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Standard test method for determining the effectiveness of liquid, gel, cream, or shampoo insecticides against human louse ova (Based on ASTM Louse Method – Designation E 1517-99)

i2LResearch USA, Inc.
1330 Dillon Heights Avenue
Baltimore, MD 21228-1199
USA

Samuel J. Grimard
April 2013

Certification

This report represents a true and accurate record of all data obtained.

Signed..... Date..April 18, 2013.....
Samuel J. Grimard
Study Director

Approved by..... Date..... April 18, 2013
Dr. Robin G. Todd, BCE
Executive Director

Report circulated to: TEC Laboratories (1 copy)
i2LResearch USA, Inc. (1 copy)

Contents

Study Information

Standard test method for determining the effectiveness of liquid, gel, cream, or shampoo insecticides against human louse ova (Based on ASTM Louse Method – Designation E 1517-99[1])

i. Testing facility:

i2LResearch USA, Inc.
1330 Dillon Heights Ave.
Baltimore, MD 21229-1199
USA

ii. Sponsor:

Jessica Smith
Tec Laboratories
7100 Tec Labs Way SW
Albany, OR 97321
USA

iii. i2LResearch Study code: 13/038

iv. Study start date: 2/13/2013
Experimental start date: 3/8/2013
Experimental end date: 3/20/2013
Study end date: 4/18/2013

v. Study Director: Samuel J. Grimard

vi. Test Substances:

Test Substance	Active ingredient	Lot Number	Physical description of test substance	Storage conditions	Expiry date	i2LResearch code number
Licefreee! Gel		NBO8784A		Upright, Ambient conditions	19/02/2013	13022704-13022707

[1] This standard is issued under the fixed designation E1517; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. The number in parentheses indicates the year of last re-approval. A superscript epsilon (ε) indicates an editorial change since the last revision or re-approval.

Summary

One formulation, Licefreee! Gel, was tested for ovicidal effectiveness against body lice nits, *Pediculus humanus humanus,* using ASTM protocol E 1517-99. Egg-infested hairs were immersed in the formulation for 1 hour. When all control replicates have hatched (after 12 days), treated nits were examined under a dissecting microscope to determine the numbers of nits hatching and failing to hatch. Failure to hatch was recorded as mortality. Eggs were categorized as follows: Early stage (no visible differentiation of the embryo when viewed under 30x), Late stage (visible differentiation of the embryo when viewed under 30x, typically eye spot is visible) and Emergent (nymphal louse has open operculum and begun to emerge, but died before emerging completely – part of nymph's body still within egg shell).

Results showed that Licefreee! Gel was effective against body lice nits in providing complete (100%) mortality.

Aim

This test method determines the effectiveness of ovicidal materials in liquid, gel, cream, or shampoo form against the ova (that is, eggs or nits) of the human louse, *Pediculus humanus humanus*; the surrogate for the human head louse (*P. h. capitis*).

Methodology

Test systems

Body lice nits, *Pediculus humanus humanus*, were used. The i2LResearch USA strain was established from the USDA Gainesville strain. It is a susceptible strain, and through selection, has adapted to a rabbit host. Five replicates of 30 eggs were used in the experiments for each formulation and control tested.

Test treatments and application

The study consisted of a one formulation, Licefreee! Gel, tested at a 1 hour immersion time. Control replicates were treated in tap water in a consistent manner.

Experimental design

Five replicates of 30 eggs were attached to human hair (one to three hair shafts) to a wood applicator stick with duct tape for each control and test formulation to be tested. The ovicide to be tested was poured into a 100mL beaker and set aside. The ovicide was not heated, per the sponsor's direction. The applicator sticks were inserted (hair side down) into the test samples for 60 minutes. After the 60 minute interval, the eggs were washed in 900mL of 32°C-+ 2°C tap water for 1 minute by vigorous up and down movement of the applicator sticks with the hairs attached. At the end of 1 minute, the applicator sticks were removed and nits were gently washed in a stream of water (32°C-+ 2°C) from a wash bottle for one minute. Excess water was blotted and absorbed with paper towels. The hairs were then transferred to a clean 4x4cm patch of dark corduroy cloth; this cloth containing the nits was housed in a Petri dish, which was placed in an incubator maintained at 31.7°C +/- 3.0°C and 60% +/- 10% RH and kept for the remainder of the study.

When all control replicates hatched (12 days), treated nits were examined under a dissecting microscope to determine the numbers of nits hatching and failing to hatch. Failure to hatch was recorded as mortality. Eggs were categorized as follows: Early stage (no visible

differentiation of the embryo when viewed under 30x), Late stage (visible differentiation of the embryo when viewed under 30x, typically eye spot is visible) and Emergent (nymphal louse has open operculum and begun to emerge, but died before emerging completely – part of nymph's body still within egg shell).

Each control replicate was subjected to the same procedures outlined above; except that nits were treated with water. The controls were housed in the same area as those treated for the duration of the prescribed observation period.

Raw data analyses

The numbers of nits that failed to hatch per replicate were added together for a total mortality count shown in Appendix I. Abbott's Formula was used to correct for any mortality among the controls.

Results

Complete data analyses are presented in Appendix I. Mortality observations are summarized in Table 1 below.

Formulation	% Mortality
Tap Water Control	2.69
Licefreee! Gel	100

Table 1. Mortality for Licefreee! Gel against body lice nits.

Licefreee! Gel was effective against body lice nits in providing complete (100%) mortality. The majority of treated nits (75.86%) did not develop past the late egg stage. Only 2.76% died in the emergent stage (Appendix I).

Appendix I – Data Analysis

Control								
Replicate	Total # Nits	Egg Stage			Total Dead	Total % Mortality	Standard Deviation	Standard Error
		Early	Late	Emergent				
1	30	0	0	3	3	10		
2	31	0	0	0	0	0		
3	30	0	0	0	0	0		
4	30	0	0	0	0	0		
5	29	0	1	0	1	3.4482759		
Total	150	0	1	3	4	13.448276		
Average	30	0	0.2	0.6	0.8	2.6896552	4.3508442	1.945757

Licefreee! Gel								
Replicate	Total # Nits	Egg Stage			Total Dead	Total % Mortality	Standard Deviation	Standard Error
		Early	Late	Emergent				
1	28	11	17	0	28	100		
2	30	8	22	0	30	100		
3	30	2	25	3	30	100		
4	27	7	19	1	27	100		
5	30	3	27	0	30	100		
Total	145	31	110	4	145	500		
Average	29	6.2	22	0.8	29	100	0	0

	Mortality	Corrected Mortality
Control	2.69	
Licefreee! Gel	100	100

Appendix II –Protocol

Study Code: 13/038

Study Protocol

Study Code: 13/038	Sponsor: Tec Laboratories, Inc.
Proposal approval date: February 12, 2013	Proposal approval method: Email
Test system(s): *Pediculus humanus humanus* .	Study Director: Samuel Grimard
Title of Study **Standard test method for determining the effectiveness of liquid, gel, cream, or shampoo insecticides against human louse ova (Based on ASTM Louse Method – Designation E 1517-99[1])**	
Test substances Test item(s): 1. Sponsor supplied Nit sample 2. Tap water - Control	Study start date: February 13, 2013 Experimental start date (Month/year): 02/13 Experimental end date (Month/year): 02/13 Study end date (Month/year): 02/13

Distribution: Sponsor (1 copy), i2LResearch USA, Inc. (1 copy)

Study director:..... Date: 2/13/13

Management:..... Date: 2-13-13

Sponsor:..... Date: 2.14.13

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Product testing and development for the pest control industry

Standard test method for determining the effectiveness of liquid, gel, cream, or shampoo insecticides against human louse ova (Based on ASTM Louse Method – Designation E 1517-99[3])

Testing facility **i2LResearch USA, Inc.**
1330 Dillon Heights Avenue
Baltimore, MD 21228-1199
USA

Sponsor **Jessica Smith**
Tec Laboratories, Inc.
7100 Tec Labs Way SW
Albany, OR 97321
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Phone Number 541-926-4577 ext. 351

Submitted by: **Kristine Styer, Study Director and Technical Writer**

Study Director: **Samuel Grimard**
Samuel@i2lresearch.com
SD Phone # 410-747-4500

Proposal submitted: 1st February, 2013

[3] This standard is issued under the fixed designation E1517; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. The number in parentheses indicates the year of last re-approval. A superscript epsilon (ε) indicates an editorial change since the last revision or re-approval.

Aim

This test method determines the effectiveness of ovicidal materials in liquid, gel, cream, or shampoo form against the ova (that is, eggs or nits) of the human louse, *Pediculus humanus humanus*; the surrogate subspecies of the human head louse (*P. h. capitis*). (Only gels or creams that liquefy at 32°C (90°F) can be tested).

Use of i2L Research USA, Inc.'s name in promotional releases

Sponsor agrees not to use i2L Research USA's name in any promotional literature, TV, radio, web-based or other media, without the express written permission of i2L Research USA, Inc. management. I2L Research USA, Inc. reserves the right to grant this permission to the sponsor based on the relation of the promotional text and images to the data generated for the sponsor.

Test species

Test species will be obtained from i2L Research USA, Inc.'s body lice colony. Five replicates of 30 eggs will be used in the experiments for each formulation and control tested.

Test treatments and application

The sponsor will supply one formulation for testing. A tap water control will also be tested. A positive control will not be tested.

Nits will be emerged in the test formulation or tap water control for 1 hour.

Apparatus and materials

Applicators -- Egg-infested hairs are attached to the end of a wooden applicator stick with duct tape such that 30 nits are on l to 3 hairs. Each replicate of 30 eggs is examined under a dissecting microscope to confirm viability. Any eggs that are shrunken or with other indications of being nonviable will be excluded.

Beakers -- A 100-mL beaker is used to contain 60 mL of test ovicide and another to contain 60 mL of water (control), into which the applicators are dipped. A 1000-mL

beaker is used for washing the eggs.

Heating Surface -- A slide dryer that provides heat of approximately 32°C (90°F).
Incubator, capable of maintaining a temperature of 31.7 ~ 0.5°C (89°F) and a relative humidity of 60 -+ 10 %.

Water Bath, capable of maintaining a temperature of 32°C (90°F).

Wash Bottle, .Slop Watch, and Dissecting Scope.

Experimental design

Five replicates of 30 eggs will be attached to human hair (one to three hair shafts) to the wood applicator stick with duct tape for each control and test formulation to be tested. The ovicide to be tested will be poured into a 100mL beaker and will be set aside. The ovicide will not be heated. The applicator sticks will be inserted (hair side down) into the test samples for 60 minutes. After the 60 minute interval, the eggs will be washed in 900mL of 32°C-+ 2°C tap water for 1 minute by vigorous up and down movement of the applicator sticks with the hairs attached. At the end of 1 minute, the applicator sticks will be removed and nits will be gently washed in a stream of water (32°C-+ 2°C) from a wash bottle for one minute. Excess water will then be blotted and absorbed with paper towels. The hairs will then be transferred to a clean 4x4cm patch of dark corduroy cloth; this cloth containing the nits will be housed in a Petri dish, which will be placed in an incubator maintained at 31.7°C +/- 3.0°C and 60% +/- 10% RH and kept for the remainder of the study.

When all control replicates have hatched (after approximately 12 days), treated nits will be examined under a dissecting microscope to determine the numbers of nits hatching and failing to hatch. Failure to hatch is recorded as mortality. Eggs will be categorized as follows: Early stage (no visible differentiation of the embryo when viewed under 30x), Late stage (visible differentiation of the embryo when viewed under 30x, typically eye spot is visible) and Emergent (nymphal louse has open operculum and begum to emerge, but died before emerging completely – part of nymph's body still within egg shell).

Statistical analyses

The number of nits that failed to hatch will be added together for a total mortality count. Abbott's Formula will be used to correct for any mortality among the controls.

Protocol amendments and deviations

Any protocol amendments and/or deviations will be documented, fully justified and maintained with the protocol. All protocol amendments will be approved by the study director and sent to the sponsor.

Archiving records

True copies of the raw data, final report and any amendments will be filed at i2LResearch USA, Inc for a period of five years. The final report, including any protocol amendments or deviations as well as raw data sheets, will be forwarded to the Sponsor. Any unused test substances will be either be returned to the sponsor or disposed of with the sponsor's consent.

Proposed dates (month/year)

Proposed study start date: February 2013
Proposed study completion date: February 2013
Proposed experimental start date: February 2013
Proposed experimental completion date: February 2013

Please note that these are proposed dates, based on the date the proposal was submitted; for actual study dates please refer to the first page of the signed protocol.

RAW DATA COLLECTION SHEET

Sponsor: _____

Date: _____

Formulation: _____

Equipment Used: _____

Rep	Total # of Nits	Egg Stage			Total Dead
		Early	Late	Emergent	
1					
2					
3					
4					
5					
Total					

Comments:

Data Recorder:

Study Coordinator:

Appendix III –Protocol Amendment

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Protocol amendment number: 1

Study code: 13/038

Title: Standard test method for determining the effectiveness of liquid, gel, cream, or shampoo insecticides against human louse ova (Based on ASTM Louse Method – Designation E 1517-99

Description of amendment	In the protocol it states that (only gels or creams that liquefy at 32°C (90°F) can be tested) but the sample being tested will not be heated
Reason for amendment	The sample does not need to be heated
Impact on study	None

Study Director ..... Date. Feb 14, 2013

Page 1 of 1

Appendix IV – Raw Data Sheets

RAW DATA COLLECTION SHEET

Sponsor: TecLabs

Date: March 8, 2013 9:07AM

Formulation: CONTROL

Equipment Used: Microscope Slide warmer

Rep	Total # of Nits	Egg Stage			Total Dead
		Early	Late	Emergent	
1	30	0	0	3	WE 3/20/13 ~~0~~ 3
2	31	0	0	0	0
3	30	0	0	0	0
4	30	0	0	0	0
5	29	0	1	0	1
Total	150	0	1	3	4

Comments:

Eggs observed on March 20, 2013

Data Recorder: SC 3/20/13

Study Coordinator:

3/20/13

i2LResearch USA, Inc. 1330 Dillon Heights Avenue. Baltimore, MD 21228-1199, USA
Tel: 001 410 747 4500. Fax: 001 410 747 4928
Email: robin@i2lresearch.com . Web: www.i2lresearch.com

RAW DATA COLLECTION SHEET

Sponsor: Tec Labs

Date: March 8, 2013 9:40 AM

Formulation: Licefree! Gel

Equipment Used: Microscope Slide warmer

Rep	Total # of Nits	Egg Stage			Total Dead
		Early	Late	Emergent	
1	28	11	17	0	28
2	30	8	22	0	30
3	30	2	25	3	30
4	27	7	19	1	27
5	30	3	27	0	30
Total	145	31	110	4	145

Comments:

Eggs observed on March 20, 2013

Data Recorder: SC 3/20/13

Study Coordinator:

Jan G. A 3/20/13

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