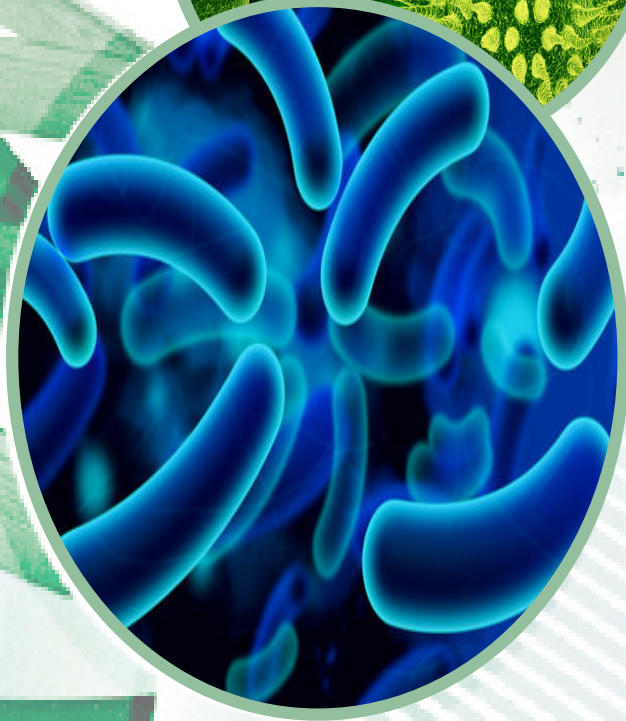
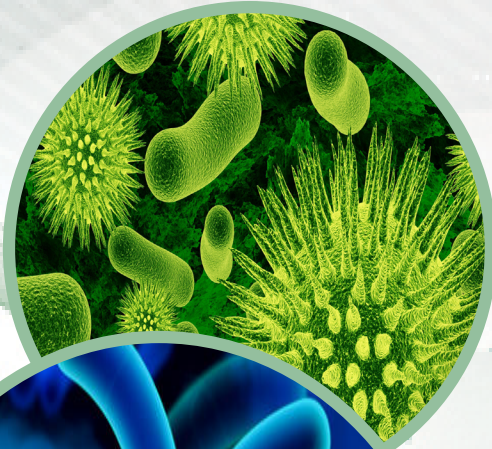


Technical Dossier



ability natural rowant technology Activity
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Improving solar choice antimicrobial

Leucidal[®] Liquid SF

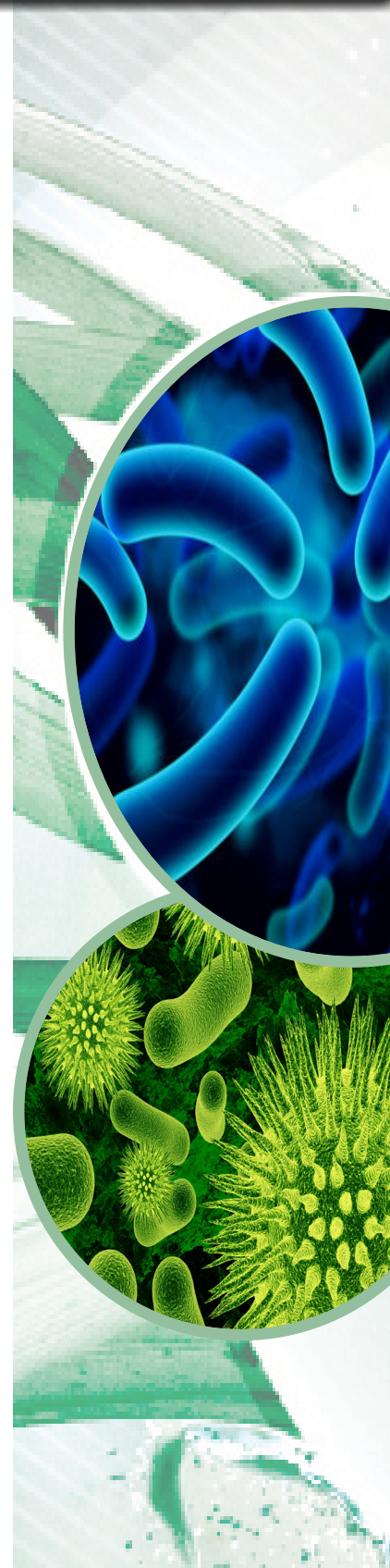
Code Number: M15019

INCI Name: Lactobacillus Ferment

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Leucidal® Liquid SF

Patent Pending: Application Number 62/013,669

Technical Data Sheet

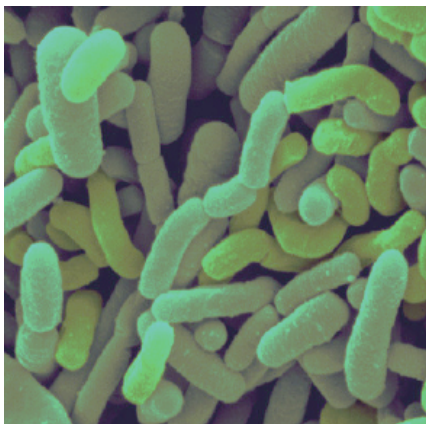
BACKGROUND

Consumer choice is the most important factor when it comes to cosmetic sales. Today, a growing number of consumers are opting to move away from synthetic preservatives such as parabens, formaldehyde donors and phenoxyethanol. In addition to public pressure, the use of these synthetic materials in cosmetics is also becoming more strictly regulated. For these reasons, formulators have been actively searching for alternatives to synthetic preservatives that can provide broad spectrum antimicrobial activity and can be added into a wide range of cosmetic applications.

Active Micro Technologies (AMT) has developed a full line of products derived from naturally occurring compounds that provide broad spectrum antimicrobial protection. As a result, these novel natural antimicrobials are considered self-preserving cosmetic actives and therefore can be used as consumer-friendly alternatives to synthetic preservatives in a wide range of cosmetic applications.

SCIENCE

Leucidal® Liquid SF is a probiotic-based ingredient created by the fermentation of *Lactobacillus* in a defined growth medium.



Lactobacillus is one of the species of microorganisms used to produce fermented products, such as kimchi and sauerkraut, a Korean dietary staple, from cabbage. Like many members of the lactic acid bacteria family, *Lactobacillus* is capable of restricting the growth of other microorganisms by acidifying its environment. However, *Lactobacillus* also produces novel antimicrobial peptides, known as bacteriocins, that are capable of providing broad spectrum antimicrobial protection. During the manufacturing process, lysozyme is added to the ferment filtrate to facilitate a controlled cell lysis to ensure the release of the antimicrobial peptides for maximized activity.

Code Number: M15019

INCI Nomenclature:

Lactobacillus Ferment

INCI Status: Approved

REACH Status: Fully Compliant

CAS Number: 1686112-36-6

EINECS Number: N/A

Origin: Biotechnology

Lactobacillus

Processing:

GMO Free

No Ethoxylation

No Irradiation

No Sulphonation

No Ethylene Oxide treatment

No Hydrogenation

Additives: None

-Preservatives: None

-Antioxidants: None

Other additives: None

Solvents used: Water

Appearance: Clear to Hazy, Colorless to Yellow Liquid

Soluble/Miscible:

Aqueous Ferment Filtrate

Suggested Use Levels: 2.0 - 4.0%

Suggested Applications:

Skin Conditioning, Antimicrobial

Leucidal® Liquid SF

Patent Pending: Application Number 62/013,669

BENEFITS

A skin moisturization study was performed using an untreated control, generic cream base, and an experimental with the same cream base containing 2.0% **Leucidal® Liquid SF**. Comparative moisturization results from this study are shown in Figure 2. As demonstrated by the results of this study, the addition of 2.0% **Leucidal® Liquid SF** improved moisture levels by 42.42% after 24 hours and by 63.00% after four weeks when compared to the untreated control. When compared to the base cream **Leucidal® Liquid SF** improved moisturization by 14.38% and after 24 hours and by 25.00% after four weeks. Based on these results, adding this innovative product provides the formulator the opportunity to capitalize on both the natural antimicrobial properties of **Leucidal® Liquid SF**, as well as its ability to provide potent moisturizing benefits to the cosmetic formulation.

Comparative Moisturization

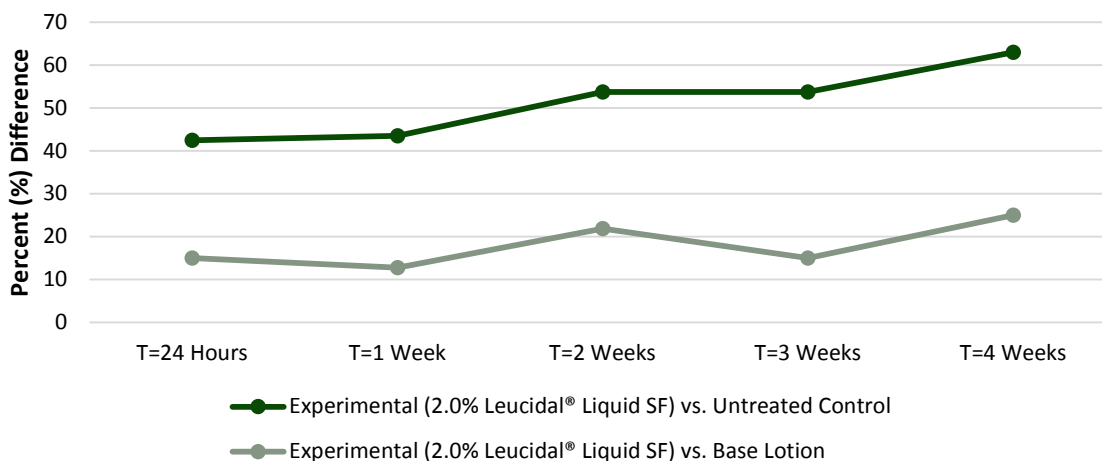


Figure 2. Percent Difference in Moisturization for **Leucidal® Liquid SF**.

The ability of **Leucidal® Liquid SF** to inhibit the growth of a variety of bacteria and fungi was determined using the Minimum Inhibitory Concentration (MIC) test. The results are illustrated in Figure 2, showing that this material provides broad spectrum antimicrobial protection.

Microorganism Tested	MIC (%)
<i>E. coli</i>	0.75
<i>P. aeruginosa</i>	1.00
<i>S. aureus</i>	1.00
<i>A. brasiliensis</i>	1.00
<i>C. albicans</i>	0.75

Figure 2. MIC data for **Leucidal® Liquid SF**.

Leucidal® Liquid SF

Patent Pending: Application Number 62/013,669

The positive MIC screening results warranted further testing to confirm its ability to provide product preservation. Double Challenge Tests were completed using either 2.0% or 4.0% **Leucidal® Liquid SF** in a generic cream base formulation at pH values of 3, 5, and 7. Samples were inoculated with *E. coli*, *P. aeruginosa*, *S. aureus*, *C. albicans*, and *A. brasiliensis*.

During the first 28-day incubation period, samples were periodically collected and tested for the presence of these microorganisms. Following this initial 28 days of incubation, the cream samples were then re-inoculated with the microbial cultures and sampled over an additional 28-day period. Figure 3 shows the positive preservation results for **Leucidal® Liquid SF** tested at 4.0% in a generic cream base formulation at pH 5.

4.0% Leucidal® Liquid SF in Cream Formula Challenge Test - pH 5

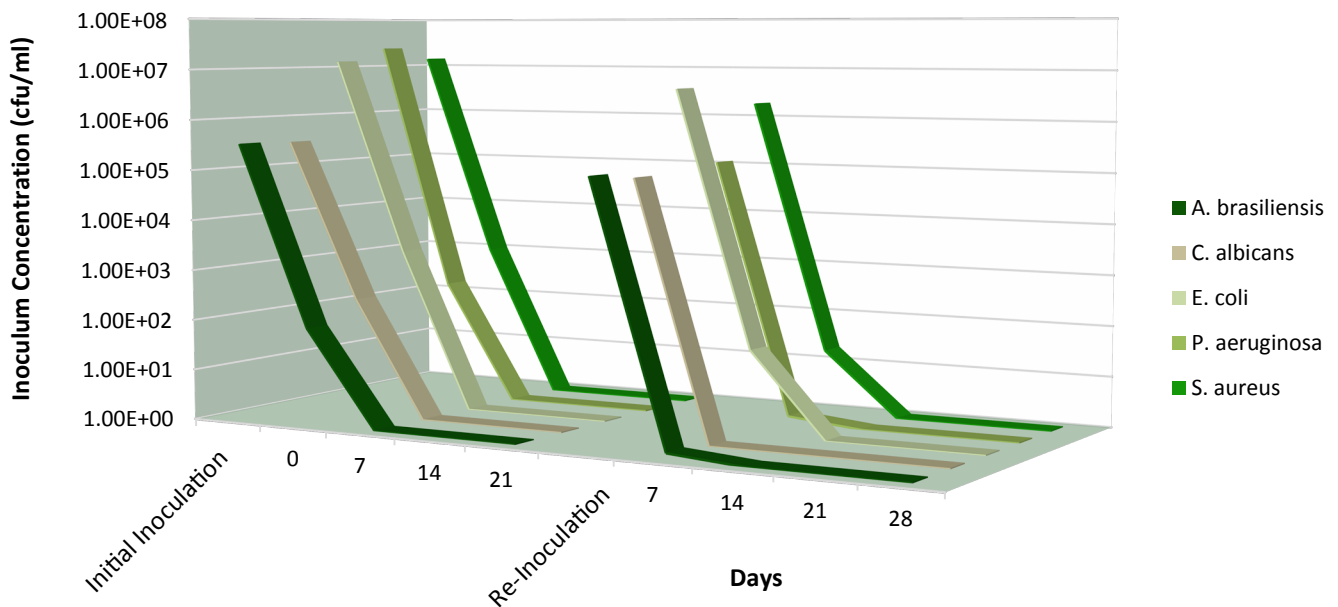


Figure 3. Challenge Test results for Generic Cream Formula pH 5 with 4.0% **Leucidal® Liquid SF** inoculated on Day 0 and re-inoculated on Day 28. Results show log reduction in viable organisms.

Leucidal® Liquid SF

Patent Pending: Application Number 62/013,669

A Time Kill Test was performed to determine the change in population of aerobic microorganisms within a specified sampling time when tested against 4.0% **Leucidal® Liquid SF** solution. The activity of the test material inoculated was evaluated at determine time intervals of 30 seconds, 1, 5, 10 and 30 minutes after the inoculation to determine quantitatively the number of viable microorganisms remaining after the incubation time. As shown in Figure 4, the Gram-positive and Gram-negative bacteria as well as the yeast and mold were reduced by 99.9% within 30 seconds interval of the test after the inoculation.

4.0% Leucidal® Liquid SF Time Kill Test

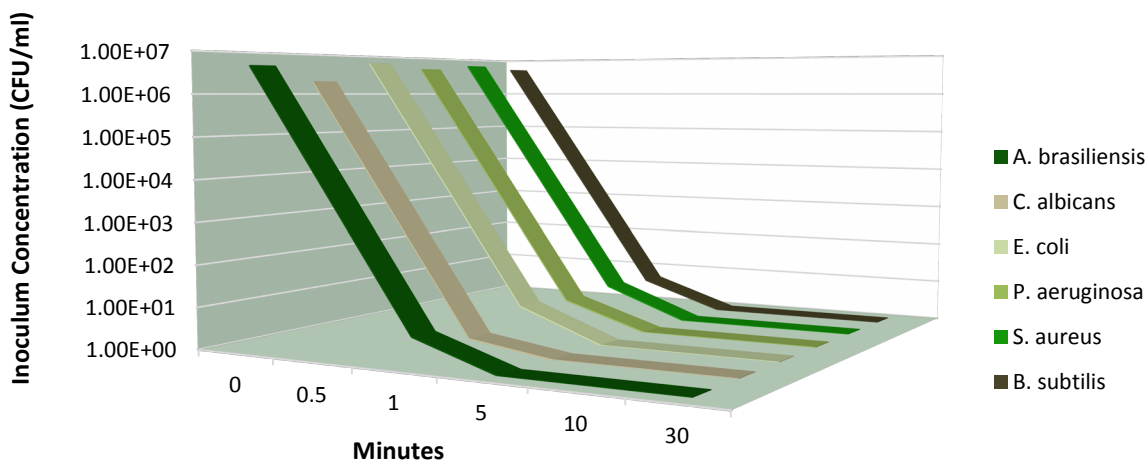


Figure 4. Time Kill Test results for 4.0% **Leucidal® Liquid SF**.

USE RECOMMENDATIONS

Leucidal® Liquid SF can be used in a wide range of cosmetic products, however to ensure optimum results we recommend using the following guidelines. Incorporate the product into formulations at a pH between 3 and 8, during the cooling phase of the process at temperatures lower than 70°C.



107 Technology Drive • Lincolnton, NC 28092
 (704) 276-7100 • Fax (704) 276-7101

Patent Pending: Application Number 62/013,669

Specification

Product Name: Leucidal® Liquid SF
Code Number: M15019
CAS #'s: 1686112-36-6
EINECS #'s: N/A
INCI Name: Lactobacillus Ferment

Specification	Parameter
Appearance	Clear to Hazy Liquid
Color	Colorless to Yellow
Odor	Characteristic
Solids (1g/1hr/105°C)	6.0 – 10.0%
pH (Direct)	3.0 – 7.0
Specific Gravity (25°C)	0.990 – 1.110
Heavy Metals	< 20 ppm
Arsenic	< 2 ppm
Minimum Inhibitory Concentration ¹ Organism (ATCC#)	
E. coli (#8739)	0.25 – 1.00%
S. aureus (#6538)	0.25 – 1.00%
P. aeruginosa (#9027)	0.25 – 1.00%
C. albicans (#10231)	0.25 – 1.00%
A. brasiliensis (#16404)	0.25 – 1.00%

**DO NOT FREEZE; Store at or near room temperature;
 May sediment upon standing; Mix well prior to use**

Note:

1) Refer to Inhibition Activity Data

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Compositional Breakdown

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Leucidal[®] Liquid SF Code: M15019

Compositional Breakdown:

Ingredient	%
Lactobacillus Ferment	100.00

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Compositional Breakdown

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This is to certify that Leucidal[®] Liquid SF does not contain allergen levels exceeding the following (Gas Chromatography-Mass Spectrometer Coupled):

ALLERGENS Dir 2003 15 CEE		
INCI NAME	CAS NUMBER	Limit (ppm)
Alpha-IsoMethyl Ionone	127-51-5	< 0.02
Amyl Cinnamal	122-40-7	< 0.10
Anise Alcohol	105-13-5	< 0.00
Benzyl Alcohol	100-51-69	< 0.01
Benzyl Benzoate	120-51-4	< 0.09
Benzyl Cinnamate	103-41-3	< 0.30
Benzyl Salicylate	118-58-1	< 0.06
Butylphenyl Methylpropional	80-54-6	< 0.50
Cinnamal	104-55-2	< 0.01
Cinnamyl Alcohol	104-54-1	< 0.30
Citral	5392-40-5	< 1.00
Citronellol	106-22-9	< 1.00
Coumarin	91-64-5	< 0.00
Eugenol	97-53-0	< 0.70
Farnesol	4602-84-0	< 0.04
Geraniol	106-24-1	< 0.08
Hexyl Cinnamal	101-86-0	< 0.40
Hydroxycitronellal	107-75-5	< 1.00
Hydroxymethylpentyl 3-Cyclohexene carboxaldehyde	31906-04-4	< 0.30
Isoeugenol	97-54-1	< 0.06
Limonene	5989-27-5	< 0.05
Linalool	78-70-6	< 0.00
Methyl 2 Octynoate	111-12-6	< 0.20
Evernia prunastri	90028-68-5	< 0.02
Evernia furfuracea	90028-67-4	< 0.00
Amylcinnamyl Alcohol	101-85-9	< 1.00

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Compositional Breakdown

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This is to certify that Leucidal® Liquid SF does not contain pesticide levels exceeding the following (Reverse Phase High Performance Liquid Chromatography-Mass Spectrometer Coupled):

EPA Pesticide Levels	
INCI NAME	LIMIT (mg/kg)
Alachlor	< 0.02
Aldrin and Dieldrin	< 0.05
Azinphos-methyl	< 1.00
Bromopropylate	< 3.00
Chlordane(cis and trans)	< 0.05
Chlorfenvinphos	< 0.50
Chlorpyrifos	< 0.20
Chlorpyrifos-methyl	< 0.10
Cypermethrin	< 1.00
DDT	< 1.00
Deltamethrin	< 0.50
Diazinon	< 0.50
Dichlorvos	< 1.00
Dithiocarbamates	< 2.00
Endosulfan	< 3.00
Endrin	< 0.05
Ethion	< 2.00
Fenitrothion	< 0.50
Fenvalerate	< 1.50
Fonofos	< 0.05
Heptachlor	< 0.05
Hexachlorobenzene	< 0.10
Hexachlorocyclohexane	< 0.30
Lindane	< 0.60
Malathion	< 1.00

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Compositional Breakdown

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Methidathion	< 0.20
Parathion	< 0.50
Parathion-methyl	< 0.20
Permethrin	< 1.00
Phosalone	< 0.10
Piperonyl butoxide	< 3.00
Pirimiphos-methyl	< 4.00
Pyrethrins	< 3.00
Quintozene(sum of 3 items)	< 1.00

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Moisturization/Hydration Assay

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: Leucidal[®] Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 1095

Lot #: NC140725-B

Sponsor: Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

Moisturization/Hydration Assay

Introduction

An *in-vivo* study was conducted over a period of four weeks to evaluate the moisturization benefits **Leucidal[®] Liquid SF**. 10 M/F subjects between the ages of 23-45 participated in the study. Results indicate that this material is capable of significantly increasing moisturization compared to the control.

The moisturization assay was conducted to assess the moisturizing ability of **Leucidal[®] Liquid SF**.

Materials

A. Equipment: DermaLab Skin Combo (Hydration/ Moisture Pin Probe)

The moisture module provides information about the skin's hydration by measuring the conducting properties of the upper skin layers when subjected to an alternating voltage. The method is referred to as a conductance measurement and the output is presented in the unit of uSiemens (uS). A moisture pin probe is the tool used to gather hydration values.

10 volunteers M/F between the ages of 23 and 45 and who were known to be free of any skin pathologies participated in this study. A Dermalab Corneometer was used to measure the moisture levels on the subject's volar forearms. The Corneometer is an instrument that measures the amount of water within the skin. The presence of moisture in the skin improves conductance therefore results in higher readings than dry skin. Therefore the higher the levels of moisture, the higher the readings from the Corneometer will be. Baseline moisturization readings were taken on day one of the study.

Following initial measurements, all subjects were asked to apply 2 mg of each test material on their volar forearms. Measurements were taken immediately after application of test materials and then weekly for 4 weeks. The test material consisted of 2% **Leucidal[®] Liquid SF** in a base lotion.

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Moisturization/Hydration Assay

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For added perspective, measurements of an untreated test site and a site treated with a base lotion (Cetaphil Moisturizing for All Skin Types) were recorded.

Results

Leucidal[®] Liquid SF showed high moisturizing capabilities at a 2.0% concentration. Please note that each value is an average of three consecutive readings per test site.

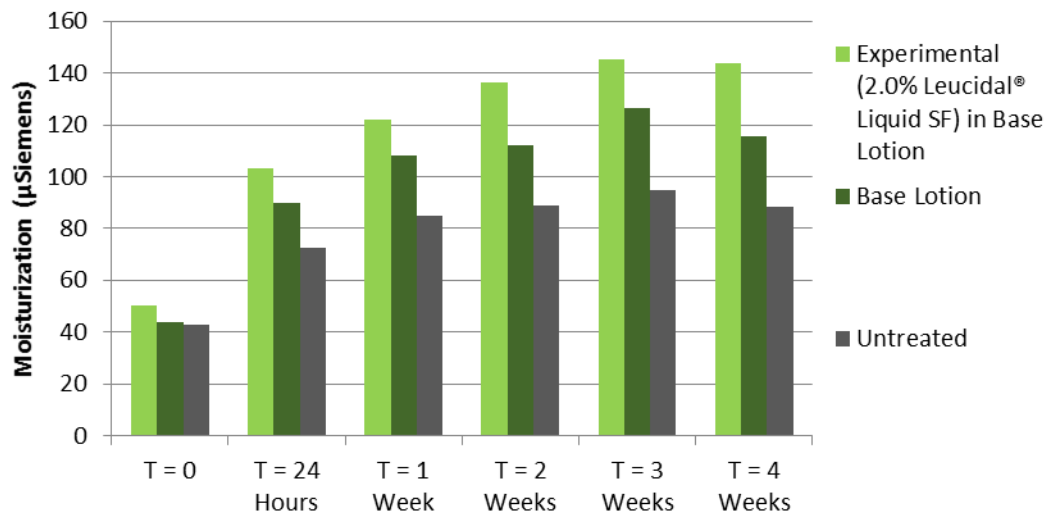
Moisturization		T = 0	T= 24 Hours	T = 1 Week	T = 2 Week	T= 3 Weeks	T= 4 Weeks
Panelist 1	Experimental	65	110	130	151	157	170
	Base Lotion	57	100	119	122	140	148
	Untreated	42	49	47	53	51	50
Panelist 2	Experimental	53	95	121	131	166	165
	Base Lotion	47	84	100	119	159	130
	Untreated	35	55	57	75	115	57
Panelist 3	Experimental	43	93	96	102	130	123
	Base Lotion	37	75	67	75	83	90
	Untreated	62	98	131	96	95	126
Panelist 4	Experimental	41	107	92	124	110	95
	Base Lotion	37	96	82	84	63	78
	Untreated	31	61	62	121	56	68
Panelist 5	Experimental	71	99	168	154	181	197
	Base Lotion	59	81	135	135	149	159
	Untreated	45	90	96	99	91	81
Panelist 6	Experimental	42	85	74	120	93	94
	Base Lotion	30	83	88	78	93	94
	Untreated	58	95	113	127	124	140
Panelist 7	Experimental	57	143	170	185	212	199
	Base Lotion	51	125	167	149	201	125
	Untreated	27	55	41	59	94	57
Panelist 8	Experimental	32	96	112	120	120	96
	Base Lotion	30	77	104	101	115	78
	Untreated	29	74	100	86	126	99
Panelist 9	Experimental	47	87	107	117	120	120
	Base Lotion	45	68	92	105	110	95
	Untreated	50	74	87	90	99	91
Panelist 10	Experimental	50	119	150	161	163	181
	Base Lotion	45	110	126	150	161	166
	Untreated	47	75	112	82	97	115
Number of Panelists		10	9	10	10	10	10

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Averages	T = 0	T = 24 Hours	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Experimental (2.0% Leucidal® Liquid SF) in Base Lotion	50.1	103.4	121.8	136.5	145.2	144.0
Base Lotion Control	43.8	89.9	108.0	112.0	126.3	115.6
Untreated Control	42.6	72.6	84.6	88.8	94.8	88.4

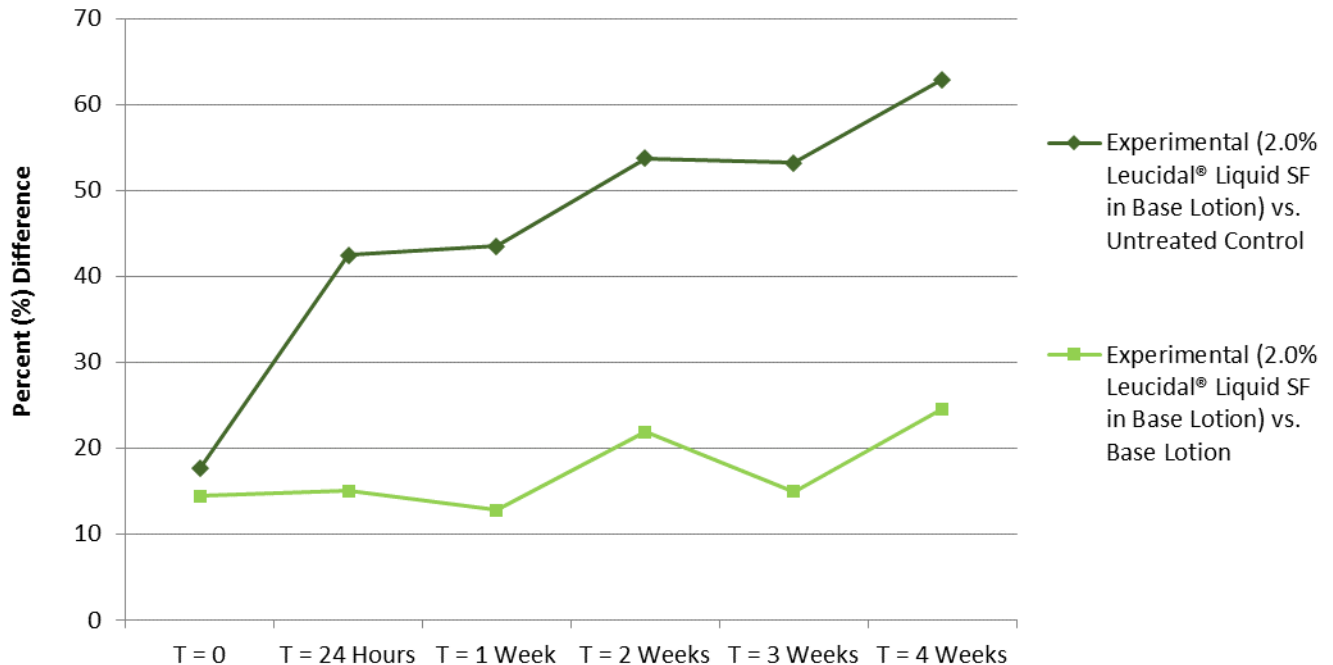
Percent (%) Change	T = 0	T = 24 Hours	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Base Lotion vs. Untreated Control	2.82	23.83	27.21	26.31	33.23	30.77
Experimental (2.0% Leucidal® Liquid SF) vs. Untreated Control	17.61	42.42	43.46	53.72	53.72	63.0
Experimental (2.0% Leucidal® Liquid SF) vs. Base Lotion	14.38	15.02	12.78	21.89	14.96	25.0

Average Moisturization



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Comparative Moisturization



Discussion

As evidenced in a four-week efficacy study of **Leucidal® Liquid SF**, moisture levels were improved by 42.42% after 24 hours and by 63% after four weeks when compared to the untreated control. When compared to the base cream **Leucidal® Liquid SF** improved moisturization by 14.38% and after 24 hours and by 25.0% after four weeks. Results indicate that **Leucidal® Liquid SF** is capable of increasing moisturization when compared to both the untreated control as well as the base lotion.

The present study confirms that **Leucidal® Liquid SF** is capable of providing strong moisturizing and skin hydrating benefits when added to cosmetic applications.



Transepidermal Water Loss (TEWL) Study

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: Leucidal[®] Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 1095

Lot #: NC140725-B

Sponsor: Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed: Transepidermal Water Loss Study

Introduction

An *in-vivo* study was conducted over a period of three weeks to evaluate the ability of **Leucidal[®] Liquid SF** to enhance barrier function through reduction in Transepidermal Water Loss (TEWL). Results indicate that this material is capable of efficiently reducing TEWL, which allows moisture retention.

Materials

A. Equipment: DermaLab Skin Combo

Methods

Ten volunteers M/F between the ages of 23 and 45 and who were known to be free of any skin pathologies participated in this study. A Dermalab Combo was used to measure TEWL on the subject's volar forearms. The instrument consists of a probe that is based upon the vapor gradient with an open chamber. This open chamber design maintains the free natural evaporation from the skin without interfering with the environment over the measurement area. This ensures unbiased and accurate readings. Operation of the water loss module is fully menu drive, allowing for pre-setting and standard deviation or measurement time. Baseline TEWL readings were taken on day one of the study.

Following initial measurements, all subjects were asked to apply 5milligrams of each test material on their volar forearms. Measurements were taken immediately after application of the test materials and then weekly for three weeks. The test material consisted of 2% **Leucidal[®] Liquid SF** in a base lotion.

For added perspective, measurements of an untreated test site and a site treated with a base lotion (Cetaphil Moisturizing for All Skin Types) were recorded.

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Transepidermal Water Loss (TEWL) Study

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(704) 276-7100 • Fax (704) 276-7101

Following initial measurements, all subjects were asked to apply 2 mg of each test material on their volar forearms. Measurements were taken immediately after application of test materials and then weekly for four weeks. The test material consisted of 2% **Leucidal® Liquid SF** in a base lotion.

For added perspective, measurements of an untreated test site and a site treated with a base lotion (Cetaphil Moisturizing for All Skin Types) were recorded.

Results

Leucidal® Liquid SF showed very effective moisture retention capabilities at a 2.0% concentration. Please note, each value is an average of three consecutive readings per test site.

Averages	T = 24 Hours	T = 1 Week	T = 2 Weeks	T = 3 Weeks
Untreated Control	-8.98	-8.14	-7.95	-7.38
Base Lotion Control	-9.26	-9.11	-8.83	-9.16
Experimental (2.0% Leucidal® Liquid SF) in Base Lotion	-10.02	-10.44	-9.62	-9.76

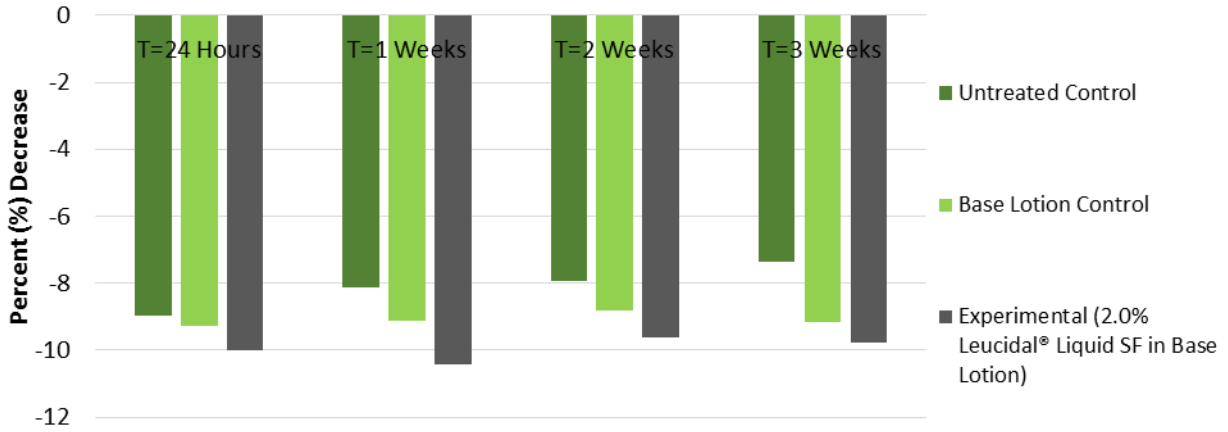
Chart 1. Average Transepidermal Water Loss of Individual Test Sites

Percent (%) Change	T = 24 Hours	T = 1 Week	T = 2 Weeks	T = 3 Weeks
Experimental (2.0% Leucidal® Liquid SF) vs. Untreated Control	9.3%	22.1%	22.9%	24.2%
Experimental (2.0% Leucidal® Liquid SF) vs. Base Lotion	3.4%	10.3%	11.0%	13.0%

Chart 2. Comparative Transepidermal Water Loss Results between Individual Test Sites

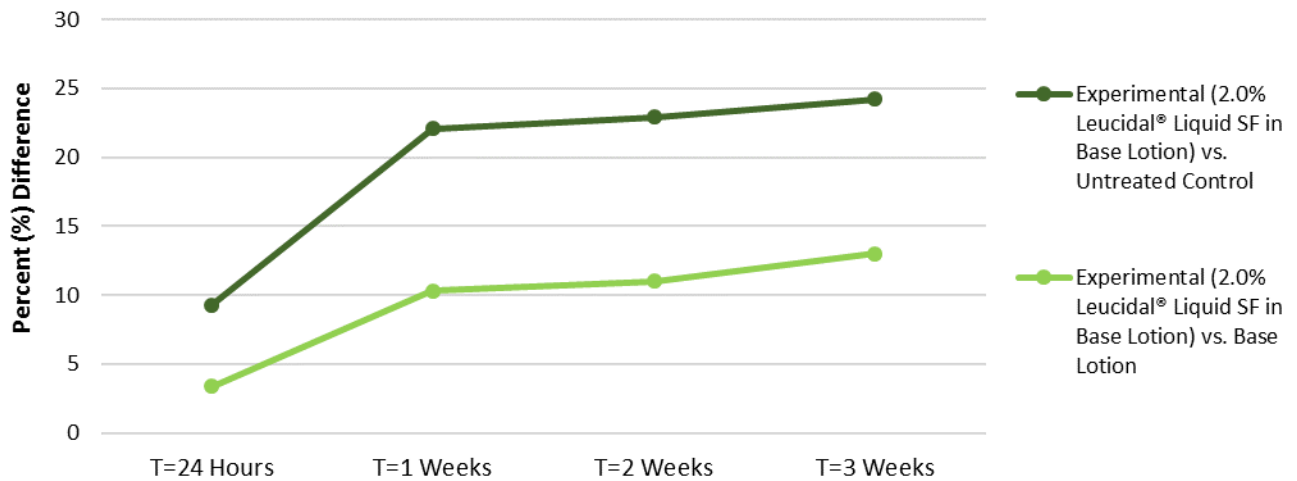
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Decrease in TEWL



Graph 1. Average Decrease in TEWL per Individual Test Site

TEWL Comparison Overtime



Graph 2. Comparison of TEWL Changes between Two Test Sites

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Transepidermal Water Loss (TEWL) Study

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Discussion

As shown, the results indicate continuous improvements in the barrier of the skin throughout the three week test period. After one week, the solution containing 2.0% **Leucidal[®] Liquid SF** decreased TEWL 10% more effectively than the base lotion alone. After three weeks, the solution containing 2.0% **Leucidal[®] Liquid SF** demonstrated even more effective barrier protection, decreasing TEWL 13% better than the base lotion alone.

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High Resolution Ultrasound Skin Imaging Assay

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: Leucidal[®] Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 1095

Lot #: NC140725-B

Sponsor: Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

High Resolution Ultrasound Skin-Imaging Assay

Introduction

An *in-vivo* study was conducted over a period of four weeks to evaluate the effect on skin density of **Leucidal[®] Liquid SF**. 10 M/F subjects between the ages of 23-45 participated in the study. Results indicate that this material is capable of significantly improving skin density compared to the control.

Materials

Equipment: DermaLab Skin Combo (Ultrasound Probe)

Ultrasound skin imaging is based on measuring the acoustic response after an acoustic pulse is sent into the skin. The energy of the acoustic pulse is low and will not affect the skin in any way. When the acoustic pulse is emitted and hits different areas of the skin, part of the pulse will be reflected and part will be transmitted further into the skin. The reflected signal travels back and is picked up by the ultrasound transducer. After processing the signal, a cross-sectional image appears on the screen. This image represents an intensity, or amplitude, analysis of the signals.

The intensity of the signals that are received refer to a color scale. Dark colors represent areas of the skin with low reflection. This means that there are no changes or very small changes in density between the structures in the skin. Bright colors represent areas with strong reflections, signifying substantial changes in density between structures.

Following initial measurements, all subjects were asked to apply 2 mg of each test material on their volar forearms. Measurements were taken immediately after application of test materials and then weekly for four weeks. The test material consisted of 2% **Leucidal[®] Liquid SF** in a base lotion.

For added perspective, measurements of an untreated test site and a site treated with a base lotion (Cetaphil Moisturizing for All Skin Types) were recorded.

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High Resolution Ultrasound Skin Imaging Assay

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Results

Leucidal® Liquid SF showed improvements in skin density at a 2.0% concentration. Please note that each value is an average of three consecutive readings per test site.

Averages	T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Experimental (2.0% Leucidal® Liquid SF) in Base Lotion	61.5	72	40.1	42.9	75.2
Base Lotion Control	58.2	62.3	61.4	67	68.3
Untreated Control	62.1	62.9	60.5	67.2	63.2

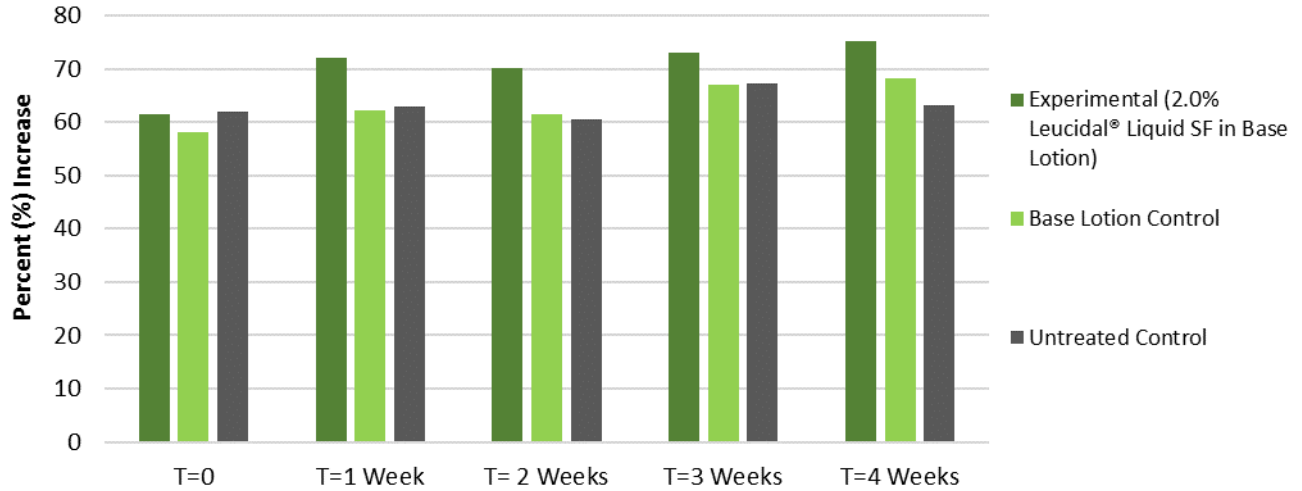
Chart 1. Average Increase in Skin Density per Individual Test Site

Percent (%) Change	T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Experimental (2.0% Leucidal® Liquid SF) vs. Untreated Control	9.16%	12.22%	12.97%	11.61%	17.33%
Experimental (2.0% Leucidal® Liquid SF) vs. Base Lotion	10.57%	10.02%	13.57%	15.35%	15.96%

Chart 2. Comparison of Skin Density Changes between Two Test Sites

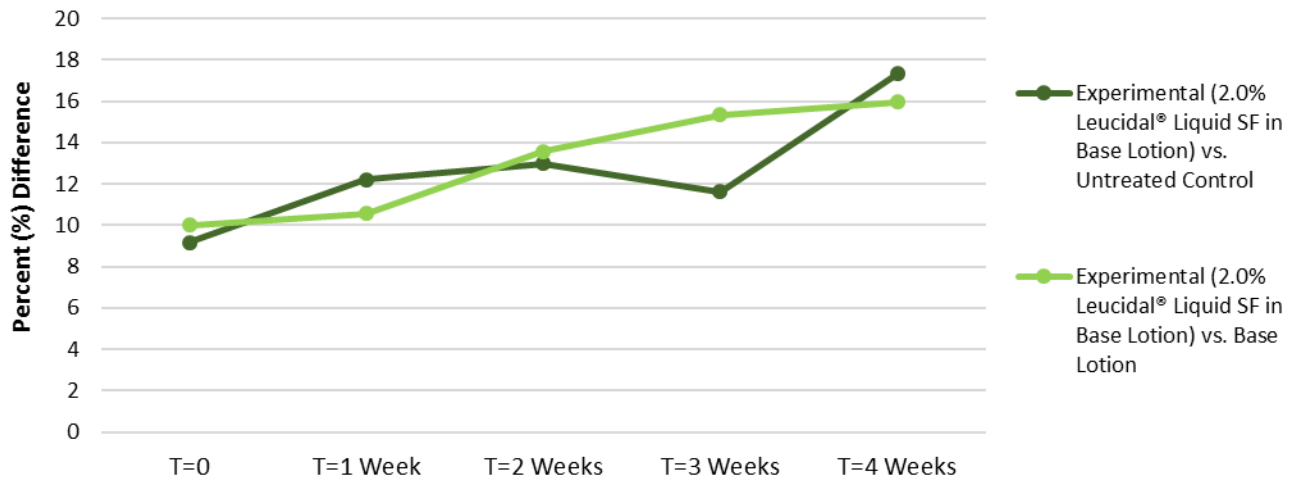
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Increase in Skin Density



Graph 1. Average Increase in Skin Density per Individual Test Site

Skin Density Comparison Overtime



Graph2. Comparison of Skin Density Changes between Two Test Sites

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High Resolution Ultrasound Skin Imaging Assay

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Discussion

As evidenced in a four-week efficacy study of **Leucidal[®] Liquid SF**, skin density was improved by 12.22% after one week and by 17.33% after four weeks when compared to the untreated control. When compared to the base cream **Leucidal[®] Liquid SF** improved skin density during each week of the trial, working 10.02% better than the base lotion after one week and 15.96% better than the base lotion after four weeks. Results indicate that **Leucidal[®] Liquid** is capable of improving skin density when compared to both the untreated control as well as the base lotion.

Leucidal[®] Liquid SF has a strong positive effect on skin's density when used at recommended use levels.

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Inhibition Activity Data

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Product Name: Leucidal[®] Liquid SF
Code Number: M15019
Lot Number: 4870P
Test Request Number: 1494
CAS #'s: 1686112-36-6
EINECS #'s: N/A
INCI Name: Lactobacillus Ferment

Organism (ATCC #)	Minimum Inhibitory Concentration (%)
<i>E.coli</i> #8739	0.5
<i>S. aureus</i> #6538	1.0
<i>P. aeruginosa</i> #9027	1.0
<i>C. albicans</i> #10231	1.0
<i>A. brasiliensis</i> #16404	0.5

QA Signature _____ Monica Beltran _____

Date _____ 09-08-2015 _____

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Zone of Inhibition Test

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Product Name: Leucidal[®] Liquid SF
Code Number: M15019
Lot Number: 39528P
Test Request Number: 1034
CAS #'s: 1686112-36-6
EINECS #'s: N/A
INCI Name: *Lactobacillus* Ferment

Organism (ATCC #)	Zone of Inhibition (mm)
<i>E. coli</i> #8379	15.8
<i>S. aureus</i> #6538	15.6
<i>P. aeruginosa</i> #9027	15.7
<i>C. albicans</i> #10231	25.0
<i>A. brasiliensis</i> #16404	17.0

QA Signature _____ Monica Beltran _____

Date _____ 01-28-2015 _____

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Challenge Test with 4.0% Leucidal® Liquid SF

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

Leucidal® Liquid SF
M15019

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on October 23rd, 2013 and was completed on December 26th, 2013.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Test Method

Fifty grams of Generic Cream Formula pH 3 with 4% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

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Challenge Test with 4.0% Leucidal® Liquid SF

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The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	1.3 x 10 ⁷	2.4 x 10 ⁷	1.4 x 10 ⁷	3.3 x 10 ⁵	3.1 x 10 ⁵
Day 0*	99.996%	>99.999%	>99.999%	99.993%	99.941%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	4.4 x 10 ⁶	1.4 x 10 ⁵	1.9 x 10 ⁶	1.3 x 10 ⁵	1.0 x 10 ⁵
Day 7	>99.999%	99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 3 with 4% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

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Challenge Test with 4.0% Leucidal® Liquid SF

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The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 3 with 4% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast were reduced by greater than 99.9% within 7 days of each challenge. The mold was reduced by greater than 90% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 4.0% Leucidal® Liquid SF

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Phase	Ingredient	Supplier	%
I	Water	-	71.15%
	Carbopol Ultrez 10	Lubrizol	0.10%
II	Dermol 816	Alzo International	5.00%
	Sunflower Oil	Arista Industries	5.00%
	Silderm Emulsifying CS	Active Concepts, LLC	2.00%
	Dermol 2014	Alzo International	5.00%
	Lipomulse 165	Lipo Chemicals	1.60%
	Lipowax D	Lipo Chemicals	1.60%
	Stearic Acid	Acme Hardesty Oleochemicals	0.25%
III	ACB Bamboo Bioferment	Active Concepts, LLC	2.00%
IV	Organic Rice Solution	Arbor Organic Technologies, LLC	5.00%
	ACB Bio-Chelate 5	Active Concepts, LLC	2.00%

Manufacturing Process:

Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Check pH and adjust to 6.0 – 7.0 with Citric Acid (50%) or NaOH (25%).

Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 15 minutes. Begin force cooling.

Phase III:

Add ingredients at 50°C and mix until homogenous.

Phase IV:

Add ingredients and mix until homogenous.

Specifications:

Appearance: White to Off-White Emulsion

pH: 4.0 – 5.5

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

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Challenge Test with 4.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

The information contained in this report is provided by Active Micro Technologies after the exercise of all reasonable care and skill in its compilation, preparation, and issue. It is provided without liability regarding its subsequent application and use. This type of screening test will be conducted only for validation of the efficacy of the antimicrobial agent or preservative system in the specific formulation tested. It does not address the suitability of the overall formula, nor does it address the regulatory status of any component therein. This testing does not account for the possibility of environmental microorganisms and cannot be relied upon as sufficient to justify commercialization of the product tested. By submitting samples for testing, the customer acknowledges that they will not hold Active Micro Technologies responsible for products launched based solely on the support of these studies.



Challenge Test with 4.0% Leucidal® Liquid SF

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

Leucidal® Liquid SF
M15019

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on October 23rd, 2013 and was completed on December 26th, 2013.

Test Organisms

- | | |
|--------------------------------------|-------------|
| 1. <i>Escherichia coli</i> : | ATCC #8739 |
| 2. <i>Pseudomonas aeruginosa</i> : | ATCC #9027 |
| 3. <i>Staphylococcus aureus</i> : | ATCC #6538 |
| 4. <i>Aspergillus brasiliensis</i> : | ATCC #16404 |
| 5. <i>Candida albicans</i> : | ATCC #10231 |

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Test Method

Fifty grams of Generic Cream Formula pH 5 with 4% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

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The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	1.3 x 10 ⁷	2.4 x 10 ⁷	1.4 x 10 ⁷	3.3 x 10 ⁵	3.1 x 10 ⁵
Day 0*	99.988%	99.999%	99.993%	99.975%	99.929%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	4.4 x 10 ⁶	1.4 x 10 ⁵	1.9 x 10 ⁶	1.3 x 10 ⁵	1.0 x 10 ⁵
Day 7	>99.999%	99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 5 with 4% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

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Challenge Test with 4.0% Leucidal® Liquid SF

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The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 5 with 4% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

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The Gram positive and Gram negative bacteria as well as the yeast were reduced by greater than 99.9% within 7 days of each challenge. The mold was reduced by greater than 90% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 4.0% Leucidal® Liquid SF

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Phase	Ingredient	Supplier	%
I	Water	-	71.15%
	Carbopol Ultrez 10	Lubrizol	0.10%
II	Dermol 816	Alzo International	5.00%
	Sunflower Oil	Arista Industries	5.00%
	Silderm Emulsifying CS	Active Concepts, LLC	2.00%
	Dermol 2014	Alzo International	5.00%
	Lipomulse 165	Lipo Chemicals	1.60%
	Lipowax D	Lipo Chemicals	1.60%
	Stearic Acid	Acme Hardesty Oleochemicals	0.25%
III	ACB Bamboo Bioferment	Active Concepts, LLC	2.00%
IV	Organic Rice Solution	Arbor Organic Technologies, LLC	5.00%
	ACB Bio-Chelate 5	Active Concepts, LLC	2.00%

Manufacturing Process:

Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Check pH and adjust to 6.0 – 7.0 with Citric Acid (50%) or NaOH (25%).

Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 15 minutes. Begin force cooling.

Phase III:

Add ingredients at 50°C and mix until homogenous.

Phase IV:

Add ingredients and mix until homogenous.

Specifications:

Appearance: White to Off-White Emulsion

pH: 4.0 – 5.5

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

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Challenge Test with 4.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

Leucidal® Liquid SF
M15019

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on October 23rd, 2013 and was completed on December 26th, 2013.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Test Method

Fifty grams of Generic Cream Formula pH 7 with 4% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

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Organisms					
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	1.3 x 10 ⁷	2.4 x 10 ⁷	1.4 x 10 ⁷	3.3 x 10 ⁵	3.1 x 10 ⁵
Day 0*	99.971%	99.996%	99.955%	99.975%	99.919%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	4.4 x 10 ⁶	1.4 x 10 ⁵	1.9 x 10 ⁶	1.3 x 10 ⁵	1.0 x 10 ⁵
Day 7	>99.999%	99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 7 with 4% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

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Results & Discussion

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The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 7 with 4% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast were reduced by greater than 99.9% within 7 days of each challenge. The mold was reduced by greater than 90% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 4.0% Leucidal® Liquid SF

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Phase	Ingredient	Supplier	%
I	Water	-	71.15%
	Carbopol Ultrez 10	Lubrizol	0.10%
II	Dermol 816	Alzo International	5.00%
	Sunflower Oil	Arista Industries	5.00%
	Silderm Emulsifying CS	Active Concepts, LLC	2.00%
	Dermol 2014	Alzo International	5.00%
	Lipomulse 165	Lipo Chemicals	1.60%
	Lipowax D	Lipo Chemicals	1.60%
	Stearic Acid	Acme Hardesty Oleochemicals	0.25%
III	ACB Bamboo Bioferment	Active Concepts, LLC	2.00%
IV	Organic Rice Solution	Arbor Organic Technologies, LLC	5.00%
	ACB Bio-Chelate 5	Active Concepts, LLC	2.00%

Manufacturing Process:

Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Check pH and adjust to 6.0 – 7.0 with Citric Acid (50%) or NaOH (25%).

Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 15 minutes. Begin force cooling.

Phase III:

Add ingredients at 50°C and mix until homogenous.

Phase IV:

Add ingredients and mix until homogenous.

Specifications:

Appearance: White to Off-White Emulsion

pH: 4.0 – 5.5

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

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Challenge Test with 4.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Challenge Test with 2.0% Leucidal® Liquid SF

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

Leucidal® Liquid SF
M15019

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on October 23rd, 2013 and was completed on December 26th, 2013.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Test Method

Fifty grams of Generic Cream Formula pH 3 with 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

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Challenge Test with 2.0% Leucidal® Liquid SF

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The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	1.3 x 10 ⁷	2.4 x 10 ⁷	1.4 x 10 ⁷	3.3 x 10 ⁵	3.1 x 10 ⁵
Day 0*	99.999%	>99.999%	>99.999%	99.996%	99.945%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	3.5 x 10 ⁶	3.2 x 10 ⁶	1.8 x 10 ⁶	1.2 x 10 ⁵	2.9 x 10 ⁵
Day 7	>99.999%	99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 3 with 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

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Challenge Test with 2.0% Leucidal® Liquid SF

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The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 3 with 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast were reduced by greater than 99.9% within 7 days of each challenge. The mold was reduced by greater than 90% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 2.0% Leucidal® Liquid SF

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Phase	Ingredient	Supplier	%
I	Water	-	71.15%
	Carbopol Ultrez 10	Lubrizol	0.10%
II	Dermol 816	Alzo International	5.00%
	Sunflower Oil	Arista Industries	5.00%
	Silderm Emulsifying CS	Active Concepts, LLC	2.00%
	Dermol 2014	Alzo International	5.00%
	Lipomulse 165	Lipo Chemicals	1.60%
	Lipowax D	Lipo Chemicals	1.60%
	Stearic Acid	Acme Hardesty Oleochemicals	0.25%
III	ACB Bamboo Bioferment	Active Concepts, LLC	2.00%
IV	Organic Rice Solution	Arbor Organic Technologies, LLC	5.00%
	ACB Bio-Chelate 5	Active Concepts, LLC	2.00%

Manufacturing Process:

Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Check pH and adjust to 6.0 – 7.0 with Citric Acid (50%) or NaOH (25%).

Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 15 minutes. Begin force cooling.

Phase III:

Add ingredients at 50°C and mix until homogenous.

Phase IV:

Add ingredients and mix until homogenous.

Specifications:

Appearance: White to Off-White Emulsion

pH: 4.0 – 5.5

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

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Challenge Test with 2.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

Leucidal® Liquid SF
M15019

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on October 23rd, 2013 and was completed on December 26th, 2013.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Test Method

Fifty grams of Generic Cream Formula pH 5 with 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

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Challenge Test with 2.0% Leucidal® Liquid SF

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The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	1.3 x 10 ⁷	2.4 x 10 ⁷	1.4 x 10 ⁷	3.3 x 10 ⁵	3.1 x 10 ⁵
Day 0*	99.840%	99.999%	99.969%	99.969%	99.967%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	4.4 x 10 ⁶	1.4 x 10 ⁵	1.9 x 10 ⁶	1.3 x 10 ⁵	1.0 x 10 ⁵
Day 7	>99.999%	99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 5 with 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

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Challenge Test with 2.0% Leucidal® Liquid SF

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The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 5 with 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast were reduced by greater than 99.9% within 7 days of each challenge. The mold was reduced by greater than 90% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 2.0% Leucidal® Liquid SF

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Phase	Ingredient	Supplier	%
I	Water	-	71.15%
	Carbopol Ultrez 10	Lubrizol	0.10%
II	Dermol 816	Alzo International	5.00%
	Sunflower Oil	Arista Industries	5.00%
	Silderm Emulsifying CS	Active Concepts, LLC	2.00%
	Dermol 2014	Alzo International	5.00%
	Lipomulse 165	Lipo Chemicals	1.60%
	Lipowax D	Lipo Chemicals	1.60%
	Stearic Acid	Acme Hardesty Oleochemicals	0.25%
III	ACB Bamboo Bioferment	Active Concepts, LLC	2.00%
IV	Organic Rice Solution	Arbor Organic Technologies, LLC	5.00%
	ACB Bio-Chelate 5	Active Concepts, LLC	2.00%

Manufacturing Process:

Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Check pH and adjust to 6.0 – 7.0 with Citric Acid (50%) or NaOH (25%).

Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 15 minutes. Begin force cooling.

Phase III:

Add ingredients at 50°C and mix until homogenous.

Phase IV:

Add ingredients and mix until homogenous.

Specifications:

Appearance: White to Off-White Emulsion

pH: 4.0 – 5.5

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

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Challenge Test with 2.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

Leucidal® Liquid SF
M15019

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on October 23rd, 2013 and was completed on December 26th, 2013.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Test Method

Fifty grams of Generic Cream Formula pH 7 with 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

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Challenge Test with 2.0% Leucidal® Liquid SF

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Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	1.3 x 10 ⁷	2.4 x 10 ⁷	1.4 x 10 ⁷	3.3 x 10 ⁵	3.1 x 10 ⁵
Day 0*	99.981%	99.992%	99.978%	99.993%	99.945%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	4.4 x 10 ⁶	1.4 x 10 ⁵	1.9 x 10 ⁶	1.3 x 10 ⁵	1.0 x 10 ⁵
Day 7	>99.999%	99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 7 with 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

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Challenge Test with 2.0% Leucidal® Liquid SF

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The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 7 with 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

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Challenge Test with 2.0% Leucidal® Liquid SF

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Phase	Ingredient	Supplier	%
I	Water	-	71.15%
	Carbopol Ultrez 10	Lubrizol	0.10%
II	Dermol 816	Alzo International	5.00%
	Sunflower Oil	Arista Industries	5.00%
	Silderm Emulsifying CS	Active Concepts, LLC	2.00%
	Dermol 2014	Alzo International	5.00%
	Lipomulse 165	Lipo Chemicals	1.60%
	Lipowax D	Lipo Chemicals	1.60%
	Stearic Acid	Acme Hardesty Oleochemicals	0.25%
III	ACB Bamboo Bioferment	Active Concepts, LLC	2.00%
IV	Organic Rice Solution	Arbor Organic Technologies, LLC	5.00%
	ACB Bio-Chelate 5	Active Concepts, LLC	2.00%

Manufacturing Process:

Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Check pH and adjust to 6.0 – 7.0 with Citric Acid (50%) or NaOH (25%).

Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 15 minutes. Begin force cooling.

Phase III:

Add ingredients at 50°C and mix until homogenous.

Phase IV:

Add ingredients and mix until homogenous.

Specifications:

Appearance: White to Off-White Emulsion

pH: 4.0 – 5.5

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

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Challenge Test with 2.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

AMTicide® Coconut
Leucidal® Liquid SF

Test Request #:

1281

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on February 25th, 2015 and was completed on April 27th, 2015.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.



Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF

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(704) 276-7100 • Fax (704) 276-7101

Test Method

Fifty grams of Generic Cream Formula pH 3 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	4.8×10^6	7.8×10^6	9.7×10^6	1.3×10^5	5.4×10^5
Day 0*	99.999%	99.999%	99.999%	99.999%	99.999%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	7.3×10^6	6.7×10^6	6.4×10^6	2.1×10^5	6.8×10^5
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 3 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

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Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF



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Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 3 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.

Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF



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Phase	Ingredient	Supplier	%
I	Water	-	85.2
	Carbopol Ultrez 10	Lubrizol	0.1
	Glycerin	PT. Musim Mas	3.0
	Tealan	RITA	0.9
II	Cetyl Alcohol	RITA	2.0
	Stearic Acid	Acme Hardesty Oleochemicals	0.8
	Glyceryl Stearate	Protameen Chem.	1.5
	Isopropyl Myristate	Alzo	1.5
	Mineral Oil	RITA	5.0

Manufacturing Process:

1. Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Add the rest of ingredients.

2. Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 30 minutes. Begin force cooling to 25°C.

3. Check the pH.

Specifications:

Appearance: White to Off-White Emulsion

pH: 6.5 – 8.0

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.



Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

AMTicide® Coconut
Leucidal® Liquid SF

Test Request #:

1282

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on February 25th, 2015 and was completed on April 27th, 2015.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.

Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF



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Test Method

Fifty grams of Generic Cream Formula pH 5 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
		4.8×10^6	7.8×10^6	9.7×10^6	1.3×10^5
Day 0*	99.901%	99.992%	99.955%	99.961%	99.970%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	7.3×10^6	6.7×10^6	6.4×10^6	2.1×10^5	6.8×10^5
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 5 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

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Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF



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Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 5 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.

Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF



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Phase	Ingredient	Supplier	%
I	Water	-	85.2
	Carbopol Ultrez 10	Lubrizol	0.1
	Glycerin	PT. Musim Mas	3.0
	Tealan	RITA	0.9
II	Cetyl Alcohol	RITA	2.0
	Stearic Acid	Acme Hardesty Oleochemicals	0.8
	Glyceryl Stearate	Protameen Chem.	1.5
	Isopropyl Myristate	Alzo	1.5
	Mineral Oil	RITA	5.0

Manufacturing Process:

1. Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Add the rest of ingredients.

2. Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 30 minutes. Begin force cooling to 25°C.

3. Check the pH.

Specifications:

Appearance: White to Off-White Emulsion

pH: 6.5 – 8.0

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.

Challenge Test with 4.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF



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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

AMTicide® Coconut
Leucidal® Liquid SF

Test Request #:

984

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on January 12th, 2015 and was completed on March 9th, 2015.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.



Challenge Test with 2.0% Leucidal® Liquid SF + 4.0% AMTicide® Coconut

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(704) 276-7100 • Fax (704) 276-7101

Test Method

Fifty grams of Generic Cream Formula pH 7 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	4.5×10^6	7.8×10^6	3.1×10^6	4.0×10^5	5.4×10^5
Day 0*	99.901%	99.992%	99.860%	99.987%	99.970%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	3.5×10^6	3.2×10^6	1.8×10^6	1.2×10^5	2.9×10^5
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 7 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

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Challenge Test with 2.0% Leucidal® Liquid SF + 4.0% AMTicide® Coconut

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Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 7 with 4% AMTicide® Coconut and 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 2.0% Leucidal® Liquid SF + 4.0% AMTicide® Coconut

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Phase	Ingredient	Supplier	%
I	Water	-	85.2
	Carbopol Ultrez 10	Lubrizol	0.1
	Glycerin	PT. Musim Mas	3.0
	Tealan	RITA	0.9
II	Cetyl Alcohol	RITA	2.0
	Stearic Acid	Acme Hardesty Oleochemicals	0.8
	Glyceryl Stearate	Protameen Chem.	1.5
	Isopropyl Myristate	Alzo	1.5
	Mineral Oil	RITA	5.0

Manufacturing Process:

1. Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Add the rest of ingredients.

2. Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 30 minutes. Begin force cooling to 25°C.

3. Check the pH.

Specifications:

Appearance: White to Off-White Emulsion

pH: 6.5 – 8.0

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.



**Challenge Test with 2.0% Leucidal® Liquid SF
+ 4.0% AMTicide® Coconut**

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Antimicrobial Efficacy (Challenge) Testing

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

AMTicide® Coconut
Leucidal® Liquid

Test Request #:

1175

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on February 25th, 2015 and was completed on April 27th, 2015.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.



Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid

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Test Method

Fifty grams of Generic Cream Formula pH 3 with 2% AMTicide® Coconut and 2% Leucidal® Liquid was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
		4.8×10^6	7.8×10^6	9.7×10^6	1.3×10^5
Day 0*	99.999%	99.999%	99.999%	99.999%	99.981%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	7.3×10^6	6.7×10^6	6.4×10^6	2.1×10^5	6.8×10^5
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 3 with 2% AMTicide® Coconut and 2% Leucidal® Liquid inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied. This information is offered solely for your investigation, verification, and consideration.



Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 3 with 2% AMTicide® Coconut and 2% Leucidal® Liquid. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid

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Phase	Ingredient	Supplier	%
I	Water	-	85.2
	Carbopol Ultrez 10	Lubrizol	0.1
	Glycerin	PT. Musim Mas	3.0
	Tealan	RITA	0.9
II	Cetyl Alcohol	RITA	2.0
	Stearic Acid	Acme Hardesty Oleochemicals	0.8
	Glyceryl Stearate	Protameen Chem.	1.5
	Isopropyl Myristate	Alzo	1.5
	Mineral Oil	RITA	5.0

Manufacturing Process:

1. Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Add the rest of ingredients.

2. Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 30 minutes. Begin force cooling to 25°C.

3. Check the pH.

Specifications:

Appearance: White to Off-White Emulsion

pH: 6.5 – 8.0

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.



Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid

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(704) 276-7100 • Fax (704) 276-7101

Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

The information contained in this report is provided by Active Micro Technologies after the exercise of all reasonable care and skill in its compilation, preparation, and issue. It is provided without liability regarding its subsequent application and use. This type of screening test will be conducted only for validation of the efficacy of the antimicrobial agent or preservative system in the specific formulation tested. It does not address the suitability of the overall formula, nor does it address the regulatory status of any component therein. This testing does not account for the possibility of environmental microorganisms and cannot be relied upon as sufficient to justify commercialization of the product tested. By submitting samples for testing, the customer acknowledges that they will not hold Active Micro Technologies responsible for products launched based solely on the support of these studies.



Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

AMTicide® Coconut
Leucidal® Liquid SF

Test Request #:

1280

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on February 25th, 2015 and was completed on April 27th, 2015.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.



Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF

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(704) 276-7100 • Fax (704) 276-7101

Test Method

Fifty grams of Generic Cream Formula pH 5 with 2% AMTicide® Coconut and 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
		4.8 x 10 ⁶	7.8 x 10 ⁶	9.7 x 10 ⁶	1.3 x 10 ⁵
Day 0*	99.960%	99.983%	99.966%	99.990%	99.988%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	7.3 x 10 ⁶	6.7 x 10 ⁶	6.4 x 10 ⁶	2.1 x 10 ⁵	6.8 x 10 ⁵
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 5 with 2% AMTicide® Coconut and 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

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Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF

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Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 5 with 2% AMTicide® Coconut and 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF

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Phase	Ingredient	Supplier	%
I	Water	-	85.2
	Carbopol Ultrez 10	Lubrizol	0.1
	Glycerin	PT. Musim Mas	3.0
	Tealan	RITA	0.9
II	Cetyl Alcohol	RITA	2.0
	Stearic Acid	Acme Hardesty Oleochemicals	0.8
	Glyceryl Stearate	Protameen Chem.	1.5
	Isopropyl Myristate	Alzo	1.5
	Mineral Oil	RITA	5.0

Manufacturing Process:

1. Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Add the rest of ingredients.

2. Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 30 minutes. Begin force cooling to 25°C.

3. Check the pH.

Specifications:

Appearance: White to Off-White Emulsion

pH: 6.5 – 8.0

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.



Challenge Test with 2.0% AMTicide® Coconut + 2.0% Leucidal® Liquid SF

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

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Antimicrobial Efficacy Test PCPC Section 20 Method 3

Determination of Preservation Adequacy of Water- Miscible Personal Care Products

Product

AMTicide® Coconut
Leucidal® Liquid SF

Test Request #:

1279

Purpose

This study was initiated to determine the efficacy of a cosmetic ingredient with antimicrobial properties in a cream formulation against bioburden as a function of time.

Study Dates

The study was started on February 25th, 2015 and was completed on April 27th, 2015.

Test Organisms

1. *Escherichia coli*: ATCC #8739
2. *Pseudomonas aeruginosa*: ATCC #9027
3. *Staphylococcus aureus*: ATCC #6538
4. *Aspergillus brasiliensis*: ATCC #16404
5. *Candida albicans*: ATCC #10231

Neutralization:

Verification of neutralization of the antimicrobial properties of the product was demonstrated prior to performing the test for microbial content by inoculating the product dilution with a low level of challenge microorganisms (100 CFU) and verifying recovery of this viable inoculum. This provides evidence that the antimicrobial has been neutralized and there are no false positive results during the Challenge Test.



Challenge Test with 2.0% Leucidal® Liquid SF + 2.0% AMTicide® Coconut

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Test Method

Fifty grams of Generic Cream Formula pH 7 with 2% AMTicide® Coconut and 2% Leucidal® Liquid SF was weighed into five individual containers. Each container was inoculated with one of the five test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 to 10^8 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition.

The inoculated samples were evaluated 0, 7, 14, 21, and 28 days after the initial inoculation to determine quantitatively the number of viable microorganisms remaining. On the 28th day of testing the samples were re-inoculated and evaluated 7, 14, 21, and 28 days after the second exposure to determine the number of viable microorganisms. The table below represents the percent reduction of viable organisms after being introduced into the test formulation.

Organisms					
Inoculum (initial) CFU/ml	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>A. brasiliensis</i>	<i>C. albicans</i>
	4.5×10^6	7.8×10^6	3.1×10^6	4.0×10^5	5.4×10^5
Day 0*	99.960%	99.983%	99.894%	99.995%	99.985%
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Inoculum (re-inoculated) CFU/ml	3.5×10^6	3.2×10^6	1.8×10^6	1.2×10^5	2.9×10^5
Day 7	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 14	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 21	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%
Day 28	>99.999%	>99.999%	>99.999%	>99.999%	>99.999%

Table 1. Challenge Test results for Generic Cream Formula pH 7 with 2% AMTicide® Coconut and 2% Leucidal® Liquid SF inoculated on Day 0 and re-inoculated on Day 28. Results show % reduction in viable organisms.

* The days listed in the first column refer to the inoculum/plating day. Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.

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Challenge Test with 2.0% Leucidal® Liquid SF + 2.0% AMTicide® Coconut

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Results & Discussion

The results obtained from the Neutralization Test of each product using Dey/Engley (D/E) broth, indicate that the neutralization steps conducted prior to performing the Challenge Test are indeed effective for avoiding false positive Challenge Test results.

The results of this Challenge Test demonstrate the effectiveness of the preservation system used in Generic Cream Formula pH 7 with 2% AMTicide® Coconut and 2% Leucidal® Liquid SF. The recommendations stated in Section 13, Determination of Preservative Adequacy in Cosmetic Formulations, in the PCPC Microbiology Guidelines are as follows:

Bacteria – There should be at least a 99.9% (3 log) reduction of vegetative bacteria within 7 days following each challenge and no increase for the duration of the test period.

Yeasts and Molds – There should be at least a 90% (1 log) reduction of yeasts and molds within 7 days following each challenge and no increase for the duration of the test period.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 7 days of each challenge. By the end of each 28-day test period Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.999% or greater.



Challenge Test with 2.0% Leucidal® Liquid SF + 2.0% AMTicide® Coconut

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Phase	Ingredient	Supplier	%
I	Water	-	85.2
	Carbopol Ultrez 10	Lubrizol	0.1
	Glycerin	PT. Musim Mas	3.0
	Tealan	RITA	0.9
II	Cetyl Alcohol	RITA	2.0
	Stearic Acid	Acme Hardesty Oleochemicals	0.8
	Glyceryl Stearate	Protameen Chem.	1.5
	Isopropyl Myristate	Alzo	1.5
	Mineral Oil	RITA	5.0

Manufacturing Process:

1. Phase I:

Charge water into main beaker and begin propeller mixing. A vortex should form. Begin heating to 75°C. Slowly sift in Carbopol while mixing. Add the rest of ingredients.

2. Phase II:

In a separate beaker, combine ingredients and heat to 75°C while mixing. Mix until homogenous. Then add to the main beaker with high-speed mixing. Maintain temperature at 75°C and mix for 30 minutes. Begin force cooling to 25°C.

3. Check the pH.

Specifications:

Appearance: White to Off-White Emulsion

pH: 6.5 – 8.0

*If a different pH is desired, adjust using Citric Acid (50%) or NaOH (25%). Formula is stable in the 3.0 – 7.0 pH range.



**Challenge Test with 2.0% Leucidal® Liquid SF
+ 2.0% AMTicide® Coconut**

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Antimicrobial Efficacy (Challenge) Testing

The intent of performing an Antimicrobial Efficacy or Challenge test is to evaluate whether an antimicrobial agent or preservation system in a given cosmetic formulation has the ability to prevent the growth of test microorganisms. The test methodology employed by Active Micro Technologies (AMT) is based on the methods published in the CTFA Microbiology Guidelines. AMT's goal is to assist our customers by providing a screening test of a product formulation that is approaching finalization. It is expected that the formulation(s) submitted for Challenge testing contain AMT antimicrobials and have already passed the customer's internal stability tests. It is also anticipated that formal challenge testing of the final formulation will subsequently be performed by the customer at an outside lab of their choosing.

The information contained in this report is provided by Active Micro Technologies after the exercise of all reasonable care and skill in its compilation, preparation, and issue. It is provided without liability regarding its subsequent application and use. This type of screening test will be conducted only for validation of the efficacy of the antimicrobial agent or preservative system in the specific formulation tested. It does not address the suitability of the overall formula, nor does it address the regulatory status of any component therein. This testing does not account for the possibility of environmental microorganisms and cannot be relied upon as sufficient to justify commercialization of the product tested. By submitting samples for testing, the customer acknowledges that they will not hold Active Micro Technologies responsible for products launched based solely on the support of these studies.

Time Kill Test

E2315

Assessment of Antimicrobial Activity Using a Time Kill Procedure

Product

Leucidal® Liquid SF

Test Request #:

1809

Purpose

This study was initiated to measure the change in population of aerobic microorganisms within a specified sampling time when tested against a cosmetic ingredient.

Study Dates

The study was started on March 8th, 2016 and was completed on March 15th, 2016.

Test Organisms

- | | |
|-------------------------------------|-------------|
| 1. <i>Escherichia coli:</i> | ATCC #8739 |
| 2. <i>Pseudomonas aeruginosa:</i> | ATCC #9027 |
| 3. <i>Staphylococcus aureus:</i> | ATCC #6538 |
| 4. <i>Bacillus subtilis</i> | ATCC #6051 |
| 5. <i>Aspergillus brasiliensis:</i> | ATCC #16404 |
| 6. <i>Candida albicans:</i> | ATCC #10231 |

Neutralization:

Inactivation of the antimicrobial activity of the test material is achieved through the dilution of the test material during the sampling time at specified sampling intervals.

Test Method

Ten grams of 4% Leucidal® Liquid SF solution was weighed into six individual containers. Each container was inoculated with one of the six test organisms. The inoculum concentration for each organism was standardized using the 0.5 McFarland turbidity standard and further diluted to yield approximately 10^6 microorganisms/ml. The amount of each inoculum added to each sample was no more than 1% of the product weight, as to not alter the product composition. Serial dilutions from each container were performed to enumerate the surviving microorganisms using the Plate Count Technique.

The activity of the test material inoculated was evaluated at determine time intervals of 30 seconds, 1, 5, 10 and 30 minutes after the inoculation to determine quantitatively the number of viable microorganisms remaining after the incubation time.

Organisms	Inoculum Concentration CFU/ml	Percentage of Reduction				
		30 seconds	1 minute	5 minute	10 minute	30 minutes
<i>E.coli</i> * ATCC# 8739	6.1×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>S.aureus</i> ATCC# 6538	6.0×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>P.aeruginosa</i> ATCC# 9027	4.6×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>B.subtilis</i> ATCC# 6051	5.0×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>A.brasiliensis</i> ATCC# 16404	4.6×10^6	99.9%	99.9%	99.9%	99.9%	99.9%
<i>C.albicans</i> ATCC# 10231	2.0×10^6	99.9%	99.9%	99.9%	99.9%	99.9%

Table 1. Time Kill Test results for 4% Leucidal® Liquid SF inoculated with 10^6 microorganisms' population. Results show % reduction in viable organisms after inoculation and sampling time intervals.

*Bacteria results are read 2 days after plating day, and mold and yeast results are read 5 days after plating day.



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Results & Discussion

The results of this Time Kill Test determine the changes in population of aerobic microorganisms within a specified sampling time when tested against 4% Leucidal[®] Liquid SF solution.

The Gram positive and Gram negative bacteria as well as the yeast and mold were reduced by 99.9% within 30 seconds interval of the test after the inoculation.

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Safety Statement

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Product Name: Leucidal® Liquid SF

Product Code: M15019

INCI Name: Lactobacillus Ferment

INCI Status: Approved

Leucidal® Liquid SF is created by the fermentation of *Lactobacillus* in a defined media under controlled conditions of pH, temperature, and time. This process creates an antimicrobial peptide that is capable of providing broad-spectrum antimicrobial activity and hydrating benefits.

Lactobacillus is a genus of microorganisms used to produce a variety of food products. It is a type of Lactic Acid Bacteria (LAB) and converts various sugars into lactic acid. Any existing LAB in Leucidal® Liquid SF is removed by filtration.

Due to its status as a product of LAB, the Federal Food, Drug and Cosmetic Act classifies materials such as Leucidal® Liquid SF as Generally Recognized as Safe (GRAS). This knowledge combined with the toxicity data provided allows us to support the safety of Leucidal® Liquid SF in cosmetic applications at use levels of 2% to 4%.

The FDA (Food and Drug Administration) states in sections 201 and 409 of the Federal Food, Drug and Cosmetic Act that “any substance that is intentionally added to food is a food additive, that is subject to review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under conditions of its use or unless the use of the substance is otherwise excluded for the definition of a food additive.”¹

To comply with global animal testing regulations (Directive 76/768/ECC), Active Micro Technologies, LLC does not test its products on animals. Therefore, Leucidal® Liquid SF has not been tested for NOAEL. The component materials that are used to make our products have not been subject to animal testing or re-testing for cosmetic purposes by us or on our behalf.

In vitro dermal and ocular irritation studies were conducted to evaluate whether Leucidal® Liquid SF would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. Test substances were applied to the tissue inserts and incubated. Cell viability was measured by dehydrogenase conversion of MTT, present in cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical was dictated by the reduction in tissue viability of exposed tissues compared to the negative control. Under conditions of this assay, the test article was considered to be non-irritating in both models. The substances used in these assays were undiluted. Please find attached a copy of these results.

In vitro phototoxicity irritation studies were conducted to evaluate whether Leucidal® Liquid SF would induce phototoxic irritation in the EpiDerm™ model assay. Test solution was applied to tissue inserts at concentrations of 0.4%, 1.3%, and 3.8%. After the required incubation, tissue inserts were irradiated for 60 minutes with 1.7 mW/cm² (=6 J/cm²). Cell viability was measured by dehydrogenase conversion of MTT, present in cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The photoirritation potential of the test chemical was dictated by the reduction in tissue viability of UVA exposed tissues compared to non-UVA exposed tissues. Under conditions of this assay the test article was considered to be non-phototoxic at tested concentrations. The negative and positive controls performed as anticipated.

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Safety Statement

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Additionally, a Human Subject Repeat Insult Patch Test Skin Irritation/Sensitization evaluation was completed to determine if Leucidal[®] Liquid SF was classified as a sensitizing agent. Under the reported testing conditions, results indicated that Leucidal[®] Liquid SF was not a primary sensitizer and a non-irritating material. Please find attached a copy of these results as well.

A *Salmonella typhimurium* reverse mutation standard plate incorporation study was conducted to evaluate whether Leucidal[®] Liquid SF would cause mutagenic changes in the average number of revertants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and WP2*uvrA* in the presence and absence of S9 metabolic activation. This study was conducted to satisfy, in part, the Genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity. Under the conditions of this assay, the test article solution was considered to be nonmutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and WP2*uvrA*. The product was tested undiluted and the negative and positive controls performed as anticipated.

In summary, several data sets exist to support the safety of Leucidal[®] Liquid SF. The molecular weight of this product is larger than what is required to penetrate skin. Therefore, hazards that may otherwise occur via this route are not an issue. It is presented in an aqueous carrier, all but eliminating its risk for inhalation. Toxicological, irritation, and sensitization assays have all been performed with favorable results for each. Therefore, it is logically concluded that Leucidal[®] Liquid SF is safe for use at the recommended use level of 2.0 - 4.0% and no further testing is required.

1. Federal Food, Drug and Cosmetic Act. U.S Food and Drug Administration. www.fda.gov.

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Dermal and Ocular Irritation Tests

107 Technology Drive • Lincolnton, NC 28092
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Sample: Leucidal® Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form/Submission #: 484

Lot #: 29436

Sponsor: Active Micro Technologies, 107 Technology Drive, Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)

EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **Leucidal® Liquid SF** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be a **non-irritant**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO₂, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(3-4,5-dimethyl thiazole 2-yl)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

II. Materials

- A. Incubation Conditions:** 37°C at 5% CO₂ and 95% relative humidity
- B. Equipment:** Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
- C. Media/Buffers:** DMEM based medium; DPBS; sterile deionized H₂O
- D. Preparation:** Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
- E. Tissue Culture Plates:** Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
- F. Reagents:** MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
- G. Other:** Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Micro Technologies, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO₂ and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO₂ and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO₂, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO₂, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO₂, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD₅₇₀) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability $\leq 50\%$ for EpiDerm™ or $\leq 60\%$ for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

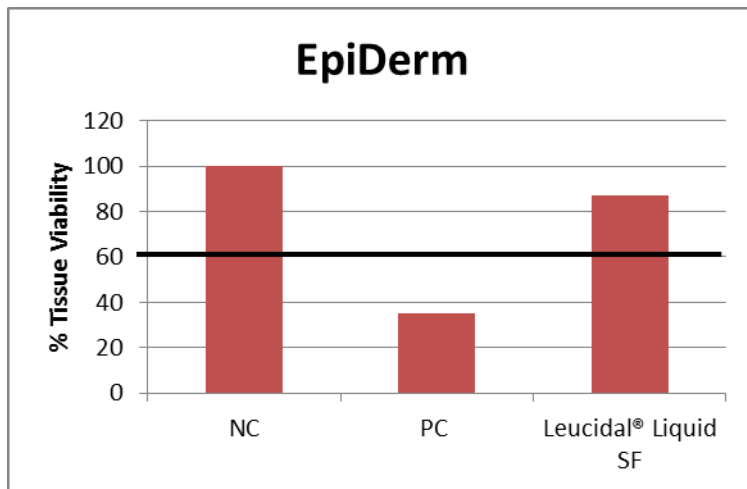


Figure 1: EpiDerm tissue viability

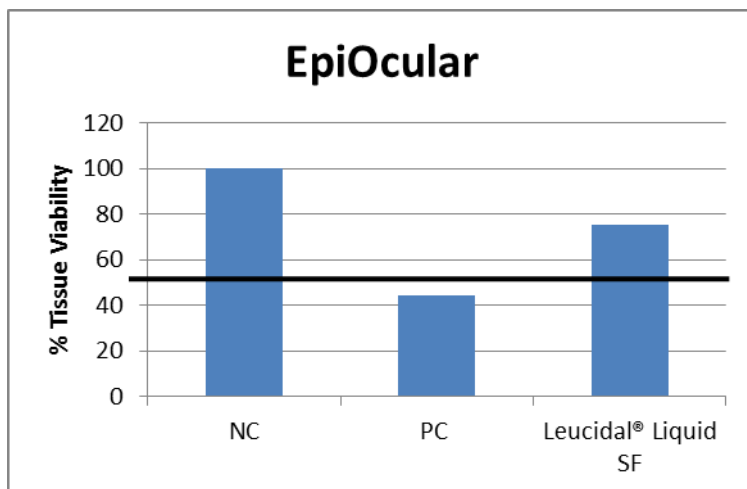


Figure 2: EpiOcular tissue viability



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50 HUMAN SUBJECT REPEAT INSULT PATCH TEST
SKIN IRRITATION/SENSITIZATION EVALUATION
(Occlusive Patch)

AMA Ref. No.: MS14.RIPT.N6324O.50.ACTC

Date: October 13, 2014

Sponsor: Active Concepts, LLC
107 Technology Drive
Lincolnton, North Carolina 28092

1.0 Objective:

Consumer products or raw materials designed for consistent reapplication to areas of the skin may, under proper conditions, prove to be contact sensitizers or irritants in certain individuals. It is the intention of a Repeat Insult Patch Test (RIPT) to provide a basis for evaluation of this irritation/sensitization potential if such exists.

2.0 Test Material:

2.1 Test Material Description:

On August 7, 2014 one test sample labeled Test Sample 1, Lot # NC140725-B was received from Active Concepts, LLC and assigned AMA Lab No. N-6324.

2.2 Handling:

Upon arrival at AMA Laboratories, Inc., the test material is assigned a unique laboratory code number and entered into a daily log identifying the lot number, sample description, sponsor, date received and tests requested.

Samples are retained for a period of three months beyond submission of final report unless otherwise specified by the sponsor or, if sample is known to be in support of governmental applications, representative retained samples are kept two years beyond final report submission.

Sample disposition is conducted in compliance with appropriate federal, state and local ordinances.

2.3 Test Material Evaluation Prerequisite:

Prior to induction of a human test panel, toxicology, microbiology or in-vitro performance spectra may be required to assess the feasibility of commencement as dictated by an Institutional Review Board (IRB) described in Section 3.0.

Sponsor purports that prior to sample submission the following tests were conducted with no adverse results and that the test data are on file on their premises and have not been made available to AMA personnel:

- USP or CTFA Preservative Efficacy Test or equivalent
- 90 Day Accelerated Stability and Container Compatibility Study

3.0 Institutional Review Board:

Reference: CFR Title 21 Part 56, Subparts A, B, C, and D. The IRB of AMA Laboratories, Inc., consists of five or more individuals, chosen from within the company for technical expertise and from the local community for lay interaction. The list of IRB members is kept on file at AMA Laboratories, Inc. and is available for inspection during the hours of operation.

4.0 Panel Selection:

4.1 Standards for Inclusion in a Study:

- Individuals who are not currently under a doctor's care.
- Individuals free of any dermatological or systemic disorder which would interfere with the results, at the discretion of the Investigator.
- Individuals free of any acute or chronic disease that might interfere with or increase the risk of study participation.
- Individuals who will complete a preliminary medical history form mandated by AMA Laboratories, Inc. and are in general good health.
- Individuals, who will read, understand and sign an informed consent document relating to the specific type of study they are subscribing. Consent forms are kept on file and are available for examination on the premises of AMA Laboratories, Inc. only.
- Individuals able to cooperate with the Investigator and research staff, willing to have test materials applied according to the protocol, and complete the full course of the study.

4.2 Standards for Exclusion from a Study:

- Individuals under 18 years of age.
- Individuals who are currently under a doctor's care.
- Individuals who are currently taking any medication (topical or systemic) that may mask or interfere with the test results.
- Subjects with a history of any acute or chronic disease that might interfere with or increase the risk associated with study participation.
- Individuals diagnosed with chronic skin allergies.
- Female volunteers who indicate that they are pregnant or lactating.

4.3 Recruitment:

Panel selection is accomplished by advertisements in local periodicals, community bulletin boards, phone solicitation, electronic media or any combination thereof.

4.4 Informed Consent and Medical History Forms:

An informed consent was obtained from each volunteer prior to initiating the study describing reasons for the study, possible adverse effects, associated risks and potential benefits of the treatment and their limits of liability. Panelists signed and dated the informed consent document to indicate their authorization to proceed and acknowledge their understanding of the contents. Each subject was assigned a permanent identification number and completed an extensive medical history form. These forms along with the signed consent forms, are available for inspection on the premises of AMA Laboratories, Inc. only. Reference 21 CFR Ch. 1 Part 50, Subpart B.

The parties agree to comply with applicable state and federal privacy laws for the use and disclosure of a subject's personal health information by taking reasonable steps to protect the confidentiality of this information. This obligation shall survive the termination or expiration of this Agreement.

5.0 Population Demographics:

Number of subjects enrolled	52
Number of subjects completing study	50
Age Range	22-68
Sex	Male 8
	Female 44
Race	Caucasian 46
	Hispanic 5
	Asian 1

6.0 Equipment:

- Patch Description: Parke-Davis Hypoallergenic Readi Bandages or the equivalent.
- 1ml volumetric syringe without a needle.

7.0 Procedure:

- Subjects are requested to bathe or wash as usual before arrival at the facility.
- As per client request, the test material N-6324 was diluted to 4% in distilled water. Dilutions were freshly prepared on each application day.
- 0.2 ml of the test material is dispensed onto the occlusive, hypoallergenic patch.
- The patch is then applied directly to the skin of the infrascapular regions of the back, to the right or left of the midline and the subject is dismissed with instructions not to wet or expose the test area to direct sunlight.
- After 24 hours the patch is removed by the panelist at home.
- This procedure is repeated until a series of nine consecutive 24 hour exposures have been made for every Monday, Wednesday, and Friday for three consecutive weeks.
- In the event of an adverse reaction, the area of erythema and edema is measured. The edema is estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin. Reactions are scored just before applications two through nine and the next test date following application nine. In most instances this is approximately 24 hours after patch removal. Clients are notified immediately in the case of adverse reaction and determination is made as to treatment program if necessary.
- Subjects are then given a 10 - 14 day rest period after which a challenge or retest dose is applied once to a previously unexposed test site. The retest dose is equivalent to any one of the original nine exposures. Reactions are scored 24 and 48 hours after application.
- Comparison is made between the nine inductive responses and the retest dose.

8.0 Results:

Please refer to attached Table.

9.0 Observations:

No adverse reactions of any kind were noted during the course of this study.

10.0 Archiving:

All original samples, raw data sheets, technician's notebooks, correspondence files and copies of final reports and remaining specimens are maintained on premises of AMA Laboratories, Inc. in limited access storage files marked "Archive". A duplicate disk copy of final reports is separately archived in a bank safe deposit vault.

11.0 Reference:

Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics, published by The Association of Food and Drug Officials of The United States, 1965 (modified).

12.0 Security Label Disclosure:

To prevent loss of and protect intellectual property, original, certified documents issued by AMA Laboratories Inc. can be identified by a proprietary, tamper evident security hologram affixed to all Conclusion/Signature pages on final reports. Any attempt to remove the hologram will irreversibly damage the label and leave an immediate trace, thus invalidating the document.

Only reports containing the AMA LABS, INC. hologram intact will be recognized by AMA Laboratories Inc. as a certified original.

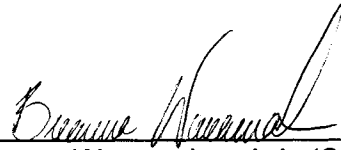
13.0 Conclusions:

The test material (AMA Lab. No.: N-6324; Client No.: Test Sample 1, Lot # NC140725-B) when tested under occlusion at a 4% dilution in distilled water as described herein, may be considered:

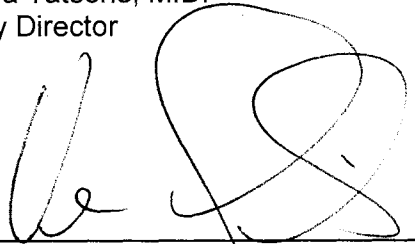
a **NON-PRIMARY IRRITANT** and **NON-PRIMARY SENSITIZER** to the skin according to the reference.



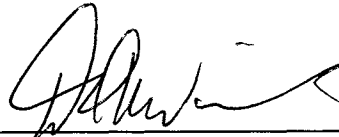
Mayya Tatsene, M.D.
Study Director



Breanna Wanamaker, A.A. (Candidate)
Technician



Vera Jelic, B.A. (Candidate)
Technician



David R. Winne, B.S.
Technical Director

10/13/14

Date



TABLE
SUMMARY OF RESULTS
(Occlusive Patch)

AMA Lab No.: N-6324
 Client No.: Test Sample 1, Lot # NC140725-B
 Dilution: 4% in distilled water

No.	Subject ID	R A C E	S E X	Response									Chall.		Score
				1	2	3	4	5	6	7	8	9	24 HR	48 HR	
1	25 0215	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
2	32 4178	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
3	36 9096	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
4	40 1274	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
5	40 2040	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
6	42 5472	C	F	0	0	0	0	0	Dc	Dc	Dc	Dc	Dc	Dc	N/A
7	44 7118	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
8	44 9339	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
9	44 9509	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
10	46 7866	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
11	48 0648	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
12	48 0738	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
13	48 0946	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
14	48 2320	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
15	48 2675	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
16	48 3564	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
17	48 4541	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
18	50 4689	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
19	52 4017	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
20	52 7983	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
21	52 9833	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
22	54 1112	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
23	54 3239	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
24	54 5868	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
25	54 9679	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
26	54 9874	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
27	56 1236	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
28	60 3008	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
29	60 9466	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0

TABLE (CONT'D)
SUMMARY OF RESULTS
(Occlusive Patch)

AMA Lab No.: N-6324
 Client No.: Test Sample 1, Lot # NC140725-B
 Dilution: 4% in distilled water

No.	Subject ID	R A C E	S E X	Response									Chall.		Score
				1	2	3	4	5	6	7	8	9	24 HR	48 HR	
30	62 5697	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
31	64 6653	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
32	64 9034	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
33	66 8507	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
34	68 6060	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
35	68 7601	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
36	70 2480	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
37	72 3555	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
38	72 3637	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
39	72 6941	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
40	73 6193	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
41	74 0600	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
42	74 1855	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
43	74 8531	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
44	76 1298	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
45	76 7056	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
46	76 8434	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
47	78 8260	A	F	0	0	0	0	0	0	0	0	0	0	0	0.0
48	80 0080	C	M	0	0	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
49	80 0847	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
50	80 8984	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
51	82 7228	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
52	90 5388	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0

Evaluation Period:

This study was conducted from September 8, 2014
 through October 10, 2014.

Scoring Scale and Definition of Symbols Shown in Table:


- 0 - No evidence of any effect
- ? - (Barely perceptible) minimal faint (light pink) uniform or spotty erythema
- 1 - (Mild) pink uniform erythema covering most of contact site
- 2 - (Moderate) pink\red erythema visibly uniform in entire contact area
- 3 - (Marked) bright red erythema with accompanying edema, petechiae or papules
- 4 - (Severe) deep red erythema with vesiculation or weeping with or without edema
- D - Patch eliminated due to reaction
- Dc - Discontinued due to absence of subject on application date
- M - Patch applied to an adjacent site after strong test reaction
- N/A - Score is not calculated for subjects discontinued before challenge
- S - Skin stained from pigment in product
- T - Tan

NOTE: All technical employees of AMA LABORATORIES, INC. are required to take and pass a visual discrimination examination conducted by a Board Certified Ophthalmologist using the Farnsworth-Munsell 100 Hue Test as published; which determines a person's ability to discern color against a black background. This test was additionally modified to include a flesh tone background more nearly approaching actual use conditions, wherein erythematous skin is graded according to intensity.

14.0 Quality Assurance Statement:

This study was inspected in accordance with the Standard Operating Procedures of AMA Laboratories, Inc. To assure compliance with the study protocol, the Quality Assurance Unit completed an audit of the study records and report.

Report reviewed by:



Tasmia Masud, B.A.
Quality Assurance Supervisor

10/13/14

Date



OECD TG 442C: In Chemico Skin Sensitization

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: Leucidal® Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 1238

Lot #: 4808P

Sponsor: Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

OECD TG 442C: *In Chemico* Skin Sensitization
Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact¹. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis². The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%)³.

This assay was conducted to determine skin sensitization hazard of **Leucidal® Liquid SF** in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report; pp 1 -74.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied.
This information is offered solely for your investigation, verification, and consideration.

Materials

- | | |
|-------------------------------|---|
| A. Equipment: | HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector);
Pipettes; Analytical balance |
| B. HPLC/Guard Columns: | Agilent Zorbax SB-C18 2.1mm x 100mm x 3.5µm; Phenomenex
Security Guard C18 4mm x 2mm |
| C. Chemicals: | Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide;
Acetonitrile; Cysteine peptide (Ac-RFAACAA-COOH); Lysine peptide
(Ac-RFAAKAA-COOH); Cinnamic aldehyde |
| D. Reagents/Buffers: | Sodium phosphate buffer (100mM); Ammonium acetate buffer
(100mM) |
| E. Other: | Sterile disposable pipette tips |

Methods

Solution Preparation:

- 0.667mM Cysteine Peptide in 100mM Phosphate Buffer (pH 7.5)
- 0.667mM Lysine Peptide in 100mM Ammonium Acetate Buffer (pH 10.2)
- 100mM Cinnamic Aldehyde in Acetonitrile
- 100mM **Leucidal® Liquid SF** in Acetonitrile

Reference Controls:

- Reference Control A: For calibration curve accuracy
- Reference Control B: For peptide stability over analysis time of experiment
- Reference Control C: For verification that the solvent does not impact percent peptide depletion

Sample, Reference Control, and Co-Elution Control Preparation:

- Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
- Each chemical should be analyzed in triplicate.

1:10 Ratio, Cysteine Peptide 0.5mM Peptide, 5mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5mM Peptide, 25mM Test Chemical
<ul style="list-style-type: none"> • 750µL Cysteine Peptide Solution (or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls) • 200µL Acetonitrile • 50µL Test Chemical Solution (or Acetonitrile for Reference Controls) 	<ul style="list-style-type: none"> • 750µL Lysine Peptide Solution (or 100mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls) • 250µL Test Chemical Solution (or Acetonitrile for Reference Controls)

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Calibration Curve:

- Standards are prepared in a solution of 20% Acetonitrile:Buffer
 - For the Cysteine peptide using the phosphate buffer, pH 7.5
 - For the Lysine peptide using the ammonium acetate buffer, pH 10.2

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

HPLC Analysis:

- HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
- Absorbance is measured at 220nm
- Flow Conditions:

Time	Flow	%A	%B
0 minutes	0.35 mL/min	90	10
10 minutes	0.35 mL/min	75	25
11 minutes	0.35 mL/min	10	90
13 minutes	0.35 mL/min	10	90
13.5 minutes	0.35 mL/min	90	10
20 minutes	End Run		

Data and Reporting
Acceptance Criteria:

1. The following criteria must be met for a run to be considered valid:
 - a. Standard calibration curve should have an $r^2 > 0.99$.
 - b. Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be <14.9 for the percent cysteine depletion and <11.6 for the percent lysine depletion.
 - c. Mean peptide concentration of reference controls A should be 0.50 ± 0.05 mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be <15.0%.
2. The following criteria must be met for a test chemical's results to be considered valid:
 - a. Maximum standard deviation should be <14.9 for percent cysteine depletion and <11.6 for percent lysine depletion.
 - b. Mean peptide concentration of the three reference control C should be 0.50 ± 0.05 mM.



OECD TG 442C: In Chemico Skin Sensitization

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Prediction Model:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	Reactivity Class	Prediction
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

If co-elution occurs with the lysine peptide, than the cysteine 1:10 prediction model can be used:

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	Reactivity Class	Prediction
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

Results and Discussion

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

Percent peptide depletion is determined by the following equation:

$$\text{Percent Peptide Depletion} = \left[1 - \left(\frac{\text{Peptide Peak Area in Replicate Injection}}{\text{Mean Peptide Peak Area in Reference Controls C}} \right) \right] \times 100$$

Based on HPLC-UV analysis of **Leucidal® Liquid SF (code M15019)** we can determine that this product is not a sensitizer and will not cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.51% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

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OECD TG 442D: In Vitro Skin Sensitization

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Tradename: Leucidal® Liquid SF

Code: M15019

CAS #: 68333-15-3

Test Request Form #: 1193

Lot #: 40015P

Sponsor: Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

OECD TG 442D: In Vitro Skin Sensitization
ARE-Nrf2 Luciferase Test Method

Introduction

Skin sensitization refers to an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals¹. Substances are classified as skin sensitizers if there is evidence in humans that the substance can lead to sensitization by skin contact or positive results from appropriate tests, both *in vivo* and *in vitro*. Utilization of the KeratinoSens™ cell line allows for valid *in vitro* testing for skin sensitization.

This assay was conducted to determine skin sensitization potential of **Leucidal® Liquid SF** in accordance with the UN GHS.

Assay Principle

The ARE-Nrf2 luciferase test method addresses the induction of genes that are regulated by antioxidant response elements (ARE) by skin sensitizers. The Keap1-Nrf2-ARE pathways have been shown to be major regulator of cytoprotective responses to oxidative stress or electrophilic compounds. These pathways are also known to be involved in the cellular processes in skin sensitization. Small electrophilic substances such as skin sensitizers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The skin sensitization assay utilizes the KeratinoSens™ method which uses an immortalized adherent human keratinocyte cell line (HaCaT cell line) that has been transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE). This test method has been validated by independent peer review by the EURL-ECVAM. The addition of a luciferin containing reagent to the cells will react with the luciferase produced in the cell resulting in luminescence which can be quantified with a luminometer.

1. United Nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013
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Materials

- | | |
|----------------------------------|--|
| A. Incubation Conditions: | 37°C at 5% CO ₂ and 95% relative humidity (RH) |
| B. Equipment: | Humidified incubator; Biosafety laminar flow hood; Microplate Reader; Pipettes |
| C. Cell Line: | KeratinoSens™ by Givaudan Schweiz AG |
| D. Media/Buffers: | Dulbecco's Modified Eagle Medium (DMEM); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS); Geneticin |
| E. Culture Plate: | Flat bottom 96-well tissue culture treated plates |
| F. Reagents: | Dimethyl Sulfoxide (DMSO); Cinnamic Aldehyde; ONE-Glo Reagent; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); sodium lauryl sulfate (SLS) |
| G. Other: | Sterile disposable pipette tips; wash bottles |

Methods

KeratinoSens™ were into seeded four 96-well tissue culture plates and allowed to grow to 80 – 90% confluency in DMEM containing 10% FBS and 500µg/mL G418 geneticin. Twelve test concentrations of **Leucidal® Liquid SF** were prepared in DMSO with a concentration range from 0.98 – 2000 µM. These 12 concentrations were assayed in triplicate in 2 independently performed experiments. The positive control was cinnamic aldehyde for which a series of 5 concentrations prepared in DMSO had final test concentrations of 4 – 64µM. The negative control was a 1% test concentration of DMSO.

24 hour post KeratinoSens™ seeding, the culture media was removed and replaced with fresh media containing 10% FBS without G418 geneticin. 50 µL of the above described test concentrations was added to the appropriate wells. The treated plates were then incubated for 48 hours at 37°C in the presence of 5% CO₂ and 95% relative humidity. After treatment incubation was complete the media was removed and the wells were washed with PBS 3 times.

One of the four plates was used for a cytotoxicity endpoint, where MTT was added to the wells and incubated for 4 hours at 37°C in the presence of 5% CO₂. SLS was then added to the wells and incubated overnight at room temperature. A spectrometer measured the absorbance at 570 nm. The absorbance values (optical density) were then used to determine the viability of each well by comparing the optical density of each test material treated well to that of the solvent control wells to determine the IC₅₀ and IC₃₀ values.

The remaining 3 plates were used in the luciferase induction endpoint of the assay. 100 µL of Promega's ONE-Glo Reagent was added to 100 µL of fresh media containing 10% FBS without geneticin. Cells were incubated for 5 minutes to induce cell lysis and release luciferin into the media. Plates were read with a luminometer and EC_{1.5} and maximum response (I_{max}) values were obtained.

Data and Reporting

Acceptance Criteria:

1. Gene induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations (from 4 to 64 µM).
2. The EC_{1.5} value should be within two standard deviations of the historical mean and the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.
3. The average coefficient of variability of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each experiment.

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A KeratinoSens™ prediction is considered positive if the following conditions are met:

1. The I_{max} is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
2. The cellular viability is higher than 70% at the lowest concentration with a gene induction above 1.5 fold (i.e., at the EC_{1.5} determining concentration)
3. The EC_{1.5} value is less than 1000 μM (or < 200 μg/ml for test chemicals with no defined MW)
4. There is an apparent overall dose-response for luciferase induction

Results

Compound	Classification	EC _{1.5} (μM)	IC ₅₀	I _{max}
Cinnamic aldehyde	Sensitizer	19	289.19 μM	31.6
DMSO	Non-Sensitizer	No Induction	243.24 μM	1.2
Leucidal® Liquid SF	Non-Sensitizer	No Induction	> 1000 μM	0.4

Table 1: Overview of KeratinoSens™ Assay Results

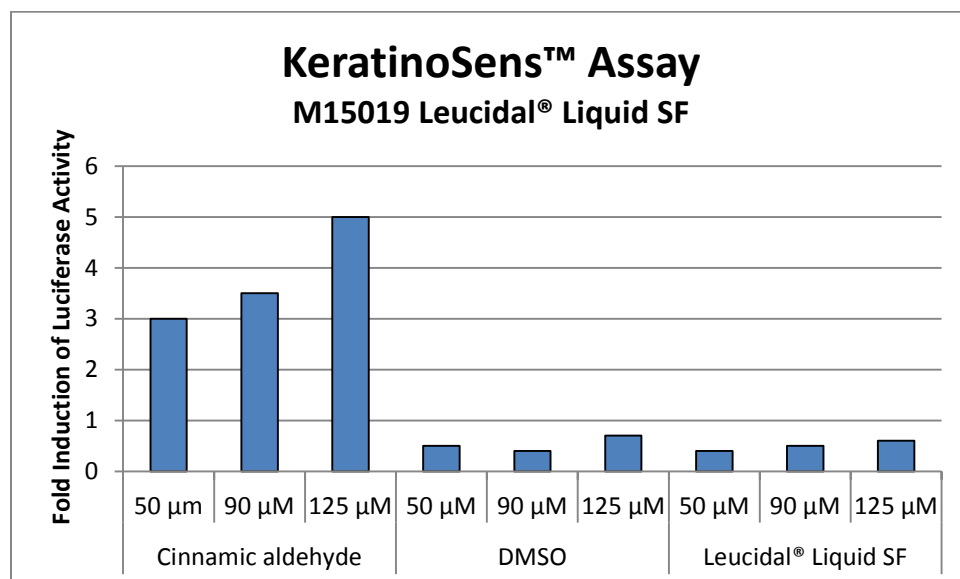


Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **Leucidal® Liquid SF (code M15019)** was not predicted to be a skin sensitizer based on the KeratinoSens™ ARE-Nrf2 Luciferase Test Method as there was not a significant increase in luciferase expression. It can be concluded that **Leucidal® Liquid SF** can be safely used in cosmetics and personal care products at typical use levels.

Test Article: Leucidal® Liquid SF
Code Number: M15019
CAS #: 1686112-36-6

Sponsor:
Active Micro Technologies, LLC
107 Technology Drive
Lincolnton, NC 28092

Study Director: *Erica Segura*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

Test Request Number: 841

SUMMARY

A *Salmonella typhimurium*/*Escherichia coli* reverse mutation standard plate incorporation study described by Ames *et al.* (1975) was conducted to evaluate whether a test article solution **Leucidal® Liquid SF** would cause mutagenic changes in the average number of revertants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2uvrA in the presence and absence of Aroclor-induced rat liver S9. This study was conducted to satisfy, in part, the Genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

The stock test article was tested at eight doses levels along with appropriate vehicle control and positive controls with overnight cultures of tester strains. The test article solution was found to be noninhibitory to growth of tester strain TA98, TA100, TA1537, TA1535 and WP2uvrA after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45°C supplemented with histidine-biotin solution for the *Salmonella typhimurium* strains and supplemented with tryptophan for *Escherichia coli* strain were inoculated with 100 µl of tester strains, 100 µl of vehicle or test article dilution were added and 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. After vortexing, the mixture was poured across the Minimal Glucose Agar (GMA) plates. Parallel testing was also conducted with positive control correspond to each strain, replacing the test article aliquot with 50µl aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37°C. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control plates for each of the strains tested. The means obtained for the positive controls were used as points of reference.

Under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* tester strain WP2uvrA. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

I. Introduction

A. Purpose

A *Salmonella typhimurium*/*Escherichia coli* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution would cause mutagenic changes in the average number of revertants for *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2uvrA in the presence and absences of the S9 metabolic activation. Bacterial reverse mutation tests have been widely used as rapid screening procedures for the determination of mutagenic and potential carcinogenic hazards.

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II. Materials

- A. **Storage Conditions:** Room temperature (23-25C).
 B. **Vehicle:** Sterile DI Water.
 C. **Preparation:** Eight different doses level were prepared immediately before use with sterile DI water.
 D. **Solubility/Stability:** 100% Soluble and Stable.
 E. **Toxicity:** No significant inhibition was observed.

III. Test System

A. Test System

Each *Salmonella typhimurium* and *Escherichia coli* tester strain contains a specific deep rough mutation (*rfa*), the deletion of *uvrB* gene and the deletion in the *uvrA* gene that increase their ability to detect mutagens, respectively. These genetically altered *Salmonella typhimurium* strains (TA98, TA100, TA1537 and TA1535) and *Escherichia coli* strain (WP2*uvrA*) cannot grow in the absence of histidine and tryptophan, respectively. When placed in a histidine-tryptophan free medium, only those cells which mutate spontaneously back to their wild type states are able to form colonies. The spontaneous mutation rate (or reversion rate) for any one strain is relatively constant, but if a mutagen is added to the test system, the mutation rate is significantly increased.

<u>Tester strain</u>	<u>Mutations/Genotypic Relevance</u>
TA98	hisD3052, Dgal chlD bio <i>uvrB rfa</i> pKM101
TA100	hisG46, Dgal chlD BIO <i>uvrB rfa</i> pKM101
TA1537	hisC3076, <i>rfa</i> , Dgal chlD bio <i>uvrB</i>
TA 1535	hisG46, Dgal chlD bio <i>uvrB rfa</i>
WP2 <i>uvrA</i>	trpE, <i>uvrA</i>

<i>rfa</i>	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
<i>uvrB</i>	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
<i>uvrA</i>	=	All possible transitions and transversions, small deletions.

B. Metabolic Activation

Aroclor induced rat liver (S9) homogenate was used as metabolic activation. The S9 homogenate is prepared from male Sprague Dawley rats. Material is supplied by MOLTOX, Molecular Toxicology, Inc.

C. Preparation of Tester strains

Cultures of *Salmonella typhimurium* TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvrA* were inoculated to individual flasks containing Oxoid broth No.2. The inoculated broth cultures were incubated at 37°C in an incubator shaker operating at 140-150 rpm for 12-16 hours.

D. Negative Control

Sterile DI water (vehicle without test material) was tested with each tester strain to determine the spontaneous reversion rate. Each strain was tested with and without S9 activation. These data represented a base rate to which the number of revertants colonies that developed in each test plate were compared to determine whether the test material had significant mutagenic properties.

E. Positive Control

A known mutagen for each strain was used as a positive control to demonstrate that tester strains were sensitive to mutation to the wild type state. The positive controls are tested with and without the presence of S9 homogenate.

F. Titer of the Strain Cultures:

Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth; to confirm this, serial dilutions from each strain were conducted, indicating that the initial population was in the range of 1 to 2×10^9 /ml.

IV. Method

A. Standard Plate Incorporation Assay:

Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* and tryptophan for *Escherichia coli* were inoculated with 100 μ l of culture for each strain and 100 μ l of testing solution or vehicle without test material. A 500 μ l aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across Minimal Glucose Agar plates labeled with strain number and S9 activation (+/-). When plating the positive controls, the test article aliquot was replaced by 50 μ l aliquot of appropriate positive control. The test was conducted per duplicate. The plates were incubated for 37°C for 2 days. Following the incubation period, the revertant colonies on each plate were recorded. The mean number of revertants was determined. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control of each strain used.

V. Evaluation

For the test solution to be evaluated as a test failure or “potential mutagen” there must have been a 2-fold or greater increase in the number of mean revertants over the means obtained from the negative control for any or all strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* tester strain used.

VI. Results and Discussion

A. Solubility:

Water was used as a solvent. Solutions from the test article were made from 0.015 to 50mg/ml.

B. Dose levels tested:

The maximum dose tested was 5000 μ g per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 μ g per plate.

C. Titer (Organisms/ml):

5×10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2×10^9 UFC/ml.

C. Standard Plate Incorporation Assay

In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA98, TA100, TA1537, TA1535 and WP2uvrA in the presence of the test solution compared with the mean of vehicle control value. The positive controls mean exhibited at least a 3-fold increase over the respective mean of the *Salmonella typhimurium* and *Escherichia coli* tester strains used. The results are summarized in Appendix 2.

VII. Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2uvrA. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

Appendix 2:

Bacterial Mutation Assay Plate Incorporation Assay Results

	Concentration µg per Plate	TA98		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	29	35	32
	1500	19	17	18
	500	21	23	22
	150	18	30	24
	50	31	22	27
	15	18	20	19
	5.0	22	21	22
	1.5	19	19	19
Test Solution w/o S9	5000	20	22	21
	1500	35	32	34
	500	17	19	18
	150	20	20	20
	50	20	23	22
	15	21	21	21
	5.0	24	21	23
	1.5	22	18	20
DI Water w/S9		34	18	20
DI Water w/o S9		4	16	10
2-aminoanthracen w/ S9		365	387	376
2-nitrofluorene w/o S9		295	211	253
Historical Count Positive w/S9		43-1893		
Historical Count Positive w/o S9		39-1871		
Historical Count Negative w/S9		4-69		
Historical Count Negative w/o S9		3-59		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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	Concentration µg per Plate	TA100		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	144	60	102
	1500	124	144	134
	500	224	163	180
	150	236	240	238
	50	180	168	174
	15	160	188	174
	5.0	148	300	224
	1.5	172	136	154
Test Solution w/o S9	5000	112	125	119
	1500	100	103	102
	500	135	140	138
	150	80	48	64
	50	92	108	100
	15	132	160	146
	5.0	108	192	150
	1.5	148	176	162
DI Water w/S9		168	144	156
DI Water w/o S9		180	46	113
2-aminoanthracen w/ S9		450	437	444
Sodium azide w/o S9		520	408	464
Historical Count Positive w/S9		224-3206		
Historical Count Positive w/o S9		226-1837		
Historical Count Negative w/S9		55-268		
Historical Count Negative w/o S9		47-250		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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	Concentration µg per Plate	TA1537		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	11	9	10
	1500	13	12	13
	500	7	14	10
	150	11	6	9
	50	10	4	7
	15	13	8	11
	5.0	8	14	11
	1.5	13	8	11
Test Solution w/o S9	5000	30	20	25
	1500	19	10	15
	500	10	8	9
	150	4	8	6
	50	24	19	22
	15	10	12	11
	5.0	18	12	15
	1.5	16	10	13
DI Water w/S9		9	3	6
DI Water w/o S9		13	16	15
2-aminoanthracen w/ S9		314	312	313
2-aminoacridine w/o S9		320	304	312
Historical Count Positive w/S9		13-1934		
Historical Count Positive w/o S9		17-4814		
Historical Count Negative w/S9		0-41		
Historical Count Negative w/o S9		0-29		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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	Concentration µg per Plate	TA1535		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	24	21	22
	1500	13	7	10
	500	28	21	25
	150	20	15	16
	50	22	20	21
	15	24	11	18
	5.0	22	32	27
	1.5	24	13	19
Test Solution w/o S9	5000	88	82	85
	1500	72	95	84
	500	81	80	81
	150	84	80	81
	50	12	8	10
	15	23	21	22
	5.0	8	18	13
	1.5	21	16	19
DI Water w/S9		18	15	17
DI Water w/o S9		18	30	24
2-aminoanthracen w/ S9		228	217	223
Sodium azide w/o S9		408	480	444
Historical Count Positive w/S9		22-1216		
Historical Count Positive w/o S9		47-1409		
Historical Count Negative w/S9		1-50		
Historical Count Negative w/o S9		1-45		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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	Concentration µg per Plate	<i>WP2uvrA</i>		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	12	12	12
	1500	13	39	26
	500	43	40	42
	150	56	25	41
	50	38	48	43
	15	49	44	47
	5.0	57	55	56
	1.5	50	65	58
Test Solution w/o S9	5000	81	65	73
	1500	40	51	46
	500	25	32	29
	150	29	16	23
	50	33	30	32
	15	40	40	40
	5.0	50	44	47
	1.5	41	54	48
DI Water w/S9		48	41	45
DI Water w/o S9		50	51	51
2-aminoanthracen w/ S9		501	522	512
Methylmethanesulfonate w/o S9		360	230	300
Historical Count Positive w/S9		44-1118		
Historical Count Positive w/o S9		42-1796		
Historical Count Negative w/S9		8-80		
Historical Count Negative w/o S9		8-84		

*CFU = Colony Forming Units

*Mean = Average of duplicate plates

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Phototoxicity Assay Analysis

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: Leucidal® Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 1120

Lot #: 31738

Sponsor: Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

In Vitro EpiDerm™ Model (EPI-200-SIT) Phototoxicity

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **Leucidal® Liquid SF** would induce phototoxic irritation in the EpiDerm™ model assay.

The product was tested according to the manufacturer's protocol. The test article solution was found to be a **non-photoirritant** at concentrations of 0.4%, 1.3%, and 3.8%. Reconstructed human epidermis was incubated in growth media for one hour to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substance was applied to the tissue inserts in five varying concentrations and incubated overnight at 37°C, 5% CO₂, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm² (=6 J/cm²). After substance incubation, irradiation, and washing were complete, the cell viability test was conducted. Cell viability was measured by dehydrogenase conversion of MTT [(3-4,5-dimethyl thiazole 2-yl)], present in the cell mitochondria, into blue formazan salt that was measured after extraction from the tissue. The photoirritation potential of the test chemical was dictated by the reduction in tissue viability of UVA exposed tissues compared to non-UVA exposed tissues.

Under the conditions of this assay, the test article was considered to be **non-phototoxic** at concentrations of 0.4%, 1.3%, and 3.8%. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal phototoxicity study was conducted to evaluate whether a test article would induce photoirritation in the EpiDerm™ model assay. MatTek Corporation's reconstructed human epidermal model is becoming a standard in determining the phototoxicity potential of a test substance. This assay is able to discriminate between photoirritants and non-photoirritants at varying concentrations.

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II. Materials

- A. Incubation Conditions:** 37°C at 5% CO₂ and 95% relative humidity
- B. Equipment:** Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; UVA-vis Irradiation Equipment; UVA meter; Pipettes
- C. Media/Buffers:** Dulbecco's Modified Eagle Medium (DMEM) based medium; Dulbecco's Phosphate-Buffered Saline (DPBS); sterile deionized H₂O
- D. Preparation:** Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
- E. Tissue Culture Plates:** Falcon flat bottom 96-well, 24-well, and 6-well tissue culture plates
- F. Reagents:** MTT (3-4,5-dimethyl thiazole 2-yl) (1.0mg/mL); Extraction Solution (Isopropanol); Chlorpromazine; Triton X-100 (1%)
- G. Other:** Wash bottle; sterile disposable pipette tips; Parafilm; forceps

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™ consists of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis. This model consists of organized basal, spinous, and granular layers, and contains a multilayer stratum corneum containing intercellular lamellar lipid layers. The EpiDerm™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile deionized water is used as the negative controls for the EpiDerm™ Phototoxicity assay.

C. Positive Control

Concentrations of chlorpromazine, ranging from 0.001% to 0.1%, were used as positive controls for the EpiDerm™ Phototoxicity assay.

D. Data Interpretation Procedure

A photoirritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance and 60 minutes of 6 J/cm² is reduced by 20% compared to the non-irradiated control tissues.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Micro Technologies, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO₂ and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and tissue insert dosing begins.

B. Test Substance Exposure

50µL of the diluted test substance in their respective concentrations are applied to 2 tissue inserts and allowed to incubate for overnight in a humidified incubator (37°C, 5% CO₂, 95% RH).

C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm² at room temperature. The non-irradiated tissue inserts are incubated at room temperature in the dark.

D. Tissue Washing and Post Incubation

After UVA-irradiation and dark incubation is complete the tissue inserts are washed using sterile DPBS and transferred to fresh 6-well plates and media for overnight incubation at 37°C, 5% CO₂, 95% RH.

E. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO₂, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD₅₇₀) is ≥ 0.8.

B. Positive Control

The assay meets the acceptance criterion if a dose dependent reduction in cell viability in the UVA-irradiated tissues is between 0.00316% and 0.0316%.

C. Standard Deviation

Since the phototoxicity potential is predicted from the mean viability of 2 tissues for the EpiDerm™ Phototoxicity Protocol, the variability of the replicates should not exceed 30%.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ assay kit were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. Cell viability is calculated for each tissue as a percentage of the corresponding vehicle control either irradiated or non-irradiated. Tissue viability was not reduced by 20% in the presence of the test substance and UVA-irradiation at concentrations of 0.4%, 1.3%, and 3.8%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay. The negative and positive controls performed as anticipated.

VII. Conclusion

Phototoxicity (photoirritation) is defined as an acute toxic response that is elicited after exposure of the skin to certain chemicals and subsequent exposure to light. Under the conditions of this assay, the test article substance was considered to be **non-phototoxic** at concentrations of 0.4%, 1.3%, and 3.8%. There is a decrease in viability at the 12% test concentration with and without irradiation. Using any test substance at this high of a concentration will have noticeable effects on cellular viability due to the fact that that substance is replacing the cell's nutrients. We can safely say that **Leucidal® Liquid SF** is not a photoirritant when used at the suggested use levels of 2.0 – 4.0%.

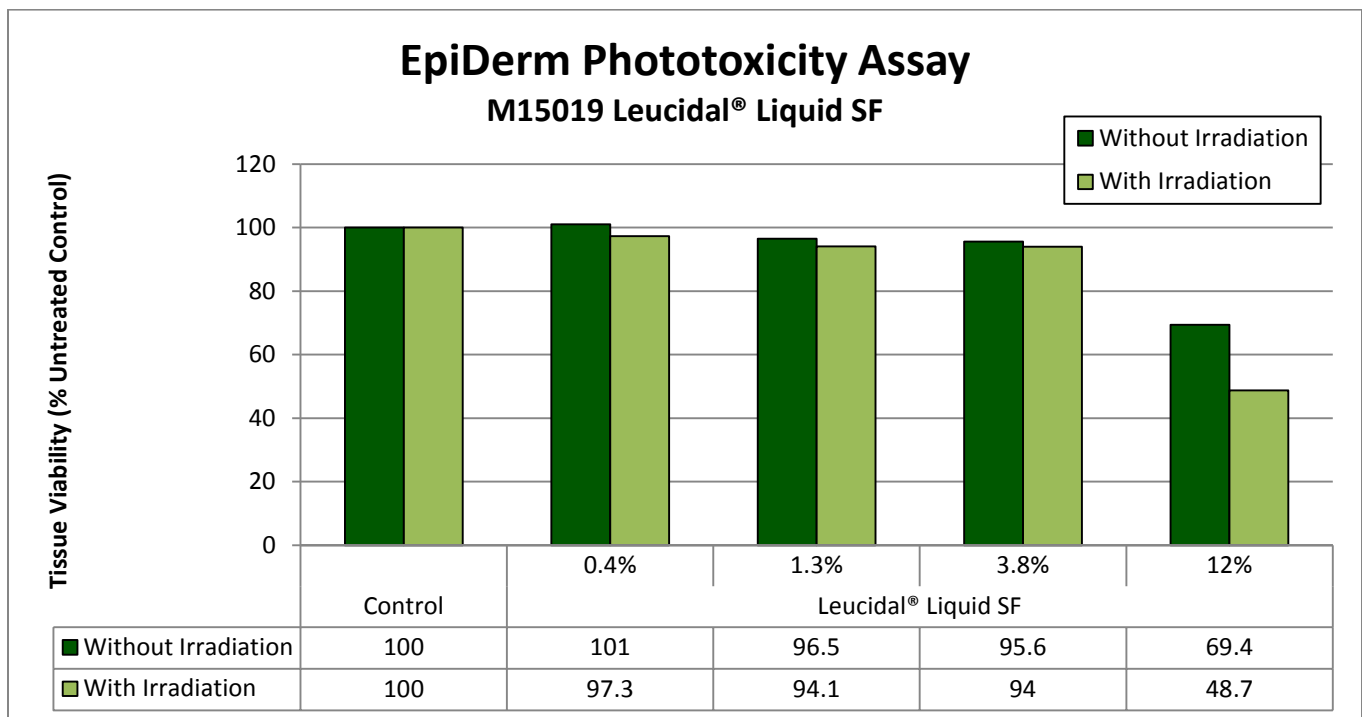


Figure 1: EpiDerm Phototoxicity Graph



OECD 202 Acute *Daphnia* Assay

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: Leucidal[®] Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 581

Lot #: 31738

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Erica Segura

Principle Investigator: Meghan Darley

Test Performed:

OECD 202

Daphnia spp. Acute Immobilization Test

Introduction

The purpose of the present study is to determine the toxicity of **Leucidal[®] Liquid SF** by exposing *Daphnia* spp. to the test substance for 48 hours and measuring the immobilization rate against the control. The present study defines an organism as being immobilized when it does not move for 15 seconds after the test vessel is gently shaken.

OECD Guideline 202 on “*Daphnia* spp., Acute Immobilization Test and Reproduction Test”, adopted in 1984, included two parts: Part I – the 24 hour EC₅₀ acute immobilization test and Part II – the reproduction test (at least 14 days). Revision of the reproduction test resulted in the adoption and publication of Test Guideline 211 on “*Daphnia magna* Reproduction Test” in September 1998. Consequently, the new version of Guideline 202 is restricted to the acute immobilization test.

Assay Principle

Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilization is recorded at 24 hours and 48 hours and compared with control values. The results are analyzed in order to calculate the EC₅₀ at 48 hours. EC₅₀ is the concentration estimated to immobilize 50% of the daphnids within a stated exposure period. Immobilization refers to those animals that are not able to swim within 15 seconds after gentle agitation of the test vessel, even if they can still move their antennae.

The water solubility and vapor pressure of the test substance should be known. A reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency and limit of determination should also be available.

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A reference substance may be tested for EC₅₀ as a means of assuring that the test conditions are reliable.

For this assay to be valid, the following performance criteria apply:

- In the control, not more than 10% of the daphnids should have been immobilized.
- The dissolved oxygen concentration at the end of the test should be at least 3 mg/L in control and test vessels.

Materials

- Glass Test Tubes and/or Beakers
- Dissolved Oxygen Meter
- pH Meter
- Temperature Control Apparatus
- Total Organic Carbon (TOC) Analyzer
- Chemical Oxygen Demand (COD) Analyzer
- *Daphnia magna* Straus
 - Use organisms less than 24 hours old. Do not use first offspring of parents.
- Water
 - Use water suitable for culturing and testing *Daphnia* spp. It can be natural water (surface water or groundwater), dechlorinated tap water, or artificially prepared water (Table 1), but must satisfy the conditions listed in Table 2. Do not use Elendt M4 or M7 media or water containing chelating agents for testing metal-containing substances.

Substance	Concentration
Particulate Matter	<20 mg/L
Total Organic Carbon	<2 mg/L
Unionized Ammonia	<1 ug/L
Residual Chlorine	<10 ug/L
Total Organophosphorus Pesticides	<50 ng/L
Total Organochlorine Pesticides plus Polychlorinated Biphenyls	<50 ng/L
Total Organic Chlorine	<25 ng/L

Table 1: Chemical Characteristics of Suitable Water

Substance	Amount Added to 1 Liter Water	To prepare the reconstituted water, add the following volumes of stock solutions to 1 liter water
Calcium Chloride	11.76 grams	25 mL
Magnesium Sulfate	4.93 grams	25 mL
Sodium Bicarbonate	2.59 grams	25 mL
Potassium Chloride	0.23 grams	25 mL

Table 2: Examples of Suitable Reconstituted Test Water

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Methods

Test Conditions

- | | |
|---|---|
| | Test Method |
| ○ | Test is performed under a static, semi-static, or flow-through condition. If test substance is unstable, a semi-static or flow-through test is recommended. |
- Exposure Period
 - 48 hours
- Test Volume
 - At least 2 milliliters
- Number of Test Organisms
 - At least 20 organisms for each test concentration and the control.
- Test Concentration
 - Adopt a concentration range of at least 5 concentrations, with the highest concentration inducing 100% immobilization and no effect at the lowest concentration.
- Culture Method
 - Illumination: The photoperiod is set to 16 hours light and 8 hours dark
 - Temperature: The temperature is between 18°C to 22°C
 - Dissolved Oxygen Concentration: Must be kept at 3mg/L or higher
 - Feeding: Do not feed test organisms

Observation

- Observe mobility of the organisms at least twice (i.e., at 24 and 48 hours after exposure).
- The organisms are considered immobilized when they do not move for 15 seconds after test vessel is gently shaken.

Measurement of Test Substance Concentrations

- At the beginning and end of exposure, measure test substance concentrations at the lowest and highest test concentration groups.
 - For volatile or adsorptive substances, additional measurements are recommended at 24 hours intervals during exposure period.

Test Condition Measurements

- Measure dissolved oxygen in the control and at the highest test concentration at the beginning and end of the exposure period.
- Measure pH in the control and at the highest test concentration at the beginning and end of the exposure period.
- Water temperature should be measured at the beginning and end of the exposure period.

Data and Reporting

I. Data

- a. Data should be summarized in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilization at each observation. The percentages immobilized at 24 and 48 hours are plotted against test concentrations. Data are analyzed by appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves and the EC₅₀ with 95% confidence limits (p = 0.95).
- b. Where the standard methods of calculating the EC₅₀ are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100% immobility should be used as an approximation for the EC₅₀ (this being considered the geometric mean of these two concentrations).

II. Test Report

- a. The test report must include the following:
 - i. Test substance:
 1. Physical nature and relevant physical-chemical properties
 2. Chemical identification data, including purity
 - ii. Test species:
 1. Source and species of *Daphnia*, supplier of source (if known), and the culture conditions (including source, kind and amount of food, feeding frequency)
 - iii. Test conditions:
 1. Description of test vessels: type and volume of vessels, volume of solution, number of daphnids per test vessel, number of test vessels (replicates) per concentration
 2. Methods of preparation of stock and test solutions including the use of any solvent or dispersants, concentrations used
 3. Details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc); composition of reconstituted water if used
 4. Incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc.
 - iv. Results:
 1. The nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported
 2. All physical-chemical measurements of temperature, pH and dissolved oxygen made during the test
 3. The EC₅₀ at 48 hours for immobilization with confidence intervals and graphs of the fitted model used for calculation, the slopes of the dose-response curves and their standard error; statistical procedures used for determination of EC₅₀.

Results

General Information:

Name of new chemical substance	Leucidal® Liquid SF		
INCI Nomenclature	<i>Lactobacillus</i> Ferment		
CAS number	1686112-36-6		
Structural or rational formula (if neither is available, summarize its formulation method)	Biotechnology: <i>Lactobacillus</i>		
Molecular weight	4890 Da		
Purity of the new chemical substance used for the test (%)	100%		
Lot number of the new chemical substance used for the test	31737		
Names and contents of impurities	n/a		
Solubility in water	100%		
Properties at room temperature	Clear to Slightly Hazy Liquid		
Stability	Heat Stable up to 70°C		
Solubility in solvents, etc.	Solvent	Solubility	Stability in solvent
	n/a	n/a	n/a

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Test Materials and Methods:

Items		Contents	
Test Organisms	Species	<i>Daphnia magna</i>	
	Source	Carolina Biological Supply Company	
	Reference substance (EC ₅₀)	Potassium dichromate (0.94 mg/L)	
Culture	Kind of Medium	Elendt Medium M4	
	Conditions (Temperature/Photoperiod)	20°C/16 Hour Light-8 Hour Dark	
Test Conditions	Test Vessel	Glass	
	Material Water	Kind	Elendt Medium M4
		Hardness	250 mg/L
		pH	7.4
	Date of Exposure	09/25/2013	
	Test Concentrations	200, 90.9, 41.3, 18.8, 8.5 mg/L	
	Number of organisms	120	
	Number of Replicates	Exposure Group	4
		Control Group	4
	Test Solution Volume	2 mL	
	Vehicle	Use or Not	N/A
		Kind	N/A
		Concentration	N/A
		Number of Replicates	N/A
	Culture Method (Static, Semi-Static, Flow-Through)	Static	
	Water Temperature	20°C ± 2°C	
	Dissolved Oxygen Concentration (DO)	3 mg/L	
Photoperiod	16 Hour Light-8 Hour Dark		
Statistical Method	Probit Analysis		

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OECD 202 Acute *Daphnia* Assay

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Test Results:

Items		Contents
Toxicity Value	48hr EC50	128 mg/L
Exposure Concentrations Used for Calculation	Nominal Values	200, 90.9, 41.3, 18.8, 8.5 mg/L
Remarks		Not harmful to aquatic organisms

Discussion

After 48 hours, the EC50 value for **Leucidal® Liquid SF** was determined to be 128 mg/L. The conditions of OECD guideline 202 for the validity of the test were adhered to: The immobility of controls in purified drinking water (dilution water) did not exceed 10%. According to the EU Directive 93/67/EEC, this product is not classified and therefore not harmful to aquatic organisms.

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OECD 301B Ready Biodegradability Assay

107 Technology Drive • Lincolnton, NC 28092
(704) 276-7100 • Fax (704) 276-7101

Tradename: Leucidal[®] Liquid SF

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 582

Lot #: 31738

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*

Study Director: *Erica Segura*

Principle Investigator: *Meghan Darley*

Test Performed:

OECD 301 B

Ready Biodegradability: CO₂ Evolution (Modified Sturm Test)

Introduction

A study was conducted to assess the readily biodegradability of **Leucidal[®] Liquid SF** in an aerobic aqueous medium. In the OECD guideline 301 for ready biodegradability, six methods are provided as options. This report uses method B, CO₂ Evolution, also known as a Modified Sturm Test. This method was chosen based on the solubility, volatility, and adsorbing capabilities of the test sample.

Assay Principle

A solution or suspension of the test substance in a mineral medium is inoculated and incubated under aerobic conditions in the dark or in diffuse light. The amount of DOC (Dissolved Organic Carbon) in the test solution due to the inoculum should be kept as low as possible compared to the amount of organic carbon due to the test substance. Allowance is made for the endogenous activity of the inoculum by running parallel blanks with inoculum but without test substance. A reference compound is run in parallel to check the procedures' operation.

In general, degradation is followed by the determination of parameters such as DOC, carbon dioxide production, and oxygen uptake. Measurements are taken at sufficiently frequent intervals to allow the identification of the beginning and end of biodegradation.

Normally this test lasts for 28 days, but it may be ended before that time if the biodegradation curve reaches a plateau for at least three determinations. Tests may also be prolonged beyond 28 days when the curve shows that biodegradation has started but the plateau has not yet been reached. In such cases the test substance would not be classified as readily biodegradable.

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The pass levels for ready biodegradability are 70% removal of DOC and 60% of ThOD (Theoretical Oxygen Demand) or ThCO₂ (Theoretical Carbon Dioxide) production for respirometric methods. They are lower in the respirometric methods since, as some of the carbon from the test chemical is incorporated into new cells, the percentage of CO₂ produced is lower than the percentage of carbon being used. These pass values have to be reached in a 10-day window within the 28-day period of the test. The 10-day window begins when the degree of biodegradation has reached 10% DOC, ThOD, or ThCO₂ and must end before day 28 of the test. Test substances which reach the pass levels after the 28-day period are not deemed to be readily biodegradable.

In order to check the procedure, reference compounds which meet the criteria for ready biodegradability are tested by setting up an appropriate vessel in parallel as part of normal test runs. Suitable compounds are freshly distilled aniline, sodium acetate, and sodium benzoate. These compounds all degrade in this method even when no inoculum is deliberately added.

Because of the nature of biodegradation and of the mixed bacterial populations used as inocula, determinations should be carried out at least in duplicate. It is usually found that the larger the concentration of microorganisms initially added to the test medium, the smaller the variation between replicates.

Materials

- Water
 - Deionized or distilled, free from inhibitory concentrations of toxic substances
 - Must contain no more than 10% of the organic carbon content introduced by the test material
 - Use only one batch of water for each series of tests
- Mineral media
 - To prepare the mineral medium, mix 10 mL of solution A with 800 mL water. Then add 1 mL each of solutions B, C, and D and make up to 1 liter with water.
 - Solution A (Dissolve in water and make up to 1 liter; pH 7.4)
 - Potassium dihydrogen orthophosphate, KH₂PO₄.....8.5g
 - Dipotassium hydrogen orthophosphate, K₂HPO₄.....21.8g
 - Disodium hydrogen orthophosphate dehydrate, Na₂HPO₄·2H₂O.....33.4g
 - Ammonium chloride, NH₄Cl.....0.5g
 - Solution B (Dissolve in water and make up to 1 liter)
 - Calcium chloride, anhydrous, CaCl₂.....27.50g
 - Or
 - Calcium chloride dehydrate, CaCl₂·2H₂O.....36.40g
 - Solution C (Dissolve in water and make up to 1 liter)
 - Magnesium sulphate heptahydrate, MgSO₄·7H₂O..... 22.50g
 - Solution D (Dissolve in water and make up to 1 liter.)
 - Iron (III) chloride hexahydrate, FeCl₃·6H₂O.....0.25g
 - Flasks, 2-5 liters each, fitted with aeration tubes reaching nearly to the bottoms of the vessels and an outlet
 - Magnetic stirrers
 - Gas absorption bottles
 - Device for controlling and measuring air flow
 - Apparatus for carbon dioxide scrubbing, for preparation of air which is free from carbon dioxide; alternatively, a mixture of CO₂-free oxygen and CO₂-free nitrogen from gas cylinders in the correct proportions (20% O₂ : 80% N₂)
 - Device for determination of carbon dioxide, either titrimetrically or by some form of inorganic carbon analyzer

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- Stock solutions of test substances
 - When solubility of the substance exceeds 1 g/L, dissolve 1-10 g, as appropriate, of test or reference substance in water and make up to 1 liter. Otherwise, prepare stock solutions in mineral medium or add the chemical directly to the mineral medium, making sure it
- Inoculum
 - The inoculum may be derived from the following sources
 - Activated sludge
 - Sewage effluents
 - Surface waters
 - Soils
 - Or from a mixture of these.
 - Inoculum may be pre-conditioned to the experimental conditions, but not pre-adapted to the test substance. Pre-conditioning consists of aerating activated sludge in mineral medium or secondary effluent for 5-7 days at the test temperature. Pre-conditioning sometimes improves the precision of the test method by reducing blank values.

Methods

- I. Preparation of flasks: As an example, the following volumes and weights indicate the values for 5-liter flasks containing 3 liters of suspension. If smaller volumes are used, modify the values accordingly.
 - a. To each 5-liter flask, add 2,400 mL mineral medium.
 - b. Add an appropriate volume of the prepared activated sludge to give a concentration of suspended solids of not more than 30 mg/L in the final 3 liters of inoculated mixture. Alternatively, first dilute the prepared sludge to give a suspension of 500-1000 mg/L in the mineral medium before adding an aliquot to the contents of the 5-liter flask to attain a concentration of 30 mg/L.
 - c. Aerate these inoculated mixtures with CO₂-free air overnight to purge the system of carbon dioxide.
 - d. Add the test material and reference compound, separately, as known volumes of stock solutions, to replicate flasks to yield concentrations, contributed by the added chemicals, of 10 – 20 mg DOC or TOC per liter. Leave some flasks without addition of chemicals as inoculum controls. Add poorly soluble test substances directly to the flasks on a weight or volume basis. Make up the volumes of suspensions in all flasks to 3 liters by the addition of mineral medium previously aerated with CO₂-free air.
 - e. If required, use one flask to check the possible inhibitory effect of the test substance by adding both the test and reference substances at the same concentrations as present in the other flasks.
 - f. If required, check whether the test substance is degraded abiotically by using a sterilized uninoculated solution of the chemical. Sterilize by the addition of a toxic substance at an appropriate concentration.
 - g. If barium hydroxide is used, connect three absorption bottles, each containing 100 mL of 0.0125M barium hydroxide solution, in series to each 5-liter flask. The solution must be free of precipitated sulfate and carbonate and its strength must be determined immediately before use.
 - h. If sodium hydroxide is used, connect two traps, the second acting as a control to demonstrate that all the carbon dioxide was absorbed in the first. Absorption bottles fitted with serum bottle closures are suitable. Add 200 mL 0.05M sodium hydroxide to each bottle. This is sufficient to absorb the total quantity of carbon dioxide evolved when the test substance is completely degraded.
 - i. In a typical run, the following flasks are used:
 - i. Flasks 1 & 2: containing test substance and inoculum (test suspension)
 - ii. Flasks 3 & 4: containing only inoculum (inoculum blank)
 - iii. Flask 5: containing reference compound and inoculum (procedure control)
 - iv. Flask 6: containing test substance and sterilizing agent (abiotic sterile control)
 - v. Flask 7: containing test substance, reference compound and inoculum (toxicity control)

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- II. Start the test by bubbling CO₂-free air through the suspensions at a rate of 30-100 mL/minute.
- III. CO₂ Determination
- It is mandatory to follow the CO₂ evolution from the test suspensions and inoculum blanks in parallel and it is advisable to do the same for the other test vessels.
 - During the first ten days it is recommended that analyses of CO₂ should be made every second or third day and then at least every fifth day until the 28th day so that the 10-day window period can be identified. On the days of CO₂ measurement, disconnect the barium hydroxide absorber closest to the test vessel and titrate the hydroxide solution with 0.05M HCl using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the test vessel and place a new absorber containing 100 mL fresh 0.0125M barium hydroxide at the far end of the series. Make titrations are needed (for example, when substantial precipitation is seen in the first trap and before any is evident in the second, or at least weekly). Alternatively, with NaOH as absorbent, withdraw a sample of the sodium hydroxide solution from the absorber nearest to the test vessel using a syringe. The sample volume needed will depend on the carbon analyzer used, but sampling should not significantly change the absorbent volume over the test period. Inject the sample into the IC part of the carbon analyzer for analysis of evolved carbon dioxide directly. Analyze the contents of the second trap only at the end of the test in order to correct for any carry-over of carbon dioxide.
 - On the 28th day withdraw samples, optionally, for DOC and/or specific chemical analysis. Add 1 mL of concentrated hydrochloric acid to each test vessel and aerate them overnight to drive off the carbon dioxide present in the test suspensions. On day 29 make the last analysis of evolved carbon dioxide.

Data and Reporting

- I. Treatment of Results
- Data from the test should be entered onto the data sheet below.
 - The amount of CO₂ produced is calculated from the amount of base remaining in the absorption bottle. When 0.0125M Ba(OH)₂ is used as the absorbent, the amount remaining is assessed by titrating with 0.05M HCl.
 - Since 1 mmol of CO₂ is produced for every mol of Ba(OH)₂ reacted to BaCl₂ and 2 mmol of HCl are needed for the titration of the remaining Ba(OH)₂ and given that the molecular weight of CO₂ is 44 g, the weight of CO₂ produced (in mg) is calculated by:

$$\frac{0.05 \times (50 - mL\ HCl\ Titrated) \times 44}{2} = 1.1 \times (50 - mL\ HCl\ Titrated)$$

Therefore, the factor to convert volume of HCl titrated to mg CO₂ produced is 1.1 in this case. Calculate the weights of CO₂ produced from the inoculum alone and from the inoculum plus test substance using the respective titration values. The difference is the weight of CO₂ produced from the test substance alone.

- d. The percentage biodegradation is calculated from:

$$\% \text{ Degradation} = \frac{\text{mg CO}_2 \text{ Produced}}{\text{ThCO}_2 \times \text{mg Test Substance Added}} \times 100$$

Or

$$\% \text{ Degradation} = \frac{\text{mg CO}_2 \text{ Produced}}{\text{mg TOC Added in Test} \times 3.67} \times 100$$

Where 3.67 is the conversion factor $\left(\frac{44}{12}\right)$ for carbon to carbon dioxide

- e. When NaOH is used as the absorbent, calculate the amount of CO₂ produced after any time interval from the concentration of inorganic carbon and the volume of absorbent used. Calculate the percentage degradation from:

$$\% \text{ ThCO}_2 = \frac{\text{mg IC from Test Flask} - \text{mg IC from Blank}}{\text{mg TOC Added as Test Substances}} \times 100$$

- f. Display the course of degradation graphically and indicate the 10-day window. Calculate and report the percentage removal achieved at the plateau, at the end of the test, and/or at the end of the 10-day window, whichever is appropriate.
- g. When appropriate, calculate DOC removals using the equation given in 301 A paragraph 27.
- h. When an abiotic control is used, calculate the percentage abiotic degradation by:

$$\% \text{ Abiotic Degradation} = \frac{\text{CO}_2 \text{ Produced by Sterile Flask After 28 Days (mg)}}{\text{ThCO}_2 \text{ (mg)}} \times 100$$

Validity of Tests

- i. The IC content of the test substance suspension in the mineral medium at the beginning of the test must be less than 5% of the TC, and the total CO₂ evolution in the inoculum blank at the end of the test should not normally exceed 40 mg/L medium. If values greater than 70 mg CO₂/L are obtained, the data and experimental technique should be examined critically.

Data Sheet

Laboratory	Active Concepts Tissue Culture Laboratory		
Test Start Date	9/25/2013		
Test Substance	Name	Leucidal® Liquid SF	
	Stock Solution Concentration	2 g/L	
	Initial Concentration in Medium	20 mg/L	
Inoculum	Source	Activated Sludge	
	Treatment Given	Centrifugation	
	Pre-conditioning	N/A	
	Suspended Solids Concentration in Reaction Mixture	4 mg/L	
Reference Material	Sodium Benzoate	Concentration	20 mg/L
CO₂ Production and Degradability	Method	Ba(OH)₂	0.0125M
		NaOH	N/A
		Other	N/A
Total Contact Time	28 Days		
Total CO₂ Evolved Measurements	Days	2, 4, 11, 17, 23, 28	
Degradation Over Time	93% and 89% after 28 days		
Remarks	Test material was readily biodegradable		
Conclusion	This test met the criteria for a valid assay		

Discussion

Based on the testing conducted in accordance with the specified test method, **Leucidal® Liquid SF** achieved 91% biodegradation after 28 days of testing. The product met method requirements for the Readily Biodegradable classification.



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Date Issued: January 23, 2015

ALLERGEN DECLARATION

RE: *Leucidal® Liquid SF (M15019)*

Please be advised that this form is to certify that the above referenced product, manufactured at Active Micro Technologies, LLC, does not contain any of the allergens listed below:

Eggs – or egg products

Milk – or milk products (includes whey, lactose, casein, milk, cream)

Peanuts – or peanut products

Fish – (includes fish: surimi, cod, pollack, whitefish)

Shellfish – (shrimp, lobster, crab, clams, etc.)

Soybeans – or soybean products (includes soya powder, protein, oil, lecithin, tofu)

Wheat – or wheat products (includes Gluten)

Tree nuts – (almond, brazil nut, cashew, chestnut, hazelnut, filbert, pine nuts (pinyon, pinon), pistachio, pecan, macadamia, walnut).

Palm Oil – or palm kernel oil

If you have any further questions or concerns, please contact us at: 1-704-276-7100



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CMR CERTIFICATE

Trade Name: Leucidal® Liquid SF (M15019)

The ingredient identified in this certificate does not contain any substances that are classified as carcinogenic, mutagenic or reprotoxic.

The labelling complies with CLP regulations.

Heather Ferguson
R&D Coordinator
Active Concepts, LLC

15-Jan-14



Certificate of Origin

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Leucidal[®] Liquid SF Code: M15019

Active Micro Technologies, LLC certifies that all raw material(s) used to manufacture the above listed ingredient originate in the United States of America.

Active Micro Technologies, LLC certifies that all raw material(s) used to manufacture the above listed ingredient are prepared from non-GMO organisms and are BSE-Free.

Active Micro Technologies, LLC certifies that the above listed ingredient is derived from fermentation using *Lactobacillus*.

Active Micro Technologies, LLC certifies that the above listed ingredient can be classified as Vegan Compliant.

Active Micro Technologies, LLC certifies that the above listed ingredient has never been tested on animals.

Active Micro Technologies, LLC certifies that neither we, nor any part of our supply chain have allowed contact with animal, milk, or grape based ingredients. We further certify that the above listed ingredient does not contain ingredients, incidental ingredients, or processing aids that have been grown on land fertilized with sewage sludge.



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SECTION 1. IDENTIFICATION

Product Name/Identifier	Leucidal [®] Liquid SF
Product Code	M15019
Recommended Use	Topical Cosmetic Use; Antimicrobial
Restrictions on Use	None
Supplier/Manufacturing Site	Active Micro Technologies, LLC
Address	107 Technology Drive Lincolnton, NC 28092, USA
Telephone No. (24hrs)	1-704-276-7100
Fax No.	1-704-276-7101
Emergency Telephone #	1-704-276-7100 (Mon-Fri: 8:00AM – 5:00PM EST)

SECTION 2. HAZARD(S) IDENTIFICATION

Classification:

GHS / CLP

Basis for Classification: Based on present data no classification and labeling is required according to GHS, taking into account the national implementation (United Nations version 2011)

USA

OSHA Regulatory Status: This material is non-hazardous as defined by the American OSHA Hazard Communication Standard (29 CFR 1910.1200).

Europe

Basis for Classification:

- According to present data no classification and labeling is required according to Directives 67/548/EEC or 1999/45/EC.
- This product is not classified as hazardous to health or environment according to the CLP regulation.

Labeling Elements:

Pictograph: No hazard symbol expected

Hazard statements/Signal Word: Not applicable

Precautionary statements:

- P233: Keep container tightly closed
- P281: Use personal protective equipment as required
- P402: Store in a dry place
- P404: Store in a closed container
- P410: Protect from sunlight
- P411: Store at temperatures not exceeding 25°C

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Other hazards which do not result in classification:

No particular fire or explosion hazard.

By mechanical effect: No particular hazards.

By hydroscopic effect: No particular hazards.

US NFPA 704 (National Fire Protection Association) Hazard Rating System:

Health hazard: Rating 0; Normal Material

Flammability: Rating 0, Will Not Burn

Reactivity: Rating 0, Stable

Other Hazard Information: None

Results of PBT and vPvB assessment:

-PBT: Not applicable

-vPvB: Not applicable

SECTION 3. COMPOSITION / INFORMATION ON INGREDIENTS

Common Chemical Name: Lactobacillus Ferment

Generic name:

Chemical Family: Ferment

Description: Substance

<u>Substance</u>	<u>CAS Numbers</u>	<u>EC Numbers</u>	<u>Percentage</u>
Lactobacillus Ferment	1686112-36-6	N/A	100.00%

Formula: Not applicable

SECTION 4. FIRST-AID MEASURES

General: In all cases of doubt, or when symptoms persist, seek medical attention.

Inhalation: Move to fresh air from exposure area. Get medical attention for any breathing difficulty.

Skin contact: Rinse with soap and water. Get medical advice if irritation develops.

Eye contact: Immediately rinse with water for at least 15 minutes, while keeping the eyes wide open. Consult with a physician.

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Ingestion: Consult with a physician.
Protection of first-aiders: No special protection required.

SECTION 5. FIRE-FIGHTING MEASURES

Fire and explosion hazards: Not considered to be a fire and explosion hazard

Extinguishing media:

Suitable: Water, dry chemicals, foam & carbon dioxide.

Not suitable: None known

Fire fighting: Move container from fire area if it can be done without risk.
Avoid inhalation of material or combustion by-products.
Stay upwind and keep out of low area

Protection for fire-fighters: Boots, gloves, goggles.

SECTION 6. ACCIDENTAL RELEASE MEASURES

Personal precautions: Avoid contact with eyes.

Personal Protective Equipment:
-Protective goggles

Environmental precautions: Prevent entry into sewers and waterways. Do not allow material to contaminate ground water system

Methods for cleaning up:

Recovery: Pick up free liquid for recycling or disposal. Residual liquid can be absorbed on an inert material.

Cleaning/Decontamination: Wash non-recoverable remainder with water.

Disposal: For disposal of residues refer to sections 8 & 13.

SECTION 7. HANDLING AND STORAGE

Handling

Technical measures: Labeling: Keep out of the reach of children.

Measures: For industrial use, only as directed.

Safe handling advice: Wash hands after use. Avoid storage near feed or food stuff.

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Storage

Technical measures: Keep container closed.
Recommended Storage Conditions: Store in a cool, dry place. This product should be stored at room temperature (23 - 25°C). It should not be exposed to excessive heat or cold. Do not freeze.

Incompatible products: Avoid contact with strong oxidizers.
Refer to the detailed list of incompatible materials (Section 10 Stability/Reactivity)

Packaging: Product may be packaged in normal commercial packaging.
Packaging materials: Recommended - Polypropylene & High Density Polyethylene

SECTION 8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Precautionary statements: Ensure adequate ventilation

Control parameters

Occupational exposure Limits:

France: Not Determined
ACGIH: Not Determined
Korea: Not Determined
UK: Not Determined

Surveillance procedures: Not Determined
Engineering measures: Not Determined

Personal Protective Equipment:

Respiratory protection: Local exhaust
Hand protection: Protective gloves made of rubber or neoprene.
Eye protection: Safety glasses.
Collective emergency equipment: Eye fountain.
Skin and Body Protection: Suitable protective clothing

Hygiene measures: Handle in accordance with good industrial hygiene and safety practice.
Measures related to the Environment: No particular measures.

SECTION 9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance: Clear to hazy liquid
Color: Colorless to yellow

Odor: Characteristic

Solids (1g/1hr/105°C): 6.0 – 10.0%

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pH (Direct): 3.0 – 7.0

Specific Gravity (25°C): 0.990 – 1.110

Heavy Metals: < 20 ppm
Arsenic: < 2 ppm

Minimum Inhibitory Concentration
Organism (ATCC#):

E. coli (#8739):	0.25 – 1.00%
S. aureus (#6538):	0.25 – 1.00%
P. aeruginosa (#9027):	0.25 – 1.00%
C. albicans (#10231):	0.25 – 1.00%
A. brasiliensis (#16404):	0.25 – 1.00%

Vapor pressure (@ 20°C): Not applicable

Vapor density: Not applicable
Boiling Point: 100°C
Freezing Point: 0°C
Melting point: Not applicable

Flash point: > 200°F
Oxidizing properties: Non oxidizing material according to EC criteria.

Solubility:

In water:	Soluble
In organic solvents:	Not determined
Log P:	Not determined

SECTION 10. STABILITY AND REACTIVITY

Stability: Stable under ordinary conditions of use and storage up to one year then re-test to full product specifications to extend shelf life

Hazardous reactions: None known

Conditions to avoid: No dangerous reactions known under use of normal conditions.
Avoid extreme heat.

Materials to avoid: No dangerous reaction known with common products.

Hazardous decomposition products: None known



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SECTION 11. TOXICOLOGICAL INFORMATION

Ingestion: Not Determined
Dermal: Non-Irritant (Dermal Irritation Model)
Ocular: Non-Irritant (Ocular Irritation Model)
Inhalation: Not Determined

Acute toxicity data: EC₅₀ (Acute Daphnia): 128 mg/L - Not harmful to aquatic organisms

Sensitization: Non-Primary Irritant & Non-Primary Sensitizer (RIPT, In-Vitro Skin Sensitization Report & Direct Peptide Reactivity Assay)

Repeated dose toxicity: No known effects
Subacute to chronic toxicity: Not Determined

Mutagenicity/genotoxicity: Non-mutagenic

Additional Toxicological Information: This product is not subject to classification according to the calculation method of the General EU Classification Guidelines for Preparations as issued in the latest version.

Specific effects:

Carcinogenicity: No known effects
Mutagenicity: No known effects
Reproductive toxicity: No known effects
Neuro-toxicity: No known effects

For more information: Does not present any particular risk on handling under normal conditions of good occupational hygiene practice.

This product has not been tested for the following:
-Primary cutaneous and corrosive irritation
-Acute oral toxicity

SECTION 12. ECOLOGICAL INFORMATION

Ecotoxicity

Effects on the aquatic environment: Not Determined

Biodegradability:

Persistence: Readily Biodegradable

Bioaccumulation:

Octanol / water partition coefficient: Not Determined

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Mobility:

Precipitation:

Expected behavior of the product: Ultimate destination of the product: Soil & sediment.

Other Adverse Effects:

None known

SECTION 13. DISPOSAL CONSIDERATIONS

Residues from product

Prohibition:

Do not allow the product to be released into the Environment.

Destruction/Disposal:

Dispose of in accordance with relevant local regulations

Contaminated packaging

Decontamination/cleaning:

Cleaning is not required prior to disposal.

Destruction/Disposal:

Note: Take all necessary precautions when disposing of this product according to local regulations.

SECTION 14. TRANSPORT INFORMATION

UN Number:

None

UN Shipping Name:

None

Transport Hazard Class:

Not classified as dangerous for transport

Land (rail/road):

Material is not restrictive for land transport and is not regulated by ADR/RID

Sea:

Material is not restrictive for sea transport and is not regulated by IMO/IMDG

Air:

Material is not restrictive for land transport and is not regulated by ICA/IATA

Marine Pollutant:

No

Transport/Additional Information:

Not regulated for US DOT Transport in non-bulk containers
This material is not dangerous or hazardous

Special Precautions for User:

None known

The above regulatory prescriptions are those valid on the date of publication of this sheet. However, given the possible evolution of transport regulations for hazardous materials and in the event of the MSDS in your possession dating back more than 12 months, it is advisable to check their validity with your sales office.

SECTION 15. REGULATORY INFORMATION

Labeling:

EC regulations:

This product does not need to be labeled in accordance with EC Directives or respective national laws

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Further regulations

United Kingdom: Handle in accordance with relevant British regulation: control of substance Hazardous to Health Regulations Environmental Hygiene Guidance: EH40
Workplace Exposure Limits (revised annually)

Korea regulations: Industrial safety and hygiene regulation: No
Hazardous material control regulation: No
Fire prevention regulation: No

Other regulations:

EINECS inventory status:	Lactobacillus Ferment	N/A
TSCA inventory status:	Exempt	
AICS inventory status:	Not Listed: 1686112-36-6	
Canadian (CEPA DSL) inventory status:	Not Listed: Lactobacillus Ferment (1686112-36-6)	
Japan (MITI list):	Lactobacillus Ferment	
Korea:	Lactobacillus Ferment	
China inventory status:	Lactobacillus Ferment	
Philippines inventory status:	Not Listed: Lactobacillus Ferment (1686112-36-6)	

Note: The regulatory information given above only indicates the principal regulations specifically applicable to the products described in this sheet. The user's attention is drawn to the possible existence of additional provision which complete these regulations. Please refer to all applicable international, national and local regulations and provisions

SECTION 16. OTHER INFORMATION

Prohibited uses: For specific uses, food industry, ask the manufacturer for more information.

Last Revision Date: 05/14/2015

Preparation Date: 08/13/2015

MSDS summary of changes

- New Logo
- Added Precautionary Statements - Section 2 (Hazards Identification)
- Added Sensitization Data – Section 11 (Toxicological Information)
- Added Mutagenicity Details – Section 11 (Toxicological Information)
- Updated pH Maximum – Section 9 (Physical & Chemical Properties)
- Added Minimum Inhibitory Concentration – Section 9 (Physical & Chemical Properties)
- Updated Transport Information – Section 14 (Transport Information)
- Added Sensitization Data – Section 11 (Toxicological Information)
- Updated CAS#'s – Section 3 (Composition / Information on Ingredients) & Section 15 (Regulatory Information)
- Added Sensitization Data – Section 11 (Toxicological Information)

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The information given is based on our knowledge of this product, at the time of publication in good faith. The attention of the user is drawn to the possible risks incurred by using the product for any other purpose other than which it was intended. This is not in any way excuse the user from knowing and applying all the regulations governing their activity. It is sole responsibility of the user to take all precautions required in handling the product. The purpose of mandatory regulation mentioned is to help the user to fulfill his obligations regarding the use of products. This information is not exhaustive, this is not exonerate the user from ensuring that legal obligations other than those mentioned, relating to the use and storage.

Leucidal[®] Liquid SF

Certificate of Compliance

Code: M15019
INCI Name: Lactobacillus Ferment
INCI Status: Conforms
CAS #: 1686112-36-6
EINECS #: N/A

The following information on regulatory clearances is believed to be accurate and is given in good faith as a guide to a global use of our ingredients in cosmetic applications. No representation or warranty as to its competences or accuracy is made. Information is offered for use in general cosmetic applications and may vary in particular applications. Users are responsible for determining the suitability of these products for their own particular use. All regulatory decisions should be made on the advice of your regulatory group or legal counsel.

Country / Regulatory Body	Status of Product
EU (REACH)	Compliant
USA (TSCA)	Exempt
Australia (AICS)	Contact Us
Japan (METI)	Compliant
Canada (DSL)	Contact Us
China (IECSC)	Compliant
Brazil (ANVISA)	Compliant
Korea (KECI)	Compliant
Philippines (PICCS)	Contact Us
Mexico (COFEPRIS)	Compliant at Suggested Use Levels



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Leucidal[®] Liquid SF Code: M15019

Attention must be paid to the use of Leucidal[®] Liquid SF in the equivalent of OTC formulations (eg. quasi-drugs in Japan, or therapeutic goods in Australia). Some countries maintain restricted inventories of raw materials that can be used in those applications so more detailed guidance may be required.

Leucidal[®] Liquid SF and any components or impurities are in compliance with the rules governing cosmetic products in the European Union (Directive 76/768/ECC & Regulation No. 1223/2009). The recommended use levels for Leucidal[®] Liquid SF is 2.00 – 4.00%.

Leucidal[®] Liquid SF is considered a non-hazardous material. All significant toxicological routes of absorption have been considered as well as the systemic effects and margin of safety (MoS) based on a no observed adverse effects level (NOAEL). Due to the restriction placed on animal testing of cosmetic raw materials, and Active Micro Technologies, LLC's internal non-animal testing policy, this product was not tested for NOAEL.

Leucidal[®] Liquid SF was tested using *in vitro* dermal and ocular irritation models. This product was found to be non-irritating in both models.

As of June 18, 2012, Leucidal[®] Liquid SF does not contain any substances present on the so called "candidate list" provided by the European Chemicals Agency (ECHA). We further certify that our product is not listed on CITES.

Leucidal[®] Liquid SF is in compliance with the standardized set of rules developed and approved by the NPA (Natural Products Association).

To our knowledge the above material is free of CMR (*) substances, as defined according to Regulation (EC) No 1272/2008 and Cosmetic Regulation (EC) No 1223/2009 as amended.

(*) Carcinogenic, Mutagenic, toxic for Reproduction

Active Micro Technologies, LLC certifies that to the best of our knowledge our product does not contain any material listed on California Proposition 65.

Active Micro Technologies, LLC certifies that Leucidal[®] Liquid SF does not contain any materials prohibited by Halal laws.

Leucidal[®] Liquid SF is REACH Compliant and free of the following:

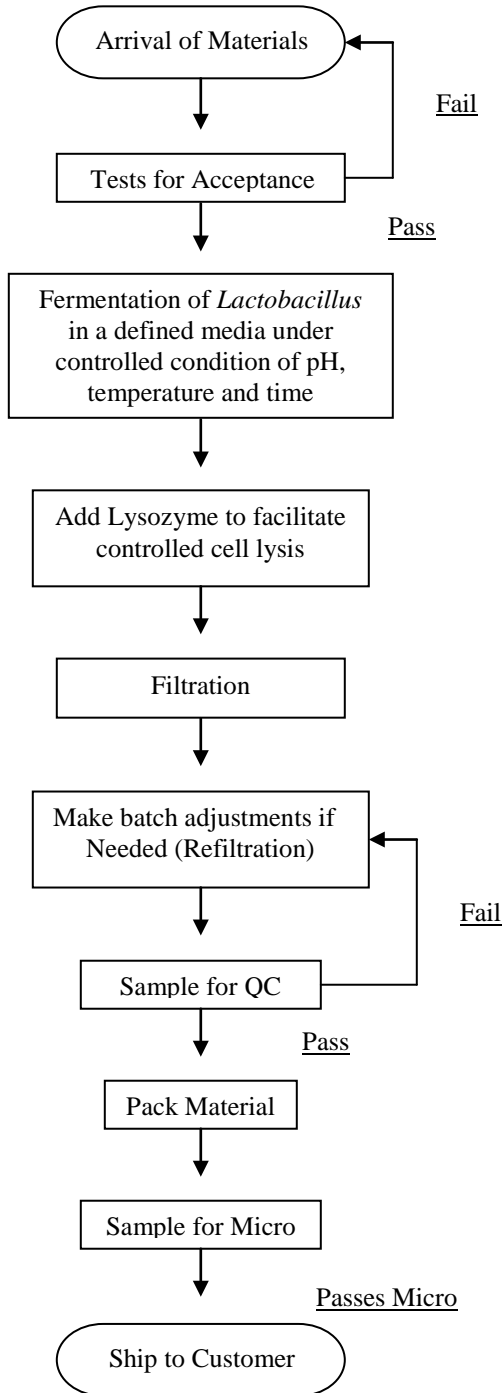
- Formaldehyde or formaldehyde donors
- Dioxin / Dioxane
- Glycol ethers
- Gluten
- Irradiation
- Lactose
- Nanoparticles
- Nitrosamines
- Nonylphenols / Alkylphenols / Phenols
- Palm oil/palm kernel oil (or derivatives)
- Parabens
- Paraffin/petroleum products
- Phthalates
- Polyethylene glycol (PEG)
- Residual solvents
- Sulfates
- Volatile organic compounds/solvents

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Leucidal[®] Liquid SF Manufacturing Flow Chart

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REACH Compliance Statement

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Trade Name: Leucidal[®] Liquid SF (M15019)

INCI Name: Lactobacillus Ferment

This is to certify that Leucidal[®] Liquid SF is REACH compliant. Lactobacillus Ferment is exempt from registration under REACH as it is currently manufactured and/or exported at less than 1 tonne per year.

If you have further questions, please feel free to contact Heather Ferguson at hferguson@activeconceptsllc.com.

**VERIFICATION OF THE RAW MATERIALS CONFORMITY TO THE
ECOCERT AND COSMOS COSMETIC STANDARDS**

THIS DOCUMENT IS NOT AN ORGANIC CERTIFICATE

Company: **ACTIVE MICRO TECHNOLOGIES LLC** Attestation n° : 5468

Page 1 on 4

The conformity (conf.) is established according to the requirements related to the raw materials contained in the applicable standard(s).

The present document is only valid for ECOCERT until official COSMOS publication of the raw materials on the website:
<http://www.cosmos-standard-rm.org/>

*reference related to the appendices II and/or V of the Cosmos standard.

AMTicide Coconut (M14003)Function: **Skin conditioning, Hair conditioning**

INCI: Lactobacillus (and) Cocos Nucifera (Coconut) Fruit Extract

Conf. ECOCERT: YES **100 %** of natural origin (**0 %** of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0 %** CPAI : **100 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

*Comments:***Leucidal Advanced - Aloe (M15015)**Function: **Moisturizing, Skin conditioning, Antimicrobial**

INCI: Water (and) Leuconostoc/Aloe Barbadosensis Leaf/Sorbus Aucuparia Fruit Ferment Filtrate

Conf. ECOCERT: YES **100 %** of natural origin (**0 %** of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0 %** CPAI : **18 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

Comments:

Drawn up in l'Isle Jourdain, valid from 01/01/2016
until 31/12/2016

Matthieu Bouffartigue
Raw Materials Service Manager



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TVA Intracommunautaire n° FR 55 509 534 095
CREDIT MUTUEL 2200 20246201 29 - SIREN 509 534 095 RCS AUCH - APE 7120B

Leucidal Advanced - Rowan (M15018)Function: **Emollient, Skin conditioning, Antimicrobial**

INCI: Water (and) Leuconostoc/Sorbus Aucuparia Fruit Ferment Filtrate

Conf. ECOCERT: YES **100 %** of natural origin (**0 %** of physically processed vegetal ingredients)
0 % synthetic**Conf. COSMOS:** YES PPAI : **0 %** CPAI : **16 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

Comments:

Leucidal Liquid (M15008)Function: **Moisturizing, Skin conditioning, Antimicrobial**

INCI: Leuconostoc/Radish Root Ferment Filtrate

Conf. ECOCERT: YES **100 %** of natural origin (**0 %** of physically processed vegetal ingredients)
0 % synthetic**Conf. COSMOS:** YES PPAI : **0 %** CPAI : **52 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

Comments:

Leucidal Liquid PT (M15021)Function: **Skin conditioning, Antimicrobial**

INCI: Lactobacillus Ferment

Conf. ECOCERT: YES **100 %** of natural origin (**0 %** of physically processed vegetal ingredients)
0 % synthetic**Conf. COSMOS:** YES PPAI : **0 %** CPAI : **18 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

Comments:

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until 31/12/2016Matthieu Bouffartigue
Raw Materials Service Manager

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Leucidal Liquid SF (M15019)Function: **Moisturizing, Skin conditioning, Antimicrobial**

INCI: Lactobacillus Ferment

Conf. ECOCERT: YES **100 %** of natural origin (**0 %** of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0 %** CPAI : **10 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

*Comments:***Leucidal Liquid SF (M15019CHI)**Function: **Skin conditioning, Antimicrobial**

INCI: Leuconostoc/Radish Root Ferment Filtrate

Conf. ECOCERT: YES **100 %** of natural origin (**0 %** of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **0 %** CPAI : **10 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

*Comments:***PhytoCide Aspen Bark Extract Powder (M16002)**Function: **Skin conditioning, Antimicrobial**

INCI: Populus Tremuloides Bark Extract

Conf. ECOCERT: YES **100 %** of natural origin (**100 %** of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **100 %** CPAI : **0 %** Petrochemical moiety : **0 %**
Non natural ingredient : **0 %**

Comments:

Drawn up in l'Isle Jourdain, valid from 01/01/2016
until 31/12/2016

Matthieu Bouffartigue
Raw Materials Service Manager



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PhytoCide Black Currant Powder (M16001)Function: **Soothing, Skin conditioning, Antimicrobial**

INCI: Ribes Nigrum (Black Currant) Fruit Extract

Conf. ECOCERT: YES **100 %** of natural origin (**100 %** of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **100 %** CPAI : **0 %** Petrochemical moiety : **0 %**
 Non natural ingredient : **0 %**

*Comments:***PhytoCide Elderberry OS (M16003)**Function: **Skin conditioning, Antimicrobial**

INCI: Sambucus Nigra Fruit Extract

Conf. ECOCERT: YES **100 %** of natural origin (**100 %** of physically processed vegetal ingredients)
0 % synthetic

Conf. COSMOS: YES PPAI : **100 %** CPAI : **0 %** Petrochemical moiety : **0 %**
 Non natural ingredient : **0 %**

Comments:

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