

FINAL REPORT

Study Title

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH  
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Test Article

EG

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**SIGNATURE PAGE**

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH  
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Initiation Date: March 2, 2007

Completion Date: May 3, 2007

Sponsor:

Sponsor's Representative:

Testing Facility: Institute for In Vitro Sciences, Inc.  
21 Firstfield Road, Suite 220  
Gaithersburg, MD 20878

Archive Location: Institute for In Vitro Sciences, Inc.  
Gaithersburg, MD 20878

Study Director: \_\_\_\_\_  
Janet W. Luczak, M.G.A. Date

Laboratory Supervisor: Gregory O. Moyer, M.B.A.

Histopathology (Principle Investigator) Stewart B. Jacobson, DVM, DACVP

**TEST ARTICLE RECEIPT**

<b>IIVS Test Article Number</b>	<b>Sponsor's Designation</b>	<b>Physical Description</b>	<b>Receipt Date</b>	<b>Storage Conditions*</b>
04AB07	EG	clear colorless non-viscous liquid	2/28/07	room temperature

\* - Protected from exposure to light

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH  
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

## INTRODUCTION

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of the test articles to isolated bovine corneas. Bovine corneas, obtained as a by-product from freshly slaughtered animals, were mounted in special holders and exposed to the test articles. An *in vitro* score was determined for each of the exposure times used for each test article based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas.

The purpose of this study was to evaluate the potential ocular irritancy of the test articles as measured by changes in opacity and permeability (to fluorescein) in isolated bovine corneas. The laboratory phase of this study was conducted from March 6, 2007 to May 2, 2007 at the Institute for In Vitro Sciences, Inc. Three corneas were treated with each test article at exposure times of 3 and 10 minutes. Based on changes in corneal opacity and permeability (relative to the control corneas), an *in vitro* score was determined at each exposure time. An evaluation of the histological changes was performed by Charles River Laboratories, Pathology Associates (PAI) - Maryland to assess the depth and degree of injury.

## MATERIALS AND METHODS

### Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TREUTH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

### Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at  $32 \pm 1^\circ\text{C}$  for a minimum of 1 hour.

### Controls

The positive control used in this study was ethanol (Pharmco). The negative control used in this study was sterile, deionized water (Quality Biological).

### Test Article Preparation

As instructed by the Sponsor, each test article was administered to the test system without dilution.

### Test Article pH Determination

The pH of each test article was determined using pH paper (EMD Chemicals Inc.). Initially, each test article was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each test article was added to 0-6 pH paper with 0.5 pH unit increments, to obtain a more accurate pH value. The pH values obtained from the narrower range pH paper are presented in Table 1.

### Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer. Three corneas, whose initial opacity readings were close to the median opacity for all the

corneas, were selected as the negative control corneas. The treatment of each cornea was identified with the test article number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test article, positive control, or negative control.

#### Method for Testing Liquid or Surfactant Materials

The liquid test articles, EG, were tested neat. An aliquot of 750  $\mu\text{L}$  of the test article, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. A group of three corneas was incubated in the presence of each test article at  $32 \pm 1^\circ\text{C}$  for 3 minutes. A second group of three corneas was incubated in the presence of each test article at  $32 \pm 1^\circ\text{C}$  for 10 minutes. The positive and negative controls were tested in three corneas at  $32 \pm 1^\circ\text{C}$  for 10 minutes. After the 3 and 10-minute exposure times, the control or test article treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test articles. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM (without phenol red) and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at  $32 \pm 1^\circ\text{C}$ . At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360  $\mu\text{L}$  from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm ( $\text{OD}_{490}$ ) was determined using a Molecular Devices Vmax kinetic microplate reader. If the  $\text{OD}_{490}$  value of a control or test article sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the  $\text{OD}_{490}$  value within the linear range of the platereader). A 360  $\mu\text{L}$  sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

#### Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours.

#### Histological Evaluation

The fixed corneas were transferred to Charles River Laboratories, Pathology Associates (PAI)-Maryland for embedding, sectioning, and staining. Each cornea was paraffin-embedded, bisected, and the two halves mounted in the paraffin block so that a section of each half could be

cut and placed on a single slide. Each slide was then stained with hematoxylin and eosin. The slides were provided to the pathologist for evaluation.

### Presentation of Data

**Opacity Measurement:** The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

**Permeability Measurement:** The mean OD<sub>490</sub> for the blank wells was calculated. The mean blank OD<sub>490</sub> was then subtracted from the raw OD<sub>490</sub> of each well (corrected OD<sub>490</sub>). Any dilutions that were made to bring the OD<sub>490</sub> readings into the linear range of the platereader (OD<sub>490</sub> should be less than 1.500), had each diluted OD<sub>490</sub> reading multiplied by the dilution factor. The final corrected OD<sub>490</sub> of the test articles and the positive control was then calculated by subtracting the average corrected OD<sub>490</sub> of the negative control corneas from the corrected OD<sub>490</sub> value of each treated cornea:

$$\text{Final Corrected OD}_{490} = (\text{raw OD}_{490} - \text{mean blank OD}_{490}) - \text{average corrected negative control OD}_{490}$$

The mean OD<sub>490</sub> value of each treatment group was calculated by averaging the final corrected OD<sub>490</sub> values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

$$\text{In Vitro Score} = \text{Mean Opacity Value} + (15 \times \text{Mean OD}_{490} \text{ Value})$$

### Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that fell within two standard deviations of the historical mean.

## RESULTS AND DISCUSSION

### Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for each test article. Table 2 summarizes the opacity, permeability and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 39.4 to 64.3), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

**Table 1**  
**BCOP Results of the Test Articles**

Assay Date	IIVS Test Article Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD <sub>490</sub> Value	<i>In Vitro</i> Score	pH
3/6/07	04AB07	EG	Neat	3 minutes	35.7	0.925	49.5	2.0
				10 minutes	54.3	1.640	78.9	

**Table 2**  
**BCOP Results of the Positive Control**

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD <sub>490</sub> Value	<i>In Vitro</i> Score
3/6/07	Ethanol	10 minutes	37.0	1.376	57.6

### Histological Evaluation

Histopathological evaluation of the corneas from this study was performed at Charles River Laboratories, Pathology Associates-Maryland. The report detailing the findings can be found in Appendix C.

## **APPENDIX A**

## **APPENDIX B**

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY**

**OPACITY SCORE**

<u>TA #</u>	<u>CORNEA #</u>	<u>INITIAL</u>	<u>FINAL</u>	<u>CHANGE</u>	<u>CORRECTED</u>	<u>AVG</u>	<u>STDEV</u>
04AB07	13	6	41	35	33.3		
Neat	14	3	40	37	35.3		
3 minutes	15	3	43	40	38.3	35.7	2.5
04AB07	16	3	63	60	58.3		
Neat	17	4	58	54	52.3		
10 minutes	18	2	56	54	52.3	54.3	3.5
Neg. Control	1	3	4	1	NA		
Sterile, DI water	2	3	5	2	NA		
60 minutes	3	4	6	2	NA	1.7	
Pos. Control	4	4	49	45	43.3		
Ethanol	5	3	40	37	35.3		
10 minutes	6	3	37	34	32.3	37.0	5.7
	*20	2					
	*25	4					
	*26	4					
	*27	4					
	*28	4					
	*30	1					
	*31	2					
	*32	5					
	*33	4					
	*35	2					
	*36	3					
	*37	4					
	*38	3					
	*39	2					
	*41	3					
	*43	4					
	*44	5					
	*46	4					
	*47	4					
	*49	4					
	*50	5					
	*51	4					

**Initial corneal opacity average:** 3

\* - Corneas not used in this assay, but used to find initial opacity average.  
 NA - Not Applicable

**PERMEABILITY SCORE**

**Neg. Control  
Sterile, DI water  
60 minutes**

Cornea #	OD490
1	0.009
2	0.004
3	0.007
-----	
Avg.	0.007

**04AB07  
Neat  
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
13	0.570	1	0.563
14	1.230	1	1.223
15	0.995	1	0.988
-----			
Avg. =			0.925
STDEV=			0.335

**Pos. Control  
Ethanol  
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
4	1.250	1	1.243
5	1.154	1	1.147
6	0.349	5	1.738
-----			
Avg. =			1.376
STDEV=			0.317

**04AB07  
Neat  
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
16	0.348	5	1.733
17	0.357	5	1.778
18	1.416	1	1.409
-----			
Avg. =			1.640
STDEV=			0.201

**IN VITRO SCORE**

**In Vitro Score = Mean Opacity Value + (15 x Mean OD490)**

<b>Test Article</b>	<b>Concentration</b>	<b>Exposure Period</b>	<b>Mean Opacity</b>	<b>Mean OD490</b>	<b>In vitro Score</b>
<b>04AB07</b>	Neat	3 minutes	35.7	0.925	49.5
<b>04AB07</b>	Neat	10 minutes	54.3	1.640	78.9
<b>Ethanol</b>	Neat	10 minutes	37.0	1.376	57.6

## **APPENDIX C**

**FINAL HISTOPATHOLOGY REPORT**

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH  
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

**Testing Facility Study Number:** 07AB48, 04AB07, 03AG15.350056

**HISTOLOGY SITE:**

Charles River Laboratories,  
Pathology Associates (PAI) – Maryland  
15 Worman's Mill Court, Suite I  
Frederick, MD 21701

**TESTING FACILITY:**

Institute for In Vitro Sciences, Inc.  
21 Firstfield Road, Suite 220  
Gaithersburg, MD 20878

**SPONSOR:**

**May 2, 2007**

## 1.0 Introduction

### 1.1 Overview

This report presents the pathology results of bovine corneas exposed to test and control substances for Institute for In Vitro Sciences, Inc (IIVS) Study Number 07AB48, 04AB07, 03AG15.350056. All test procedures and tissue harvests were performed at IIVS under the direction of Janet Luczak, M.G.A., Study Director. Histopathology was completed at Charles River Laboratories, Pathology Associates (PAI) - Maryland by Stewart B. Jacobson, DVM, DACVP.

### 1.2 Objective

The objective of this study was to evaluate the potential ocular irritancy/toxicity of a test article as measured by test article's ability to induce opacity and permeability to fluorescein in an isolated bovine cornea. This report addresses the histopathology portion of the bovine corneal opacity and permeability (BCOP) assay.

## 2.0 Methods

### 2.1 Compliance Statement

The portion of this study performed by PAI was not conducted under the US Food and Drug Administration's Good Laboratory Practice (GLP) regulations for non-clinical laboratory studies, 21 CFR Part 58 (FDA). There were no protocol or SOP deviations during the pathology portion of this study. Although digital photomicrographs are included as part of this report, the diagnoses and findings contained herein were obtained using the original histologic preparations, not the images. An electronic copy of this report (PDF format) is included. It is a representation of the pathology report hard copy; however, only the signed hard copy of the pathology report is considered raw data.

### 2.2 Archives

All pathology data and materials are maintained at Charles River Laboratories, Pathology Associates (PAI) – Maryland during the conduct of the histology portion of the study. Within three months of the issuance of the draft pathology report, or upon completion of the final pathology report, all data and materials generated by Charles River Laboratories, Pathology Associates (PAI) - Maryland, will have been shipped or delivered to the Institute for In Vitro Sciences, Inc. for archiving with the study.

### 2.3 Tissue Collection

According to the protocol, following treatment, corneas were placed in pre-labeled cassettes in 10% neutral buffered formalin fixative. After 24 hours, the fixed tissues were transferred to PAI for slide preparation. Tissues were processed, bisected, embedded in paraffin, and cut

to approximately 5 microns. Slides were stained with hematoxylin and eosin (H&E).

## 2.4 Basis for Histological Evaluation

Damage to the epithelial layer is generally the first change seen in the cornea since the test materials are applied topically to this “unprotected” epithelium. Each “layer” of the epithelium is scored for cell loss or damage. Figure 1 shows the structure of the epithelium from a control cornea. Changes to the surface epithelium (squamous and upper wing cell epithelium) in the BCOP assay are usually not predictive of lasting corneal changes *in vivo*. The loss of the squamous and upper wing cell layers in the excised bovine cornea by surfactant-based test articles appears to coincide with mild to moderate damage to the conjunctiva of the rabbit *in vivo*<sup>1</sup>. This type of damage is typically reversible in the rabbit. Many types of surfactants (e.g., sodium lauryl sulfate) are expected to lyse these cells so that they are progressively lost from the epithelial surface. The positive control, ethanol, provides an example of a different type of damage. In this case, the surface squamous epithelial cells are coagulated and fixed in place. The coagulated tissue stains heavily with eosin (see Figure 5). In addition, the wing and basal cells are vacuolated and the adhesion between the basal cells and the basal lamina is disrupted. Such changes can lead to sloughing of the epithelium. Lesions in the deep wing cell and basal cell layers, either by cell lysis or coagulation, are associated with more damage *in vivo*. Much of the available data come from studies on surfactants and show that loss of the bovine corneal epithelium is predictive of some corneal opacity in the rabbit.

Special effort has been made to detect changes in the stromal elements of the corneas. Jester<sup>2</sup>, Maurer<sup>3,4</sup> and others have shown for a range of chemical classes that depth of injury in the early hours after exposure can be predictive of the eventual degree and duration of the ocular lesions. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Deep injury to the stroma has more serious consequences. Stromal damage may be manifest by several types of changes within the tissue. These may include swelling within the stromal collagen matrix and loss/damage to the keratocytes. These changes do not necessarily occur together. Stromal swelling may be detected by the presence of vacuole-like “holes” in the organized collagen matrix. Their

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<sup>1</sup> Gettings, SD, Lordo, RA, Hintze, KL, Bagley, DM, Casterton, PL, Chudkowski, M, Curren, RD, Demetriulias, JL, DiPasquale, LC, Earl, LK, Feder, PI, Galli, CL, Glaza, SM, Gordon, VC, Janus, J, Kurtz, PJ, Marenus, KD, Moral, J, Pape, WJW, Renskers, KJ, Rheins, LA, Roddy, MT, Rozen, MG, Tedeschi, JP, and Zyracki, J. (1996) The CFTA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. **Food and Chemical Toxicology** 34(1):79-117.

<sup>2</sup> Jester, JV, Li, HF, Petroll, WM, Parker, RD, Cavanaugh, HD, Carr, GJ, Smith, B, and Maurer, JK. (1998) Area and depth of surfactant-induced corneal injury correlates with cell death. **Investigative Ophthalmology & Visual Science** 39(6):922-936.

<sup>3</sup> Maurer, JK and Parker, RD. (1996) Light microscopic comparison of surfactant-induced eye irritation in rabbits and rats at three hours and recovery/day 35. **Toxicologic Pathology** 24(4):403-411.

<sup>4</sup> Maurer, JK, Parker, RD, and Jester, JV. (2002) Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. **Regulatory Toxicology and Pharmacology** 36:106-117.

appearance suggests that liquid has entered the matrix expanding space between the fibers. Examples of these vacuoles may be seen in Figure 7 where the positive control exposure has induced some stromal swelling. The depth and degree of vacuolization can be indicative of the degree of injury to the cornea and/or penetration of the test article into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix vacuolization (swelling). Viable epithelium or endothelium will not only retard entry of the water but actively transport it out of the stroma. As the viability of epithelium declines, both the barrier and transport capacities are reduced. Thus, the amount of water accumulating in the upper stroma reflects the damage to the epithelium. When the full epithelial layer is lost over a section of stroma, stromal swelling can increase the stromal thickness by 50% or more over the control corneal stroma. Such changes are often observed when corneas treated with severe irritants are incubated for extended periods after exposure. Loss of the endothelium can also lead to appreciable deep stromal swelling. The loss may result from test article penetration or mechanical damage. Mechanical damage tends to be more focal (patchy) in nature while the true toxic response to the test article exposure tends to extend across much of the cornea. When focal (mechanical) damage is present, the majority of the collagen matrix vacuolization will be located in the deep stroma (just above Descemet's Membrane). In contrast, test article-induced toxicity to the endothelium should include both epithelial as well as endothelial damage so that collagen matrix vacuolization will be observed throughout the stroma. Such extreme damage may increase the stromal thickness two fold over the control corneal stroma thickness.

*In vivo*, the inflammatory response that follows deep initial injury can, itself, lead to permanent lesions through "scar" collagen deposition or neovascularization in the corneal stroma. The authors cited above have suggested that the damaged keratocytes are involved in initiating this inflammatory process. Classic inflammatory changes (e.g., inflammatory cell infiltration) are possible only in the presence of active circulation through the limbus. Since the isolated cornea has no source of inflammatory cells, the potential for test article-induced inflammation is judged by the changes in the extracellular matrix and particularly the keratocytes. Some forms of damage are more easily recognized than are others. Necrotic cell death, as might follow exposure to a strong alkaline, would be quite apparent since the cellular components rapidly break down. More subtle damage could also lead to a delayed cell death and release of inflammatory mediators. Nuclear changes (vacuolization [swelling], punctate chromatin condensation, pyknosis or karyorrhexis) are signs of this process. Cytoplasmic changes can also be informative. Vacuole formation and/or loss of basic elements (mRNA for example) are also indicative of the beginning of the degenerative process. The cell cytoplasm normally stains with both basophilic (hematoxylin) and acidophilic (eosin) stains. When the basic elements are lost, eosinophilic staining predominates. This type of change is reported as cytoplasmic eosinophilia. Harbell and Curren<sup>5</sup> have reported that mechanical removal of the corneal epithelium leads to marked stromal swelling (marked collagen matrix vacuolization). The keratocytes in the zone of marked swelling undergo changes as well. The nuclei become enlarged and the cytoplasmic staining is very eosinophilic. Thus, when test article-treated corneas show only this type of keratocyte change

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<sup>5</sup> Harbell, J and Curren, R (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. ALTEX 22 (Special Issue):236.

(with stromal swelling), the change may be the result of the stromal swelling rather than direct action of the test article on the cells.

Since depth of injury (both extracellular and cellular) to the stroma is important in the analysis, a means to describe the depth of injury is necessary as part of this analysis. Determining this depth is not always a straightforward process. Because of the topical application of the test article to the epithelium, one would expect that exposure to the stroma would progress from the area just under Bowman's Layer down through the stroma to Descemet's Membrane. There is no external inflammatory process *in vitro*, so one might also expect the progression of damage to follow the progression of exposure. That means that damage to the stroma should first appear close to Bowman's Layer. As the damage to the stroma increases, deeper layers might be involved. This implies that one would want to express damage as progressing from the anterior (Bowman's Layer) to the posterior (Descemet's Membrane) and express it as a fraction of the total stromal depth involved. However, collagen matrix vacuolization can (and often does) increase total stromal thickness. Thus, measurements of the depth of a stromal lesion can be complicated by the change in overall stromal thickness. To account for stromal swelling, this depth may be estimated from the percentage of the stromal cross section that remained undamaged (starting at the posterior border). For this report, depth of stromal damage is reported simply in terms of relative depth (e.g., upper, middle and deep stroma).

Photomicrographs of the epithelium and stroma are intended to illustrate the degree of damage at the indicated depth. Where little or no damage to the stroma was detected, the area directly under Bowman's Layer was photographed since that is where one would expect to see damage if it had occurred. Images were captured using a Micropublisher 5.0 Digital Camera connected to an Olympus BX45 microscope and processed with Image-Pro Plus software version 5.1 (MediaCybernetics, Inc., Silver Spring, MD). The white balance of each image was corrected to better represent the colors that are seen through the microscope.

With the digital camera and associated software, it is possible to measure distances within the captured image. This feature can be used to measure corneal stromal thickness directly. Such measurements require a true cross-section of the cornea (i.e., a perpendicular section relative to the corneal surface plane). In some cases, a true cross-section is not available. The Descemet's Membrane thickness is used to estimate how close the section is to a true cross section. The appearance of an unusually thick membrane suggests that the section was cut at a tangent to the true cross section (or from a more mature animal). If the measured corneal section did not appear to be a true cross section, the observation was noted. Even in a true cross section, the isolated cornea is not of uniform thickness but rather shows peaks and valleys. Therefore, an effort has been made to select "representative" cross-sections that are neither extreme "peaks" nor "valleys" for measurement of corneal thickness. The values obtained should be considered "representative" of the treatment group rather than strict quantitative measures. Many more measurements would be required to provide a quantitative comparison. In some cases, the treated corneas will have lost most of their epithelium or it has been compromised (i.e., ethanol treatment). In those cases, the stromal thickness of the treated corneas should be compared to the stromal thickness of the negative control-treated corneas.

## 2.5 Histopathologic Results

The negative control corneas were treated for 60 minutes (60 minutes post incubation time) with sterile, deionized water (slides C1837-1839). The negative control-treated epithelium was composed of three layers. The basal cell layer was a well-formed, columnar-cell region directly attached to the Bowman's Layer. The basal cells were always tightly attached to each other. Several layers of wing cells covered the columnar basal layer. In both of these layers, the cell nuclei showed diffuse chromatin without clear nucleoli. Asymmetric clear spaces ("nuclear halos") were occasionally observed around nuclei in the wing and basal cell layers. These were an artifact of tissue processing and unrelated to treatment. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei (Figure 2).

The stromal elements in the negative control corneas showed well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei showed a range of morphologies from moderate sized (smaller than the epithelial nuclei) with diffuse basophilic staining, to narrow, elongated and condensed with dark basophilic staining. Cytoplasmic staining, when it was visible, was moderately basophilic. Rarely cells with eosinophilic cytoplasmic staining were observed. Collagen bundles were generally parallel and well-ordered (Stroma just under Bowman's Layer, Figure 3).

The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well-maintained.

The cross section of a negative control-treated cornea, showing the general thickness of the whole cornea and stroma, is shown in Figure 4.

The positive control corneas (slides C1840-C1842), treated for 10 minutes with 100% ethanol (120 minutes post incubation time), showed the classic pattern of squamous layer coagulation (darkening) and marked vacuolization in the wing and basal cell nuclei (Figure 5). The loss of adhesion between the basal cells (cell to cell) and the basal lamina was marked. The epithelium was probably not viable at the time of fixation. Overall, the positive control-treated corneas were thicker than the negative control-treated corneas (Figure 6). In the stroma directly below Bowman's Layer, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate/marked collagen matrix vacuolization extended past 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 7) as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization (Figure 8). In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei and cytoplasmic eosinophilia (Figure 9). The endothelial cells were generally intact (similar to the negative control-treated corneas).

Table 1 presents the results for the test articles evaluated.

**Table 1**  
**Histopathological Observations of the Test Article-Treated Corneas**

IIVS Number	Sponsor's Designation	Exposure Time (minutes)/ Post-Exposure Incubation (minutes)	Observations	Figure #
04AB07 Slides C1849- C1851	EG	3/120	<p>All corneas were similar histopathologically.</p> <p><b>Epithelium:</b> There was coagulative necrosis of the squamous layer, characterized by hypereosinophilic cytoplasm and loss of nuclear detail. There was multifocal cytoplasmic hypereosinophilia throughout the wing layer and multifocal separation of basal cells from the basal lamina. (Figure 10).</p> <p><b>Stroma:</b> The stroma was of intermediate thickness between positive and negative controls (Figure 11). In the upper third of the stroma, there was mild to moderate expansion of the collagen matrix and numerous keratocytes with pyknotic nuclei and/or eosinophilic cytoplasm (Figure 12). The stroma and keratocytes in the lower stroma resembled negative controls.</p>	10-12
			<p><b>Endothelium:</b> The endothelium was generally intact and resembled negative controls.</p>	

IIVS Number	Sponsor's Designation	Exposure Time (minutes)/ Post-Exposure Incubation (minutes)	Observations	Figure #
04AB07 Slides C1852- C1854	EG	10/120	<p>All corneas were similar histopathologically.</p> <p><b>Epithelium:</b> There was coagulative necrosis of the squamous layer and upper wing layer, characterized by hypereosinophilic cytoplasm and loss of nuclear detail. There was diffuse cytoplasmic hypereosinophilia throughout the lower wing layer and basal layers and multifocal separation of basal cells from the basal lamina. (Figure 13).</p> <p><b>Stroma:</b> The stroma was notably thicker than positive controls (Figure 14). There was moderate to marked expansion of the entire collagen matrix and numerous keratocytes with vacuolated nuclei throughout the stroma (Figure 15). In the lower stroma adjacent to Descemet's membrane there were scattered keratocytes with pyknotic nuclei (Figure 16).</p>	13-16
			<p><b>Endothelium:</b> Endothelial cells were diffusely hypereosinophilic with condensed, hyperchromatic nuclei (Figure 16).</p>	

The figures displayed on the subsequent pages of this pathology report are representative H&E-stained cross-sections presented at the magnifications indicated in each image caption. A 100 um bar appears on each micrograph for reference. Arrows included in some of the figures identify examples of the lesions noted, however not all lesions in all photomicrographs are marked.



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Date

Figure 1. An example of a control cornea showing the three layers of the epithelium, Bowman's Layer and the upper stroma (H&E)

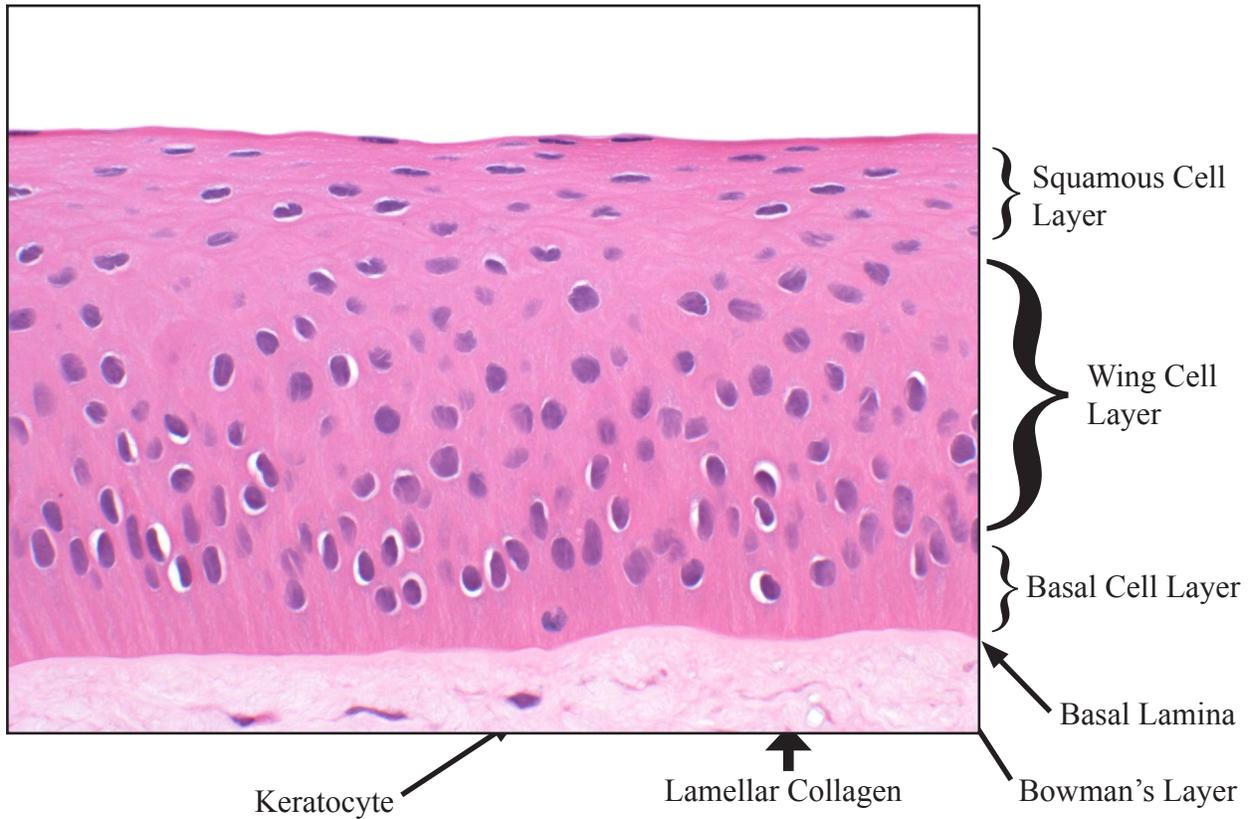


Figure 2. Negative Control (sterile, deionized water, 60 minute exposure/60 minute post incubation) - Epithelium (Slide C1838-1, 40x, H&E)

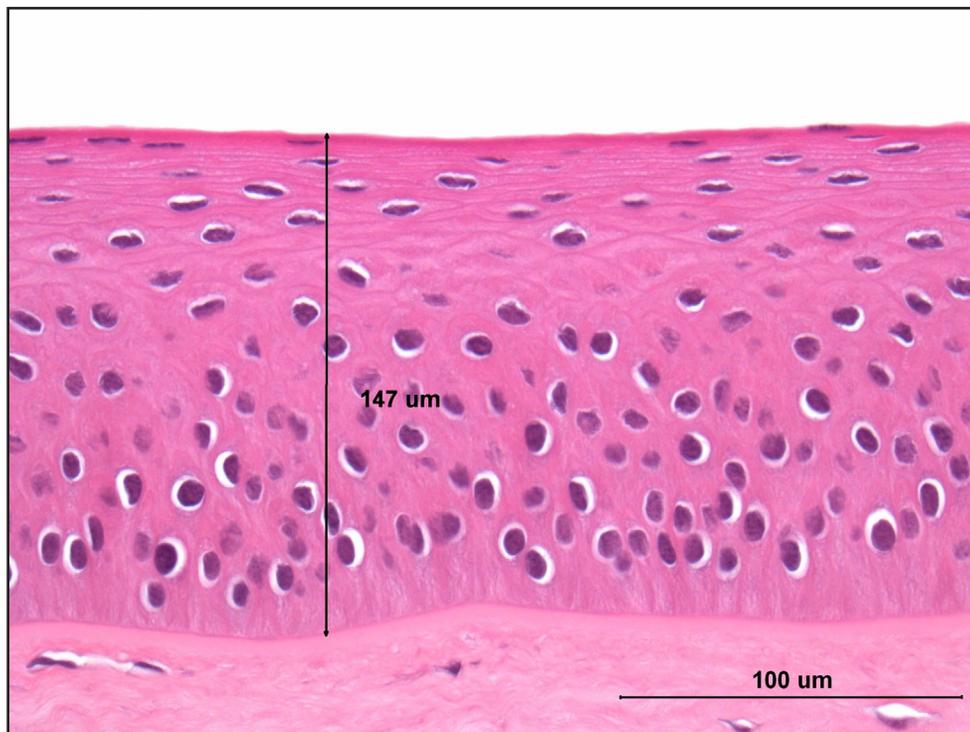


Figure 3. Negative Control (sterile, deionized water, 60 minute exposure/60 minute post incubation) - Stroma directly below Bowman's Layer (Slide C1838-1, 40x, H&E)

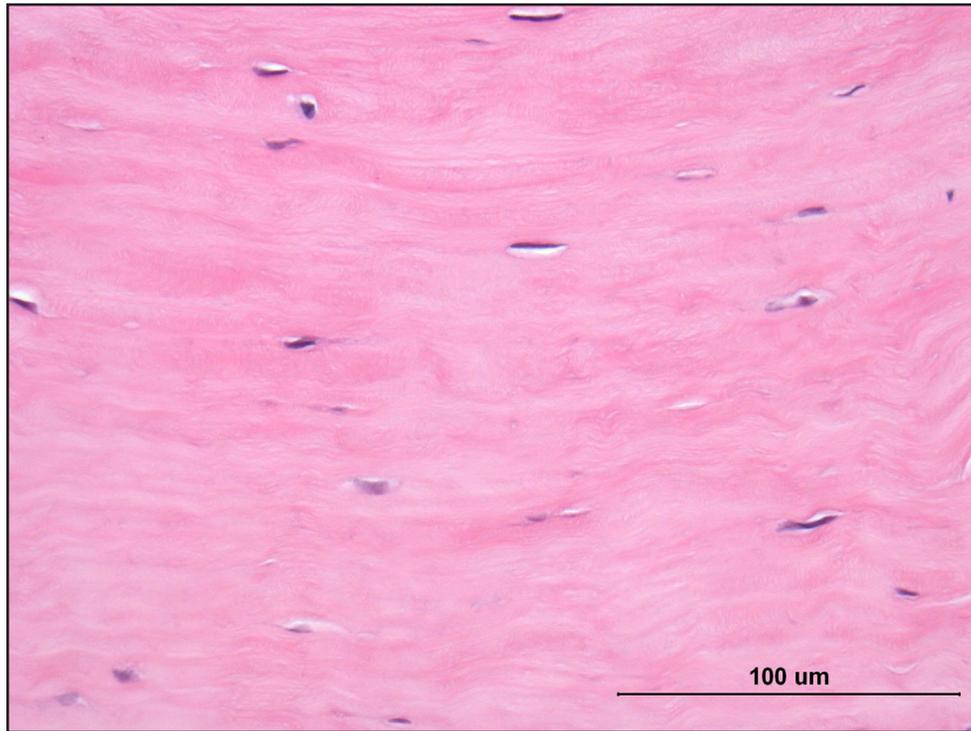


Figure 4. Negative Control (sterile, deionized water, 60 minute exposure/60 minute post incubation) - Full thickness (Slide C1838-1, 4x, H&E)

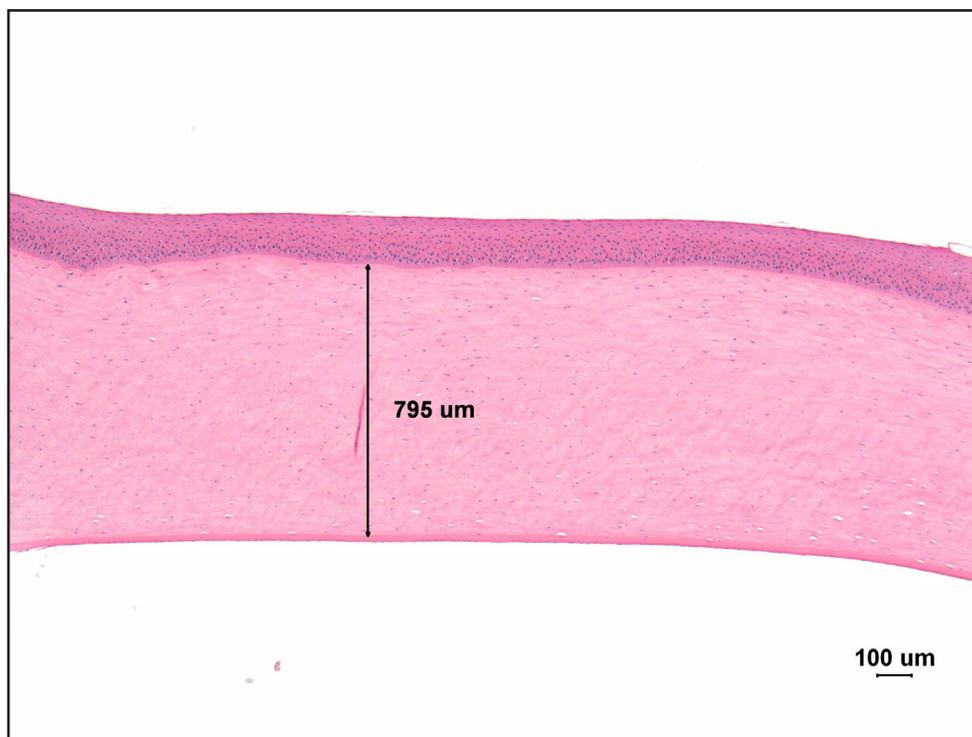


Figure 5. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Epithelium with coagulation of the squamous layer (A →) and vacuolation of wing and basal cells (B →) (Slide C1841-1, 40x, H&E)

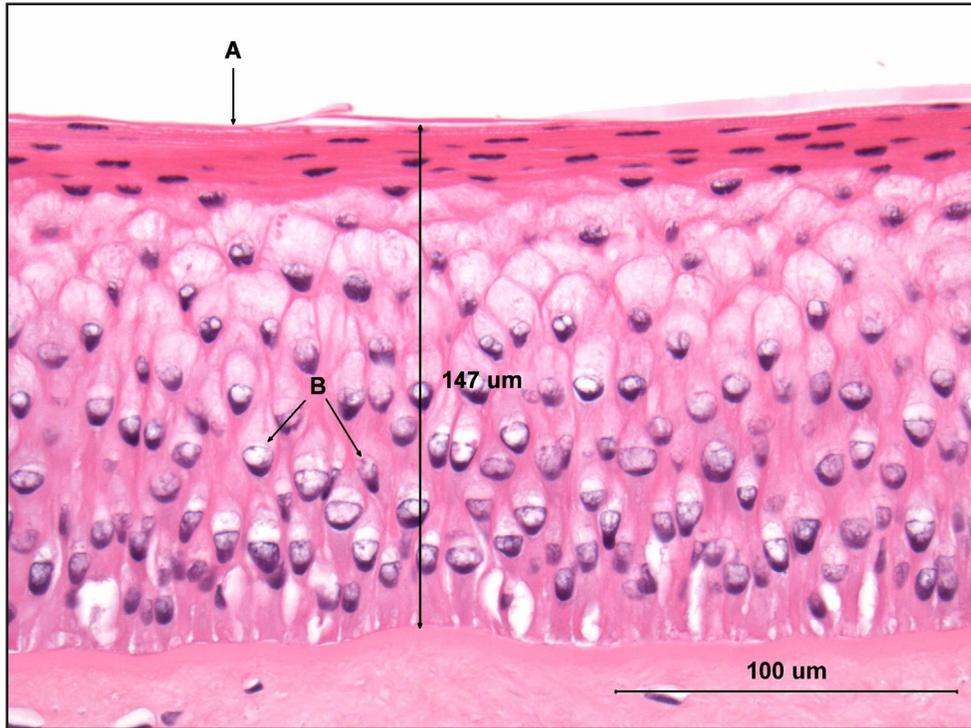


Figure 6. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Full thickness (Slide C1842-1, 4x, H&E)

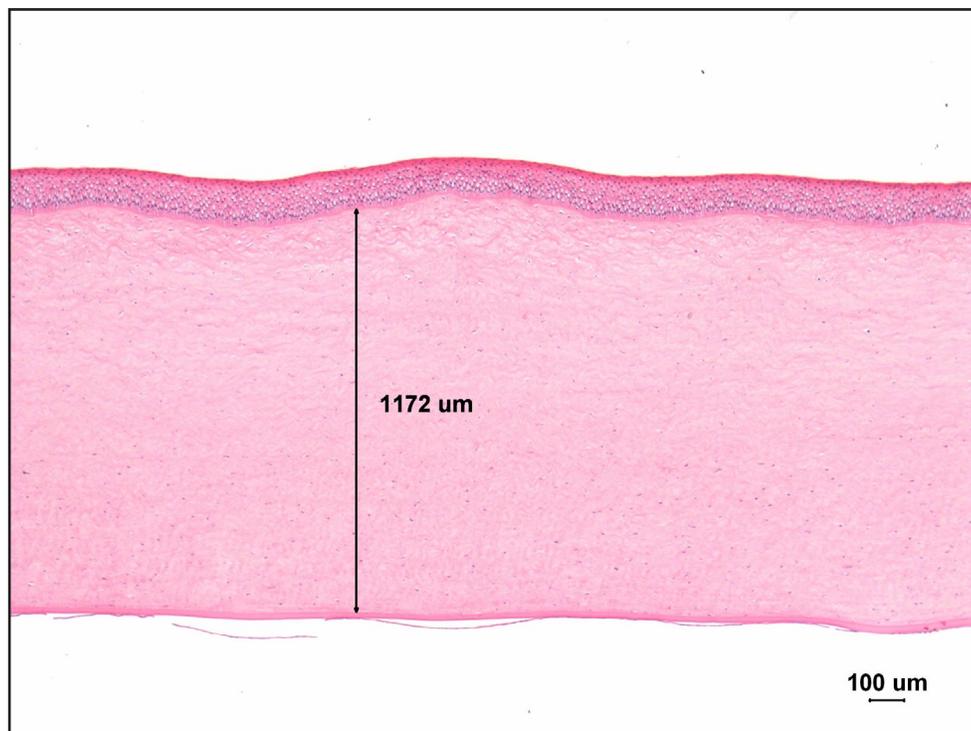


Figure 7. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Upper stroma with vacuolation and decreased density of viable keratocytes (→) (Slide C1842-1, 40x, H&E)

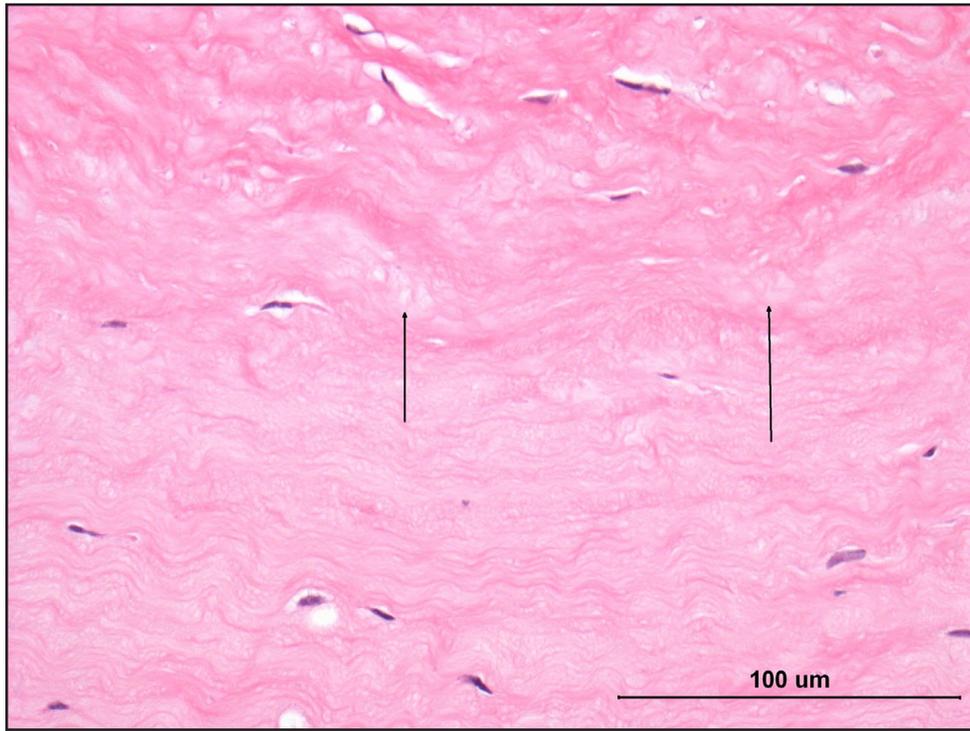


Figure 8. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Upper stroma with vacuolated keratocyte nuclei (→) (Slide C1842-1, 40x, H&E)

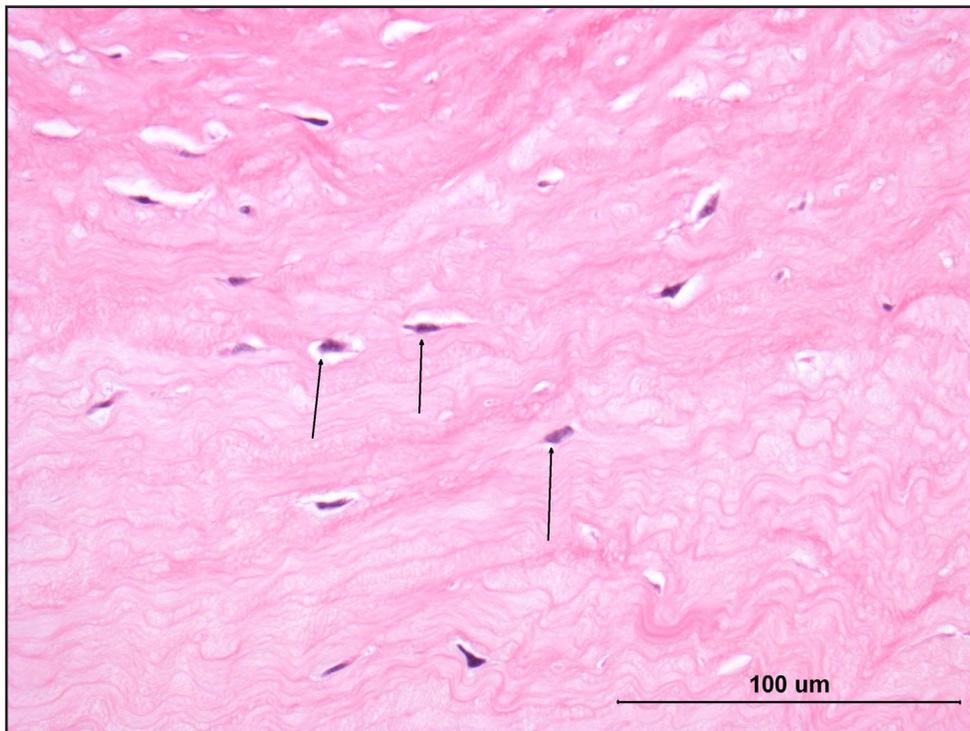


Figure 9. Positive Control (100% ethanol, 10 minute exposure/120 minute post incubation) - Keratocytes with eosinophilic cytoplasm (—→) in mid stroma (Slide C1842-1, 40x, H&E)

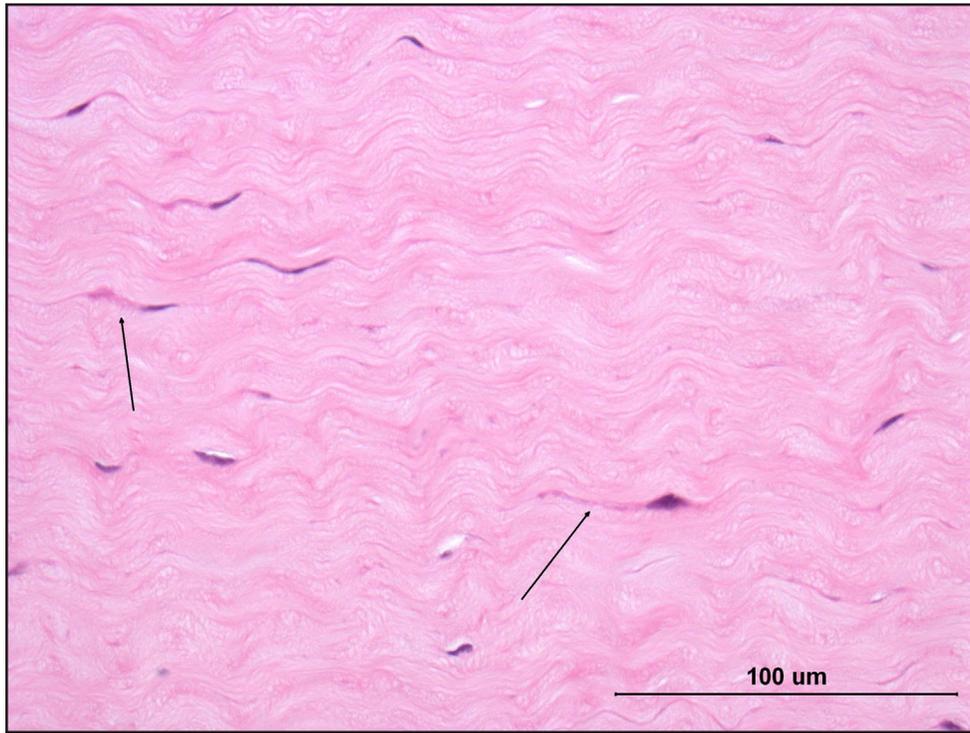


Figure 10. 04AB07, EG (3 minute exposure/120 minute post incubation) - Epithelium with coagulation of the squamous layer (A —→) and separation of the basal layer (B —→) (Slide C1849-1, 40x, H&E)

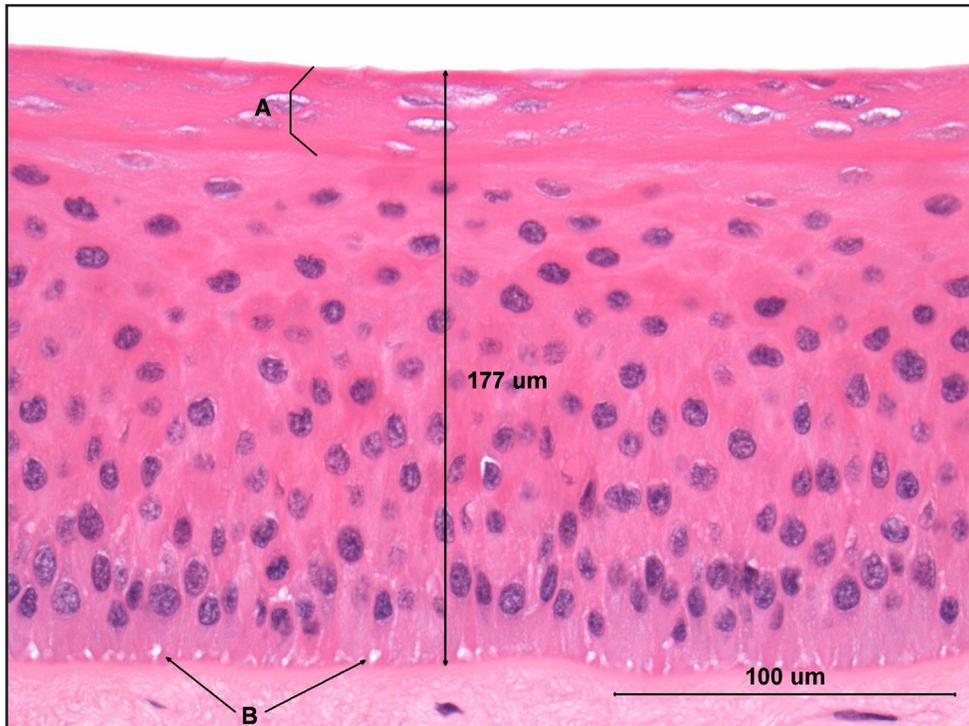


Figure 11. 04AB07, EG (3 minute exposure/120 minute post incubation) - Full thickness (Slide C1849-1, 4x, H&E)

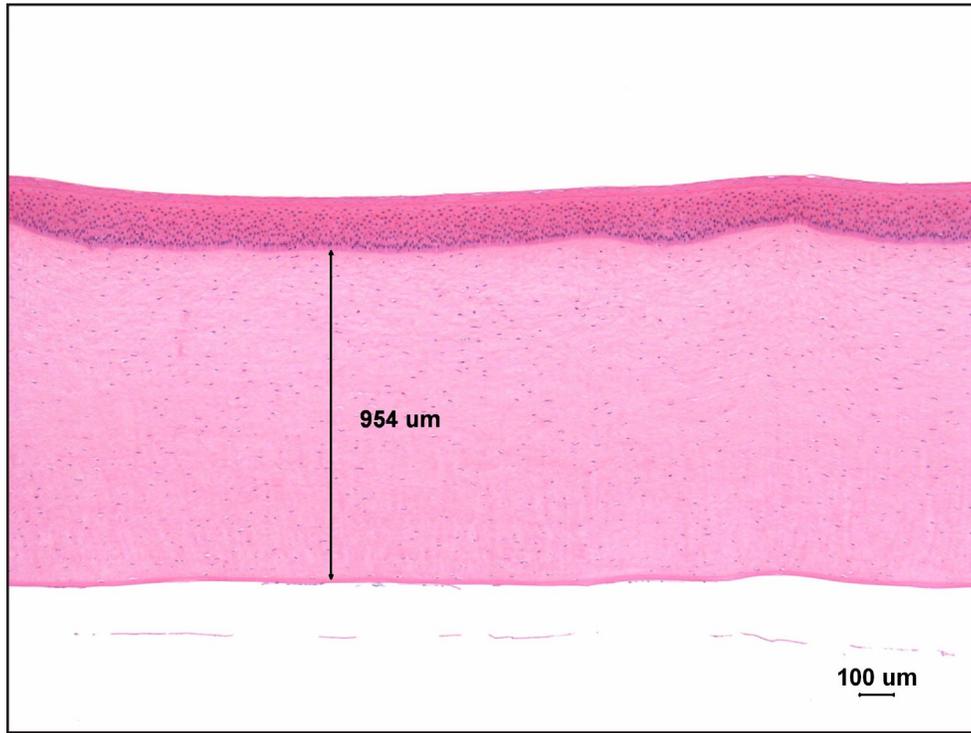


Figure 12. 04AB07, EG (3 minute exposure/120 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →), pyknotic keratocyte nuclei (B →), and eosinophilic cytoplasm (C →) (Slide C1849-1, 40x, H&E)

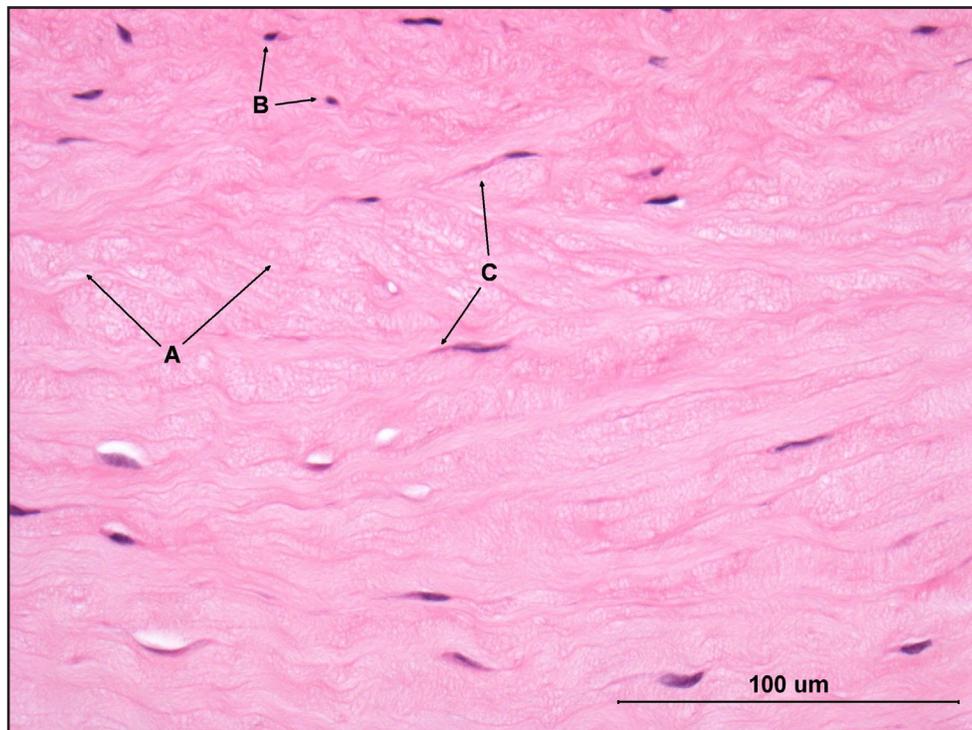


Figure 13. 04AB07, EG (10 minute exposure/120 minute post incubation) - Epithelium with coagulation of the squamous layer (A →) and separation of the basal layer (B →) (Slide C1852-1, 40x, H&E)

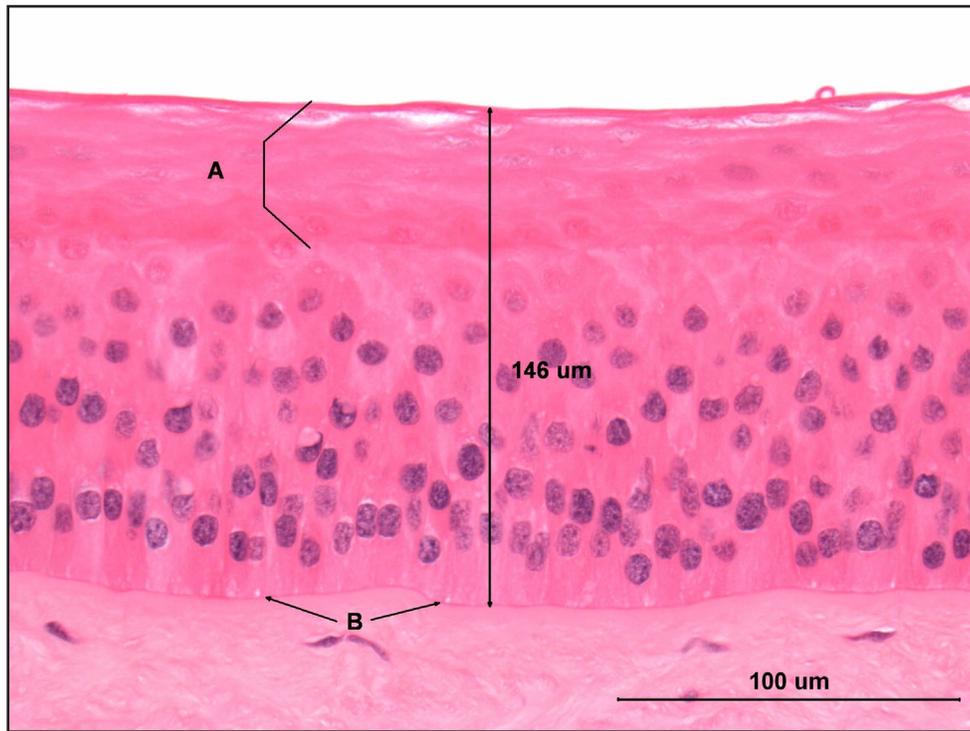


Figure 14. 04AB07, EG (10 minute exposure/120 minute post incubation) - Full thickness (Slide C1852-1, 4x, H&E)

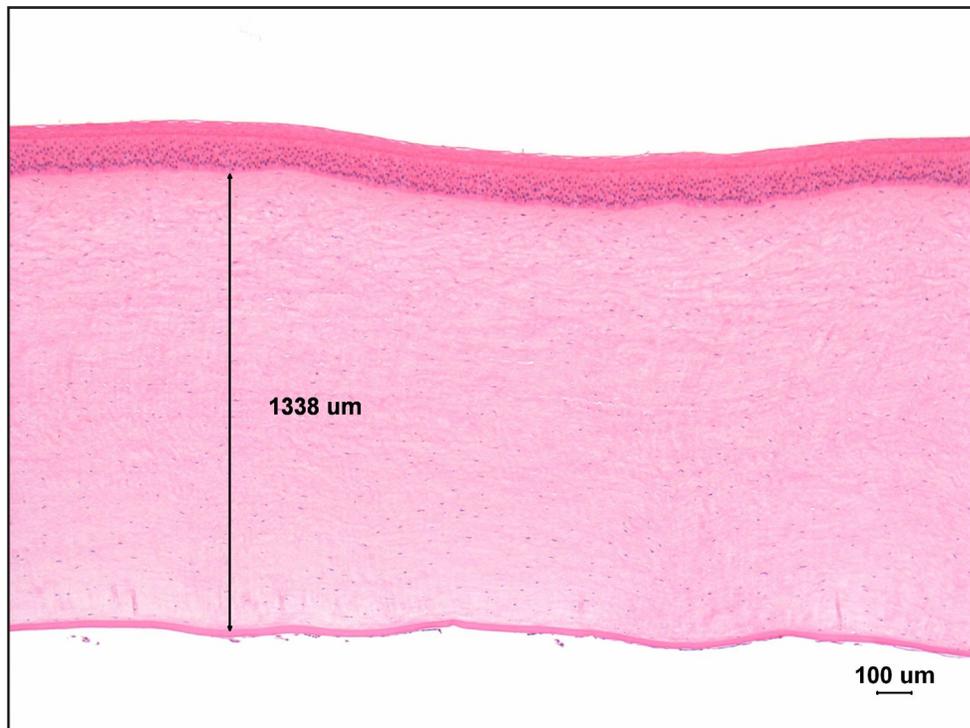


Figure 15. 04AB07, EG (10 minute exposure/120 minute post incubation) - Upper stroma with expansion of the collagen matrix (A →) and vacuolated keratocyte nuclei (B →) (Slide C1852-1, 40x, H&E)



Figure 16. 04AB07, EG (10 minute exposure/120 minute post incubation) - Lower stroma with expansion of the collagen matrix (A →) and pyknotic keratocyte nuclei (B →) and endothelium with hyper eosinophilic cytoplasm (C →) (Slide C1854-1, 40x, H&E)

