard 6-well tissue culture plate. Transfer the inserts to be measured to the 6-well plate and gently rinse the apical surface of tissues by adding ~0.5 mL of KCl and then aspirating, then add 250 uL KCl to each insert. Transfer the individual tissues into the Endohm Tissue Resistance Measurement Chamber and replace the top electrode (make certain that the electrode does not touch the tissue and that is in contact with the KCl). With the Mode knob set to R, record the resistance. Change the Mode knob to V and record the PD reading.

e) Calculate TEER value: Subtract the blank insert reading from the resistance measurement of the tissue and multiply by the area of the tissue culture insert (For KID-100: area = 0.6 cm^2).

f) Decant the KCl from the apical surface of the tissue insert and return the tissue to the culture vessel for longer term studies. Alternatively, process the tissue for histology, transmission electron microscopy, RNA isolation, etc.

> ***Note**: For more information regarding TEER measurements, ask Customer Service for protocol MK-24-007-0051: "Measurement of TEER and Potential Difference."*

VI. MTT Assay

a) Apply dose: Expose tissues to test articles or other treatment conditions as per Section III a)-b). For testing of potential nephrotoxins, both time to toxicity (test article concentration remains constant and exposure time is varied) and concentration to toxicity (exposure time remains constant and test article concentration is varied) are possible.

b) Prepare MTT solution: Approximately 1 hour prior to the end of the first dosing/ treatment period, prepare the MTT solution. Thaw the MTT concentrate and dilute it with the MTT diluent (provided in the MatTek MTT Assay Kit, Part # MTT-100). Centrifuge the MTT solution at 300 x g for 5 minutes to remove any precipitate. Store the remaining MTT solution in the dark at 4°C for the later time points. *Note: The MTT solution should not be stored for more than 1 day.*

c) Prepare MTT plate: 15 minutes before the first dosing period is complete, pipet 300 µL of the MTT solution into the appropriate number of wells of a 24-well plate to accommodate all the inserts for the experiment. Label the 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.

d) Transfer samples to MTT plate: After exposure of the EpiKidney samples to the test material(s)/treatment is complete, decant any liquid remaining atop the EpiKidney. Remove each insert individually and gently rinse twice with PBS (provided) to remove any residual test material. Shake off excess liquid and place the EpiKidney tissues in the MTT containing 24-well plate making sure that no air bubbles are trapped underneath the cell culture insert.

e) Media for inflammatory mediator analysis (optional): Save the media from the 12-well plates in the labeled vials for subsequent LDH or cytokine analysis. Store samples for LDH 4°C, samples for cytokine analysis at -20°C; samples to be assayed for PGE-2 under nitrogen and frozen.

f) MTT loading: Return the EpiKidney 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.*

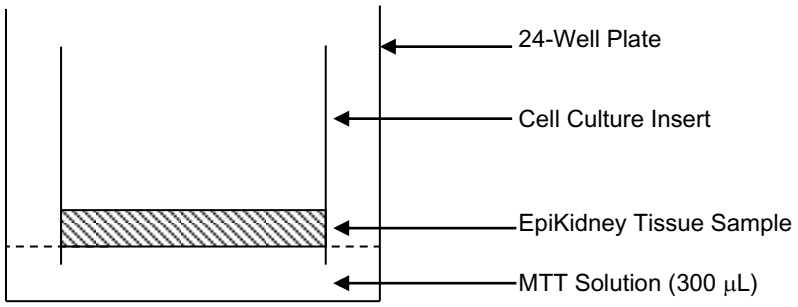

Figure 4: Incubation in the MTT Solution (37°C, 95% RH, 3 hrs, 5% CO_2

g) 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)/treatment directly reduces MTT. Prepare a 1.0 mg/mL MTT solution as above and add 100 µL of the neat TA to 1 mL of the MTT solution; alternatively, apply the treatment condition to the MTT solution. Incubate the tissue 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/ treatment has reduced the MTT; the absence of darkening indicates that the TA or treatment does not directly reduce MTT. If the MTT is reduced, a false viability measurement may be obtained. Please contact MatTek technical assistance for further guidance.

h) Transfer samples to extraction plate: After the 3 hour MTT incubation period is complete, remove each insert individually and gently blot the bottom on an absorbent material (e.g. Kimwipe). Place the inserts into the pre-labeled 24-well extraction plate.

i) Add extractant: Add 2.0 mL of the extractant solution to each well to completely immerse the EpiKidney tissues. **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.

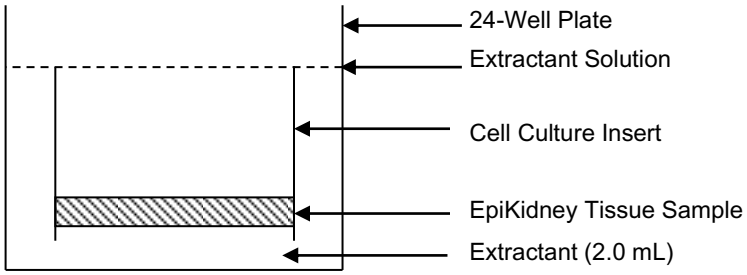

Figure 5: Extraction Configuration (room temperature in dark)

j) 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.

k) Decant extractant back into 24-well plate: After the extraction is complete, remove each insert and decant the liquid within the apical chamber 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.

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

m) 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).

n) 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.

o) Calculate % viability: Determine the % viability at each of the dosed concentrations using the following formula: % viability = 100 x [OD (sample)/OD (negative control)].

p) Construct dose response curve: Using a semi-log scale, plot the % viability (linear y axis) versus the dosing time or dose concentration (log x axis). By interpolation, the time or concentration at which the % viability has dropped to 50% is considered the ET-50 or EC-50 value. Note: An excel spreadsheet is available from MatTek technical service for calculation of ET-50 and EC-50.

VII. Materials Provided

EpiKidney™ (Part No. KID-100)

Quantity	Description	Part No.
24	EpiKidney tissues	KID-100
2	Hanging top 12-well plates	HNG-TOP-12
1	PBS rinse solution, 125 mL	TC-PBS
2	24-well plates (sterile)	MW-15-003-0028*
1	Assay medium, w/o antibiotics and antimycotics, 250 mL	KID-100-MM-AFAB
1	EpiKidney KID-100 Use Protocol	MK-24-007-0151

*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 (5X), 2 mL	MTT-100-CON

Additional Materials

Quantity	Description	Part No.
1	24-well plates, 5-pack	MW-15-003-0028-PK
1	Maintenance medium with antibiotics and antimycotics, 250 mL	KID-100-MM
1	Maintenance medium w/ only antibiotics, 250 mL	KID-100-MM-AFF
1	Maintenance medium w/ only antimycotics	KID-100-MM-ABF
1	Blank insert (min order: 6 inserts)	MILCEL-MTK-PTFE
1	Collagen coated blank insert (min order: 6 inserts)	MILCEL-ECM-MTK-PTFE

IX. Technical References

1003. Development of an in vitro 3D Human Kidney Proximal Tubular Epithelial Tissue Model. Kaluzhny Y, Finelli J, Stevens Z, Armento A, Ayehunie S. Society of Toxicology annual meeting, Nashville, TN (2023).