

MelanoDerm™ Tissue Model

Procedures for use of MEL-300-A, MEL-300-B, MEL-300-C
& MEL-606-A, MEL-606-B, MEL-606-C

I. Shipping and Storage of MelanoDerm Tissues and Kit Components

a) **Shipping:** MelanoDerm is produced so that tissue is ready for shipment on Mondays and Thursdays. Delivery into your lab will depend on your location and the mode of transport as given in the following table.

Destination	Mode of Shipping	Day of shipment	Day of Arrival*
USA	FedEx/DHL	Monday	Tuesday
Europe	FedEx/DHL	Monday	Wednesday
Europe	Special Handling	Monday	Tuesday
Japan	FedEx/DHL	Thursday	Tuesday
Japan	Special Handling	Thursday	Monday

* Assumes normal handling in customs.

b) **Storage:** We recommend returning the tissue to culture and beginning experiments on the day that the tissues are received. If experiments will not commence upon receipt, place the sealed 24-well plate containing the MEL-300 or the 6-well plate containing the MEL-606 tissue samples and the maintenance medium into the refrigerator (2-8°C). The tissues and maintenance medium should be stored unopened at 2-8°C until experiments begin. If the MTT assay is to be run, please refer to the MTT ET-50 protocol for EpiDerm (MK-24-007-001 or MK-24-007-0007 for EPI-200 and EPI-606, respectively) for storage conditions of the MTT kit and assay procedures.

<u>Part #</u>	<u>Description</u>	<u>Storage Conditions</u>	<u>Shelf Life</u>
MEL-300-A, MEL-300-B, MEL-300-C	MelanoDerm tissues	Refrigerate (2-8°C)	6 days*
MEL-606-A, MEL-606-B, MEL-606-C	MelanoDerm tissues	Refrigerate (2-8°C)	6 days*
EPI-100-LLMM, -NMM, -NMM-113	Maintenance Medium	Refrigerate (2-8°C)	14 days

*Note: Refers to storage time @ 2-8°C in unopened package.

c) **Background:** MelanoDerm experiments typically extend for 7-21 days. To maintain good skin morphology, MelanoDerm tissues must be fed with 5.0 ml of medium underneath each tissue and replaced with fresh medium every other day. Use of the hanging top plates (HNG-TOP-12, **Figure 1**) facilitates culture of MEL-300 tissues for melanogenesis and skin lightening studies. Culture stands (part #: **MEL-STND**) or washers (part #: **EPI-WSHR**) can also be used for culturing MEL-300 tissues (including previous versions of MelanoDerm tissues). For culturing MEL-600, see protocol for culturing EPI-606, protocol # MK-24-007-0007.

Notes:

- Each hanging top (HNG-TOP-12) can accommodate 12 tissues. 2 x HNG-TOP-12 plates are included with each MEL-300 order and are sufficient to handle the tissues over the course of a standard melanogenesis or skin lightening experiment (7-21 days). If additional hanging top plates are needed, order 2 x HNG-TOP-12 per kit of 24 tissues.
- Hanging tops cannot be autoclaved and are not recommended for re-use.

II. Preparation of MelanoDerm tissues

a) **Pre-warm media:** Pre-warm the required volume of maintenance medium (provided) to 37°C. Label the 12-well plates indicating the treatment/ exposure conditions to be used. *Note: At this step and for each feeding, warm only the required volume of medium.*

b) **Transfer MelanoDerm samples:** Using sterile technique, pipet 5.0 ml of the pre-warmed maintenance medium into each well of the sterile 12-well HNG-TOP-12 plates (provided). Place the hanging top lid on the plate bottom containing the maintenance medium. Remove the MEL-300 tissue from the agarose-containing package using sterile forceps and transfer the MEL-300 inserts into the hanging top lid (**Figure 1C**). Cover the plate with the regular plate lid (**Figure 1D**) *Note: When removing the tissue samples from the agarose shipping plate, care should be taken to remove any adherent agarose sticking to the outside of the cell culture inserts containing the MEL-300 samples.*

c) **Pre-equilibration:** Incubate the 12-well plates containing the MEL-300 samples in a humidified 37°C, 5% CO₂ incubator overnight (18-24 hours) prior to applying treatment.

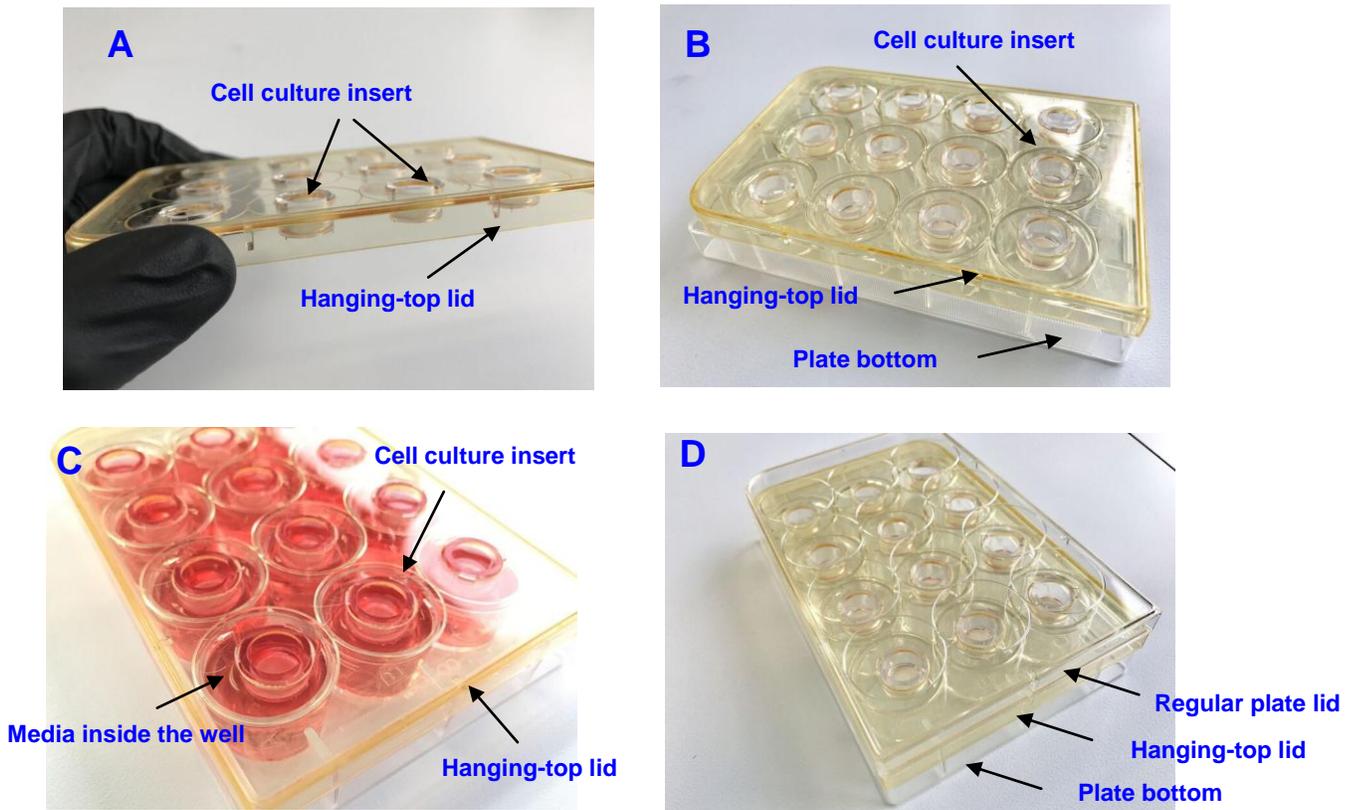


Figure 1. (A) Cell culture inserts (8.8 mm ID) in the hanging-top lid. (B) Hanging-top lid with inserts on top of the bottom plate without medium. (C) Hanging-top lid on top of the plate bottom containing medium. (D) Fully assembled HNG-TOP-12.

III. Melanogenesis/lightening studies

a) **Pre-screen treatment conditions:** A good skin lightener should inhibit melanin synthesis but not cause cytotoxicity to the tissue. Cytotoxicity testing can be performed with the less expensive EpiDerm tissue (EPI-200). Follow procedures in the MTT ET-50 protocol except: i) apply 25 and 10 µl of each test article to duplicate (n=2) EpiDerm tissues, ii) use exposure times of 2 and 7 days, and iii) negative control tissues (n=2) should be treated with 25 µl of sterile, ultrapure water for the same time points used for the test articles. After exposure is complete, the tissues are rinsed with DPBS and the MTT assay is run. Calculate the % viability for each treatment from the formula:

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

In order to avoid cytotoxicity in the skin lightening study, the viability should be >90% at 48 hours. If both the 25 and 10 µl dose are > 90% viability, use the 25 µl dose since the positive control (2% kojic acid) is run at 25 µl. *Note: If the % viability for the 10 µl treatment is <90%, it is likely that such a material would be an irritant.*

b) **Treatment conditions:** Liquid or lotion materials can be applied directly to the MEL-300 stratum corneum by pipetting with a positive displacement pipette into the cell culture insert which contains the MEL-300 tissue. 25 or 10 µl of liquid sample is applied (25 mg of solids) based on the results of the cytotoxicity pre-screen. *Note: The 25 µl represents a dose of ~40 mg/cm² – for many lotions (e.g. sunscreens are typically tested at 2 mg/cm²), this represents an exaggerated exposure and a lower, more *in vivo*-like dose may be desirable. Alternatively, dosing can be applied using the intended end-use application in mg/cm²; the surface area of the MEL-300 tissues is 0.6 cm². If you are using the “Recommended lightening study design” described in Section V.d, 8 tissues per treatment condition will be required.*

c) **Negative control:** Once placed at 37°C, the MEL-300 tissue will continue to undergo melanogenesis and differentiation. Hence, for time course or histological studies it is important to allow for ample non-treated controls. For the recommended lightening study (Section V.d), 8 tissues should be treated with 25 µl of sterile, ultrapure water to serve as negative controls. All manipulations with these negative controls should be identical to those treated with test articles.

d) **Replace maintenance medium:** Following the pre-equilibration period, under sterile conditions grasp both the regular lid and the hanging-top lid (containing the inserts) and remove them together from the 12-well plate. Aspirate the medium from the 12-well plate and replace with 5.0 ml/well of fresh, pre-warmed medium. Return the regular top and the hanging top lid containing the tissues to the 12-well bottom containing the fresh medium. Place the fully assembled HNG-TOP-12 plate in the incubator (37°C, 5% CO₂). *Note: Slight adjustments to medium volume in the 12-well plates may be necessary to ensure complete medium contact with the bottom of the insert. If following the recommended skin lightening protocol, the medium is EPI-100-NMM-113.*

e) **Apply treatment:** Pipet the test material into the cell culture insert containing the MEL-300 tissue. Do not add the test material to the medium in the well unless you are interested in effects directly on the melanocytes (i.e. bypassing the epidermal barrier). Negative controls (not exposed) should be treated in an identical manner to the treated inserts. Prior to re-application of test article on subsequent days, gently rinse the surface of the tissue with sterile DPBS to remove any residual test article.

f) **Non-pipettable materials:** Many viscous materials can be applied using a positive displacement pipette. For materials that cannot be pipetted, applicator pins (part # PIN-24) should be used to provide a reproducible means of application. For solids, a 1:1 slurry/paste of material and ultrapure H₂O is made and 50 mg of the slurry is applied using the applicator pin. *Note: Different solid/H₂O ratios can be used to improve the consistency of the paste; however, the amount of slurry applied to the applicator pin should be adjusted so that 25 mg of the original material are applied.*

g) **Length of treatment:** Treatments can be extended for up to 21 days and re-applied as often as desired. Medium should be changed every other day with 5.0 ml of fresh medium. For treatment periods extending over the weekend, it is preferable if the tissues are fed once either on Saturday or Sunday. If this is not possible, medium should be replaced on Friday as late as possible and then changed on Monday morning as early as possible. *Note: 625 ml of maintenance medium are supplied with each kit of MEL-300. The pre-equilibration and each feeding requires 120 ml (5.0 ml/tissue * 24 tissues). Thus, sufficient medium is supplied to perform the pre-equilibration step along with 4 additional feedings (e.g. day 0, day 2, day 4, day 6). For experiments extending beyond day 6, additional maintenance medium must be ordered.*

h) **Choice of assay procedures:** At this point, the tissues can be observed using light microscopy (top view –see next section) and then used in the MTT assay, fixed for histology, or extracted for melanin content. In addition, the medium samples can be collected for cytokine release studies.

IV. Assay Methods

Utilization of a number of different assay methods will increase the information which can be obtained from experiments using MEL-300. For instance, certain treatments may cause a reduction or increase in melanin production but may also be cytotoxic or have deleterious effects on epidermal morphology. A non-comprehensive list of assay methods which are useful for MelanoDerm studies is given below.

a) **Top view microscopic observation:** The melanocytes within MEL-300-A and MEL-300-B can be observed in a non-destructive, non-perturbing manner. These observations can give information regarding: a) the success of lightening/ darkening treatments, or b) cytotoxic effects on the melanocytes (i.e. if melanocytes are rounded up or if they exhibit non-dendritic morphology). Note: Because the melanocytes are actively undergoing melanogenesis, visualization of the melanocytes in control cultures is possible without the addition of L-dopa. The melanocytes within MEL-300-C tissue, however, are not readily observable without the addition of L-dopa.

Top view observation: Place the cell culture insert containing the MEL-300-B or MEL-300-A tissue or Dopa-treated MEL-300-C tissue in a petri dish with enough DPBS below the cell culture insert to wet the bottom of the cell culture insert. Using an inverted microscope, focus through the tissue. Melanocytes should appear highly dendritic and brown. Use of a glass bottom microwell dish (Part # P50G-0-14-F) from MatTek Corporation will provide additional working distance so that higher magnification objectives can be utilized (e.g. 40 X). Fixed samples can also be returned to MatTek for photo-microscopy analysis (part # MEL-PHO).

Dopa reaction (for use with MEL-300-C tissue): At room temperature, the cultures are: 1) briefly washed with Dulbecco's phosphate buffered saline (DPBS), b) fixed for 15 minutes in 10% formalin, c) exposed to 0.1% L-dopa in DPBS for 1 hour, d) exposed to fresh L-dopa solution for an additional 4 hours, and e) post-fixed in 10% formalin overnight. Melanocytes can then be observed using the top view described above.

b) **Macroscopic darkening:** If left in culture for 7 days or more, macroscopic darkening of control MEL-300-B or MEL-300-A tissues will be observable to the naked eye. Macroscopic darkening and difference between treated (e.g. lightened) tissues and controls will be accentuated by using longer culture periods of up to 14 days. For such experiments, it is important to supply 5.0 ml of fresh EPI-100-NMM-113 every other day. In addition, topical application of test articles should be limited to 25 μ l or less so that epidermal morphology is not perturbed. It is often useful to take a time course of control and samples every 3 or 4 days (e.g. day 0, day 4, day 8, day 12 etc.). Use of colorimeter or other image analysis techniques can be used to quantify levels of darkening; alternatively, melanin content can be quantified as described below.

c) **Histology:** This method will provide information regarding the melanosome/pigment granule and melanocytes distribution throughout the tissue and will give an overall picture of any histological changes which have occurred as a result of the treatment. Use H&E stained cross-sections to view effects on epidermal morphology and unstained cross-sections to view melanosome and melanocyte distribution throughout the tissue. Samples can be fixed and returned to MatTek Corporation for H&E processing (part # MEL-HIS) and photo-microscopy services (Part # MEL-PHO). Typical turn-around times are less than 2 weeks following receipt of samples at MatTek. **Method:** Refer to the "Histology" protocol available from MatTek Corporation. Better visualization of melanin distribution throughout the tissue may be obtained using **Fontana Mason staining**. As a service, MatTek can perform this staining (at cost) on treated tissues.

d) **MTT tissue viability assay:** It is possible that a material can have significant effects on pigmentation due to cytotoxicity. Thus, we strongly recommend that exposure conditions are tested in the EpiDerm (EPI-200) or MelanoDerm tissue model to ensure that the viability of the tissue is not compromised due to the lightening/ darkening treatment. Treatment conditions are described above in Section III.a. The tissue viability is assessed using the MTT viability assay (MTT Kit part #: MTT-100). Refer to Section III.a (above). *Note: For tissue viability assay using EpiDerm (EPI-200), order the MTT-100 kit; for tissue viability assays using Melanoderm tissue, order the MTT-100-MEL kit.*

e) **Melanin assay:** A number of melanin assays have been proposed in the literature. See: a) Bessou-Touya, S, et al. (*J. Invest. Dermatol.*, 111:1103-1108 (1998), b) Schmidt, R., et al., *Anal. Biochem.*, 235, 113-118 (1996) or c) Ito, S., *J. Invest. Dermatol.*, 100(2), 166S (1993). The assay that we recommend is the Solvable Melanin assay:

Solvable Melanin Assay

Notes:

- a) It is recommended that at least n=2 tissues are used for this assay. MatTek will perform this assay as a service to its customers (at cost). Two frozen tissues per treatment condition are returned to MatTek on dry ice via overnight express. Use part # MEL-ASSY (minimum order = 12 tissues).

A. Sample preparation

- After treatment of MelanoDerm tissues, submerge the inserts (containing the tissues) in Dulbecco's phosphate buffered saline (DPBS) for at least 10 minutes to remove any residual phenol red and test article from the tissue.
- Decant the DPBS and freeze the tissues (-20 ±5 °C). It is preferable to seal the tissues in a plastic bag to avoid dehydration of the tissues.
- Thaw the tissues by placing them at room temperature. Fill the cell culture inserts with 1% Na-bicarbonate (~300 µl/insert). After 30 minutes, decant the Na-bicarbonate and continue with the next step.
- Remove the tissues from the inserts using the fine-point forceps. Blot the tissue dry and place each tissue in a 1.7 ml microfuge tube.
- Add 500 µl of Solvable™ (Tissue and Gel Solubilizer 0.5 M—Packard BioScience Co. Catalogue No. 6NE9100) to each tube.
- Close the tube and make sure that the tissue is completely submerged.
- Incubate at 95°C overnight along with melanin standards (see below).
- In the morning, vortex the samples. Sometimes thick tissues will require additional time to complete the solution process.

B. Prepare stock melanin solution

- Dissolve Melanin (Sigma cat. M 8631) in Solvable at 1mg/ml to make stock solution.
- Place solution in a water bath or heating block at 95 degrees Celsius until all melanin is dissolved. Mix regularly during incubation. Store stock solution in dark.

C. Prepare dilutions for standard curve

- Prepare dilutions for the standard curve using the stock solution as given in Table 1.
- Incubate the dilutions of melanin overnight along with samples.

D. Read assay

- Cool samples and standards.
- Centrifuge at 13,000 rpm for 5 minutes to pellet.
- Fill microwell plate (C-96) with 300 µl of each sample and standard.
- If there is some foaming of samples when pipetted, blow gently across the samples to break bubbles prior to reading the plate.
- Read the plate at 490nM.

Table 1: Dilutions of stock solution to make solutions for the standard curve			
#	Stock Solution (µl)	Solvable (µl)	Melanin Content (µg)
0	-	500	0
1	2.5	497.5	2.5
2	5	495	5
3	10	490	10
4	25	475	25
5	50	450	50
6	100	400	100

V. Tissue ordering choices

a) **Seeding ratio:** Standard MEL-300 tissue is seeded at a keratinocyte: melanocyte ratio of 10:1 even though in normal human skin the ratio is in slight excess of 30:1. The 10:1 seeding ratio is used to hasten effects of lightening or darkening agents on the tissue, thus often minimizing the length of end-use experiments. If desired, however, tissue seeded at the more physiologic concentration of 30:1 can also be ordered. Part #'s MEL-30010 and MEL-30030 signify the 10:1 or 30:1 seeding ratio, respectively.

b) **Melanocyte source:** MEL-300 can be ordered containing melanocytes derived from either Asian, Black, or Caucasian donor tissue and are distinguished by addition of the suffix “-A”, “-B”, or “-C” in the part # (e.g. MEL-300-B). Following 1-2 weeks in culture, untreated “-A” and “-B” tissues becomes noticeably darker, melanocytes are readily visible in top view microscopy, and melanin granules are readily observable throughout the tissue in non-stained cross-sections. “-B” tissue will darken more than corresponding “-A” tissue. Untreated “-C” tissue, on the other hand, remains considerable lighter in color, melanocytes are generally visible (top view) only after the DOPA reaction, and very few melanin granules are observable in non-stained cross-sections.

c) **Choice of maintenance medium:** Three distinct maintenance media can be used with MEL-300 tissue, EPI-100-LLMM, EPI-100-NMM, or EPI-100-NMM-113. The –LLMM medium contains β -fibroblast growth factor (β FGF) and α -melanocyte stimulating hormone (α -MSH), both of which are known to stimulate melanogenesis and melanocyte growth. The –NMM medium contain keratinocyte growth factor (KGF) but omits any direct stimulators of melanogenesis such as β FGF and α -MSH. The EPI-100-NMM-113 combines the positive aspects of the –NMM and –LLMM media. All media result in the retention of good epidermal morphology when used as per instructions in this protocol. Tissues cultured with EPI-100–LLMM will be the darkest, EPI-100-NMM will have the least pigmentation, and tissues cultured in EPI-100–MM-113 will be of intermediate darkening. For lightening studies, EPI-100-NMM-113 is recommended. For darkening studies, use EPI-100-MM (which omits all melanogenesis stimulators) is recommended. *Note:* EPI-100-MM, which contains no stimulators of melanogenesis, can also be used (for instance for darkening studies) but the number of viable tissue layers will decrease in experiments in excess of 3 days.

d) **Recommended lightening study design:** Use the MEL-300-B-NMM-113 or MEL-300-A-NMM-113 tissue. Prepare tissues (Section II) and apply the initial treatment (Day 0) on Wednesday (or on the day following overnight equilibration) and then continue to apply the treatment every Monday, Wednesday, and Friday (e.g. Days 0, 1, 3, 6, and 8). Include tissues treated with 2% kojic acid (25 μ l) as a positive control and sterile, ultrapure water (25 μ l) as a negative control. For MEL-300-B, single tissues are harvested and fixed with 10% formalin on Day 3, 7, 10, and 14; for MEL-300-A, apply test articles on Days 0, 1, 3, 6, 8, and 10 and fix tissues on Day 7, 10, 14, and 18. In addition to these fixed tissues, on Days 10 & 14, 2 additional MEL-300-B tissues for each treatment are frozen for the melanin assay; for a study utilizing MEL-300-A, tissues should be frozen on Day 14 & 18. *Note:* Towards the end of the experiment, application of the test article is omitted to verify that “skin lightener suppressed” melanocytes (sometimes of rounded or abnormal morphology) are viable and that they will return to normal melanocyte (dendritic) morphology and melanin production once the skin lightener is removed.

e) **Analysis:** The fixed tissues are lined up and photographed for an evaluation of the macroscopic lightening (Section IV.b) as a function of time and are used for top view microscopic observation of the melanocytes (Section IV.a). These same tissues are then processed for histology (Section IV.c). The frozen tissues are used to quantify melanin content (Section IV.e).

f) **Tissue/media requirements:** Using this study design, 8 tissues each are required for the negative control, the positive control (2% kojic acid), and for each treatment condition. Thus, from 1 MEL-300 kit, 1 lightening treatment/formulation plus the negative and positive controls can be evaluated. If more formulations are to be evaluated, 3 additional formulations per MEL-300 kit can be evaluated in the same experiment utilizing the same negative and positive controls (*Note:* The treated tissues and controls must be run simultaneously). To perform the recommended study, 1 additional bottle of EPI-100–NMM-113 is required. An initial pre-screen of the lightening treatment conditions to ensure that no cytotoxicity results from the treatment is also strongly recommended (Sections III.a & IV.d). The less expensive EpiDerm tissue (EPI-200) can be used for this purpose.

VI. Materials Provided

MelanoDerm® (Part No. MEL-300)

<u>Quantity</u>	<u>Description</u>	<u>Part No.</u>
24	MelanoDerm tissue samples	MEL-300-B, -A, -C
2	12-well plates with hanging top (sterile)	HNG-TOP-12
1	DPBS rinse solution, 100 mL	TC-PBS
1	Maintenance medium, 625 mL	EPI-100-NMM-113
1	MelanoDerm protocol – <i>delivered electronically</i>	MK-24-007-0019

MelanoDerm® (Part No. MEL-606)

<u>Quantity</u>	<u>Description</u>	<u>Part No.</u>
6	MelanoDerm tissue samples	MEL-606-B, -A, -C
4	6-well plates (sterile)	MW-15-003-0027
6	100-mm petri dishes (sterile)	MK-15-003-0031
1	DPBS rinse solution, 100 mL	TC-PBS
1	Maintenance medium, 810 mL	EPI-100-NMM-113
1	MelanoDerm protocol – <i>delivered electronically</i>	MK-24-007-0019

VII. Optional Materials / Services

<u>Quantity</u>	<u>Description</u>	<u>Part No.</u>
24	EpiDerm tissue samples (for viability studies)	EPI-200
48	Stainless steel washers, sterile (1 pack/kit required)	EPI-WSHR
6	Stainless steel washers, sterile (1 pack/kit required)	EPI-WSHR-606
1	Culture stands, sterile (24/kit required)	MEL-STND
10	Glass bottom microwell dish	P50G-0-14-F.S
1	Maintenance medium, 250 ml	EPI-100-LLMM, -EPI-NMM-113
1	H&E histology processing i) H&E stained, ii) unstained	MEL-HIS
1	Fontana Mason staining of fixed tissue	MEL-FM
1	Photography of H&E and unstained slide(s)	MEL-PHO
24	Applicator pins	PIN-24
1	Melanin assay (2 tissue required/treatment)	MEL-ASSY
2	24-well plates (sterile)	MEL-P24 (MW-15-003-0028)

MTT Kit for MelanoDerm (part # MTT-100)

<u>Quantity</u>	<u>Description</u>	<u>Part No.</u>
1	MTT assay kit	MTT-100