

FINAL REPORT

Study Title

**TOPICAL APPLICATION OCULAR IRRITATION SCREENING ASSAY
USING THE EPIOCULAR™ HUMAN CELL CONSTRUCT**

Test Substances

AT, AU,
BL, BB, BM, BK

Reference Substance

AV

Authors

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Study Completion Date

January 23, 2006

Performing Laboratory

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

Study Number

05AD96-AD98, AE08-AE10, AE14-AE15.015033

Laboratory Project Number

4278

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STATEMENT OF COMPLIANCE

The Topical Application Ocular Irritation Screening Assay Using The EpiOcular™ Human Cell Construct of the test substances, AT, AU, BL, BB, BM, and BK, and the reference substance AV, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test and reference substances have not been determined by the testing facility.

The stability of the test and reference substances under the test conditions has not been determined by the testing facility and is not included in the final report.

Greg Mun, B.A.
Study Director

Date

QUALITY ASSURANCE STATEMENT

Study Title: Topical Application Ocular Irritation Screening Assay Using the EpiOcular™
Human Cell Construct

Study Number: 05AD96-AD98, AE08-AE10, AE14-AE15.015033

Study Director: Greg Mun, B.A.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	07-Dec-05	07-Dec-05	08-Dec-05
Rinsing of the Tissues/ Addition to MTT	07-Dec-05	07-Dec-05	08-Dec-05
Final Report and Data	20-Jan-06	23-Jan-06	23-Jan-06

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Amanda K. Ulrey, RQAP-GLP
Quality Assurance

Date

SIGNATURE PAGE

**TOPICAL APPLICATION OCULAR IRRITATION SCREENING ASSAY
USING THE EPIOCULAR™ HUMAN CELL CONSTRUCT**

Initiation Date: December 7, 2005

Completion Date: January 23, 2006

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:

Greg Mun, B.A.

Date

TEST/REFERENCE SUBSTANCE RECEIPT

IIVS Test/Reference Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
05AD96	AT	clear light yellow non-viscous liquid	6/23/05	room temperature
05AD97	AU	clear light yellow non-viscous liquid	6/23/05	room temperature
05AD98	AV	clear light yellow non-viscous liquid	6/23/05	room temperature
05AE08	BL	clear colorless non-viscous liquid	7/1/05	room temperature
05AE09	BB	clear colorless non-viscous liquid	7/1/05	room temperature
05AE10	BM	clear colorless non-viscous liquid	7/1/05	room temperature
05AE14	BK	clear colorless non-viscous liquid	7/1/05	room temperature

* - Protected from exposure to light

**TOPICAL APPLICATION OCULAR IRRITATION SCREENING ASSAY
USING THE EPIOCULAR™ HUMAN CELL CONSTRUCT**

INTRODUCTION

The EpiOcular™ Human Cell Construct was used to assess the potential ocular irritation of the test and reference substances. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) conversion assay, which measures the NAD(P)H-dependent microsomal enzyme reduction of MTT (and to a lesser extent, the succinate dehydrogenase reduction of MTT) to a blue formazan precipitate, was used to assess cellular metabolism after exposure to the test and reference substances for various exposure times¹. The duration of exposure resulting in a 50% decrease in MTT conversion in test substance-treated EpiOcular™ human cell constructs, relative to control cultures, was determined (ET₅₀).

The purpose of this study was to evaluate the potential toxicity of the test and reference substances as measured by the conversion of MTT by EpiOcular™ human cell constructs after exposure to each test or reference substance for various exposure times. The laboratory phase of the study was conducted from December 7, 2005 to December 8, 2005 at the Institute for In Vitro Sciences, Inc. The test and reference substances were tested in a screening assay to determine the duration of exposure to each test substance, which resulted in the ET₅₀ endpoint.

¹ Berridge, M.V., Tan, A.S., McCoy, K.D., Wang, R. (1996) The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. **Biochemica** 4:14-19.

MATERIALS AND METHODS

Receipt of the EpiOcular™ Human Cell Construct Model

Upon receipt of the EpiOcular™ Human Cell Construct Kit (MatTek Corporation), the solutions were stored as indicated by the manufacturer. The EpiOcular™ human cell constructs were stored at 2-8°C until used. On the day of dosing, EpiOcular™ Assay Medium was warmed to approximately 37°C. Nine-tenths mL of Assay Medium were aliquoted into the appropriate wells of six-well plates. The six-well plates were labeled to indicate test substance and exposure time. The constructs were inspected for air bubbles between the agarose gel and Millicell® insert prior to opening the sealed package. Cultures with air bubbles covering greater than 50% of the Millicell® area were not used. The 24-well shipping containers were removed from the plastic bag and their surfaces were disinfected with 70% ethanol. The EpiOcular™ human cell constructs were transferred aseptically into the six-well plates. The constructs were then incubated at 37±1°C in a humidified atmosphere of 5±1% CO₂ in air (standard conditions) for at least one hour. The medium was then aspirated and 0.9 mL of fresh Assay Medium were added to each assay well below the EpiOcular™ human cell construct. The plates were returned to the incubator until treatment was initiated.

Assessment of Direct Test/Reference Substance Reduction of MTT

Each test/reference substance was added to a 1.0 mg/mL MTT (Sigma) solution in warm Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (MTT Addition Medium) to assess its ability to directly reduce MTT. Approximately 100 µL of each test or reference substance were added to 1 mL of the MTT solution and the mixtures were incubated in the dark at standard conditions for approximately one hour. A negative control, 100 µL of sterile, deionized water (Quality Biological), was tested concurrently. If the MTT solution color turned blue/purple, the test substance was presumed to have reduced the MTT. Water insoluble test materials may show direct reduction (darkening) only at the interface between the test substance and the medium.

The test substances, AT, AU, BL, BB, BM, and BK, and the reference substance AV, were not observed to reduce MTT in the absence of viable cells.

pH Determination

The pH of each liquid test or reference substance was measured using pH paper. Initially, the test and reference substances were added to pH paper (EMD Chemicals Inc./EM Science) with a 0-14 pH range in 1.0 pH unit increments to approximate a narrow pH range. Next, each neat test or reference substance was added to pH paper with a narrower range of 7.5-14 pH units 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.

MTT Assay

The EpiOcular™ cultures were treated in duplicate with the test and reference substances, AT, AU, AV, BL, BM, and BK for 1, 5, 10, and 20 minutes. The test substance, BB, was treated in duplicate for 20, 45, 90, and 240 minutes. One hundred microliters of each liquid test or

reference substance were applied to each EpiOcular™ human cell construct. Duplicate cultures of the negative control (exposure time control), 100 µL of sterile, deionized water (Quality Biological), were exposed for 15 and 240 minutes. Duplicate cultures of the positive control, 100 µL of 0.3% Triton®-X-100 (Fisher), were exposed for 15 and 45 minutes. The exposed cultures were then incubated for the appropriate amount of time at standard conditions.

After the appropriate exposure time, the EpiOcular™ cultures were extensively rinsed with Calcium and Magnesium-Free Dulbecco's Phosphate Buffered Saline (Ca⁺⁺Mg⁺⁺Free-DPBS) and the wash medium was decanted. After rinsing, the tissues were transferred to 5 mL of Assay Medium for a 10 to 20 minute soak at room temperature to remove any test substance absorbed into the tissue. A 1.0 mg/mL solution of MTT in warm MTT Addition Medium was prepared no more than 2 hours before use. Three-tenths mL of MTT solution were added to designated wells in a prelabeled 24-well plate. The EpiOcular™ constructs were transferred to the appropriate wells after rinsing with Ca⁺⁺Mg⁺⁺Free-DPBS. The trays were incubated for approximately three hours at standard conditions.

After the incubation period with MTT solution, the EpiOcular™ cultures were blotted on absorbent paper, cleared of excess liquid, and transferred to a prelabeled 24-well plate containing 2.0 mL of isopropanol in each designated well. The plates were sealed with parafilm and stored in the refrigerator (2-8°C) until the last exposure time was harvested. The plates were then shaken for at least two hours at room temperature to extract the MTT.

At the end of the extraction period, the liquid within the Millicell® inserts was decanted into the well from which the Millicell® insert was taken. The extract solution was mixed and 200 µL were transferred to the appropriate wells of a 96-well plate. Two hundred microliters of isopropanol were added to the two wells designated as the blanks. The absorbance at 550 nm (OD₅₅₀) of each well was measured with a Molecular Devices Vmax plate reader.

Presentation of Data

The raw absorbance values were captured. The mean OD₅₅₀ value of the blank control wells was calculated. The corrected mean OD₅₅₀ of the exposure time controls was determined by subtracting the mean OD₅₅₀ of the blank control from their mean OD₅₅₀ values. The corrected OD₅₅₀ of each individual test or reference substance exposure times and the positive control exposure times was determined by subtracting the mean OD₅₅₀ of the blank control from their OD₅₅₀ values. All calculations were performed using an Excel spreadsheet. The following percent of control calculations were made:

$$\% \text{ of Control} = \frac{\text{corrected OD}_{550} \text{ of Test or Reference Substance or Positive Control Exposure Time}}{\text{appropriate corrected mean OD}_{550} \text{ Negative Control}} \times 100$$

Exposure time response curves were plotted with the % of Control on the ordinate and the test substance or positive control exposure time on the abscissa. The ET₅₀ value was interpolated from each plot. To determine the ET₅₀, two consecutive points were selected, where one exposure time resulted in a relative survival greater than 50%, and one exposure time resulted in less than 50% survival. Two select points were used to determine the slope and the y-intercept for the equation $y=m(x) + b$. Finally, to determine the ET₅₀, the equation was solved for $y=50$.

When all of the exposure time points showed greater than 50% survival, the ET_{50} value was presented as greater than the maximum exposure time. When all of the exposure times showed less than 50% survival, the ET_{50} value was presented as less than the minimum exposure time.

Criteria for a Valid Test

The assay results were accepted when: 1) The ET_{50} value of the positive control fell within two standard deviations of the historical mean (updated every three months) and 2) The corrected mean OD_{550} value for the minimum negative control exposure time was within 20% of the corrected mean OD_{550} value for the maximum negative control exposure time (up to 240 minutes).

RESULTS AND DISCUSSION

MTT Assay

The EpiOcular™ cultures were treated in duplicate with the test and reference substances, AT, AU, AV, BL, BM, and BK for 1, 5, 10, and 20 minutes. The test substance, BB, was treated in duplicate for 20, 45, 90, and 240 minutes. The negative control was exposed in duplicate for 15 and 240 minutes. Table 1 summarizes the ET₅₀ results of the Topical Application Ocular Irritation Screening Assay using the EpiOcular™ Human Cell Construct for the test and reference substances and the positive control, 0.3% Triton®-X-100. The exposure time response curves are included in Appendix B. Since the positive control fell within two standard deviations of the historical mean (15.5 – 39.1 minutes), and the corrected mean OD₅₅₀ value for the minimum negative control exposure time (1.488) was within 20% of the corrected mean OD₅₅₀ value for the maximum negative control exposure time (up to 240 minutes) (1.463), the assay results were accepted.

The test substances, AT, AU, BL, BB, BM, and BK and reference substance AV were not observed to reduce MTT in the absence of viable cells.

Table 1

Assay Date	IIVS Test/Reference Substance Number	Sponsor's Designation	Conc.	ET ₅₀ (minutes)	pH
12/7/05	05AD96	AT	Neat	< 1	12.5
	05AD97	AU	Neat	< 1	13.0
	05AD98 ^a	AV ^a	Neat	< 1	13.0
	05AE08	BL	Neat	6.7	9.5
	05AE09	BB	Neat	> 240	9.0
	05AE10	BM	Neat	4.9	11.0
	05AE14	BK	Neat	9.4	10.0
	Positive Control	0.3% Triton®-X-100	NA	22.6	NA

NA - Not Applicable

^a – Reference Substance for AT and AU

APPENDIX A

APPENDIX B

EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05
 TEST MATERIAL: AT
 TEST ARTICLE: 05AD96

Study No.
 05AD96-AD98, AE08-AE10, AE14-AE15.015033

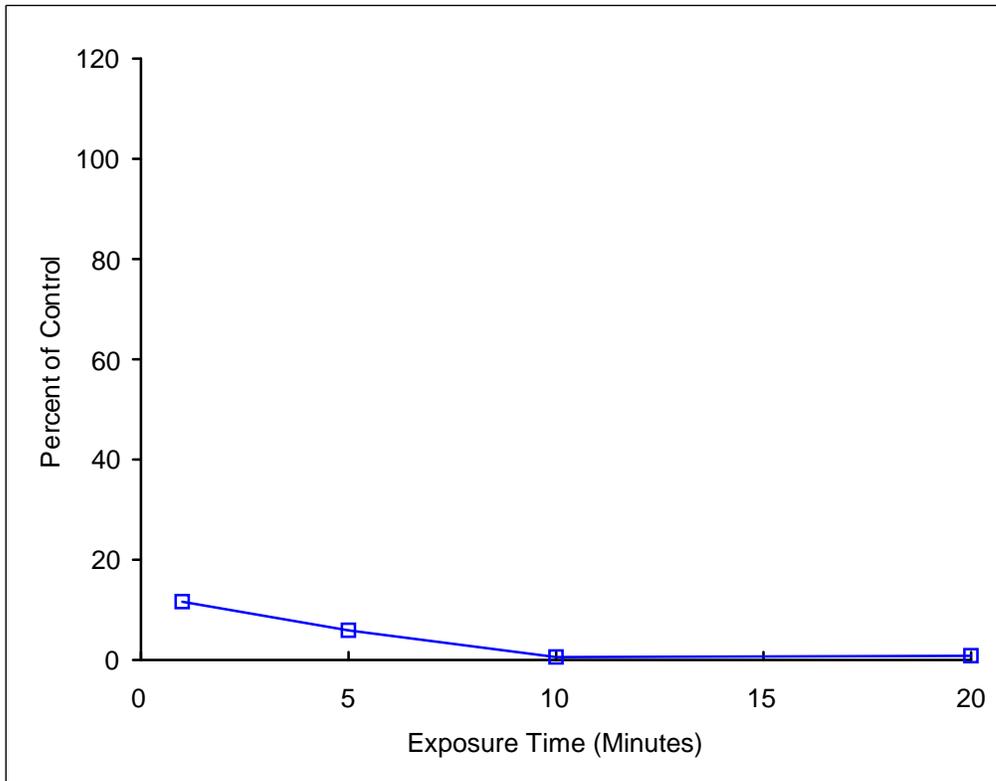
ET₅₀ = < 1 Minute

TRIAL 1
 CONCENTRATION: 100%.

			y = Percent Viable	
			x = Exposure Time	
			slope=rise/run=(y1-y2)/(x1-x2)	
			y intercept=y-(slope*x)	
			X	Y
TIME	PERCENT			
EXPOSURE	VIABLE			
(Minutes)				
1	11.6	1	1.0	11.6
5	5.9	2	1.0	11.6
10	0.6	3	#DIV/0!	50
20	0.8			
			slope =	#DIV/0!
			y intercept =	#DIV/0!

AT

CONCENTRATION: 100%.
 TRIAL 1



EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05
 TEST MATERIAL: AU
 TEST ARTICLE: 05AD97

Study No.
 05AD96-AD98, AE08-AE10, AE14-AE15.015033

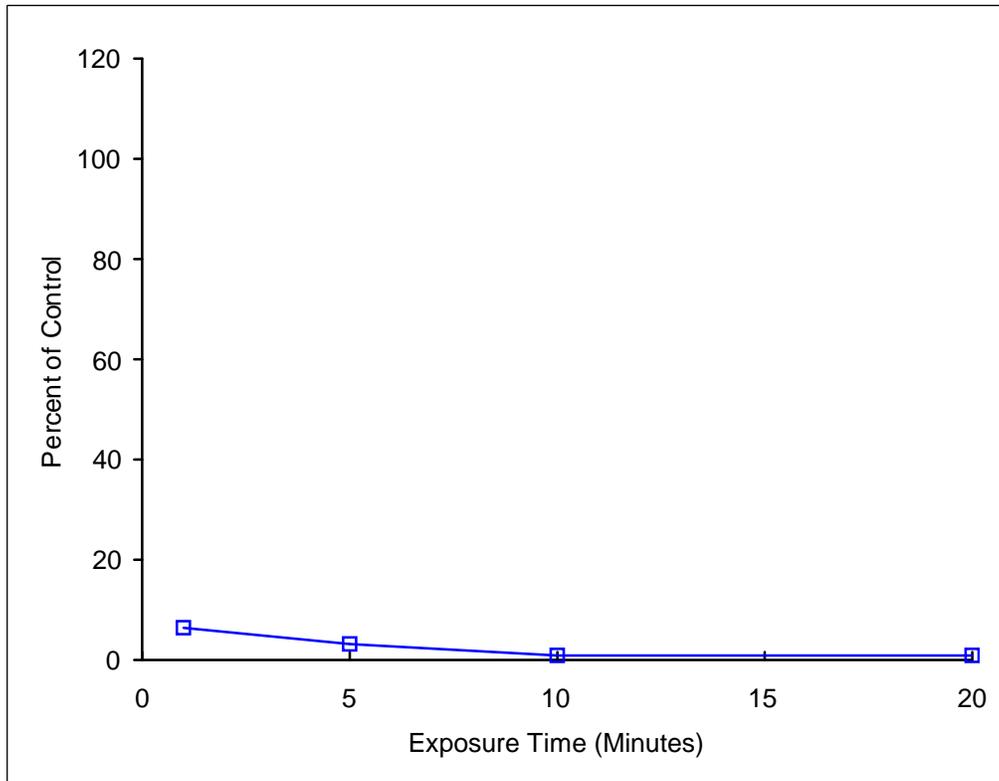
ET₅₀ = < 1 Minute

TRIAL 1
 CONCENTRATION: 100%.

			$y = \text{Percent Viable}$ $x = \text{Exposure Time}$ $\text{slope} = \text{rise/run} = (y_1 - y_2) / (x_1 - x_2)$ $y \text{ intercept} = y - (\text{slope} * x)$	
TIME	PERCENT		X	Y
EXPOSURE	VIABLE			
(Minutes)				
1	6.4	1	1.0	6.4
5	3.2	2	1.0	6.4
10	0.9	3	#DIV/0!	50
20	0.9			
			slope =	#DIV/0!
			y intercept =	#DIV/0!

AU

CONCENTRATION: 100%.
 TRIAL 1



EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05
 TEST MATERIAL: AV*
 TEST ARTICLE: 05AD98

Study No.
 05AD96-AD98, AE08-AE10, AE14-AE15.015033

ET₅₀ = < 1 Minute

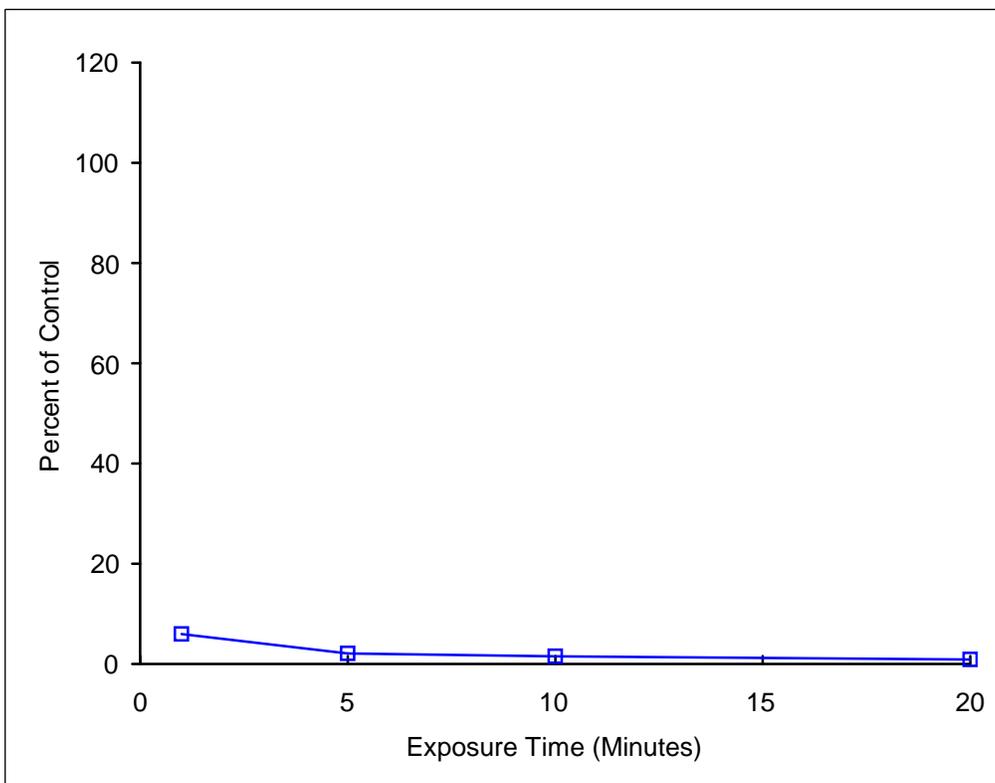
TRIAL 1
 CONCENTRATION: 100%.

		$y = \text{Percent Viable}$ $x = \text{Exposure Time}$ $\text{slope} = \text{rise/run} = (y_1 - y_2) / (x_1 - x_2)$ $y \text{ intercept} = y - (\text{slope} * x)$
TIME	PERCENT	
EXPOSURE	VIABLE	
(Minutes)		X Y
1	6.0	1 1.0 1 6
5	2.1	2 1.0 2 6
10	1.5	3 #DIV/0! 3 50
20	0.9	slope = #DIV/0!
		y intercept = #DIV/0!

*- Reference Substance

AV*

CONCENTRATION: 100%.
 TRIAL 1



EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05
 TEST MATERIAL: BL
 TEST ARTICLE: 05AE08

Study No.
 05AD96-AD98, AE08-AE10, AE14-AE15.015033

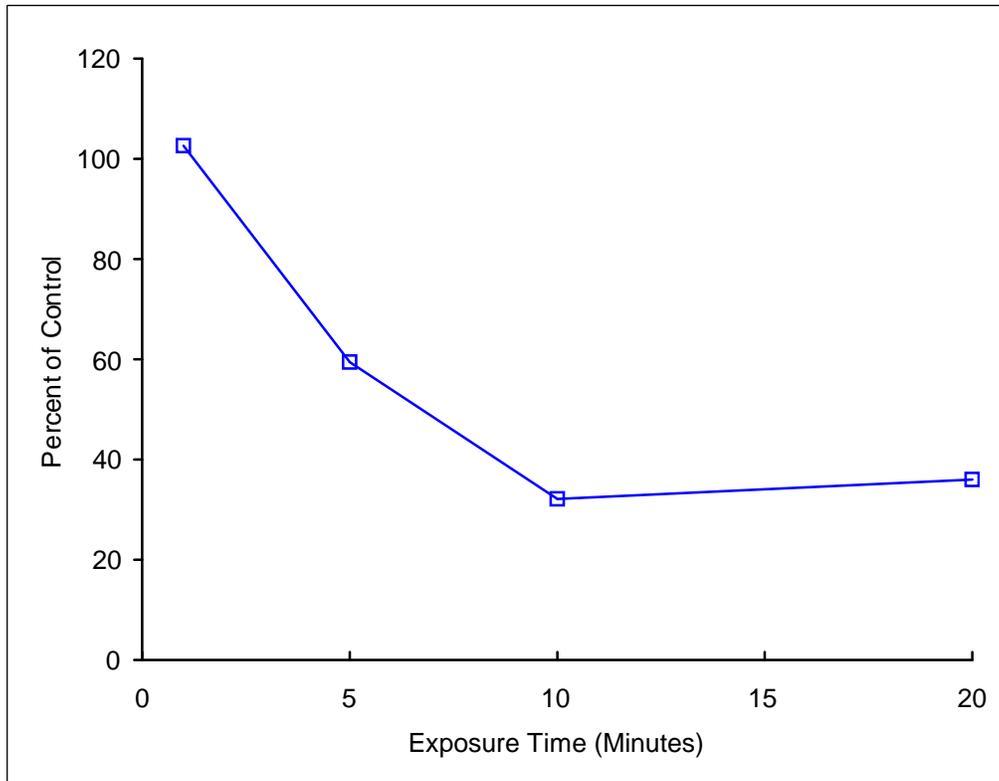
ET₅₀ = 6.7 Minutes

TRIAL 1
 CONCENTRATION: 100%.

			$y = \text{Percent Viable}$ $x = \text{Exposure Time}$ $\text{slope} = \text{rise/run} = (y_1 - y_2) / (x_1 - x_2)$ $y \text{ intercept} = y - (\text{slope} * x)$	
TIME	PERCENT		X	Y
EXPOSURE	VIABLE			
(Minutes)				
1	102.6	1	5.0	59.4
5	59.4	2	10.0	32.1
10	32.1	3	6.7216117	50
20	36.0			
			slope =	-5.46
			y intercept =	86.7

BL

CONCENTRATION: 100%.
 TRIAL 1



EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05
 TEST MATERIAL: BB
 TEST ARTICLE: 05AE09

Study No.
 05AD96-AD98, AE08-AE10, AE14-AE15.015033

ET₅₀ = > 240 Minutes

TRIAL 1
 CONCENTRATION: 100%.

TIME EXPOSURE (Minutes)	PERCENT VIABLE
20	100.2
45	96.1
90	105.6
240	99.2

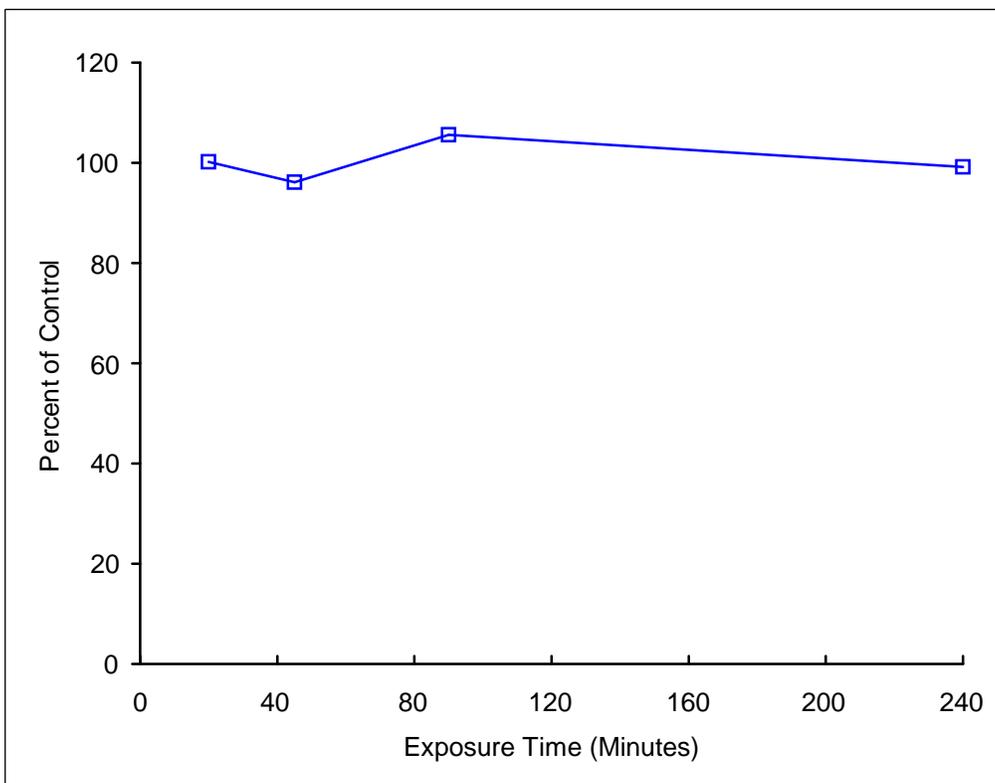
y = Percent Viable
 x = Exposure Time
 slope=rise/run=(y1-y2)/(x1-x2)
 y intercept=y-(slope*x)

X	Y
1 240.0	1 99.2
2 240.0	2 99.2
3 #DIV/0!	3 50

slope = #DIV/0!
 y intercept = #DIV/0!

BB

CONCENTRATION: 100%.
 TRIAL 1



EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05
 TEST MATERIAL: BM
 TEST ARTICLE: 05AE10

Study No.
 05AD96-AD98, AE08-AE10, AE14-AE15.015033

ET₅₀ = 4.9 Minutes

TRIAL 1
 CONCENTRATION: 100%.

TIME EXPOSURE (Minutes)	PERCENT VIABLE
1	98.7
5	48.4
10	18.4
20	15.4

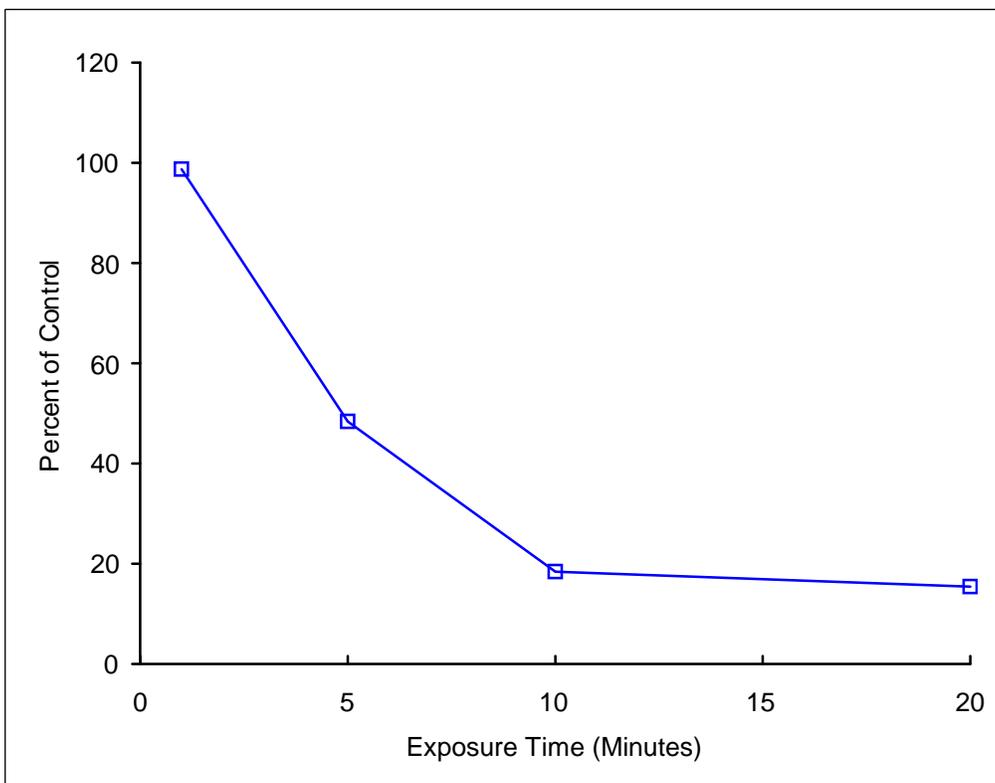
y = Percent Viable
 x = Exposure Time
 slope=rise/run=(y1-y2)/(x1-x2)
 y intercept=y-(slope*x)

X	Y
1	98.7
2	48.4
3	50

slope = -12.575
 y intercept = 111.275

BM

CONCENTRATION: 100%.
 TRIAL 1



EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05
 TEST MATERIAL: BK
 TEST ARTICLE: 05AE14

Study No.
 05AD96-AD98, AE08-AE10, AE14-AE15.015033

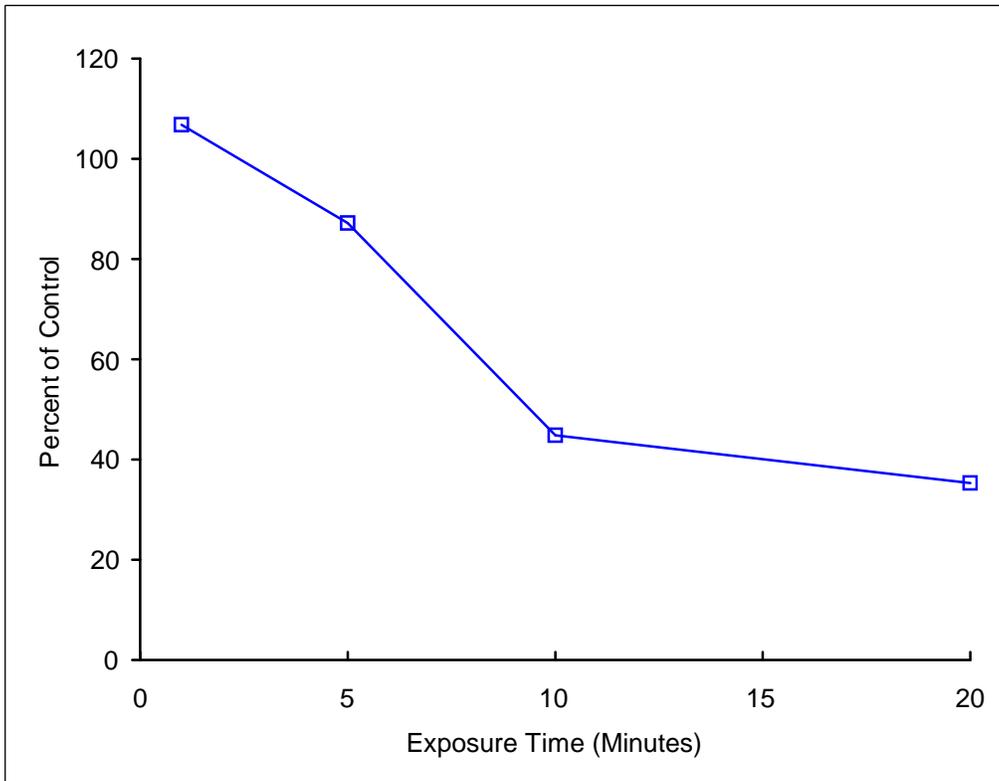
ET₅₀ = 9.4 Minutes

TRIAL 1
 CONCENTRATION: 100%.

			$y = \text{Percent Viable}$ $x = \text{Exposure Time}$ $\text{slope} = \text{rise/run} = (y_1 - y_2) / (x_1 - x_2)$ $y \text{ intercept} = y - (\text{slope} * x)$	
TIME	PERCENT		X	Y
EXPOSURE	VIABLE			
(Minutes)				
1	106.8	1	5.0	87.2
5	87.2	2	10.0	44.8
10	44.8	3	9.3867925	50
20	35.3			
			slope =	-8.48
			y intercept =	129.6

BK

CONCENTRATION: 100%.
 TRIAL 1



EPIOCULAR™ BIOASSAY

EXPERIMENT DATE: 7-Dec-05

TEST MATERIAL: 0.3% TRITON®-X-100

ET₅₀ = 22.6 Minutes

			y = Percent Viable	
			x = Exposure Time	
			slope=rise/run=(y1-y2)/(x1-x2)	
			y intercept=y-(slope*x)	
TIME	PERCENT		X	Y
EXPOSURE	VIABLE			
(Minutes)				
15	60.4	1	15.0	60.4
45	19.4	2	45.0	19.4
		3	22.609756	50
			slope =	-1.366667
			y intercept =	80.9

0.3% TRITON®-X-100

7-Dec-05

