November 4, 2020

PROTOCOL #2009680-201

# A NON-GLP EVALUATION OF ONE TEST PRODUCT FOR ITS ANTIMICROBIAL PROPERTIES AT THREE EXPOSURE TIMES FOLLOWING THE ASTM E2783 IN-VITRO TIME-KILL METHOD

Prepared for:

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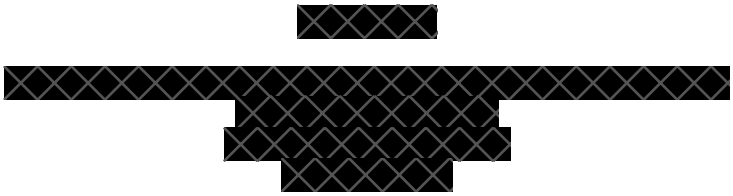

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**1.0 TITLE:** **NON-GLP EVALUATION ONE TEST PRODUCT FOR ITS ANTIMICROBIAL PROPERTIES AT THREE EXPOSURE TIMES FOLLOWING THE ASTM E2783 IN-VITRO TIME-KILL METHOD**

**2.0 SPONSOR:** **ENVISION PRODUCT DEVELOPMENT GROUP, LLC**
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**3.0 TESTING FACILITY:** **BIOSCIENCE LABORATORIES, INC.**
1755 South 19th Avenue
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**4.0 STUDY DIRECTOR:** Danielle Goveia

**5.0 PURPOSE:**

This study will use an In-Vitro Time-Kill Method to evaluate the antimicrobial properties of one test product when challenged with one bacterial strain. This procedure is based upon the methodology described in ASTM E2783-11 (2016), *Standard Test Method for Assessment of Antimicrobial Activity for Water Miscible Compounds Using a Time-Kill Procedure*. All testing will *not* be performed in accordance with Good Laboratory Practices, as specified in 21 CFR Part 58, and the characterization of the identity, strength, purity, composition, stability, and solubility of the test materials remains the responsibility of the Study Sponsor and will not be performed by the Testing Facility (GLP 58.105 and GLP 58.113).

**6.0 SCOPE:**

An In-Vitro Time-Kill evaluation of one test product will be performed versus *Cutibacterium* acnes (ATCC #6919). The percent and $\log_{10}$ reductions will be determined following exposure to the test product for 1 minute, 3 minutes, and 30 minutes. Testing will be performed in triplicate and all agar-plating will be performed in duplicate.

**7.0 TEST PRODUCT:**

The test product will be provided to the Testing Facility by the Study Sponsor, complete with the appropriate documentation. Responsibility for determination of the identity, strength, purity, composition, solubility, and stability of the test product, as well as responsibility for retention of the test product, rests with the Study Sponsor.

Test Product #1: Benzoil Peroxide (75%)
Active Ingredient:
Lot Number:

The test product will be prepared to a final use concentration of 0.075% (w/v) in Sterile Water for Irrigation (WFI) as follows; 1.0 g of the test product will be weighed and added to a 1 L volumetric flask, which will be filled to the mark with WFI. A sterile stir bar may be added, and the solution allowed to stir for approximately 2 to 3 hours, or until dissolution is achieved and the solution is visibly clear. Once fully dissolved, the test solution will be sterilized by vacuum filtration and may be stored at 2 – 8 °C before use. The final test solution may be prepared a day in advance of testing, as needed.

## 8.0 CHALLENGE MICROORGANISM:

The challenge microorganism strain (American Type Culture Collection (ATCC) to be evaluated is designated below.

8.1 *Cutibacterium acnes* (ATCC #6919)

**TABLE 1: CHALLENGE MICROORGANISM**

| Microorganism Challenge Strain | Incubation Time * | Incubation Temperature | Media |
|---|---|---|---|
| *Cutibacterium acnes* (ATCC #6919) | 2 – 5 days | 35 ± 2 °C Anaerobic | RCM/CAE |

***** Incubation times are nominal, but in practice, incubation will continue until good growth is observed.

## 9.0 MEDIA:

9.1 Chocolate Agar with Enrichment (CAE)
9.2 Reinforced Clostridial Medium (RCM)
9.3 Neutralizing Formulation – Dey-Engley Neutralizing Broth (D/E Broth)
9.4 0.9% Sodium Chloride Irrigation, USP (SCI)
9.5 Sterile Water for Irrigation (WFI)

## 10.0 INOCULUM PREPARATION:

Inoculum Preparation

10.1 Approximately 6 days prior to testing, a sterile tube containing Reinforced Clostridial Medium (RCM) will be inoculated from a lyophilized vial containing the challenge species. These broth cultures will be incubated anaerobically at 35 °C ± 2 °C for approximately 72 hours, or until sufficient growth is observed.

10.2 Approximately 72 hours prior to testing, the broth cultures will be subcultured into additional tubes of RCM and incubated anaerobically at 35 °C ± 2 °C. The purity of the culture will be verified by preparing isolation streaks on plates of Chocolate Agar with Enrichment (CAE), and incubating both aerobically and anaerobically.

## 11.0 NEUTRALIZATION STUDIES:

A Neutralization study will be performed versus *Cutibacterium acnes* to ensure that the neutralizing solution employed is effective in neutralizing the antimicrobial properties of the test product and is non-toxic to the challenge species.

11.1 A Neutralization Challenge Suspension containing approximately 1 x $10^{4-5}$ CFU/mL will be prepared for *Cutibacterium acnes* (ATCC #6919) by diluting the initial suspension in additional RCM, as necessary.

Neutralization Effectiveness (Test A)

11.2 One replicate of this procedure will be performed.

11.3 A 0.1 mL aliquot of a Neutralization Challenge Suspension will be transferred to a test tube containing 8.9 mL of D/E Broth ($10^0$ dilution). A 1.0 mL aliquot of a test product will be added to the tube containing inoculum/D/E Broth and will be mixed thoroughly.

11.4 The inoculum/D/E Broth/product mixture will be immediately diluted 10-fold in RCM and mixed (e.g., $10^{-1}$).

11.5 1.0 mL and/or 0.1 mL aliquots of the suspension will be spread plated, in duplicate, using CAE to produce final plated dilutions of e.g., $10^{0}$, $10^{-1}$, and $10^{-2}$. The plates will be incubated anaerobically at 35 ± 2 °C for 2-5 days, or until sufficient growth is observed.

Neutralizer Toxicity (Test B)

11.6 One replicate of this procedure will be performed.

11.7 A 0.1 mL aliquot of a Neutralization Challenge Suspension will be transferred to a test tube containing 8.9 mL of D/E Broth ($10^{0}$ dilution). A 1.0 mL aliquot of SCI will be added to the tube containing inoculum/D/E Broth and mixed ($10^{0}$ dilution).

11.8 The inoculum/D/E Broth/SCI mixture will be immediately diluted 10-fold in RCM and mixed (e.g., $10^{-1}$).

11.9 1.0 mL and/or 0.1 mL aliquots of the suspension will be spread plated, in duplicate, using CAE to produce final plated dilutions of e.g., $10^{0}$, $10^{-1}$, and $10^{-2}$. The plates will be incubated anaerobically at 35 ± 2 °C for 2-5 days, or until sufficient growth is observed.

Test Organism Viability (Test C)

11.10 One replicate of this procedure will be performed.

11.11 A 0.1 mL aliquot of the Neutralization Challenge Suspension will be transferred to a test tube containing 9.9 mL of SCI and mixed ($10^{0}$ dilution).

11.12 The inoculum/SCI mixture will be immediately diluted 10-fold in RCM and mixed (e.g., $10^{-1}$).

11.13 1.0 mL and/or 0.1 mL aliquots of the suspension will be spread-plated, in duplicate, using CAE to produce final plated dilutions of e.g., $10^{0}$, $10^{-1}$, and $10^{-2}$. The plates will be incubated anaerobically at 35 ± 2 °C for 2-5 days, or until sufficient growth is observed.

Data Collection

11.14 Following incubation, the colonies on the plates will be counted manually using a hand-tally counter. Counts in the range of 30 to 300 CFU will be used preferentially in the data calculations. If no counts in this range are observed, those plates with colony counts closest to those ranges will be used for the data calculations. If colonies on one of the plates are uncountable, the count from the remaining plate will be used.

Acceptance Criterion

11.15 The $\log_{10}$ of the number of survivors of each challenge strain from Test A and Test B will be compared to those from Test C. If the average $\log_{10}$ number of survivors for Test A are no more than 0.2 $\log_{10}$ lower than that of Test C, neutralization of the test product will be considered effective. If the average $\log_{10}$ number of survivors for Test B are no more than 0.2 $\log_{10}$ lower than that of Test C, the neutralizing formulation will be considered non-toxic.

## 12.0 TIME-KILL METHODOLOGY:

Initial Population

12.1 Following incubation of the inoculum cultures, a suspension may be prepared by centrifuging the broth culture tubes, combining the resulting pellets, and re-suspending them in RCM. A challenge suspension containing approximately $10^9$ CFU/mL will be prepared. Prior to use in testing, the initial population of each challenge suspension will be determined by preparing 10-fold dilutions (e.g., $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$) in RCM. Using CAE, spread plates will be prepared, in duplicate, from the inoculum dilutions by plating 0.1 mL of the final dilutions to achieve plated dilutions (e.g., $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$). The plates will be incubated anaerobically at 35 °C ± 2 °C or until sufficient growth is observed.

Numbers Control

12.2 One replicate of this procedure will be performed at the longest exposure duration.

12.3 A 0.1 mL aliquot of a challenge suspension will be transferred to a sterile tube containing 10.0 mL of SCI and mixed thoroughly using a vortex mixer ($10^0$ dilution).

12.4 The challenge microorganism strain will be exposed for 30 minutes, timed using a calibrated minute/second timer.

12.5 After the exposure time has elapsed, 1.0 mL will be transferred from the tube containing SCI/challenge suspension to a tube containing 9.0 mL D/E Broth ($10^{-1}$ dilution) and will be mixed thoroughly using a vortex mixer. Ten-fold dilutions (e.g., $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$) will be prepared in RCM, mixing thoroughly using a vortex mixer between dilutions.

12.6 From the final dilutions of the SCI/neutralizer/challenge suspension, 0.1 aliquots will be spread plated, in duplicate, using CAE, producing final plated dilutions of (e.g. $10^{-4}$, $10^{-5}$, $10^{-6}$, and $10^{-7}$). The plates will be incubated anaerobically at 35 °C ± 2 °C or until sufficient growth is observed.

Time-Kill Testing Methodology

12.7 Three replicates of this procedure will be performed.

12.8 A 0.1 mL aliquot of a challenge suspension will be transferred to a sterile tube containing 10.0 mL of the test product and will be mixed thoroughly using a vortex mixer and/or positive displacement pipette ($10^0$ dilution).

12.9 The challenge microorganism will be exposed to the test product for 1 minute ± 10 seconds, 3 minutes ± 10 seconds, and 30 minutes ± 30 seconds, timed using a calibrated minute/second timer.

12.10 After each exposure time has elapsed, 1.0 mL will be transferred from the tube containing product/challenge suspension to a separate sterile test tube containing 9.0 mL D/E Broth ($10^{-1}$ dilution) and mixed thoroughly using a vortex mixer. Ten-fold dilutions (e.g., $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$) will be prepared in RCM, mixing thoroughly using a vortex mixer between dilutions.

12.11 From the final dilutions of the product/neutralizer/challenge suspension, 0.1 aliquots will be spread plated, in duplicate, using CAE, producing final plated dilutions of (e.g. $10^{-4}$, $10^{-5}$, $10^{-6}$, and $10^{-7}$). The plates will be incubated anaerobically at 35 °C ± 2 °C or until sufficient growth is observed.

Data Collection

12.12 Following incubation, the colonies on the plates will be counted manually using a hand-tally counter. Counts in the range of 30 to 300 CFU will be used preferentially for the data calculations.

If no counts in this range are observed, those plates with colony counts closest to those ranges will be used for the data calculations. If colonies on one of the plates are uncountable, the count from the remaining plate will be used.

## 13.0 CALCULATIONS:

13.1 The Initial Population (IP) of the challenge suspension will be calculated as follows:

$CFU/mL\ (IP) = (C_i \times 10^{-D})$

$Log_{10}\ (IP) = Log_{10}\ (C_i \times 10^{-D})$

Where:

$C_i$ = Average of the Two Plates Counted
D = Dilution Factor of the Plates Counted

13.2 The Numbers Control (NC) population recovery (CFU/mL and $Log_{10}$ CFU/mL) will be calculated as follows:

$CFU/mL\ (NC) = (C_i \times 10^{-D})$

$Log_{10}\ (NC) = Log_{10}\ (C_i \times 10^{-D})$

Where:

$C_i$ = Average of the Two Plates Counted
D = Dilution Factor of the Plates Counted

13.3 The Post-Exposure Population ($P_{EX}$) following each timed exposure will be calculated as follows:

$CFU/mL\ (P_{EX}) = (C_i \times 10^{-D})$

$Log_{10}\ (P_{EX}) = Log_{10}\ (C_i \times 10^{-D})$

Where:

$C_i$ = Average of the Two Plates Counted
D = Dilution Factor of the Plates Counted

13.4 The $Log_{10}$ Reductions attributable to the test product will be calculated as follows:

$Log_{10}\ Reduction = Log_{10}\ (NC) - Log_{10}\ (P_{EX})$

Where:

NC = Numbers Control Population (CFU/mL)
$P_{EX}$ = Post-Exposure Population (CFU/mL)

13.5 The Mean $Log_{10}$ Reductions attributable to the test product will be calculated as follows:

$$\text{Mean } Log_{10}\ \text{Reduction} = \frac{\sum Log_{10}\ \text{Reductions}}{3}$$

Where:

3 = Number of replicates

13.6 The Percent Reduction attributable to the test product will be calculated as follows:

$$\text{Percent Reduction} = \frac{NC - P_{EX}}{NC} \times 100$$

Where:

NC = Numbers Control Population (CFU/mL)
$P_{EX}$ = Post-Exposure Population (CFU/mL)

## 14.0 STATISTICAL ANALYSIS:

A statistical analysis will not be performed on the data derived from the Neutralization or Time-Kill portions of this evaluation.

## 15.0 FINAL REPORT:

Final data will be provided in a summary table and will be issued presenting the results of this evaluation in a clear, concise manner.

## 16.0 EXCEPTIONAL CONDITIONS:

The Study Sponsor will be notified by telephone, email, and/or letter of any exceptions encountered in this study. The exceptional conditions or occurrences will be detailed in full and formally recorded. Exceptional conditions that occur and are not addressed in this protocol will be subject to Out-of-Scope charges (see Proposal/Contract).

## 17.0 DOCUMENTATION AND RECORD-KEEPING:

All documentation and records will be compiled, analyzed, and retained by BioScience Laboratories, Inc. at its facility in Bozeman, Montana. All raw data for this study, as well as the Final Report, will be retained in safe storage by the Testing Facility for a period of at least 2 years. BioScience Laboratories, Inc. will notify the Study Sponsor before any documents or records are destroyed.

## 18.0 REFERENCES:

18.1 ASTM E2783-11 (2016), *Standard Test Method for Assessment of Antimicrobial Activity for Water Miscible Compounds Using a Time-Kill Procedure*

18.2 ASTM E 1054-08 (2013), *Standard Test Methods for Evaluation of Inactivators of Antimicrobial Agents*