6-2024

Transport of The Fluorescent Substrate ASP+ by Organic Cation Transporter-3 in Human Corneal Epithelial Cells

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TRANSPORT OF THE FlUORESCENT SUBSTRATE ASP$^+$ BY ORGANIC CATION TRANSPORTER-3 IN HUMAN CORNEAL EPITHELIAL CELLS

by

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A THESIS

Presented to the Faculty of the University of the Incarnate Word
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE IN APPLIED PHARMACEUTICAL SCIENCES

UNIVERSITY OF THE INCARNATE WORD

August,2024

ACKNOWLEDGMENTS

Firstly, I would like to acknowledge Dr. Rheaclare Fraser-Spears for all her teachings and for guiding me through this master's program. Her time, energy, and encouragement through this life-changing experience and being my first mentor on my scientific journey.

Secondly, I would like to thank Dr. Adeola Coker for accepting me into the applied pharmaceutical science program, trusting her intuition that I was the right choice for this program, and giving me my chance.

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ABSTRACT

Organic cation transporter-3 (OCT3) is expressed abundantly throughout the body, but little is known about the presence of these transporters within the eye and, more specifically, the cornea epithelium. An extensive library of compounds interacts with OCT3, including the fluorescent molecule ASP^+ and metformin. Metformin is an OCT3 substrate and antidiabetic medication that can lower the risk of eye-related diseases like keratopathy and retinopathy linked to hyperglycemia. Utilizing high throughput microplate assays in a human cornea epithelial cell line (HCE-S), the time-dependent saturation of ASP^+ uptake and the competition of ASP^+ uptake by corticosterone (CORT), a known selective inhibitor of OCT3 was evaluated. The hypothesis is that OCT3 is present and functional in human corneal epithelial HCE-S cells. The specific aims are to determine the functionality of OCT3 using saturation and competition assays that provide uptake capacity, transporter kinetics, and potency information. Saturation (B_{max} and K_d) and competition (IC_{50}) data help to establish the expression and function of OCT3 in HCE-S cells as a model to understand better the transporter's function in the human cornea epithelial cells. The findings suggest OCT3 is robustly expressed in HCE-S cells and can function to transport ASP^+. At baseline (25 mM glucose levels), the HCE-S cell line expresses transporters like OCT3 (and likely other isoforms) that can bind and transport the fluorescent substrate ASP^+, which can be blocked by CORT and decynium22 (D22). The ASP^+ saturation assay and time trial 30-minute uptake experiments are better for uptake

capacity and affinity (B_{max} ≅ 1100 RFU; K_d ≅ 272 μM). CORT competition assay using higher glucose levels may potentially decrease the potency of CORT to inhibit ASP^+ uptake in HCE-S cells (IC_{50} ≅ 283 μM (baseline); ≅ 1717 μM (50 mM glucose, 24 hrs.). Our results also imply that other non-OCT3 transporters are present in this endogenous cell model capable of ASP^+ uptake, as evidenced by our non-specific binding measurements using a non-selective inhibitor, D22. The next stage of competition experiments will use metformin to confirm that OCT3 is active in the HCE-S cell line. This work has future implications for establishing corneal cells as a new model to study OCT3 activity, test the pharmacological characteristics of ligands, and develop alternate administration routes for metformin (e.g., as eye drops).

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Background

The human solute carrier family, known as the organic ion transporter family (SLC22), is made up of 28 membrane-spanning proteins.[1] Three distinct groups in the SLC22 family are the organic cation, organic anion, and zwitterion transporters. The groups are determined based on charges related to the protein structure and the substrates associated with the specific transporter. There are three primary subtypes of organic cation transporters (OCTs) within the SLC22 family: the organic cation transporter-1 (OCT1), -2 (OCT2), and -3 (OCT3). OCTs are secondary transporters with a high capacity but low affinity for molecule binding and transport.[2] Capacity is the maximum amount of molecules the transporter can bind to (indicated by maximum binding capacity or B_{max}), and affinity is how well a compound will inhibit the action of the transporter[3] (equilibrium constant K_d or inhibitor concentration rendering half the transporters uptake capacity (IC_{50})), such that smaller affinity values indicates greater attraction between the ligand and transporter. This project will focus on determining the function and expression of OCT3 in a human corneal epithelial cell line by evaluating B_{max}, K_d, and IC_{50} values obtained from functional assays measuring OCT-mediated uptake.

The human corneal epithelial cell line, HCE-S, was chosen because it is immortalized from human cornea tissue, has resilient growth, and has been shown as a useful tool for studying drug molecules.[4] Like OCT1 and OCT2, OCT3 interacts with a wide array of endogenous and exogenous small organic cations, including vitamins, neurotransmitters, environmental chemicals, and prescription drugs.[5] However, while OCT1 is dominantly expressed in the liver and OCT2 is primarily expressed in the kidneys, OCT3 is expressed pervasively throughout the body and brain.[1,6] OCT3 is also abundantly expressed in the blood-brain barrier.[5] OCTs are functionally active in blood-ocular barriers and transport substrates from blood to vitreous humor.[7] A few studies have used animal ocular tissue to show the presence of OCT transporters, particularly OCT1.[8,9,10] OCTs,

including OCT3, are reported to be involved in drug transport in the mouse eye and human retinal pigment epithelium cell lines[11,12]. However, the role of OCT3 in corneal cells has not been identified. This project tests the hypothesis that the OCT3 isoform is expressed and functional in the human corneal epithelial HCE-S cell line.

An extensive library of molecules interacts with OCT3 as substrates or inhibitors, including the antidiabetic medication metformin.[5,13] Diabetes, or elevated blood glucose, is a known cause of several health complications, such as diabetic retinopathy (retina disease) or keratopathy (cornea disease), which are common complications of diabetes mellitus and are a major cause of vision loss.[14,15] Diabetic keratopathy is generally described as a deviation from the standard wound healing mechanism, leading to persistent corneal epithelial defects and unresponsiveness to treatments in the hyperglycemic environment. [16]

Metformin, a substrate of OCT3[5,17], shows a significant ability to lower diabetic retinopathy in patients with type 2 diabetes[18] and reduce corneal endothelial cell density in patients with glaucoma.[19] It is possible that OCT3 could function as a transporter responsible for getting metformin into corneal cells to mitigate complications in eye tissue that are present due to hyperglycemia and diabetic retinopathy. Note that the specific action of metformin within the cornea epithelium is outside this project's parameters but is an interesting future direction. The expression and function of OCT3 in corneal cells are not well-researched both under baseline conditions or in the presence of a hyperglycemic environment. The baseline culture condition for the human corneal epithelial (HCE-S) cells includes 25 mM glucose in the growth media. An extension of this project will compare OCT3 activity using the *in vitro* HCE-S cell-based model maintained in normal (baseline) versus hyperglycemic (elevated glucose) culture conditions.

Radioligand binding is a commonly used and widely accepted assessment method to determine the density of transporter expression or ligand properties. It uses "a radioactively labeled

compound (e.g., ^{3}H or ^{14}C), which binds at the target binding site".[20] To assess the function of the transporters, researchers have often used radiolabeled substrates like [^{3}H]MPP^{+} or [^{3}H]metformin to evaluate the uptake activity of OCTs.[21,22,23] While radioisotopes provide extensive information about OCT binding properties, the use of hazardous radioactive material imposes limitations such as expensive controlled disposal of waste and the need for specific licensing.[20] To circumvent these constraints, this project will use a microplate method to measure the uptake of the fluorescent molecule 4-Di-1-ASP (4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP^{+}). ASP^{+} is a fluorescent derivative of 1-methyl-4-phenylpyridinium (MPP^{+}) used to study transporter kinetics.[24] The benefits of ASP^{+} are its cost-effectiveness, reduced licensing and hazardous waste disposal, and its adaptability to high throughput/large sample quantity measurements.

ASP^{+} is a known substrate for OCT3 and was used to confirm the function of OCT3 in different cell lines.[25] In this thesis project, the fluorescent ASP^{+} microplate method was used to determine the functional activity of OCT3 in corneal cells. Human corneal epithelial cells (HCE-S) are commercially available immortalized cell lines used in all experiments described herein. The experiments provide evidence that the isoform OCT3 is expressed and functional in human corneal epithelial cells. The first aim evaluated the functionality of OCT3 using saturation time course assays in the HCE-S cell line. Saturation assays provide information about these cells' ASP^{+} uptake capacity (B_{max}) and transporter affinity (K_d). The time trial experiments were next utilized to determine if longer exposure times had a statistically significant effect on the uptake and transport effect of OCT3. The second aim utilized competition assays to provide potency information (IC_{50}) about CORT binding interactions with the transporter and its inhibition of OCT3-mediated ASP^{+} uptake in the corneal cell line. Results from each aim provide information on the function of OCT3 in human corneal epithelial cells and the interactions with these specific drugs. Furthermore, elevated extracellular glucose levels may alter OCT expression or function, based on preliminary findings

herein. The collective results from these studies give rise to a new avenue to investigate the role of OCTs in an *in vitro* ocular model.

Materials and Methods

This study was conducted from the summer of 2023 to spring 2024. All experiments and research were performed at the University of the Incarnate Word Feik School of Pharmacy in San Antonio, Texas. This project was funded through an internal Feik School of Pharmacy Research grant and funding support from the MS in Applied Pharmaceutical Sciences Program.

Materials

All chemicals and laboratory supplies were purchased from Millipore-Sigma (USA), Thermo Fischer Scientific (USA), Stellar Scientific (USA), or Applied Biological Materials (CAN).

Cell culture maintenance

The human corneal epithelial cells (HCE-S), PriGrow III media, and applied extracellular matrix are purchased from Applied Biological Materials (CAN). The cells were maintained at 37°C/5% CO_2 in complete growth media (CGM) consisting of Pri Grow III supplemented with 10% non-heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 2 mM glutamine. Cells were fed with CGM every 2-3 days and passaged into new 100 mM culture dishes when they were ~90 % confluent. Cells were seeded onto 24- or 96-well, black, clear-bottom plates for ASP^+ assays. Detailed steps for cell culture maintenance are provided to prepare CGM (Appendix I), apply the extracellular matrix (Appendix II), and pass the cells (Appendix IV).

Hyperglycemic growth conditions

HCE-S cells were grown in the standard CGM for baseline glucose (25 mM) or in CGM containing a moderately higher glucose concentration (~50 mM) of glucose. Future studies will

include exposure to a high-level hyperglycemic (≧ 60-100 mM) state.[29] Cells exposed to higher glucose conditions were evaluated in CORT competition assays.

ASP^+ uptake saturation and time trial assays

The ASP^+ uptake saturation assay was conducted to determine the function of the organic cation transporters in the human cornea epithelial cells. The initial experiments were performed in a 24-well format and then scaled to a 96-well format following the ASP^+ saturation standard operating procedure (Appendix VI). Appendices III and VI describe detailed protocols for counting cells and plating the uptake experiments, respectively. Briefly, the plated cell density for experiments was 100,000 cells/well (24-well plate) or 50,000 cells/well (96-well) incubated at 37°C for 24 hrs. The 96-well format allowed for having more replicates and concentration ranges per plate. Following a 10-minute acclimation in 1X Krebs-Ringer HEPES (KRH) uptake buffer, the total ASP^+ uptake was measured at a range of substrate concentrations 0-10 mM for 10 min at 37°C. For non-specific uptake, ASP^+ was prepared with CORT, a selective OCT3 inhibitor, or decynium-22 (D22), a non-selective specific inhibitor of all OCTs. ASP^+, CORT and D22 were prepared in KRH uptake buffer containing a final concentration of 1% DMSO to help dissolve the drugs. D22 and CORT at 100 μM were used for the nonspecific binding. After the uptake assay time (10 min or 30 min) was complete, cells were washed 3x in cold KRH buffer to remove residual ASP^+ and stop the uptake process. Cells were lysed by adding 1% SDS to each well, and ASP^+ fluorescence was detected with a microplate reader. Time-course experiments of ASP^+ saturation ranging from 0 to 60 minutes were also performed.

Competition assays

Competition assays were performed to determine the ability of CORT to compete for OCT3-mediated ASP^+ uptake in human corneal epithelial cells. The competition assay used CORT concentrations ranging from 0-100 μm (at baseline, 25 mM glucose) or 0-1 mM (at 50 mM glucose),

mixed with a constant ASP^+ concentration of 1 mM. Appendix VII explains the protocol for the competition assay, which follows instructions similar to those for the saturation assay. The cells were plated at 50,000 cells/well and incubated for 24 hours before running the experiment. Based on the knowledge of the time trial experiments, the competition assays were performed with 10-minute acclimation times in the 1x KRH buffer, a 10-minute preincubation with nonspecific CORT, and exposure to ASP^+ with competing CORT for 30 minutes. Afterward, cells were washed 3x in cold KRH buffer to remove residual ASP^+ and stop the competition process. Cells were lysed by adding 1% SDS to each well, and ASP^+ fluorescence was detected with a microplate reader.

Data analysis

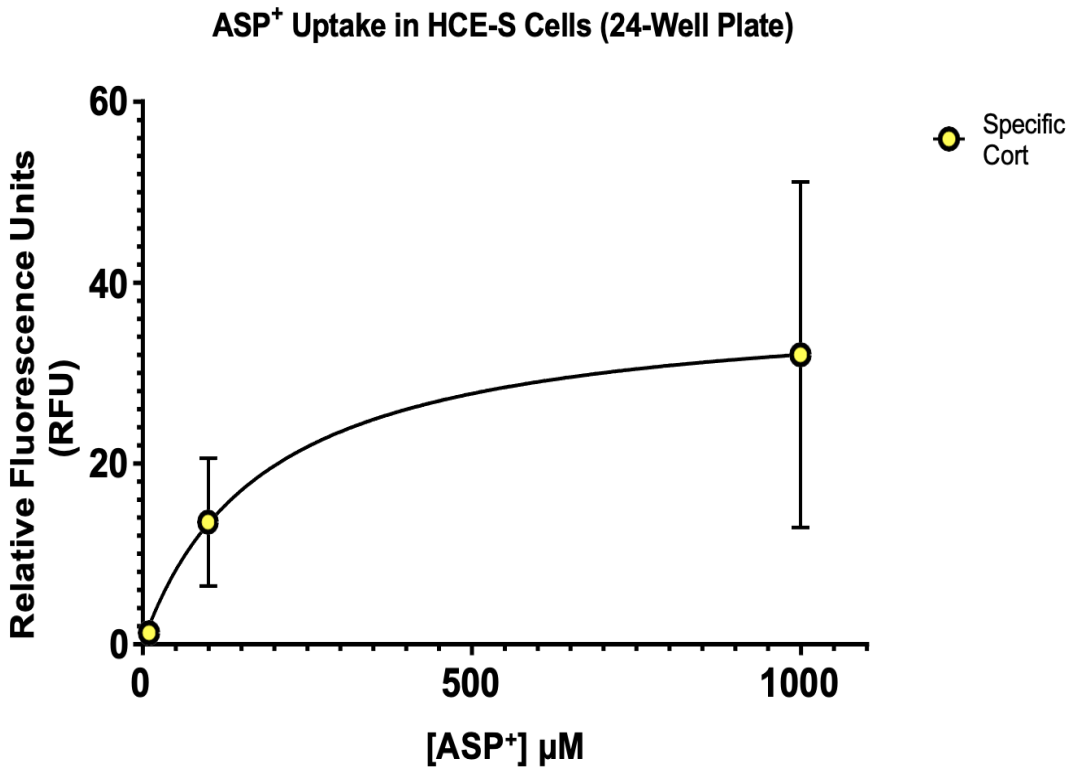
The relative fluorescence units (RFUs) were measured by a microplate reader, SynergyMax by BioTek, with set parameters of 485 excitation/590 emission using an area scan (24-well) or end-point scan (96-well) with the optics reading from the bottom and temperature registered at 37°C. The RFU data from the plate reader was organized in Microsoft Excel into columns of ASP^+ concentrations (saturation assays, μM or M) or competing compound concentrations (competition assays, log M) that correspond to total ASP^+ uptake RFU or nonspecific RFU determined with CORT or D22. Each concentration was run in four replicates (96-well plate) and then averaged for the total and non-specific uptake. To determine specific binding, the total ASP^+ concentration average was subtracted from the average of the non-specific CORT or D22 at each concentration. For the competition assay, an additional step was taken to calculate the specific uptake for each competing concentration as a percent of control (0 mM competing drug). These specific uptake values were transferred into GraphPad Prism, version 10 software, for further analysis. For the saturation experiments, the data was fit to a one-site specific binding and plotted to a nonlinear regression to determine the B_{max} (maximum binding) and K_d (affinity constant). For the competition assays, the percent of control-specific uptake was fit to a one-site fit log IC_{50} and plotted on a

nonlinear regression curve to create the concentration-inhibition curve and calculate the IC_{50} values of the competing agent. To make comparisons of the B_{max}, K_d, or IC_{50}, the null hypothesis of the parameters being the same was rejected if the p-value was less than or equal to 0.05, and a statistical difference between the compared conditions was accepted.

Results and Discussion

ASP^+ saturation experiments – 24-well format

The RFU of uptake by 6 concentrations of ASP^+ (0, 0.1, 1, 10, 100, and 1000 μM) was measured for 10 minutes at 37°C in HCE-S cells using the 24-well microplate method. Optics were positioned from the bottom, and the microplate temperature was set at 37°C. OCT3-mediated uptake was determined by utilizing CORT at 100 μM for the nonspecific interaction with ASP^+. The RFU for total uptake (without CORT) was expected to be higher than the nonspecific RFU counts. However, the trends were sporadic, especially at the highest 1000 μM concentration of ASP^+, where the margin of error was wide (Figure 1). Previous work in the lab using an OCT3 overexpressed cell line has shown the 96-well format to be more reproducible and suitable for ASP^+ uptake compared to the 24-well format (unpublished data). Therefore, in this project, ASP^+ uptake in the HCE-S cell was developed for 96-well plates (Figure 2).



One site -- Specific binding	
Best-fit values	
Bmax	37.98
Kd	185.6
95% CI (profile likelihood)	
Bmax	23.05 to +infinity
Kd	32.14 to +infinity

Figure 1: Graph of an ASP^+ saturation assay, 24-well microplate. The average specific relative fluorescence units for ASP^+ concentrations are from (0-1000 µM). Concentrations of ASP^+ are (0, 0.1, 1, 10, 100, 1,000 µM). Nonspecific ASP^+ uptake was measured with 100 µM CORT. Uptake performed at 37°C for 10 minutes, n = 2. The table presents information on B_{max} and K_d.

ASP^+ saturation experiments – 96-well format

After initially using 24-well plates (Figure 1), the ASP^+ saturation assays were modified to a 96-well format following a similar ASP^+ saturation standard operating procedure (Appendix VI), but with 8 varying concentrations of ASP^+ (0, 1, 10, 100, 300, 1000, 3000, and 10000 μM). In later experiments, a few low concentrations were removed, and the 8-range of ASP^+ concentrations included more points at the higher levels (0, 100, 300, 500, 1000, 3000, 5000, and 10000 μM). Some experiments included D22 to determine non-specific ASP^+ uptake as a point of comparison between the selective OCT3 inhibitor CORT (Figure 2). Decynium-22 is a non-selective inhibitor of all 3 isoforms of OCTs and another low-affinity transporter plasma membrane monoamine transporter (PMAT).[23,27,28] Differences observed using D22 would suggest ASP^+ uptake can happen through OCT3 and OCT1, OCT2, or PMAT. The change from 24-well to 96-well was determined based on our data and literature[5], demonstrating that the smaller surface area of the 96-well format is suitable for uptake detection using ASP^+. Our preliminary studies supported this, considering the 24-well format gave inconsistent data, while the change to the 96-well format gave clearer RFU values for total and nonspecific readouts. Figure 3 shows the 96-well format at 10 minutes, comparing the nonspecific block of ASP^+ uptake by CORT and D22, and the B_{max} was not statistically different (476 and 335 RFU, respectively). There was a statistical difference in K_d (p-value = 0.003) but not in B_{max} for specific ASP^+ uptake at 10 min when measured with D22 versus CORT as the non-specific blocker (Figure 3). This suggests the maximum capacity for ASP^+ transport did not vary when uptake was blocked from OCT3 or all OCTs. Comparing the K_d, the data showed a significant difference in the affinity of ASP^+ for OCT3 versus all OCT subtypes. CORT had a larger affinity value (6617 μM) that was statistically different from the specific uptake measured by blocking uptake with D22 (1011 μM) (Figure 3). This implies a higher ASP^+ affinity is measured for specific uptake after blocking all OCT subtypes.

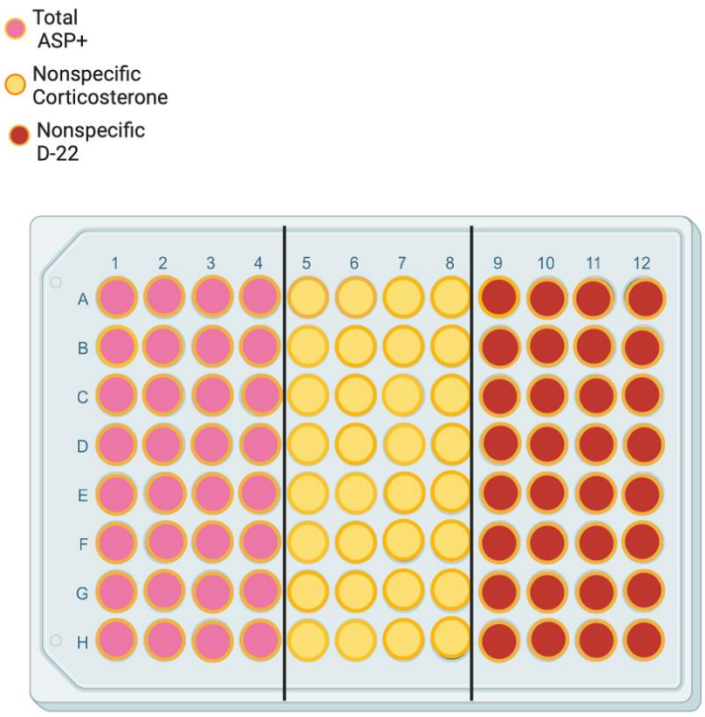
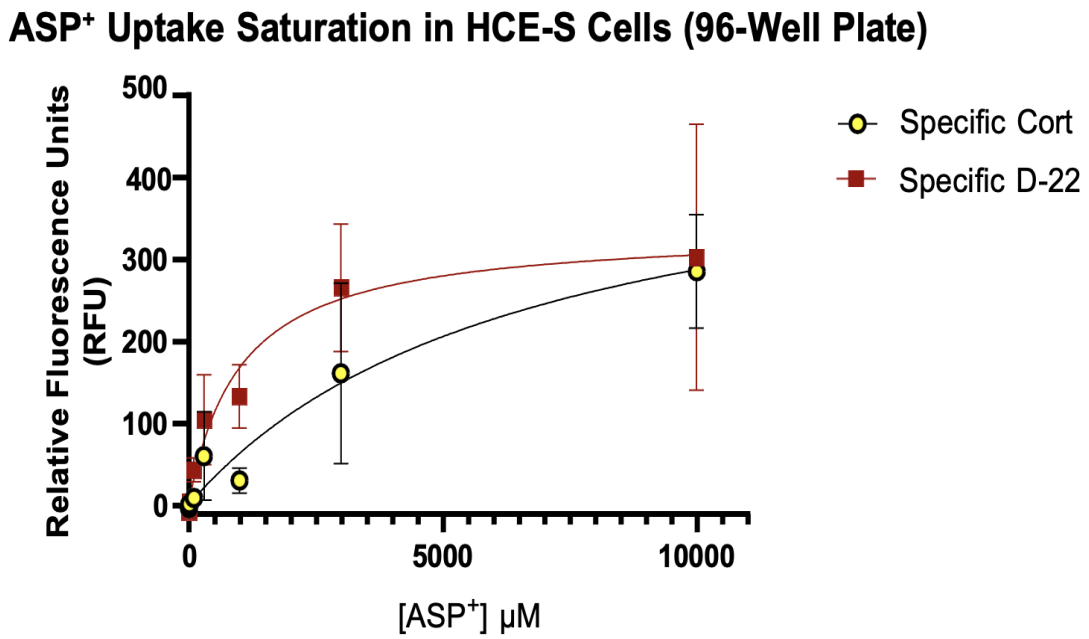


Figure 2: 96-well microplate setup for a saturation assay. The plate is divided into three sections: the Total ASP^+ concentration (pink), 100μM CORTCORT nonspecific (yellow), and the 100μM D22 nonspecific (red). The plate is incubated at 37°C. ASP^+ concentrations ranged from (0, 1, 10, 100, 300, 1000, 3000, and 10000μM in corresponding rows A, B, C, etc.).

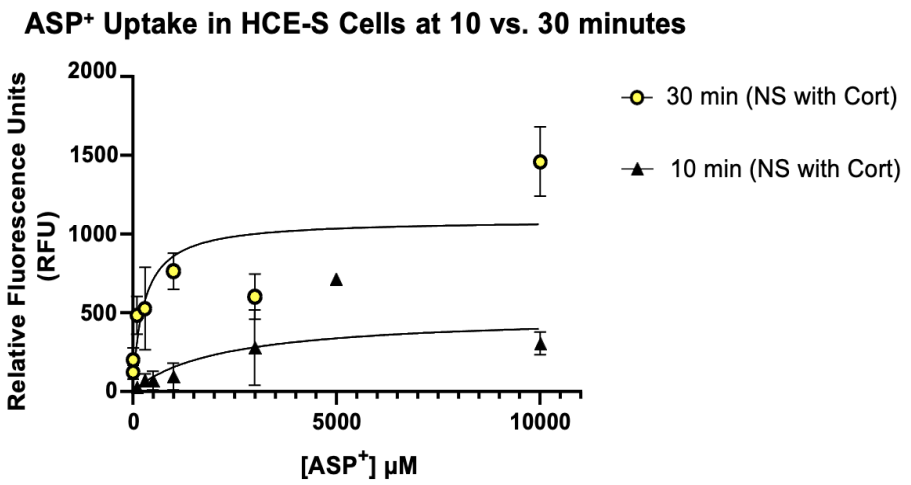


	Specific Cort	Specific D-22
One site -- Specific binding		
Best-fit values		
Bmax	476.2	335.2
Kd	6617	1011
95% CI (profile likelihood)		
Bmax	313.6 to 1218	260.4 to 436.9
Kd * p = 0.003	2619 to 29883	415.1 to 2306

Figure 3: ASP^+ uptake saturation assay, 96-well microplate. The average relative fluorescence units for ASP^+ concentrations are from (0-1000 µM). n = 4 total experiments with 4 replicates per condition (Total ASP^+, NS with CORT, NS with D22) that were averaged. The uptake was conducted at 37°C for 10 minutes. The table presents information on B_{max} and K_d. Concentrations of ASP^+ were 0 µM, 1 µm, 10 µM, 100 µM, 300 µM, 1000 µM, 3000 µM, 10,000 µM.

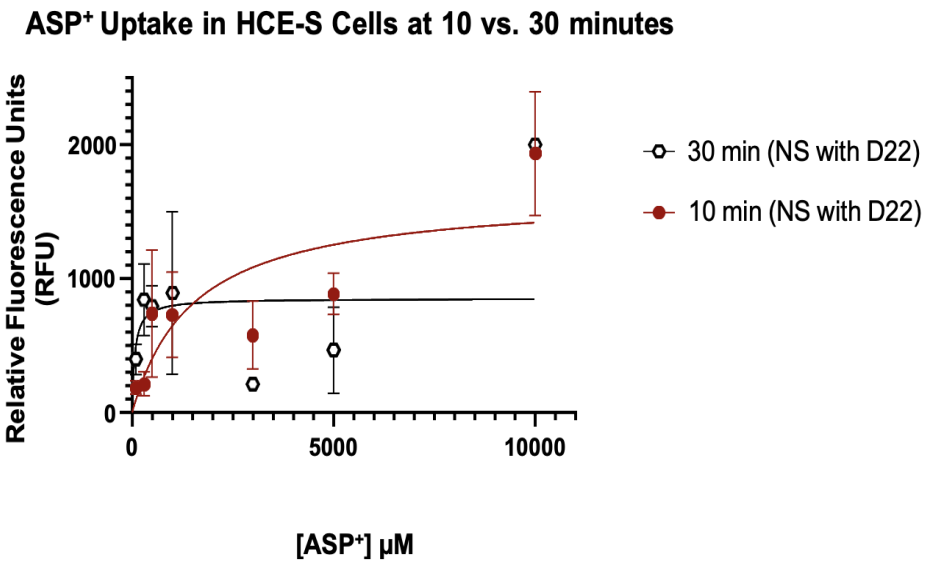
ASP^+ time trial experiments

Time trial experiments were performed to determine the assay time could impact ASP^+ uptake saturation in the HCE-S cells. Time trials at 0, 10, 30, or 60 minutes were performed at 37°C using the 96-well format. It is plausible that cells with endogenous OCT3 expression may require longer times for saturation, as was seen in one study that measured ASP^+ uptake in a placenta cell line for 30 minutes.[26] The analyzed data from the time-course experiments for ASP^+ uptake saturation using CORT to determine nonspecific uptake is shown in Figure 4. There was a significant difference between the B_{max} and K_d at the 10-minute versus 30-minute uptake assay (p-value < 0.0001). There was a greater B_{max} (1092 RFU) and higher affinity (K_d 272 μM) when ASP^+ uptake was measured for 30 minutes compared to B_{max} of 485 RFU and K_d 2224 μM at 10 minutes. The analyzed data from the time-course experiments for ASP^+ uptake saturation using decynium-22 to determine nonspecific uptake is shown in Figure 5. The D22 experiment also had statistically significant differences between the 10- and 30-minute treatments. Both the B_{max} and K_d had a shared p-value of 0.044. Interestingly, at 30 minutes the B_{max} (852 RFU) was lower while the affinity was higher (K_d 67.7 μM) compared to the 10-minute time point (1634 RFU, 1524 μM). Using this information, the competition assays were performed for 30 minutes using CORT to block non-specific uptake.



	30 min (NS with Cort)	10 min (NS with Cort)
Different curve for at least one data set		
Best-fit values * p < 0.0001		
Bmax	1092	485.2
Kd	271.8	2224
95% CI (profile likelihood)		
Bmax	807.9 to 1704	346.5 to 707.7
Kd	42.28 to 2329	922.1 to 5464

Figure 4: Graph of an ASP^+ time trial saturation assay with CORT for non-specific uptake in a 96-well microplate. The average relative fluorescence units for ASP^+ concentrations are from (0-10,000 µM). n = 4 total experiments with 4 replicates per condition (Total ASP^+ or with CORT) that were averaged. The table presents information on B_{max} and K_d.



	10 min (NS with D22)	30 min (NS with D22)
Different curve for at least one data set		
Best-fit values * p = 0.044		
Bmax	1634	852.6
Kd	1524	67.72
95% CI (profile likelihood)		
Bmax	1001 to +infinity	549.1 to 1235
Kd	297.7 to 12840485303887	-27.95 to 438.4

Figure 5: Graph of an ASP+ saturation assay time trial with D22 for nonspecific uptake in a 96-well microplate. The average relative fluorescence units for ASP+ concentrations are from (0-10,000 µM). n = 4 total experiments with four replicates per condition (Total ASP+ or with D22) that were averaged. The table presents information on B_{max} and K_d.

CORT competition of ASP^+ uptake

The competition assays (layout in Figure 6) followed the competition assay standard protocol (Appendix VII). ASP^+ uptake in HCE-S cells was determined by increasing amounts of CORT with a fixed concentration of ASP^+ (1 mM). These experiments were planned to establish the binding characteristics (i.e., IC_{50}) of CORT as a known OCT3 inhibitor in the human corneal epithelial HCE-S cell line. After analyzing the results to create a CORT inhibition curve (Figure 7), there was a linear trend at the start of the competition curve, indicating competition of ASP^+ uptake is not occurring at low concentrations of CORT. Increasing the CORT concentrations helped produce more descending points of the competition curve and gave better insight into the binding kinetics of CORT in HCE-S cells. A preliminary IC_{50} of 282.6 μM was obtained for CORT to inhibit ASP^+ uptake in the HCE-S cells (Figure 7). The HCE-S cells were grown in media that contained 25 mM of glucose. Finally, to test the effect of short-term exposure to a small increase in glucose in the cell culture environment, the cells were cultured in media with a final glucose concentration of 50 mM for 24 hrs. Cells grown in higher glucose were used in a CORT competition assay. The CORT competition experiments showed an IC_{50} of ≅ 283 μM (baseline, Figure 7) that increased to ≅ 1717 μM after cells were exposed to a higher glucose concentration (50 mM glucose, 24 hrs.) (Figure 8). The ability of the drug molecules to displace the ASP^+ from its binding site in a way that was detectable by the microplate plate reader was promising for the continued generation of competition curves both at baseline and varied glycemic environments.

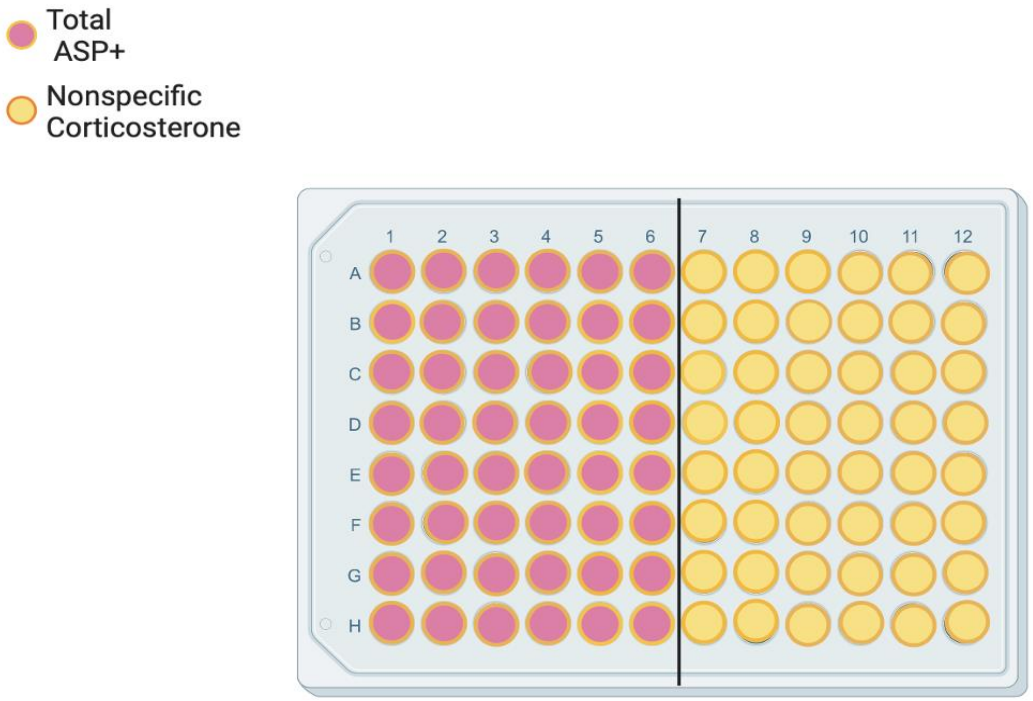
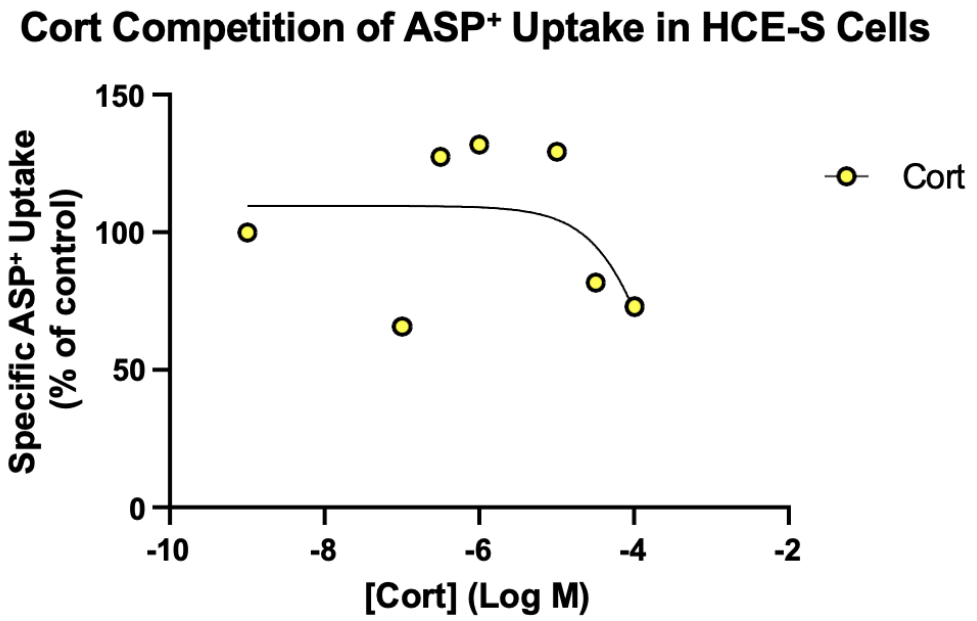
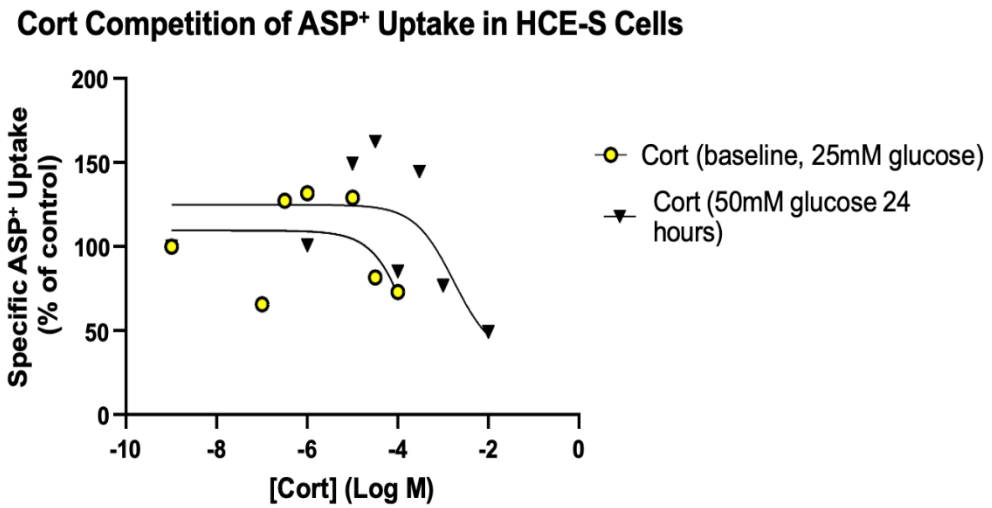


Figure 6: The CORT competition assay layout in a 96-well microplate. The plate is divided into two sections with 6 replicated per concentration: the Total ASP$^+$ concentration (pink) and CORT nonspecific (yellow). Competition of 1 mM ASP$^+$ was measured at 37°C for 30 minutes. CORT concentrations were applied in rows A-H and set at 0, 0.1, 1, 3, 10, 30, 100, 1000 μM.



One site - Fit logIC50	
Best-fit values	
Top	109.7
Bottom	-36.48
LogIC50	-3.549
IC50	0.0002826

Figure 7: The competition assay of CORT in a 96-well microplate. The y-axis shows RFU plotted as specific ASP^+ uptake as a percent of the control, and the x-axis has the concentration of CORT in LogM units. ASP^+ concentration set at 1 mM. CORT competing concentrations were 0 (control), 0.1, 1, 3, 10, 30, 100, 1000 μM, n = 1. The table presents information on IC_{50}.



	Cort (baseline, 25mM glucose)	Cort (50mM glucose 24 hours)
One site - Fit logIC50		
Best-fit values		
Top	109.7	125.0
Bottom	-36.48	34.11
LogIC50	-3.549	-2.765
IC50	0.0002826	0.001717

Figure 8: Comparison of CORT competition with HCE-S cells cultured in glucose at baseline (25 mM), yellow circles, or with a moderate glucose level (50 mM) in the growth media for 24-hour, black downward triangles. Competition was measured for 30 minutes at 37°C, n = 1.

Conclusions and Future Directions

The ASP^+ assays confirmed the putative presence of organic cation transporters in human cornea epithelial cells. This conclusion was drawn from the successful microplate fluorescence indicating uptake of the ASP^+ molecules. CORT, an OCT3 selective inhibitor at lower concentrations than for inhibition of OCT1 and OCT2, provided further evidence of OCT3-mediated transport in HCE-S cells through the saturation and competition assays performed. Utilizing D22, an inhibitor of all OCTs, showed evidence that other endogenous transporters in the HCE-S cells contribute to ASP^+ uptake. The ASP^+ affinity was lower in this endogenous cell line but expected when compared to overexpressed OCT cell-based systems.[30]

The first aim of this research focused on the ASP^+ saturation and time course assays in HCE-S corneal epithelial cells. The results showed ASP^+ at 10 minutes when comparing nonspecific uptake determined with CORT, or D22 had no statistical difference in the B_{max} or maximal uptake but impacted ASP^+ affinity with a statistically different K_d (p-value 0.003). In the 10-minute versus 30-minute time trial experiments using nonspecific uptake with CORT, both the B_{max} and the K_d were statistically different (p-value of < 0.0001). The 30-minute time point was deemed a better choice for performing saturation and subsequent competition assays. This is sensible considering the nature of secondary transporters like OCT3, which have a low affinity but increased capacity for transporting molecules. After 60 minutes, the corneal epithelial cells had a more challenging time staying attached to the plate, resulting in loss of cells during washing and inconsistent data (not shown). It was concluded that 30 minutes is optimal for ASP^+ uptake with non-specific uptake blocked with CORT (Figure 4) to focus on OCT3-mediated transport. Interestingly, specific ASP^+ uptake was calculated with non-specific uptake by D22 at 10 minutes, with both the B_{max} and K_d being statistically different compared to the 30-minute interval, with a p-value of 0.044 (Figure 5). The CORT competition assays performed at 30 minutes, required measurement with higher

competing CORT concentrations to demonstrate inhibition (Figure 7, IC_{50} = 283 μM). The points graphed towards the top of the competition curve were linear, indicating using higher CORT levels should show improved displacement of the ASP^+ to produce a downward trend for the competition curve parameters. Glucose exposure may also impact the binding and inhibitory effects of CORT in HCE-S cells as shown by its reduced potency to inhibit ASP^+ uptake after growing cells in 50 mM glucose for 24 hours (Figure 8).

It is known that the human eye is a high glucose-demanding organ in the body, but it is difficult to measure in patients; thus, typical and pathological glucose levels in the extracellular environment of the cornea are currently unclear.[31] While thorough manipulation studies of glucose levels in the HCE-S cell culture environment could not be fully performed for this project, it is an important future direction. Changing the glucose environment in which the cornea epithelial cells are grown could alter the activity of the OCT transporters, impacting their uptake function and ability to bind molecules. One preliminary CORT competition of ASP^+ uptake was done after manipulating glucose levels from baseline (25 mM) to moderate (50 mM levels) for 24 hours. Increasing glucose exposure of the cells for this short period appeared to produce a rightward shift or decreased potency of CORT (Figure 8, IC_{50} 272 μM vs. 1717 μM). However, the duration of exposure and glucose concentrations in the culture media should be further characterized to learn more about the effects of hyperglycemic environments on OCT3 activity in HCE-S cells. If time allowed, HCE-S would have been grown in 0 mM glucose and varying levels, such as the 50 mM mid-level hyperglycemic conditions and 120 mM hyperglycemic conditions, and testing the cells in various time constraints, such as a 24-hour growth period for acute exposure and possibly one week to assess the effect of chronic glucose exposure. During the project, some competition experiments were performed at the 50 mM and 120 mM glucose levels with one week of growth and 24-hour

growth exposure; however, the data was inconclusive, and time constraints did not allow further exploration of this avenue.

Looking ahead, the future directions for this project hold promise for complete testing of the initial hypothesis that OCT3 is a dominant mediator of corneal transport. CORT competition assays need further replication to finalize the uptake-inhibition curves. Additional studies, including competition assays, would use metformin, an OCT3 substrate, to evaluate OCT3 binding interactions with other ligands in the HCE-S cell line.

Initial OCT3 western blot experiments presented unexpected challenges. Regrettably, the protein bands did not appear, and the time required to optimize and run the protein quantification experiments was not conducive to our project's timeline. The challenges of the western blot experiment could be overcome by establishing a collaboration to utilize a Jess automated western blot analysis. This equipment is expensive but is an innovative approach that would allow running multiple western blots in a fraction of the time and with fewer materials, potentially yielding better results for the secondary transporters that are known to be difficult to probe. Alternatively, quantifying messenger RNA (mRNA) expression using real-time PCR is another approach that could confirm the expression of the genetic components necessary for HCE-S cells to produce the OCT3. This could further improve the understanding of the OCT3 machinery naturally present in human corneal epithelial cells.

Despite their discovery nearly six decades ago, these transporters, referred to as 'uptake 2' transporters, remained largely unstudied until relatively recently, likely because in foundational studies, uptake 2 transport mechanisms did not become apparent until substrate concentrations reached the micromolar range, which was believed to be supraphysiological at the time.[31] This work has future implications for establishing corneal epithelial cells as a reliable model to study OCT3 activity, test the pharmacological characteristics of ligands, evaluate the effects of hyperglycemic

growth conditions on OCT3 function, and develop alternate administration routes for metformin (e.g., as eye drops).

APPENDICES

APPENDIX A

Preparing Complete Growth Media For Cornea Epithelial Cells-HCE-S (ABM catalog #: T0737)
Version: 1

1. Purpose
   1.1 Preparing complete growth media to maintain human cornea epithelial cells.

2. Scope
   2.1 Laboratory personnel will utilize this protocol to prepare the complete growth media required to grow the human cornea epithelial cells-HCE-S.

3. Definitions/Acronyms
   3.1 Penicillin-Streptomycin Antibiotics: (Pen-Strep) Antibiotic solution maintains healthy media and cells and prevents bacterial growth.
   3.2 Fetal Bovine Serum: (FBS) The USDA research grade origin fetal bovine serum. Note that the HCE-S cells from ABM require non-heat-inactivated FBS.
   3.3 Growth Media, 500 mL: PriGro III medium designed by the manufacturer to grow primary cells.
   3.4 Complete Growth Media: (CGM) Growth media supplemented with 10% FBS and 1% Pen-Strep.

4. Materials
   4.1 Basic PPE: lab coat, gloves, closed-toed shoes, long pants
   4.2 Dry bath set at 37°C
   4.3 Sterile Plastics: conical tubes (15mL, 50mL) pipette tips and media dispenser
   4.4 BLS2 Hood
   4.5 FBS
   4.6 Pen-Strep
   4.7 Growth media
   4.8 Glutamine
   4.9 CGM

5. Procedures
   5.1 Put on gloves and place the FBS, Pen-Strep, and Growth media in the dry bath to warm. When warmed, collect and sterilize before putting items into the sterile hood.
   5.2 Turn on the hood to cycle air, and clean the inside hood with 70% alcohol to ensure a sterile environment.
   5.3 Collect the sterile plastics (Pipette tips, media dispenser, conical tubes), sterilize and place them in the hood.
   5.4 Open the complete growth media, and with the pipette, remove 50mL into a sterile conical tube for later use to rinse plated cells.
   5.5 To prepare the complete growth media (CGM)
      5.5.1 open the conical tube containing non-heat treated fetal bovine serum with a pipette, take out 50mL, and add it to the media bottle.
      5.5.2 Open the Pen-Strep container, remove 5mL with a pipette, and add it to the growth media container.

5.5.3 Open the glutamine container, remove 5mL with a pipette, and add it to the growth media container.

5.5.4 Close the Complete growth media container, invert to mix, and label with CGM, date, and supplements 10% FBS and 1% pen-strep.

5.5.5 Aliquot out the desired amount of complete growth media (e.g., 10 aliquots of 50 mL); Store at 4°C until needed.

5.6 Prepare 50 mL aliquots of the remaining FBS; Store at -20°C until needed.

5.7 Prepare 5 mL aliquots of the remaining Pen-Strep; Store at -20°C until needed.

5.8 Prepare 5mL aliquots of glutamine; Store at -20°C until needed.

5.9 Clean the BSL hood, remove PPE, and wash hands.

5.10 Growth media can be modified with extra glucose to create environments of varying glycemic conditions.

APPENDIX B

Applying Extracellular Matrix
Version:1

1. Purpose
   1.1 To coat cell culture vessels with the applied extracellular matrix to improve attachment of adherent cell lines (e.g., human corneal epithelial cells HCE-2) to the container surface.

2. Scope
   2.1 Laboratory personnel will utilize this protocol to coat appropriate containers for growing adherent cells.

3. Definitions/Acronyms
   3.1 Extracellular Matrix (ECM) solution liquid (ABM catalog# GM422) is added to containers to improve cell adhesion.

4. Materials
   4.1 Basic PPE: lab coat, gloves, closed-toed shoes, long pants
   4.2 Sterile Plastics: Pipette tips and media dispenser
   4.3 TrypLE Express
   4.4 Fridge 4°C
   4.5 Appropriate size of sterile cell culture vessel (T25, T75, 100 mmx20mm, 24-well plate, 96-well plate).
   4.6 ECM solution
   4.7 BSL2 Hood: A class 2 hood for an isolated and sterile environment

5. Procedures
   5.1 Turn on the BSL2 hood to begin fan cycling, raise it to the required sash level, and sterilize the environment.
   5.2 Glove up, turn on the Sterile hood, collect required materials (Pipettes, media dispenser, conical tubes), sterilize all items with 70% ethyl alcohol, and collect samples from the incubator.
   5.3 Open the containers, open the ECM, and place the pipette tip on the pipette.
   5.4 Bring the appropriate amount of ECM needed into the pipette and then expel the ECM into the appropriate container. Repeat this process until all containers are coated with ECM.
   5.4.1 T25 flask recommends 3mL, T75 flask recommends 5mL, 100mm dish recommends 3mL
   5.5 Let the containers sit in ECM for 1 hour without lids
   5.6 After the hour has passed, pipette any remaining ECM liquid and collect this leftover fluid into a container to be kept and stored in the fridge to be used again in the future.
   5.7 Let the containers dry for another 1 hour without lids.
   5.8 After the drying hour, label all containers appropriately, put the lids back on, parafilm them, and place them in the fridge until they are to be used.
   5.9 The coated containers can be used for up to 2 weeks after coating.

6. References

1. www.abmgood.com. Accessed May 22, 2023. https://www.abmgood.com/pub/media/productdocument/document//g/2/g238_datasheet_v4-3_1.pdf.

APPENDIX C

Counting Cells
Version:1

1. Purpose
   1.1 Counting cells via the automated cell counter.

2. Scope
   2.2 Laboratory personnel will utilize this protocol to use the automated cell counter to count cells.

3. Definitions/Acronyms
   3.1 Countess 3: automated cell counter, counts cells automatically.
   3.2 Trypan blue stain (0.4%): used to dye cells and determine alive vs. dead cells.

4. Materials
   4.1 Basic PPE: lab coat, gloves, closed-toed shoes, long pants
   4.2 Sterile Plastics: Pipette tips, conical tubes, microcentrifuge tubes, and media dispenser
   4.3 Trypan Blue
   4.4 BSL2 Hood: A class 2 hood for an isolated and sterile environment
   4.5 Countess 3
   4.6 Countess chamber slide
   4.7 Coverslip
   4.8 Complete growth media
   4.9 Dry bath set to 37°C
   4.10 Centrifuge

5. Procedures
   5.1 Turn on the hood, glove up, and collect sterile plastic materials (pipettes and media dispenser).
   5.2 Place Trypan Blue in the dry bath with complete growth media. Trypan blue, when warmed, reduces crystals on the cell plate imaging.
   5.3 Take the cells from the incubator to the hood and begin the process of subculturing the cells.
   5.4 After the cells have been dislodged with TrypLE express and inactivated with CGM pipette, the cell mixture to a conical tube to be centrifuged.
   5.5 After centrifuging, aspirate the supernatant and add fresh media to mix the cell pellet back into the solution.
   5.6 After thoroughly mixing, take a 10ul sample from the conical tube.
   5.7 The 10ul sample is placed in a small microcentrifuge tube; add 10ul of the trypan blue and mix.
   5.8 Turn on the Countess 3 cell counter.
   5.9 Take 10ul of this sample and add it to the countess chamber slide with the cover slip resting on top of it. vacuum force will distribute the trypan/cell mix across the plate.
   5.10 Place the slide in the Countess 3, the cells will then automatically be counted.
   5.11 The cell will be displayed and the count of live cells vs dead cells will be displayed.

APPENDIX D

Subculture Protocol for Human Cornea Epithelial Cells (HCE-S)
Version:1

1. Purpose
   1.1 To split cells from one confluent container to a new container for an experiment or continued growth.

2. Scope
   2.1 Laboratory personnel will utilize this protocol to dislodge cells, transfer cells, and plate cells in new containers for continued growth and experiments.

3. Definitions/Acronyms
   3.1 Extracellular Matrix (ECM) solution liquid (ABM catalog# GM422) is added to containers to improve cell adhesion.
   3.2 TrypLE Express: recombinant enzyme used to detach adherent cell lines.
   3.3 Phosphate-buffered saline (PBS): water-based salt solution helps maintain PH levels.
   3.4 Complete Growth Media: (CGM) Growth media supplemented with 10% FBS and 1% Pen-Strep.
   3.5 Countess 3: automated cell counter, counts cells automatically.
   3.6 Trypan blue stain (0.4%): used to dye cells and determine alive vs. dead cells.

4. Materials
   4.1 Basic PPE: lab coat, gloves, closed-toed shoes, and long pants
   4.2 Dry bath set at 37°C
   4.3 Sterile Plastics: conical tubes, pipette tips, and media dispenser
   4.4 BLS2 Hood
   4.5 CGM, ECM, TrypLE Express
   4.6 Light Microscope
   4.7 Countess automated cell counter
   4.8 Trypan Blue Stain (0.4%)
   4.9 Cell Aspirator vacuum
   4.10 Centrifuge

5. Procedures
   5.1 Put on PPE, place the PBS and CGM in the dry bath to warm, and while warming, turn on the BLS2 hood to also warm up.
   5.2 When CGM and PBS are warm, spray with alcohol and place in the hood, spray gloves with alcohol, and retrieve cells from the incubator to be placed inside the hood.
   5.3 Retrieve pipette equipment and dispensers, spray them with alcohol, and place them in the hood. Begin by aspirating the cells, then add the PBS to the cells for one minute to rinse the cells. Aspirate the PBS, add the TrypLE Express to cells, and place cells back in the incubator for five minutes.
      5.3.1 For a 100mmx20mm dish add 3mL PBS and 3mL of TrypLE Express.
   5.4 After 5 minutes elapsed, check the cells with the light microscope to determine the number of cells dislodged. Usually, only some have dislodged at this time; wait 5 more

minutes and check again. The cells should be dislodged by this time; tapping the dish to encourage dislodging also helps.

5.5 When the cells are dislodged, return them to the hood and add CGM to stop the effects of the TripLE Express; using a pipette, transfer the cells to a centrifuge tube to be centrifuged at 1500rpm for 3 minutes.

5.6 After centrifuging, return to the hood and aspirate the TripLE Express/CGM from the tube to not disturb the cell pellet.

5.7 After removal, add fresh CGM and, using a fine pipette tip, redistribute the cell pellet back into the media.

5.8 At this point, add cells to a new container to continue the growth process with more fresh media.

5.9 Or take a sample (10 μL) of the cell mixture to be counted with the Countess automatic cell counter to get an accurate cell count to calculate how many cells are needed to plate for an experiment.

APPENDIX E

Human Cornea Epithelial (HCE-S) Cryopreserve/Thaw Protocol
Version:1

1. Purpose
   1.1 Procedure to freeze and thaw cells needed for experiments or for storage.

2. Scope
   2.1 Laboratory personnel will utilize this protocol to Freeze and thaw cells as needed for experiments and storage.

3. Definitions/Acronyms
   3.1 Immortalized Human cornea epithelial cells (HCE-S):

4. Materials
   4.1 Basic PPE: lab coat, gloves, long pants, close-toed shoes
   4.2 BLS2 Hood
   4.3 Incubator set at 37°C and 5% CO_2
   4.4 FBS
   4.5 Glutamine
   4.6 ECM
   4.7 Glutamine
   4.8 Liquid nitrogen storage tank
   4.9 Dry bath 37°C
   4.10 Sterile plastic wear
   4.11 Centrifuge

5. Procedure
   5.1 Cell removal from storage container and thaw:
      5.1.1 Remove the cryovials from the liquid nitrogen storage tank/ -80°C freezer.
      5.1.2 Thaw the cells in the 37°C dry bath for one minute.
      5.1.3 Spray the vial with alcohol and place it in the turned-on and warmed-up BLS2 hood.
      5.1.4 Resuspend the cells and pipette them into a centrifuge set at 1500rpm for three minutes to pellet the cells.
      5.1.5 Aspirate the supernatant off the pellet without disturbing it, then pipette warmed fresh media to redistribute the cell.
      5.1.6 Perform a cell count and, plate the appropriate amount of cells onto the prepared container, and place in the incubator until needed.
   5.2 Cryopreserve cells for storage:
      5.2.1 Dislodge cells from adherent plates and collect cells into a centrifuge tube.
      5.2.2 Perform a cell count to determine cell density and volume of cryopreservation.
      5.2.3 The cells should be frozen at around 1.0 to 2.5x10^6 cells/mL
      5.2.4 Centrifuge the cell suspension at 1500rpm for 3 minutes to pellet the cells and aspirate the supernatant without disturbing the cell pellet.

5.2.5 Resuspend the cell pellet in cryopreservation media at the appropriate cell density.

5.2.6 Dispense cells into cryovials and freeze. (Control the freezing rate to around 1°C drop per minute, and transfer cells into a liquid nitrogen tank after 24 hours of controlled freezing.)

6. References

6.1 Notara M, Daniels JT. Characterization and functional features of a spontaneously immortalized human corneal epithelial cell line with progenitor-like characteristics. Brain Res Bull. 2010 Feb 15;81(2-3):279-86. doi: 10.1016/j.brainresbull.2009.08.009. Epub 2009 Aug 21. PMID: 19699783.

6.2 General guidelines for thawing cryopreserved cells. https://test.abmgoodchina.com/document/t/h/thawing_protocol_1_.pdf. Accessed April 28, 2023.

6.3 Freezing Protocol. http://new.abmgoodchina.com/document/Freezing%20Cells%20Final_%20111315.pdf. Accessed April 28, 2023.

APPENDIX F

ASP^+ Saturation Assay in Human Cornea Epithelial Cells (HCE-S)
Version:1

1. Purpose
   1.1 To perform an ASP^+ saturation assay with human cornea epithelial cells (HCE-S).

2. Scope
   2.1 Laboratory personnel will utilize this procedure to perform an ASP^+ saturation assay experiment.

3. Definitions/Acronyms
   3.1 4-Di-1-ASP (4-(4-(dimethylamino)styryl)-*N*-methylpyridinium) (ASP^+): Fluorescent molecule substrate of OCT3
   3.2 Krebs-Ringers HEPES (KRH): Used to maintain cells during experiments
   3.3 Sodium dodecyl sulfate lysis buffer (SDS): solution to denature and break the cell membrane.
   3.4 Dimethyl sulfoxide (DMSO): solvent that is miscible in water and many organic solvents.
   3.5 Decynium-22 (D-22): Non-selective organic cation transporter inhibitor.
   3.6 CORTicosterone(CORT): selective inhibitor of the isoform organic cation transporter 3

4. Materials
   4.1 Basic PPE: Lab coat, long sleeves, long pants, close-toed shoes, gloves.
   4.2 Plastic wear: (weigh boats, channel pipette, pipette tips, conical tubes)
   4.3 Aluminum foil: light-sensitive compounds need to be protected (Dim lights/Cover tubes with compounds)
   4.4 96-well plate seeded with cells
   4.5 Channel pipette
   4.6 Heated plate at 37°C

5. Procedures
   5.1 Prepare 10x KRH (without HEPES and Glucose)
      5.1.1 To prepare 500mL, weigh out: 36.53g (125mM) NaCl, 1.789g (4.8mM) KCl, 0.8165g (1.2mM) KH_2PO_4, 0.9556g (1.3mM) $CaCl_2$-$2H_2O$, 1.479g (1.2mM) $MgSO_4$-$7H_2O$
   5.2 When it is time for the experiment, Prepare the 1x KRH solution
      5.2.1 To prepare 250mL of 1x KRH, weigh out 1.49g (25mM) HEPES, 0.252g (5.6mM) Glucose
      5.2.2 Measure out 25mL of the 10x KRH and mix all items with 225mL of water.
      5.2.3 P.H. test the solution and add NaOH to stabilize the solution to P.H. 7.4
      5.2.4 The reason to make 1x KRH when experimenting is to preserve the solution for longer due to the glucose. To store, place the solution in 4°C.
   5.3 Prepare 20% SDS lysis solution
      5.3.1 To prepare the 20% SDS solution dissolve 40g electrophoresis-grade SDS in 180mL of H_2O.

5.4 Prepare 1% SDS solution for Experimenting
- 5.4.1 To prepare the 1% SDS lysis solution for the experiment, take 2.5mL of the 20% SDS stock made and add it to 47.5mL of water to make 50mL of the 1% SDS solution

5.5 Stock Solution Preparation
- 5.5.1 Weigh out around 15mg ASP^+, around 6mg CORT, and around 6mg
- 5.5.2 Calculate the amount of DMSO for each amount weighed to make the stock solutions.
- 5.5.3 The Concentration of stock solutions is calculated to 100mM

5.6 Pre-incubation CORT and Pre-incubation D-22 solutions
- 5.6.1 Take 40ul of the stock CORT solution and add it to 3.96mL of 1x KRH to make the preincubation solution in a labeled tube.
- 5.6.2 Take 40ul of D-22 stock solution and add it to 3.96mL of 1x KRH to make the preincubation solution in a labeled tube.
- 5.6.3 Calculations to determine the amount of 1x KRH were based on the volume of the 96-well format.

5.7 ASP^+ solutions
- 5.7.1 Take the Stock ASP^+ solution and proceed to dilute the concentration to the desired levels for the experiment in separate labeled tubes. (Ex: 100mM-30mM-10mM-3mM-1mM-100um-10um)
- 5.7.2 Prepare and label 8 tubes corresponding with the concentrations needed, and pipette 200ul of the diluted ASP^+ concentrations into each tube.
- 5.7.3 In each tube pipette, 1.8mL of 1x KRH into each of the 8 new tubes to make the ASP^+ solutions for the assay.

5.8 Non-Specific CORT and Non-Specific D-22 solutions
- 5.8.1 Prepare and label 8 new tubes for non-specific CORT, then take 5ul of the stock CORT solution and add it to each of the 8 labeled tubes.
- 5.8.2 Then, take 495ul of the ASP^+ dilution solutions and pipette them into each corresponding tube, creating ASP^+ mixed with CORT.
- 5.8.3 Prepare and label 8 new tubes for the non-specific D-22, then take 5ul of the stock D-22 solution and add it to each of the labeled 8 tubes.
- 5.8.4 Then, take 495ul of the ASP^+ dilution solutions and pipette them into each of the corresponding 8 tubes, creating ASP^+ mixed with D-22.

5.9 Performing the Experiment Saturation Assay
- 5.9.1 The plate is divided into three sections: the ASP^+ solutions (1-4), the non-specific CORT (5-8) and Nonspecific D-22 (9-12).
- 5.9.2 Turn on the warm plate and set it to 37°C
- 5.9.3 Take 50mL of 1x KRH in a conical tube and place on the plate to warm.
- 5.9.4 Gather a tub of ice and place the remaining 1x KRH on ice for the experiment
- 5.9.5 Retrieve the 96-well plated cells from the incubator and place the plate on the warmed plate.
- 5.9.6 Aspirate every well and pipette 100ul of warm 1x KRH into every well.
- 5.9.7 Aspirate every well and pipette 100ul of warmed 1x KRH into every well and turn on the timer for 10 minutes of acclimation.
- 5.9.8 After the 10 minutes, reset the timer and begin with the ASP^+ diluted solutions.
- 5.9.9 Aspirate the corresponding row of 4 wells and pipette 100ul the concentration ASP^+ dilution solution into each well.

5.9.10 Every 30 Seconds, you do a row of wells moving down the plate, aspirating the 4 corresponding wells, and pipetting the ASP^+ dilution solutions in descending order.
5.9.11 After the last row of wells, you wait till the timer reaches 10 minutes, then begin the washing phase.
5.9.12 Aspirate the ASP^+ out of the wells and wash the wells with cold 1x KRH, repeating this process 3 times, then use the 1% SDS to lysis the cells to finish.
5.9.13 After completing this for every well, we reset the clock and move to the next section of the plate.
5.9.14 Start the clock and aspirate the 1x KRH out of the row of 4 wells, then pipette the Pre-CORT incubation solution into the wells.
5.9.15 Every 30 seconds, move down a row, aspirate the 1x KRH, and pipette the pre-CORT solution into the wells.
5.9.16 After the last row, move to the next section and continue with the timer every 30 seconds aspirate the 1x KRH, and this time pipette the Pre-D-22 solution into the wells, working down the plate until you finish the last row of cells.
5.9.17 Then, back to the CORT Section of the plate, and when the timer reaches 10 minutes, aspirate the Pre-CORT solution and add 100ul the Non-specific CORT solution to the well at the appropriate concentration tube.
5.9.18 Continue this process down the plate every 30 seconds, aspirating the pre-CORT solution and adding the 100ul of the non-specific CORT into the wells.
5.9.19 When you finish the last row of cells change to the D-22 section of the plate and continue this process every 30 seconds aspirating the Pre-D-22 solution out of the wells and adding the Non-specific D-22 solution to the cells, working down the plate.
5.9.20 After the last row is completed, change to the washing phase and at the 20-minute mark on the timer begin washing the wells in the CORT section of the plate.
5.9.21 Aspirate the non-specific CORT out of the wells and wash the wells with 100ul of cold 1x KRH 3 times (aspirate/wash/aspirate/wash/aspirate/wash/aspirate) then after the last aspiration add the 1% SDS solution to lysis the cells. Move down the plate repeating this process until the CORT section of the plate is finished, then change to the D-22 section of the plate.
5.9.22 Repeat this process with the D-22 side of the plate, aspirate the nonspecific D-22 solution, and wash the wells with 100ul of cold 1x KRH (aspirate/wash/aspirate/wash/aspirate/wash/aspirate) after the last aspiration pipette 100ul of the 1% SDS solution to lysis the cells.
5.9.23 Continue this process until the D-22 side of the plate is finished.
5.9.24 Once the palate is completed, pop any bubbles present in the wells to help with the reading of the plate and put the plate into the cell reader.
5.9.25 The cell reader is turned on and warmed up to read the cells.
5.9.26 After reading the cells, the data is collected, and the plate can be discarded.

APPENDIX G

Competition Assay in Human Cornea Epithelial Cells (HCE-S)
Version: 1

1. Purpose
   1.1 To perform a competition assay with human cornea epithelial cells (HCE-S).

2. Scope
   2.1 Laboratory personnel will utilize this procedure to perform a competition assay experiment.

3. Definitions/Acronyms
   3.1 4-Di-1-ASP (4-(4-(dimethylamino)styryl)-*N*-methylpyridinium) (ASP^+): Fluorescent molecule substrate of OCT3
   3.2 Krebs-Henseleit buffer (KRH): Used to maintain cells during experiments
   3.3 Sodium dodecyl sulfate lysis buffer (SDS): solution to denature and break the cell membrane.
   3.4 Dimethyl sulfoxide (DMSO): solvent that is miscible in water and many organic solvents.
   3.5 Decynium-22 (D-22): Non-selective organic cation transporter inhibitor.
   3.6 (CORT): a selective inhibitor of the isoform organic cation transporter 3

4. Materials
   4.1 Basic PPE: Lab coat, long sleeves, long pants, close-toed shoes, gloves.
   4.3 Plastic wear: (weigh boats, channel pipette, pipette tips, conical tubes)
   4.3 Aluminum foil: light-sensitive compounds need to be protected (Dim lights/Cover tubes with compounds)
   4.4 96-well plate seeded with cells
   4.5 Channel pipette
   4.6 heated plate 37°C

5. Procedures
   5.3 Prepare 10x KRH (without HEPES and Glucose)
      5.3.1 To prepare 500mL, weigh out: 36.53g (125mM) NaCl, 1.789g (4.8mM) KCl, 0.8165g (1.2mM) KH_2PO_4, 0.9556g (1.3mM) $CaCl_2$-$2H_2O$, 1.479g (1.2mM) $MgSO_4$-$7H_2O$
   5.4 When it is time for the experiment, Prepare the 1x KRH solution
      5.4.1 To prepare 250mL of 1x KRH, weigh out 1.49g (25mM) HEPES, 0.252g (5.6mM) Glucose
      5.4.2 Measure out 25mL of the 10x KRH and mix all items with 225mL of water.
      5.4.3 P.H. test the solution and add NaOH to stabilize the solution to P.H. 7.4
      5.4.4 The reason for making 1x KRH when experimenting is to preserve the solution for longer due to the glucose. To store, place the solution in 4°C.
   5.5 Prepare 20% SDS lysis solution.

5.5.1 To prepare the 20% SDS solution, dissolve 40g electrophoresis-grade SDS in 180mL of H_2O.

5.6 Prepare 1% SDS solution for Experimenting.

5.6.1 To prepare the 1% SDS lysis solution for the experiment, take 2.5mL of the 20% SDS stock made and add it to 47.5mL of water to make 50mL of the 1% SDS solution

5.7 Stock Solution Preparation

5.7.1 Weigh out however much ASP^+ and CORT needed for experiment

5.7.2 Calculate the amount of DMSO for each amount weighed to make the stock solutions.

5.7.3 The Concentration of stock solutions is calculated to 100mM

5.8 Pre-incubation CORT solutions

5.8.1 Take 40ul of the stock CORT solution and add it to 3.96mL of 1x KRH to make the preincubation solution in a labeled tube.

5.8.2 Calculations to determine the amount of 1x KRH were based on the volume of the 96-well format.

5.9 ASP^+ solutions

5.9.1 Take the Stock ASP^+ solution and proceed to dilute the concentration to the desired levels for the experiment in separate labeled tubes. (Ex: 100mM-30mM-10mM-3mM-1mM-100um-10um)

5.9.2 Prepare and label 8 tubes corresponding with the concentrations needed, and pipette 200ul of the diluted ASP^+ concentrations into each tube.

5.9.3 In each tube pipette, 1.8mL of 1x KRH into each of the 8 new tubes to make the ASP^+ solutions for the assay.

5.10 Non-Specific CORT solution

5.10.1 Prepare and label 8 new tubes for non-specific CORT, then take 5ul of the stock CORT solution and add it to each of the 8 labeled tubes.

5.10.2 Then, take 495ul of the ASP^+ dilution solutions and pipette them into each corresponding tube, creating ASP^+ mixed with CORT.

5.11 Performing the Experiment Competition Assay

5.11.1 The plate is divided into two sections: the ASP^+ solutions (1-6), the non-specific CORT (7-12)

5.11.2 Turn on the warm plate and set it to 37°C

5.11.3 Take 50mL of 1x KRH in a conical tube and place on the plate to warm.

5.11.4 Gather a tub of ice and place the remaining 1x KRH on ice for the experiment

5.11.5 Retrieve the 96-well plated cells from the incubator and place the plate on the warmed plate.

5.11.6 Aspirate every well and pipette 100ul of warm 1x KRH into every well.

5.11.7 Aspirate every well and pipette 100ul of warmed 1x KRH into every well and turn on the timer for 10 minutes of acclimation.

5.11.8 After the 10 minutes, reset the timer and begin with the ASP^+ diluted solutions.

5.11.9 Aspirate the corresponding row of 6 wells and pipette 100ul the concentration ASP^+ dilution solution into each well.

5.11.10 Every 30 seconds, you do a row of wells moving down the plate, aspirating the 6 corresponding wells, and pipetting the ASP^+ dilution solutions in descending order.

5.11.11 After the last row of wells, you wait till the timer reaches 30 minutes, then begin the washing phase.

5.11.12 Aspirate the ASP^+ out of the wells and wash the wells with cold 1x KRH, repeating this process 3 times, then use the 1% SDS to lysis the cells to finish.
5.11.13 After completing this for every well, we reset the clock and moved to the next section of the plate.
5.11.14 Start the clock and aspirate the 1x KRH out of the row of 6 wells, then pipette the Pre-CORT incubation solution into the wells.
5.11.15 Every 30 seconds, move down a row, aspirate the 1x KRH, and pipette the pre-CORT solution into the wells.
5.11.16 When the timer reaches 10 minutes, aspirate the Pre-CORT solution and add 100ul the Non-specific CORT solution to the well at the appropriate concentration tube.
5.11.17 Continue this process down the plate every 30 seconds, aspirating the pre-CORT solution and adding 100ul of the non-specific CORT into the wells.
5.11.18 After the last row is completed, change to the washing phase, and at the 30-minute mark on the timer, begin washing the wells in the CORT section of the plate.
5.11.19 Aspirate the non-specific CORT out of the wells and wash the wells with 100ul of cold 1x KRH 3 times (aspirate/wash/aspirate/wash/aspirate/wash/aspirate) then after the last aspiration add the 1% SDS solution to lysis the cells. Move down the plate repeating this process until the CORT section of the plate is finished.
5.11.20 Once the palate is completed, pop any bubbles present in the wells to help with the reading of the plate and put the plate into the cell reader.
5.11.21 The cell reader is turned on and warmed up to read the cells.
5.11.22 After reading the cells, the data is collected, and the plate can be discarded.

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