ZenSkin RHE Care Manual

INSTRUCTION MANUAL ZBM0105.00

SHIPPING CONDITIONS

RHE-24

Orders are delivered via Federal Express courier. All US and Canada orders are shipped via Federal Express Priority service and are usually received the next day. Please inquire if alternate couriers are needed.

Must be processed upon shipment receipt.

STORAGE CONDITIONS

Kit: See storage information of kit components on page 3.

All Zen-Bio Inc. products are for research use only. Not approved for human or veterinary use or for use in diagnostic or clinical procedures.

Ordering Information and Technical Services

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Electronic mail (e-mail)	information@zenbio.com
World Wide Web	http://www.zenbio.com

THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

RHE-24	ZENSKIN RHE KIT

PRECAUTIONS

This product is for research use only. *It is not intended for human, veterinary, or in vitro diagnostic use*. Proper precautions and biological containment should be taken when handling cells of human origin, due to their potential biohazardous nature. **Always wear gloves and work behind a protective screen when handling primary human cells.** All media, supplements, and tissue culture ware used in this protocol should be sterile.

LIMITED PRODUCT WARRANTY

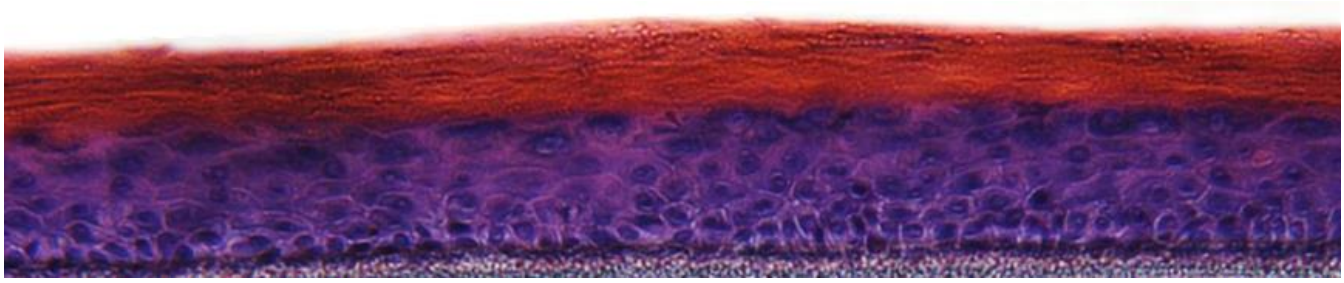
This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Zen-Bio, Inc. warrants its cells only if Zen-Bio media are used and the recommended protocols are followed without amendment or substitution.

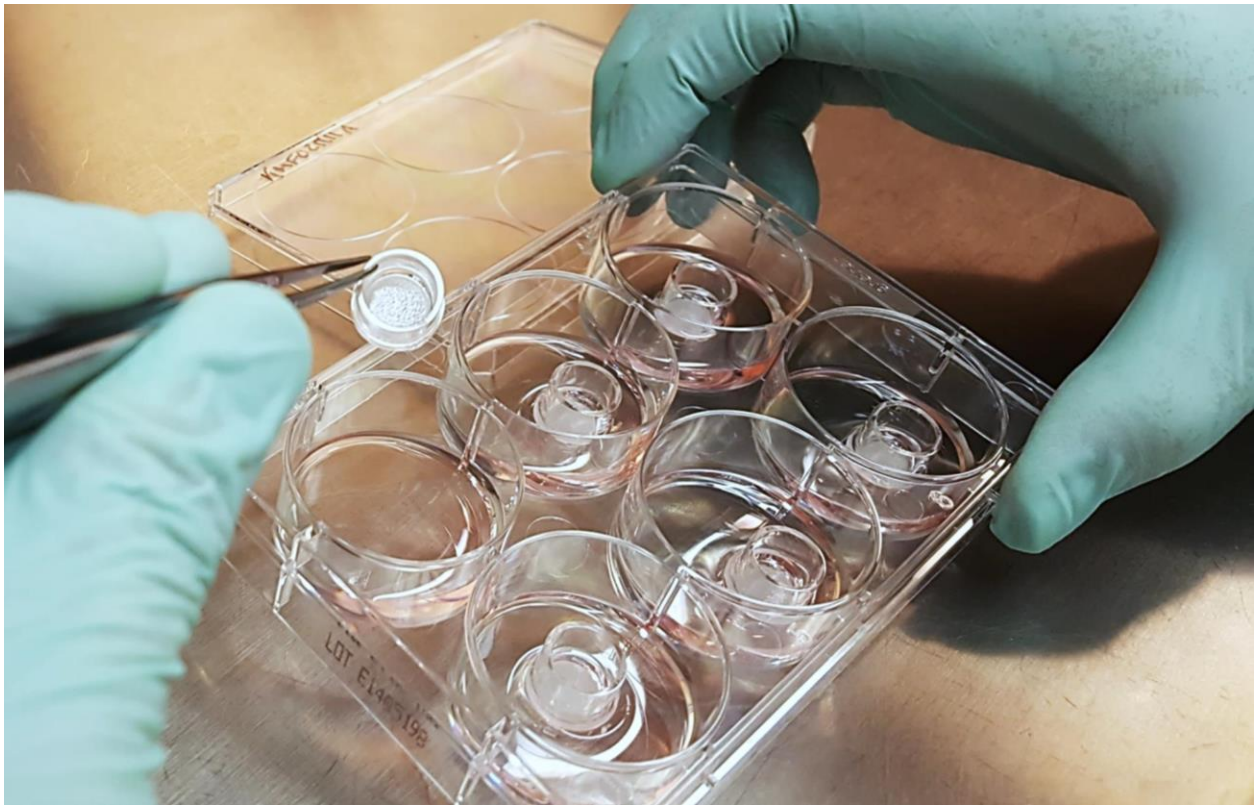
Contact ZenBio, Inc. within no more than 24 hours after receipt of products for all claims regarding shipment damage, incorrect ordering or other delivery issues. Delivery claims received after 7 days of receipt of products are not subject to replacement or refund.

INTRODUCTION

ZenBio's Reconstructed Human Epidermis (RHE), called ZenSkin RHE, is generated from keratinocytes isolated from parental-consent donated human neonatal foreskin tissue in the United States. Each sample has validated parental consent on file for donation of tissues for non-clinical research. ZenSkin RHE is an excellent platform for scientists interested in investigating the effect of experimental compounds and formulations topically and systemically. The ZenSkin RHE is a three-dimensional model replicating and mimicking the human epidermis, which consists of a stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC). Included in the RHE Kit are 24 ZenSkin RHEs, RHE Assay Medium, MTT, 1% Triton-X 100, and cultureware.



A. H&E staining of a ZenSkin RHE.



B. ZenSkin RHEs in culture format

MATERIALS PROVIDED FOR EACH CATALOG ITEM_____

❖ZenSkin RHE Kit (Catalog# RHE-24)

ITEM	DESCRIPTION	UNIT	QTY	STORAGE
Cells	24 ZenSkin RHEs	PLATE	1	4°C
Assay Plates	6 well assay plates, blank	PLATE	4	---
Assay Plates	24 well assay plates, blank	PLATE	2	---
Assay Medium	RHE Assay Medium (cat# RHE-1), 60ml	BOTTLE	1	4°C
Buffer	DPBS, 125ml	BOTTLE	1	RT
Positive Control	1% Triton-X100, 10ml	VIAL	1	RT
MTT	MTT Powder, 10mg	VIAL	1	4°C, protected from light
Forceps	Disposable Sterile Polystyrene Forceps, Sterile	EACH	1	--

**Place RHEs in culture media upon arrival. Testing should begin the following day.
RT= Room Temperature

MEDIUM COMPOSTION

RHE Assay Medium (catalog# RHE-1)	Storage and Expiration Date
MCDB153 Custom DMEM/F12 (1:1) Insulin, human Human Epidermal Growth Factor Bovine Pituitary Extract (BPE) Other Proprietary Ingredients	If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label.

ZenSkin RHE Topical Treatment Protocol

1. Upon receipt of the ZenSkin RHE kit, warm the RHE Assay Medium to 37°C.

2. Add 1ml/well of pre-warmed RHE Assay Medium to the appropriate number of 6 well plates.

3. Carefully remove the cell culture inserts from the nutrient supplemented agarose gel and place 1 insert per well. Incubate at 37°C, 5% CO_2 overnight.

4. Replace Assay Medium: Following the overnight incubation, remove the assay media contained within the 6-well plates and replace with 1 ml (per well) of pre-warmed, fresh Assay Medium.

Note: Any air bubbles trapped underneath the cell culture insert should be released (tilt the cell culture insert with sterile forceps) so that adequate nutrients are supplied to the tissue samples.

5. Apply test material: Topically apply the test material onto the ZenSkin RHE. 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 vehicle) should be handled in an identical manner to the dosed tissues.

Exposure times: For ZenSkin RHEs, time range finding dose between 1 and 72 hours recommended. *Additional media may be required for longer experiments.

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.

Note: If investigating multiple time points, back-calculate appropriately to ensure all samples are harvested at the same time!

6. Return the 6-well plates containing the RHEs to the incubator for the desired time period.

Media for inflammatory mediator analysis: Collect and save the assay media from the 6-well plates for subsequent analysis. Store at -80°C.

PCR and/or histological analysis: The RHEs can be removed from the inserts and stored in RNA stabilization reagent for future PCR analysis, or fixed in 10% neutral buffered formalin for histological analysis.

MTT ET-50 Protocol (Optional)

1. Approximately 1 hour prior to the end of the dosing period, prepare 1mg/ml of MTT solution by adding 10ml of pre-warmed RHE Assay Media to 10mg MTT powder (provided).

2. Prepare MTT plate: 15 minutes before each dosing period is complete, prepare a 24-well plate with MTT solution. Pipet 300ul of the MTT solution into the appropriate number of wells. Label the 24-well plate top to indicate which wells the samples will be transferred.

3. Transfer samples to MTT plate: After treatment(s) are complete, decant any liquid remaining within the RHE insert. Remove each insert individually and gently rinse with pre-warmed DPBS to remove any residual test material. Repeat this rinse a second time. Shake off excess liquid prior to placing the

insert into the MTT containing 24-well plate, making sure that no air bubbles are trapped underneath the cell culture insert.

4. MTT loading: Return the tissue samples in the 24-well MTT plate to the incubator for 3 hours. 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.

5. After the 3 hour MTT incubation period is complete, remove each insert individually and gently blot the bottom with a Kimiwipe. Finally, place the inserts into the pre-labeled 24-well extraction plate.

6. Immerse the cell culture inserts with 2.0ml of the 200 proof EtOH (not provided) per well to completely cover the cell culture insert. Cover the extraction plate to reduce evaporation.

Note: If the test article is colored and does not completely rinse off, pipet 1.0 ml of EtOH 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 1.0ml of EtOH to bring the total volume to 2.0ml.

Note: If 200 proof EtOH is unavailable, 70% Isopropyl Alcohol can be used.

7. 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.

8. 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 EtOH in the well). The inserts can be discarded.

Construction of Dose Response Curve

1. Pipet the EtOH solution up and down at least 3 times to insure that the extraction solutions are well mixed.

2. Pipet 200ul of the mixed extraction solution into a 96-well microtiter plate.

3. Determine the optical density of the extracted samples at 570nm using 200ul EtOH as a blank.

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

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.

Choose new times (if necessary): Based on the time range finding plot, additional time points may be necessary or desirable.

FREQUENTLY ASKED QUESTIONS

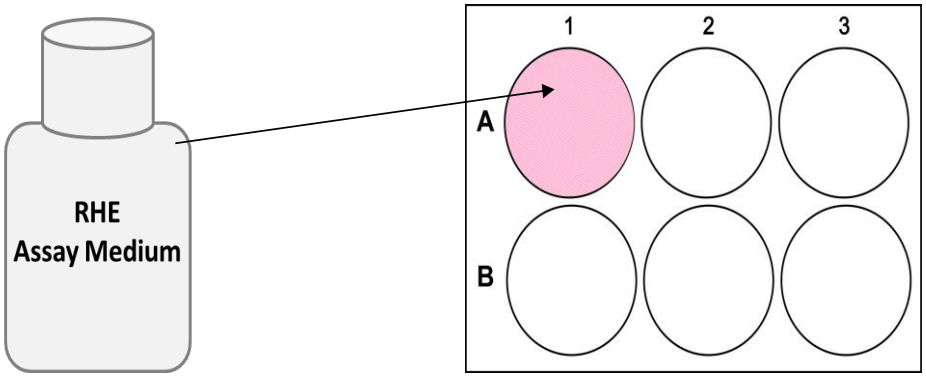
1. **From where are the cells obtained?** Human keratinocytes are isolated from the foreskins of healthy male newborns in the United States. Each sample has validated parental consent on file for the donation of tissues for non-clinical research. The cells are isolated by centrifugal force following enzymatic treatment.
2. **Do I have to use the RHE's immediately upon arrival**? Yes. We recommend using the RHE immediately upon arrival. Contact ZenBio, Inc to coordinate shipment arrival with your schedule at the time of order.
3. **Can I perform multiple media changes?** Yes. However, additional RHE Assay Medium (Cat# RHE-1) will be needed for multiple media changes. Contact Zen-Bio for details.

PATHOGEN TESTING

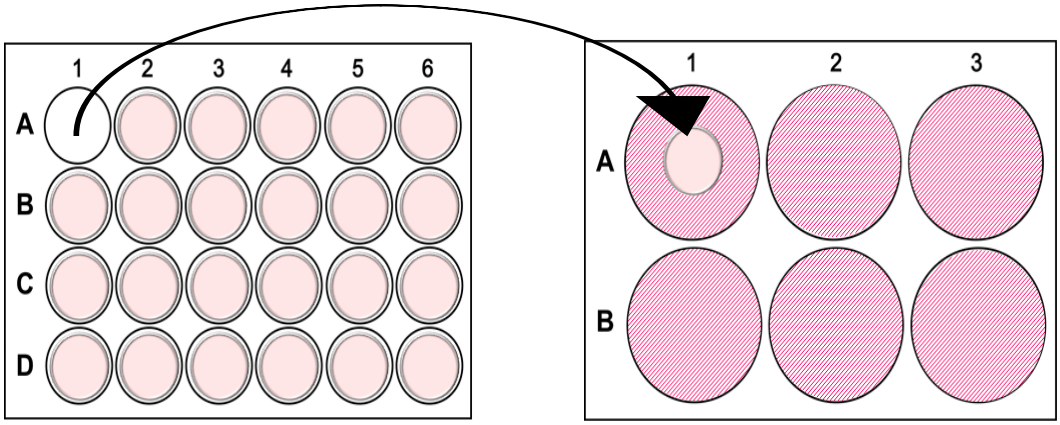
Each lot of primary cells is tested via PCR and found non-reactive to viral DNA from HIV and hepatitis B and viral RNA from Hepatitis C. However, no known test can offer complete assurance that these viruses are not present. Since we cannot test all pathogens, always treat the culture as a potentially infectious reagent. We recommend using the US Centers for Disease Control (CDC) Universal Precautions for prevention of blood-borne pathogens as a minimum guideline for standards of practice at Biosafety Level 1 or higher. Our cells are tested for mycoplasma contamination via direct plating and DNA fluorochrome staining; mycoplasma contamination is not detected.

APPENDIX A: ASSAY PROCEDURE FLOWCHART

Warm RHE assay medium to 37°C.
Add 1 ml of warm assay medium to each well of the provided 6-well plates



Using the provided forceps, gently remove RHEs from the supplemented agarose gel and place 1 per each well.
Equilibrate overnight in a 37°C 5% CO_2 incubator

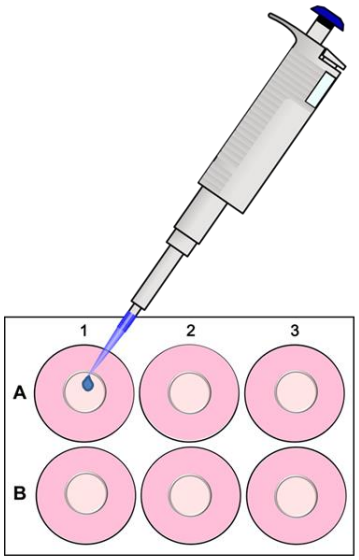


Following the overnight incubation, aspirate the assay media contained within the 6-well plates and replace with 1ml (per well) of pre-warmed, fresh assay media

Apply test articles using on of the following methods:

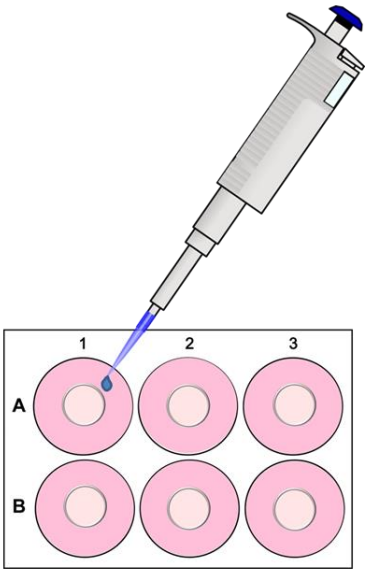
Topical Liquid:

Apply desired amount of test article to the surface of the RHE



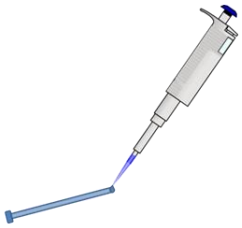
Systemic Liquid:

Apply desired amount of test article to the Assay Medium

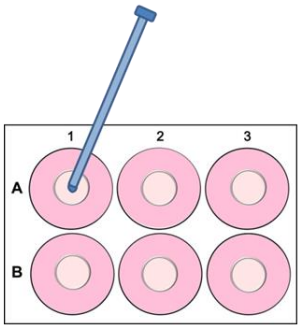


Topical Cream or Lotion:

Using a positive displacement pipette, apply the desired amount of test article to a clean glass rod or sterile 1cc syringe plunger



Gently apply the test article to the surface of the RHE using a circular motion, working from the inside outward



Incubate at 37°C, 5% CO_2 for the desired time period

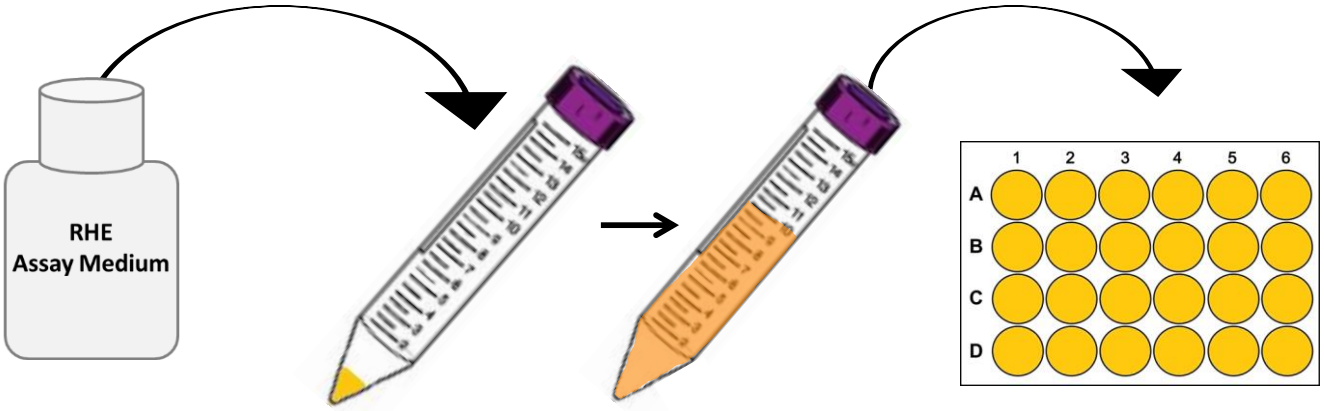
Collect media and store at -80°C. **PCR and/or histological analysis**: The RHEs can be removed from the inserts and stored in RNA stabilization reagent for future PCR analysis, or fixed in 10% neutral buffered formalin for histological analysis.

(If performing MTT, proceed to Appendix B)

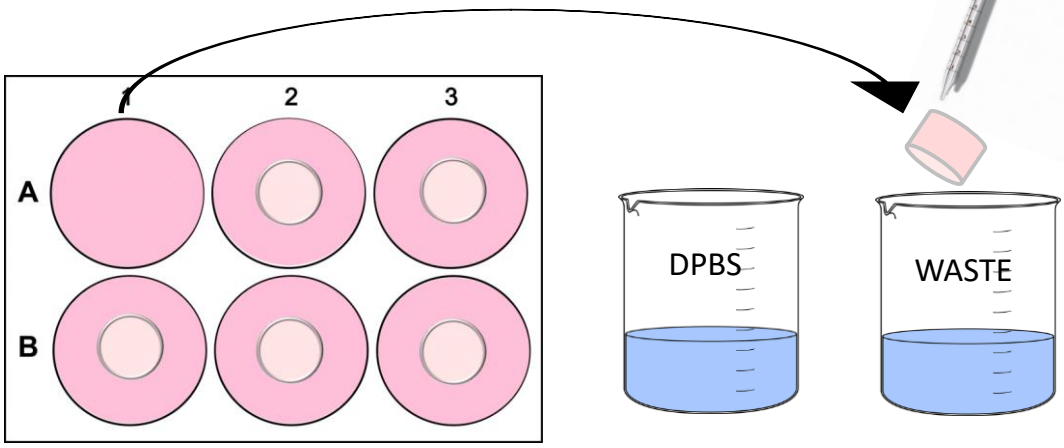
APPENDIX B: MTT PROCEDURE FLOWCHART

Warm RHE assay medium to 37°C.
Add 10 ml of warm assay medium to 10mg of the provided MTT powder

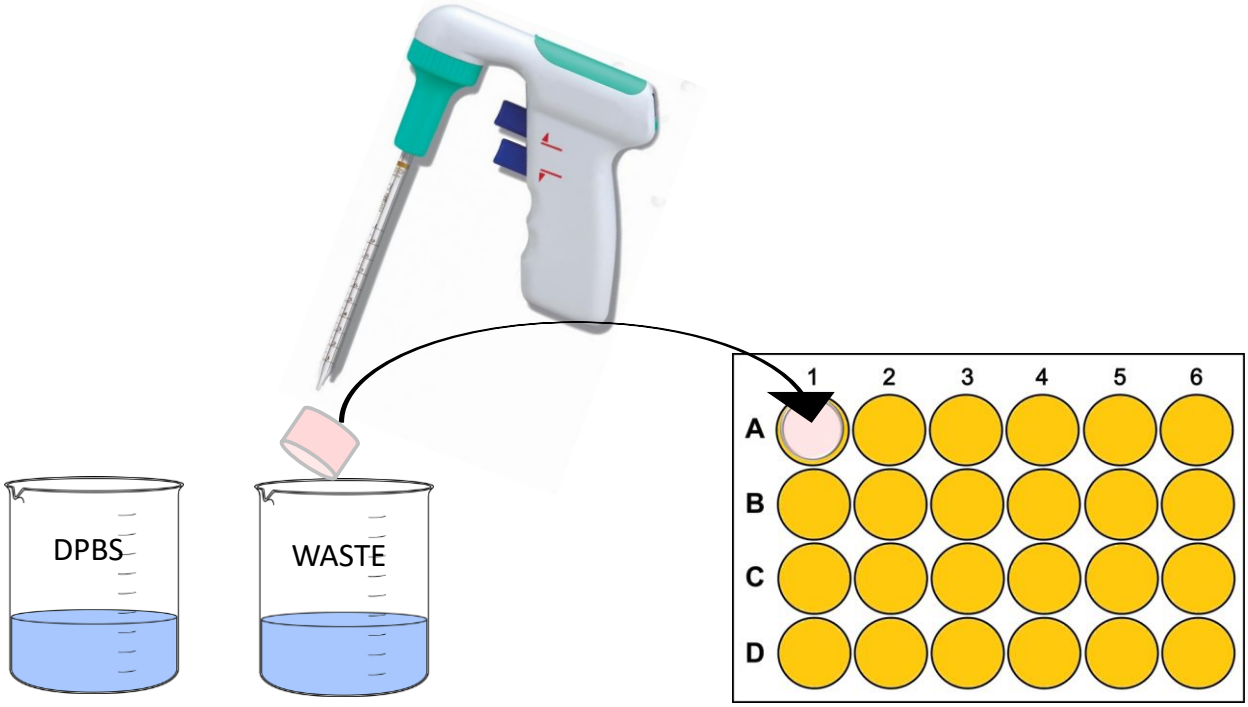
Add 300ul of MTT solution to each well (Protect from light)



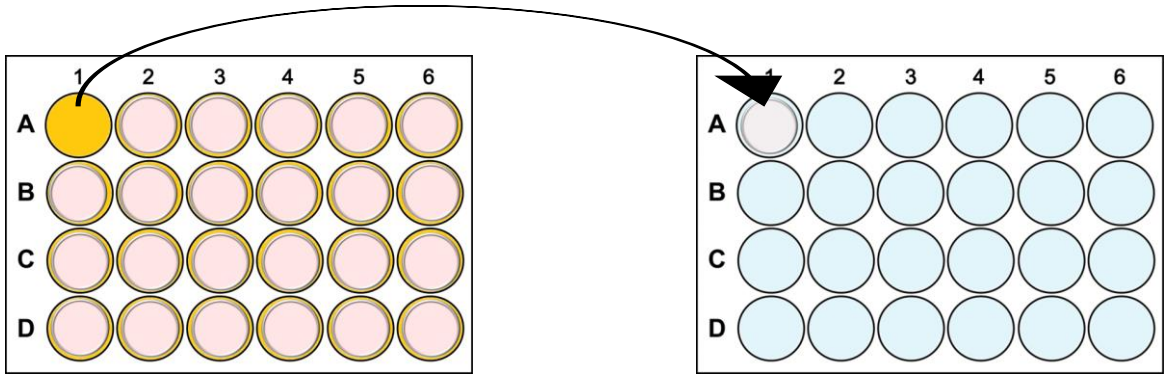
Gently decant remaining liquid within the RHE insert and rinse 2x with pre-warmed DPBS. **Gently** pour off excess liquid



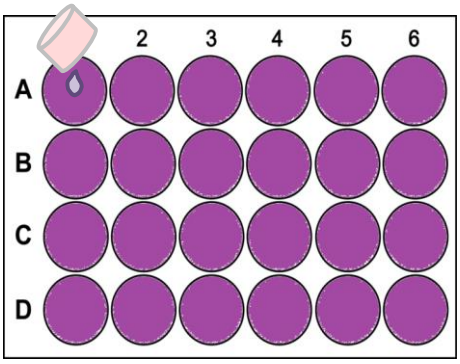
Place RHE inserts into the MTT containing 24-well plate. Make sure that no air bubbles are trapped underneath the insert. Incubate at 37°C, 5% CO_2 for 3 hrs.



Following the 3hr incubation, transfer and completely submerge RHE inserts in 2ml of 200pf EtOH. Seal plate and protect from light. Extract for 2hrs at RT on an orbital shaker (~30rpm) or overnight at RT without shaking.



Following extraction, remove the RHE inserts and decant the liquid back into the well from which it was taken



Mix thoroughly by pipetting

Transfer 200ul of the mixed extraction solution into a 96-well optically clear, flat bottom plate. Determine optical density @ **570nm**. Use 200ul of EtOH as a blank